EFFECTS OF RATE AND NUMBER OF APPLICATIONS ON RESIDUAL TOXICITY OF NEEM-BASED INSECTICIDES TO THE LEAFMINER, *Liriomyza trifolii* (BURGESS) (DIPTERA: AGROMYZIDAE) ON SNAP BEAN

By

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To my parents and my wife for their love, support and encouragement

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
cm	Centimeters
d.f	Degree of freedom
F	Statistical F-Value
g/lw	Grams per liter water
L:D	Relation of light to darkness
ml/lw	Milli-liters per liter water
oz/gal	Ounce per gallon
P	Statistical probability value
RH	Relative humidity
SAS	Statistical Analysis Software
SE	Standard error

Abstract of Thesis Presented to the Graduate School
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EFFECTS OF RATE AND NUMBER OF APPLICATIONS ON RESIDUAL TOXICITY OF NEEM-BASED INSECTICIDES TO THE LEAFMINER, *Liriomyza trifolii* (BURGESS) (DIPTERA: AGROMYZIDAE) ON SNAP BEAN

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The American serpentine leafminer, *Liriomyza trifolii* (Burgess) is a polyphytophagous pest of greenhouse crops and ornamentals that has developed resistance to most synthetic insecticides. Neem-based insecticides have shown promising attributes in management of leafminers. However, no attempt has been made to evaluate the effects of number of foliar applications on residual toxicity of neem products to leafminer. The toxicity of commercial neem (*Azadirachta indica* Juss) products, Aza-Direct (Gowan Company) and NimBioSys (The Ahimsa Alternative, Inc.) neem oil, was evaluated against different stages of the leafminer. Observations were done on number of mines, larval mortality and pupal mortality. Treatments consisted of one, two, and three foliar applications of each product at two rates. In addition to foliar application, drench application was evaluated for both products at two rates.

Residual toxicity of both neem products was highly prevalent in snap beans using both foliar and drench application. Persistency was observed for at least 10 days from foliar application, while it lasted longer (14 days) in the drench application. In the foliar

application experiment, numbers of mines were greatly reduced and larval mortality was very high with multiple applications compared to single application. Pupal mortality was 100% in all applications regardless of product type and different rates. Numbers of mines in drench application did not differ among neem treatments and water treated control. Larval and pupal mortality in drench application was higher in neem treatments compared to control, but lower than foliar application. Increasing the number of foliar applications of neem increased mortality of different stages of leafminer under greenhouse conditions.

CHAPTER 1
INTRODUCTION

Snap Bean

Snap bean (*Phaseolus vulgaris* L) is an economically important vegetable crop in the family Fabaceae (Leguminosae). It is also commonly referred to as green bean, wax bean or string bean. Snap bean is believed to have originated in the Americas and was first described in Argentina and Guatemala (Gentry 1969). It gradually spread to the southwestern United States and is now distributed worldwide. Snap bean is an economically important legume crop; the fresh immature pods are a mainstay for vegetable marketing while marketing is also done in processed form by canning and freezing. Snap bean is a warm-season crop and can be successfully grown within the temperature range of 70 to 80 degrees Fahrenheit. Snap bean is categorized into two different groups, pole or bush beans, based on growth characteristics. Bush bean forms short, erect and compact plants, 1 to 2 feet in height, and have a determinate growth habit. Pole bean produces vines that can reach 8 to 10 feet and are trained on poles, fences or strings. Pole bean has indeterminate growth habit and sets pod continuously. Bush bean is ready for harvest in 45-50 days after sowing, while pole beans are harvested after 50-70 days (Olson et al. 2012).

Snap bean holds importance due to its wider geographical distribution, high production as well as economic and nutritional role (Silbernagel et al. 1991). It is an important source of dietary fiber, vitamin C, folic acid and minerals like potassium and several micronutrients. In 2011, snap bean production in the world totaled 20.39 million tons (Food and Agriculture Organization, 2011). In the U.S., snap bean was planted in 99,600 acres, of which 93,300 acres were harvested with a production of 274,434 tons

for fresh market during 2012. Similarly, for processing, snap bean was planted in 179,675 acres and 169,555 acres was harvested with production of 733,430 tons (USDA, NASS, 2012). Florida, California and Georgia are the largest producers of fresh market snap bean while Wisconsin, Oregon, Michigan and New York lead the U.S. snap bean production for processing (USDA, NASS, 2012).

Snap Bean Production in Florida

Florida has the highest production, acreage, and total value of fresh market of snap bean in the U.S. (USDA, NASS, 2012). During the year 2010-2011, snap bean was planted in 46,000 acres and harvested in 40,000 acres with production of 121.92 tons. This represents 43 percent of U.S. fresh market snap bean production worth 131.28 million dollars. Southeastern Florida is the principal snap bean production area, with Miami-Dade and Palm Beach counties leading in production.

The major planting season ranges between August 15 and April 1, but it varies with region. Florida continuously produces snap bean throughout the year and is the only state that supplies snap beans during the winter months in the U.S. Both bush-type and pole-type snap beans are produced in Florida, but the majority grown is bush-type. Pole bean is principally grown in Miami-Dade County and “Dade” and “Macaslan” are the most common varieties grown. Bush-type varieties include Bronco, Caprice, Dusky, Frontier, Valentino and Prevail (Olson et al. 2013).

Insect Pests of Snap Beans

Numerous insect pests, mites and diseases cause severe economic loss in snap bean production. The major pests in the U.S. include bean leaf beetle (*Cerotoma trifurcata* Foster), corn earworm (*Helicoverpa zea* Boddie), European corn borer (*Ostrinia nubilalis* Hubner), two-spotted spider mite (*Tetranychus urticae* Koch), cowpea

aphid (*Aphis craccivora* Koch) and melon thrips (*Thrips palmi* Karny). Silverleaf whitefly (*Bemisia argentifolii* Bellows & Perring), melon thrips (*Thrips palmi* Karny), leafminer (*Liriomyza trifolii and L. sativae*), bean leafroller (*Urbanus proteus* Linnaeus), cabbage looper (*Trichoplusia ni* Hubner) and southern green stink bug (*Nezara viridula* Linnaeus) form the major pest complex infesting snap bean in Florida.

Leafminer

Leafminers were first described from Argentina and are considered endemic to South and North America. More than 300 species of *Liriomyza* (Diptera: Agromyzidae) are recorded, of which 24 species are considered of economic significance (Spencer, 1973). *Liriomyza sativae* Blanchard, *L. trifolii* Burgess and *L. huidobrensis* Blanchard are the most destructive phytophagous leafminers of vegetables and ornamental plants worldwide (Spencer 1973, Parella 1987), with *L. trifolii* and *L. sativae* being occasionally major pests of snap bean in Florida.

L. sativae was recorded as an economically important pest of horticultural crops in Florida by 1940s (Spencer 1973). It established itself as a predominant leafminer species in Florida until 1970s but was gradually displaced by *L. trifolii* during late 1970s (Reitz and Trumble 2002). *L. trifolii*'s ability to displace *L. sativae* population is attributed to its stronger ability to develop resistance to insecticides used for its management (Schuster and Everett 1982). *L. trifolii* is now considered a major pest of field grown and greenhouse vegetable crops in Florida (Seal et al. 2002, Waddill et al. 1986). It infests numerous crops, ornamentals and weeds distributed across different families including but not limited to Cucurbitaceae, Fabaceae, Solanaceae, Brassicaceae, Asteraceae, Compositae, Caryophyllaceae and others (Capinera 2001, Musgrave et al. 1975, Parkman et al. 1989).

Biology

L. trifolii has a tremendous reproductive potential, can develop rapidly and has a short life cycle that makes it a threatening pest at all times. It undergoes complete development from egg deposition to adult emergence in less than 19 days under optimal temperature (Leibee 1984, Lanzoni et al. 2002). Optimum temperature for development ranges from 25°C to 30°C and larval mortality increases as temperature increases above 30°C. As a result, it can have multiple, overlapping generations in a single cropping season. *L. trifolii* has four distinct life-stages: egg, larva, pupa and adult. Studies have shown that different life stages of leafminer averages 2.7 days for egg hatch, 1.4, 1.4, and 1.8 days for three respective larval stages and 9.3 days for pupal development into adults at 25°C (Minkenberg, 1988).

An adult leafminer female prefers mature foliage for oviposition and usually lays eggs on the upper leaf surface. The egg is oval and small in size (1.0 mm long and 0.2 mm wide), initially clear and eventually becomes creamy white in color. Eggs are laid singly, but in close proximity to each other in punctures made by the female's ovipositor Leibee (1984) observed that a single leafminer can lay eggs at the rate of 35 to 39 per day totaling up to 400, on celery. Similar fecundity was reported by Parella (1987) in tomato, with less total number of eggs due to lower preference of the host plant.

Larval stage is comprised of three distinct instars, usually distinguished by the length of their cephalopharyngeal skeleton. The larva begins to feed soon after hatching until late third instar when it is ready to pupate. Both the eggs and larval stages are concealed internally within plant foliage. Often, a fourth instar (prepupal stage) is taken under consideration, and is a non-feeding stage between puparium formation and pupation. The late third instar exits through the feeding mine, forms a pupa and drops to

ground or substrate for further development. The leafminer pupa is initially golden brown in color and gradually becomes darker brown. Pupal stage lasts for about 9 to 12 days depending upon temperature.

The adult *L. trifolii* is small, about 2 mm in body length and 1.25 to 1.9 mm in wing length. The head is yellow and black in color with red eyes. The thorax and abdomen are gray, and the ventral surface and legs are black and yellowish. Most of the females mate within 24 hours after emergence (Parella et al. 1983). Multiple matings by female maximizes egg production. After mating, female punctures the leaves with its ovipositor. These punctures are used for laying eggs as well as for feeding on exuding sap. Female feeds from all punctures whether or not they are used for oviposition. Feeding and oviposition activities of the female peak during mid-day. Male leafminer solely depends on oviposition punctures made by female for feeding (Fagoonee and Toory, 1984). Male typically lives for about 2-3 days due to limited food source while female lives for about a week.

Damage

L. trifolii causes damage to crops in two ways. First, damage is caused by oviposition punctures made by adult female for laying eggs and for feeding (Bethke and Parella 1985). These stipples reduce the aesthetic appeal and subsequent marketability of the crop, reduce photosynthesis in foliage (Trumble et al. 1985, Johnson et al. 1983) and may even kill seedling plants. However, damage due to stippling is considered insignificant compared to the second mode of damage - larval mining.

Excessive mining reduces photosynthesis, plant vigor, growth and yield (Al-Khateeb and Al-Jabr, 2004). In addition, high numbers of leafminer larvae cause mining of bean pods and premature leaf defoliation, resulting in sunscald damage of fruits such

as tomatoes. The mines also serve as route for disease pathogens. Indirectly, it creates quarantine restrictions and causes huge economic loss. Despite the high number of leafminer infestation and mines, significant reduction on yield of crops are not reported (Kotze and Dennil, 1996). Crop damage is considered minimal as plants can tolerate significant level of mining without affecting the yield. Economic impact due to yield loss from mining has not been studied in detail, but Schuster (1978) has recorded up to 90% loss in tomato foliage due to uncontrolled leafminer infestation.

Control

Control strategies for *L. trifolii are* mainly focused on application of synthetic insecticides since its establishment as the most dominant leafminer pest (Leibee 1981, Cox et al. 1995). The most commonly used insecticides for leafminer control in Florida include abamectin, cyromazine, spinosad, dimethoate, lambda cyhalothrin, rynaxypyr, spinetoram and azadirachtin. During the early and mid-nineties, growers increased the frequency of application of these insecticides to three times per week in an attempt to control leafminer, but had little success (Waddill 1989).

Reduction in effectiveness of insecticides occurred due to their indiscriminate use resulting insecticide resistance (Leibee 1981, Keil and Parrella 1990, Ferguson 2004). Ferguson (2004) documented cyromazine, abamectin and spinosad resistance in *L. trifolii* but resistance was unstable. Parella et al (1984) also observed slight resistance of leafminers to methyl-parathion and methamidophos (organo-phosphates) and permethrin (pyrethroids) when treated on chrysanthemum. In addition, insecticides used to control leafminer were ineffective because they failed to penetrate and affect protected life stages such as egg, larvae and pupae (Parella 1987). Overuse of insecticides decimated populations of natural enemies that were successful in

maintaining leafminer population below economically damaging levels (Johnson et al. 1980). The effectiveness of newly developed insecticides lasted only for two years in Florida (Leibee 1981) due to development of resistance. A limited number of insecticides such as abamectin, cyromazine and spinosad are successfully used in management of leafminers (Civelek and Weintraub 2003, Leibee 1988, Seal et al. 2002).

Alternative measures for leafminer management are being increasingly sought in recent years. Some efforts have been made towards developing plant resistance (Jong and Rademaker 1991), using selective translaminar insecticides (Weintraub and Horowitz 1997), using biological control (Liu et al. 2009) and use of botanical derivatives such as neem (*Azadirachta indica*) products (Stein and Parella 1985, Hossain et al. 2007).

Integrated pest management (IPM) strategies provide effective and economical pest control with minimum disturbances to the natural components of the farming system. Biological control by conserving natural enemies is an important IPM strategy to combat leafminer outbreak. About 40 hymenopteran parasitoids have been studied that parasitize *Liriomyza* spp. (Waterhouse and Norris 1989) with 48.5 -68.5% parasitism on crops and 83.7% parasitism on weeds of vegetables (Chen et al. 2003). Mujica and Kroschel (2011) observed about 29.5% parasitism of leafminers by 63 parasitoids including Eulophidae (41 species.), Braconidae (11 species.), Pteromalidae (8 species.), Figitidae (1 species.) and Mymaridae (2 species.). The most dominant parasitoids found in Florida belong to the families Braconidae (*Opius dimidiatus* Ashmead and *O. dissitus* Muesebeck), Pteromalidae (*Halticoptera circulus* Walker) and

Eulophidae (*Diglyphus intermedius* Girault) (Parkman et al. 1989). Predatory insects are also found to prey on leafminers but do not significantly contribute to leafminer control.

There has been an increasing interest on use of biorational pesticides as alternatives to conventional synthetic pesticides. Biopesticides such as neem products have been studied as potential products effective in controlling numerous insect pests such as leafminers (Hossain et al. 2008, Schmutterer 1990). Appropriate integration of biopesticides with natural enemies can effectively manage leafminer population. Such products should preferably have properties such as faster degradability in the environment, low human toxicity, easy and cheap production, low impact on beneficial insects and low risk of selecting resistant pest biotypes. One possible alternative to synthetic pesticides can be the extracts from the neem tree, *Azadirachta indica* Juss.

Neem

The neem tree, *Azadirachta indica* Juss (Meliaceae) is an evergreen, fast growing plant highly recognized for its insecticidal properties and low toxicity to humans (Mordue and Nisbet 2000). It is believed to have originated in Southern Asia and gradually spread to Africa, Australia and Central and South America (Schmutterer, 1990). Ingredients obtained from neem fruit kernels constitute the most important compounds for insect control, while ingredients from the leaves, barks, and roots are also toxic to insects in various ways. Neem oil, obtained by cold pressing seeds, and extracts of seed residue after removal of oil are two types of neem products that have been scientifically studied and widely recommended as a biorational pesticides (Isman 2006).

Azadirachtin and its derivatives are the most effective active ingredients used as insecticides and primarily function as insect growth regulators (Rembold et al. 1984)

and anti-feedants (Schmutterer 1990). It is a complex tetranortriterpenoid limonoid that produces toxic effects in treated insects (Mordue and Nisbet 2000). Other active compounds such as salanin, vilasinin, nimbinen and azadirodoid show strong anti-feedant properties (Schwinger et al. 1984). Further effects of neem derivatives include olfactory repellency, anti-oviposition, reduction in fecundity and egg-fertility and reduction in vigor of adult insects. Neem products effectively control numerous pest species through different modes of action, resulting from systemic and contact activity in treated plants. They are non-toxic to vertebrates and have low toxicity to beneficial insects. These properties contribute towards the use of neem as a potential bio-rational pesticide.

Schmutterer and Singh (1995) studied toxicity of neem products and found that about 400 insect species are susceptible to neem extracts. Both holometabolous and hemimetabolous insect orders such as Thysanura, Orthoptera, Hemiptera, Hymenoptera, Coleoptera, Lepidoptera and Diptera are significantly affected. Neem causes insecticidal effects in insects primarily by two modes of action: growth regulation and feeding deterrence (Isman 2006). Studies on the physiological response of insects to neem show that azadirachtin prevents the synthesis and release of ecdysteroids, or molting hormones, from the prothoracic gland, thus interrupting ecdysis (Mordue and Blackwell 1993). Immature insects exposed to neem show reduced and delayed synthesis of juvenile hormones and ecdysone causing interference during molts (Rembold 1989). As a result, high mortality was observed during the larval-pupal and pupal-adult molts. Adult insects treated with neem show reduction in ecdysone levels,

sterility caused by reduced ovarian development and no vitellogenin synthesis (Ascher 1993; Sieber and Rembold 1983).

Schmutterer discovered antifeedant property of neem after observing desert locusts that avoided feeding on neem trees. Insect pests exposed to azadirachtin show different levels of sensitivity. Many lepidopterans are highly sensitive to azadirachtin (<1-50 ppm); Coleoptera, Hemiptera and Diptera are less sensitive (100-600 ppm), while Orthoptera show a wide range in sensitivity (0.5 -1000 ppm) (Schmutterer 1990; Mordue and Nisbet 2000). Insects feeding on neem exhibit reduced growth due to reduced trypsin activity, resulting in inhibition of digestion and utilization of proteins (Timmins and Reynolds 1992).

Other influences of neem derivatives on insects include olfactory repellent effect on settling behavior, oviposition repellent, reduction in fecundity and egg-sterility (Schmutterer 1990; Mordue and Blackwell 1993). Insect pests of medical and veterinary significance such as lice, mites, ticks, fleas, bugs, cockroaches, and flies are also effectively controlled by neem treatments (Mehlhorn et al. 2011). In addition to insect pests, neem products possess deterrent effects resulting in significant mortality, reduced fecundity and retarded development on two-spotted spider mites (Dimetry et al. 2009) and root-knot nematodes (Javed et al. 2007), growth inhibitory effects on various fungi (Singh et al. 1980), as well as antibacterial properties (Mahfuzul et al. 2007).

Phytophagous insects can be treated with neem either by foliar application or by soil drenching since neem functions both as a contact and a systemic insecticide (Holmes et al. 1999). Neem is systemically taken up by the plants and is present in the food source producing strong effects on insect pests (Larew et al. 1985, Schmutterer

1990, Mordue et al. 1998). The systemic activity of neem is very important in management of piercing and sucking insects, stem and root-feeding insects and leaf mining insects (Isman et al. 1991). Systemic effects of neem have been studied with different pests such as mountain pine beetle, *Dendroctonus ponderosae* Hopkins, (Nauman et al. 1994), two-spotted spider mite, *Tetranychus urticae* Koch, (Sundaram et al. 1995), leafminer, *L. huidobrensis* Blanchard (Weintraub and Horowitz 1997), green peach aphid, *Myzus persicae* Sulzer (Holmes et al. 1999), and on western flower thrips, *Frankliniella occidentalis* (Thoeming et al. 2003).

An important property of neem is its non-toxicity to invertebrates, which makes it a suitable alternative in integrated pest management and organic farming. Toxicological studies have shown that the rat oral acute LD50 is > 5000 mg/kg (Raizada et al. 2001) and that neem does not produce any mutagenic or carcinogenic effects. Neem-derived insecticides do not produce skin irritations or organic alterations in mice and rats, even at higher concentrations (Mehlhorn et al. 2011). Studies have also shown non-toxicity of neem to fish (Wan et al. 1996) and pollinators (Naumann and Isman 1996). However, the timing of treatment can be important; parasitoid emergence is not highly affected if treatments are made before parasitism of the pests (Hohmann et al. 2010). Neem products are highly degradable and do not leave residues in soil, having a half-life of only one day (Kleeburg and Ruch 2006). Hence, no phytotoxicity is observed in plants nor any adverse effects are seen on underground water (Mehlhorn et al. 2011). These properties make neem products a safe to use biopesticide for pest control and a viable option to replace synthetic pesticides.

The insecticidal properties of neem have been investigated for a long time and have been shown to be useful where pesticide resistance is documented and management options are limited. Pesticide resistant agricultural pests such as leafminers, are susceptible to neem treatments and have shown lower fecundity, reduced longevity of adults (Parkman and Pienkowaski 1990), and increased mortality of the larval stages (Webb et al. 1983). Neem derivatives have proven to be effective through foliar spray (Seljasen and Meadow 2006) and drench application due to their systemic properties (Weintraub and Horowitz 1997), and have potential to be adopted in integrated pest management schemes.

Objectives

Neem extracts are developed as commercial insecticides and used for management of various pests. They have shown promising attributes in the management of leafminer and have a great significance in organic production systems. Neem-based insecticides possess translaminar and systemic properties and so can be used as foliar and drench application. However, no attempt has been made to evaluate the residual effects of neem products applied at different frequencies. This study was conducted for the following purposes:

1. To evaluate the effects of different rates and number of applications of Aza-Direct and NimBioSys neem oil on residual toxicity to *L. trifolii* on snap beans using foliar application.
2. To evaluate the effects of different rates of Aza-Direct and NimBioSys neem oil on residual toxicity to *L. trifolii* on snap bean using drench application.

CHAPTER 2
MATERIALS AND METHODS

General Background

The experiments for this study were performed at Mid-Florida Research and Education Center (MREC), University of Florida, Apopka, Florida. All experiments were carried out in the greenhouse. Laboratory rearing and maintenance of the leafminer culture were done in an insectary at the same location.

Insect and Plant Sources

American serpentine leafminer, *Liriomyza trifolii* Burgess, was chosen for the study for several reasons. It is a common pest of various crops as well as ornamentals, has a shorter life cycle, is highly prolific, and relatively easy to work with. The leafminer colony used in the experiments was established and maintained from infested carrot tops collected from Zellwood, Florida about 30 years ago. The insect culture was maintained in the insectary at a room temperature of 25 ± 1°C, 50-60 % RH and 15L: 9D photoperiod: scotoperiod. The leafminers were provided with cowpea seedlings in rearing cages for oviposition. Cowpea plants containing late third instar larvae were placed on a tray containing paper towels as a substrate for pupation. The pupating larvae fell into the tray and were collected manually. This process was followed in order to obtain synchronized life-stages. The collected pupae were stored in a refrigerator at about 4-5°C for later use or allowed to develop depending on need. Two-day old adults were used in all the experiments. The snap bean variety used in the experiment was Kentucky Wonder, a pole-bean type that exhibits indeterminate growth. It was planted and maintained in the greenhouse throughout the experimental period.

Location and Conditions

Experiments were run in greenhouses and 45-day-old snap beans were used. The day-night temperature ranged from 24.4-34.6°C with relative humidity from 50-70% throughout the experimental period. The pole beans were planted in 15 cm high × 20 cm diameter pots, watered twice daily and fertilized once a week.

Neem Biopesticides

Two neem-based products, Aza-Direct (Gowan Company LLC.), containing 1.2% azadirachtin, and NimBioSys (The Ahimsa Alternatives), 100% neem oil, were tested to evaluate the residual efficacy against leafminer, *Liriomyza trifolii*. These products were applied at label rates, using the lowest and the highest concentration commercially recommended. Aza-Direct solution with dosage rates of 25 oz/100 gallons and 35 oz/100 gallons were used. Solution equivalent to 1.98 ml and 2.73 ml respectively were freshly prepared by mixing thoroughly in a liter of deionized water. For NimBioSys neem oil, 0.5% and 1% solution were prepared by dissolving 5 ml and 10 ml product in 1 liter of deionized water, respectively. Prior to mixing the neem oil in water, a nonionic surfactant (Triton®-X-100) was added to the product to maximize mixing and reduce surface tension. 1 ml of surfactant for 0.5% and 2 ml of surfactant for 1.0% neem oil was used based on product label recommendations. The solution was thoroughly mixed and freshly prepared before application.

CHAPTER 3
FOLIAR APPLICATION EXPERIMENT

Commercially available neem products Aza-Direct® (Gowan Company), containing 1.2% azadirachtin, and NimBioSys® neem oil (The Ahimsa Alternatives, Inc), containing 100% neem oil, were used for the experiments. Two recommended high rates of Aza-Direct (25 oz/100 gallons and 35 oz/100 gallons) and two recommended rates of NimBioSys neem oil (0.5% and 1.0%) were used to prepare solutions for application. All product rates were prepared using the procedures mentioned earlier.

Treatments consisted of one, two and three foliar applications of each product at two different rates and a water treated control (13 treatments). Plants receiving three applications were sprayed starting two weeks before exposure to leafminer adults, and were sprayed on a weekly interval. Similarly, plants receiving two applications were sprayed starting a week before leafminer release. Plants receiving single application of neem products were sprayed one day before exposure to leafminer adults. Each plant was considered a single treatment and each treatment was replicated four times. Treatments were arranged on a randomized complete block design. Freshly prepared neem products were used for application and sprayed until runoff with a hand-held mist sprayer. The plants were positioned in a horizontal orientation while spraying to avoid the spray dripping into the soil and to avoid uptake by roots and systemic translocation. The plants were left to air-dry for half an hour before righting and arranging into randomized blocks.

Leafminer adults were released on all plants simultaneously on the same day. First sets of leafminers were released one day after all applications were complete while second sets of leafminers were released seven days later. A trifoliate leaf was randomly

chosen and a micro perforated polypropylene bag was placed over it. Five pairs of leafminer adults (1 male: 1 female) were released into each perforated bread bag containing a trifoliate. The leaves were exposed to leafminer adults for 48 hours to ensure sufficient time for mating and oviposition. After 48 hours, the leafminer adults were removed manually by killing them in order to achieve synchronous development of immatures and adult emergence. Total number of mines was recorded five days after leafminer adults were released. The micro-perforated polypropylene bag was left intact over the trifoliate, allowing the late third instar larvae and/or prepupae to fall onto the bag for pupation. Pupae were then collected and placed in polyethylene cups until adult emergence. Observations were made on the number of mines, larval mortality and pupal mortality. The total number of adults that eclosed was used to determine pupal mortality. Larval mortality was calculated using the formula in Leibee (1988):

$$\% \text{ larval mortality} = (m-p)/m \times 100$$

Where, ‘m’ denotes the number of mines or larvae in each treatment and ‘p’ denotes the number of pupae reared from each treatment.

Pupal mortality was assessed using the same formula with a little modification:

$$\% \text{ pupal mortality} = (p-a)/p \times 100$$

Where, ‘p’ was the number of pupae in each insecticide treatment and ‘a’ was the number of adults emerged from each treatment.

The observation from treatment receiving three applications of 1% NimBioSys neem oil was discarded for analysis, because of higher phytotoxicity to the plants. Very few mines, if any, were recorded on this treatment.

Statistical Procedure

The treatments were arranged in a randomized complete block design (RCBD). SAS 9.3 (SAS Institute, 2011) was used for all statistical procedures. PROC FACTEX was used to randomize blocks and treatments within blocks. The data were checked for normality using the UNIVARIATE procedure and Shappiro-Wilk test was used as a measure of normality (Shappiro and Wilk 1965). In addition, scatter plots of residuals versus predicted values were used to determine normality and homogeneity of variances. In case of non-normality, data were transformed. Data with numbers (count values) were transformed using square root, and percentage data were transformed using arcsine-square root before running an ANOVA. ANOVA was performed using the MIXED procedure in SAS. Four treatment (two neem products with two different rates each) means along with three application frequencies in two releases of leafminer were analyzed for differences of means. ANOVA means were separated using Tukey's test ($P < 0.05$) as appropriate after a significant F-test. In addition, contrast analysis was performed after ANOVA to compare between control versus rest of the treatments, as well as the difference between two neem products and its rates. Analysis between control and neem treatments was performed by treating each combination of number of application and rate as a unique treatment. All tests were performed at 5% level of significance.

Results

Number of Mines

Observations were based on numbers of mines formed by the first generation of leafminer larvae when adults were released one day and seven days after neem application. There was no significant difference in the number of mines when adult

leafminers were released one day and seven days after application of two neem products at different rates and frequencies ($F = 1.22$; $df = 1$; $P = 0.2722$). Therefore, data collected on number of mines were pooled for analysis. The number of mines formed from first and second release of leafminer adults (seven days after neem application) is depicted in Fig. 3-1. This indicated extended residual toxicity of two neem products against leafminer in treated plants.

When number of mines between the different treatments (product and rates) was analyzed, a significant difference was seen ($F = 5.68$; $df = 3$; $P = 0.0014$). So, contrast analysis was used to test difference in means between the two products and their rates. Significant difference was obtained when Aza-Direct and NimBioSys products were compared ($t = 3.92$; $P = 0.0002$). However, no significant differences existed between different rates of Aza-Direct ($t = 1.24$; $P = 0.2200$) and different rates of NimBioSys ($t = 1.11$; $P = 0.2715$). Outcome based on number of mines show that effect of Aza-Direct and NimBioSys were different (Fig. 3-2).

Of particular significance was the result on number of mines when different numbers of application were made. Highly significant difference in number of mines was observed between the three application frequencies ($F = 22.81$; $df = 2$; $P < 0.0001$). Tukey's test was used to compare significant difference in mean number of mines from different frequency of neem applications. Multiple applications resulted in significantly lower numbers of mines compared to single application while there was no significant difference in mine numbers when two and three applications were compared (Fig. 3-3). Contrast analysis was used to compare number of mines between neem treatments and

water treated control. Analysis showed a significant difference between control and neem treatments ($t = -6.26$; $P < 0.0001$).

Mean numbers of mines from various combinations are depicted in Table 3-1. Comparative significant differences on number of mines when adult leafminers were released one day and seven days after neem application, different treatments and number of applications are shown in Table 3-3.

Larval and Pupal Mortality

Observations on larval mortality from two leafminer releases were pooled for analysis. Results showed that there was no significant difference in larval mortality when leafminer adults were released one day and seven days after neem application ($F = 0.63$; $df = 1$; $P = 0.4290$). Larval mortality was high in both releases indicating high residual effect (Fig. 3-4). Contrary to the number of mines, no significant difference in larval mortality was observed among treatments (two product and their rates) ($F = 0.90$; $df = 3$; $P = 0.4427$) (Fig 3-5).

Contrast analysis was used to compare larval mortality between water treated control and neem treatments. A significant difference in larval mortality ($t = 10.42$; $P < 0.0001$) was observed indicating that difference in larval mortality was due to water treated control. Both neem products applied at multiple frequencies showed significantly different larval mortality ($F = 24.54$; $df = 2$; $P < 0.0001$). A single application resulted in a lower proportion of larval mortality as compared to multiple applications (Fig. 3-6). Mean larval mortality from various combinations are depicted in Table 3-2. Comparative significant differences in larval mortality between number of releases, treatments and number of applications are shown in Table 3-4.

Both neem products were highly effective against pupal stage of leafminer irrespective of different rates and application frequency. All pupae developed from neem treated snap bean foliage were dead and no adult eclosion occurred. A total mortality of the generation was observed. On the other hand, in water treated control, all pupae except a few eclosed into adults. Hence, no analysis of variance could be performed on observations from pupal mortality.

Discussion

This study presents the first attempt to evaluate the residual effects of different neem products with varying rates applied at different frequencies against leafminer under greenhouse conditions. It is clearly evident that multiple applications of neem-derived insecticides are highly effective in controlling leafminer population compared to products applied once. However, neem products applied two and three times did not result in significant differences in mean number of mines and larval mortality. The outcome indicated anti-ovipositional property of neem based on the number of mines formed. Similarly, larval and pupal mortality could be attributed to translaminar property of neem and high toxicity against different immature stages of leafminer. Increasing application frequency is negatively correlated with leafminer numbers and has extended residual effect for at least 10 days.

Effect on Formation of Mines

The results obtained from the foliar application of different neem products at different rates and frequencies showed that residual effect was highly prevalent for at least two weeks and posed toxic effect to different developmental stages of leafminer. It was observed that there was an inverse relationship between increasing the rate and frequency of application of neem products to the number of larval mines formed on the

treated foliage. An explanation for this phenomenon could be due to anti-oviposition property of neem products shown with different pests.

Evidence of anti-oviposition property of neem are shown from studies on lepidopterous pests such as *Sesamia calamistis* Hampson and *Eldana saccharina* Walker (Bruce et al. 2004) and dipteran pests like melon fly, *Bactrocera cucurbitae* Coquillett and oriental fruit fly, *Bactrocera dorsalis* Hendel (Singh and Singh 1998). Bruce et al. conducted an experiment studying ovipositional behavior of noctuids on maize using 0, 0.075, 0.1 and 0.15 ml/plant neem oil and tested persistency at 0, 5 and 10 days after application. They observed that neem treatments resulted up to 88% reduction in numbers of eggs laid on maize leaves compared to control. There was no difference in oviposition between days after application of neem demonstrating long-term persistency of the neem treatments. Similarly, Singh and Singh (1998) experimented with different neem seed kernel extracts at different concentrations against fruit flies and observed oviposition deterrency. Such anti-ovipositional property could be one reason for reduction in number of larval mines in the experiment.

Studies conducted on the effects of neem on egg hatch have supported that neem insecticides do not reduce egg hatch. Hossain and Poehling (2006) studied effect of different rates of neem (1, 3, 5, 7 and 10 ml/lw) on egg mortality of *L. sativae* on tomatoes and found that nearly all eggs hatched irrespective of treatment. Similar studies on cabbage moth using 0.5% concentration of commercial neem extract (NeemAzal-T) also showed that number of egg hatched on cabbage plant were not affected (Seljasen and Meadow 2005). This study also reported inhibition of oviposition,

reduced larval development, anti-feeding and high mortality for at least two weeks providing evidence of residual properties of neem.

Mortality of Larval and Pupal Stages

Results on larval mortality indicated that neem products were highly toxic to leafminer larvae when multiple applications were made. This was due to translaminar property of Azadirachtin. Azadirachtin containing neem products sprayed as topical application successfully penetrated the foliage and affected larval stages of leafminer. Similar conclusions were found in studies conducted by Hossain and Poehling (2006) who recorded up to 100% mortality of leafminer larvae through topical application of the commercial neem product NeemAzal®-T/S (Trifolio-M GmbH, Germany). NeemAzal treatments at the rates of 1, 3, 5, 7, and 10 ml/l were used to evaluate larval mortality in *L.* sativae and up to 100% larval mortality was observed in all larval stages.

In similar studies, Webb et al. (1983) demonstrated that 100% larval mortality of *L. trifolii* was achieved through application of neem seed extract (Vikwood Ltd, Sheboygan, Wisconsin). Aqueous solutions of neem seed extract (0.1 to 0.05%) used as dip on primary leaves of Henderson bush lima beans showed insecticidal properties that resulted in 91 to 100% larval mortality of *L. trifolii*. Larew et al. (1986) also reported 98.1% larval mortality of *L. trifolii* on bean leaves painted on both sides with a 1% aqueous solution of neem extract. The mortality of larvae hatching from eggs deposited 7 days after the neem treatment was still significant, and since about 3 days are required for larvae to hatch, the mortality recorded in 7-day leaves actually represented a 10-day residual effect. Weathersbee and McKenzie (2004) studied effects of neem products on immatures of citrus psyllids. Neemix 4.5 was used at a concentration range

of 11-180 ppm, where psyllid nymphs showed susceptibility even at very low concentration.

In this study, larvae that survived on neem treated snap beans were all killed during the pupal stage. Adult emergence was greatly influenced by all Aza-Direct and NimBioSys neem oil concentrations in both direct and residual toxicities from foliar application. No adult eclosion occurred even with the lowest concentration of neem products used. This finding is similar with the findings of several other studies. Hossain and Poehling (2006) observed no adult emergence from plants treated with NeemAzal one, three or seven days before infestation indicating high residual effectiveness of the neem product. Similar reports were obtained from Larew (1988) where pupal mortality was 100 percent when plants were treated with neem seed kernel extracts (1.6% Margosan-O). Weintraub and Horowitz (1997) also obtained very low adult emergence ranging from 0.8 to 1.8% from pupae collected from plants sprayed with 15 ppm azadirachtin (Neemix-45).

It was observed from this study and several other studies that neem products are effective in controlling the leafminer population and have extended residual effects. In this study, insecticidal effect from neem residues lasted at least 10 days. Growth inhibitory effects of neem were observed until two weeks, particularly during development of larvae and pupae. Multiple application of neem exhibited greater toxicity to various stages of leafminer. Hence, foliar application of neem can be considered as a potential control measure for leafminer management under greenhouse conditions when multiple applications are used.

Table 3-1. Effect of different rates and number of applications of Aza-Direct and NimBioSys neem oil on *L. trifolii* mines on snap beans.

Effect	Categories	Number of mines (Mean ± SE)
Leafminer release	First	26.0221±3.99
	Second	31.4085±3.99
Treatment	Aza- Direct 25 oz/100 gal	41.2083±4.85
	Aza- Direct 35 oz/100 gal	34.6667±4.85
	Nimbiosy 0.5%	22.7083±4.85
	Nimbiosys 1%	16.2778±5.93
Number of applications	1	49.2813±4.38
	2	23.4688±4.38
	3	13.3958±5.07

Note: Mean number of mines are the least square mean values.

Table 3-2. Effect of different rates and number of applications of Aza-Direct and NimBioSys neem oil on larval mortality of *L. trifolii*.

Observation	Categories	Larval mortality (Mean ± SE)
Leafminer release	First	80.69±4.26
	Second	77.81±4.26
Treatment	Aza- Direct 25 oz/100 gal	75.01±5.23
	Aza- Direct 35 oz/100 gal	77.80±5.23
	Nimbiosy 0.5%	77.42±5.23
	Nimbiosys 1%	86.76±6.44
Number of applications	1	54.79±4.70
	2	90.35±4.70
	3	92.61±5.48

Note: Mean larval mortality are the least square mean values.

Table 3-3. Mean comparisons between different release of leafminer, rates and application frequency of Aza-Direct and NimBioSys neem oil on *L. trifolii* mines using Tukey-test.

Observation	Tukey-Test	P-value
Leafminer release	First vs second	0.2722
Treatment	Aza-Direct 25 oz/100 gal vs Aza-Direct 35 oz/100 gal	0.2200
	Aza-Direct 25 oz/100 gal vs NimBioSys 0.5%	0.0039
	Aza-Direct 25 oz/100 gal vs NimBioSys 1%	0.0004
	Aza-Direct 35 oz/100 gal vs NimBioSys 0.5%	0.0863
	Aza-Direct 35 oz/100 gal vs NimBioSys 1%	0.0108
	NimBioSys 0.5% vs NimBioSys 1%	0.2715
Number of applications	1 vs 2	0.0002
	1 vs 3	<0.0001
	2 vs 3	0.0909

Table 3-4. Mean comparision between different release of leafminer, rates and application frequency of Aza-Direct and NimBioSys neem oil on larval mortality of *L. trifolii* using Tukey-test.

Observation	Tukey-test	P-value
Leafminer release	First vs second	0.4290
Treatment	Aza-Direct 25 oz/100 gal vs Aza-Direct 35 oz/100 gal	0.5113
	Aza- Direct 25 oz/100 gal vs NimBioSys 0.5%	0.7282
	Aza- Direct 25 oz/100 gal vs NimBioSys 1%	0.1140
	Aza- Direct 35 oz/100 gal vs NimBioSys 0.5%	0.7566
	Aza- Direct 35 oz/100 gal vs NimBioSys 1%	0.3076
	NimBioSys 0.5% vs NimBioSys 1%	0.1986
Number of applications	1 vs 2	< 0.0001
	1 vs 3	< 0.0001
	2 vs 3	0.8165

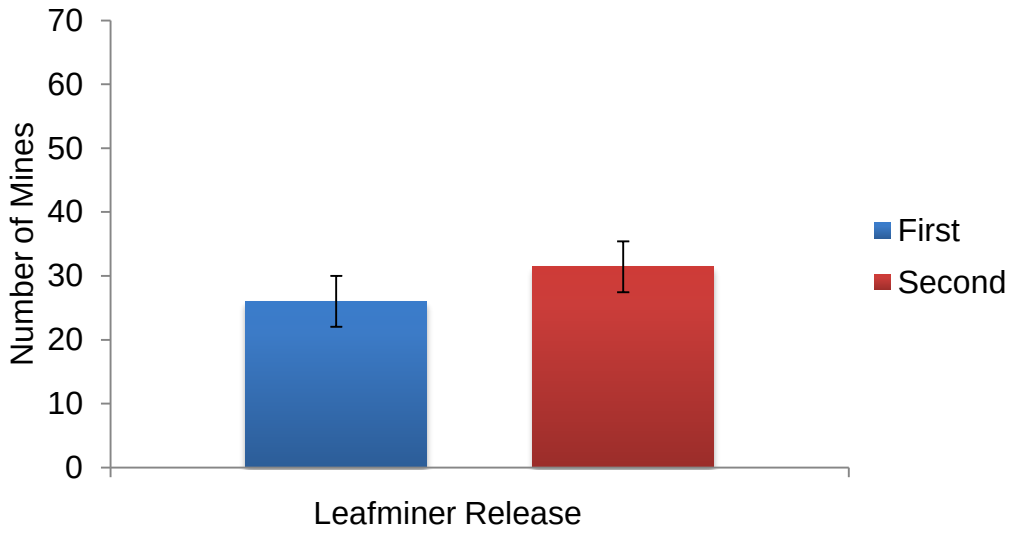

Figure 3-1. Mean (± SE) number of *L. trifolii* mines from various treatments of Aza-Direct and NimBioSys neem oil from leafminer adults released 1 and 7 days after foliar application.

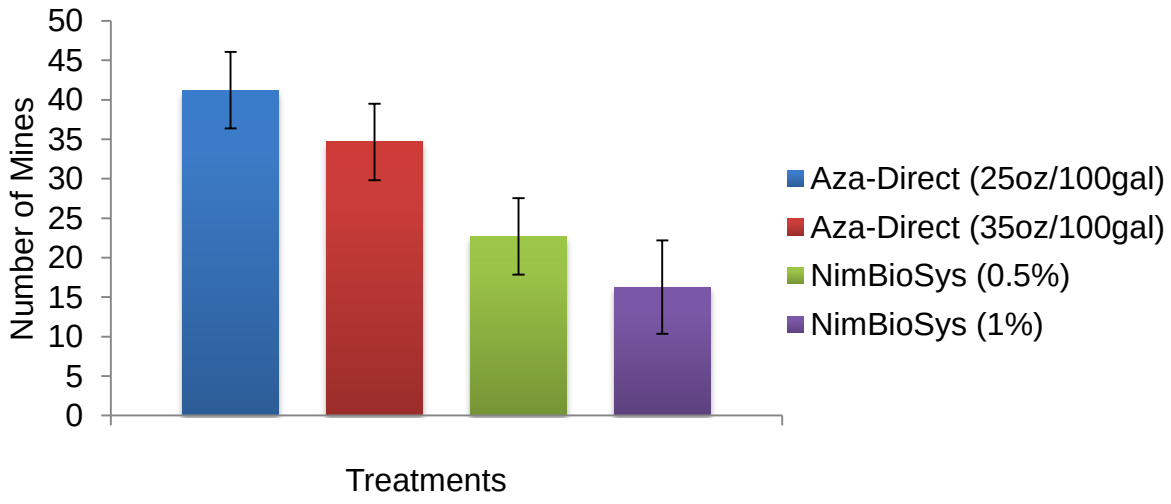

Figure 3-2. Mean (± SE) number of *L. trifolii* mines from different treatments of Aza-Direct and NimBioSys using foliar application.

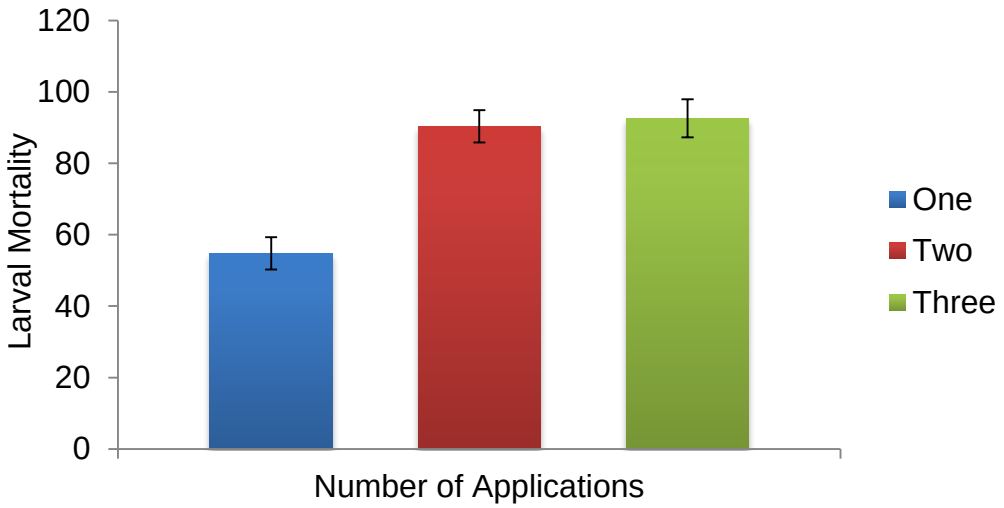

Figure 3-3. Mean (± SE) number of mines developed from different number of applications (Aza-Direct and NimBioSys data pooled).

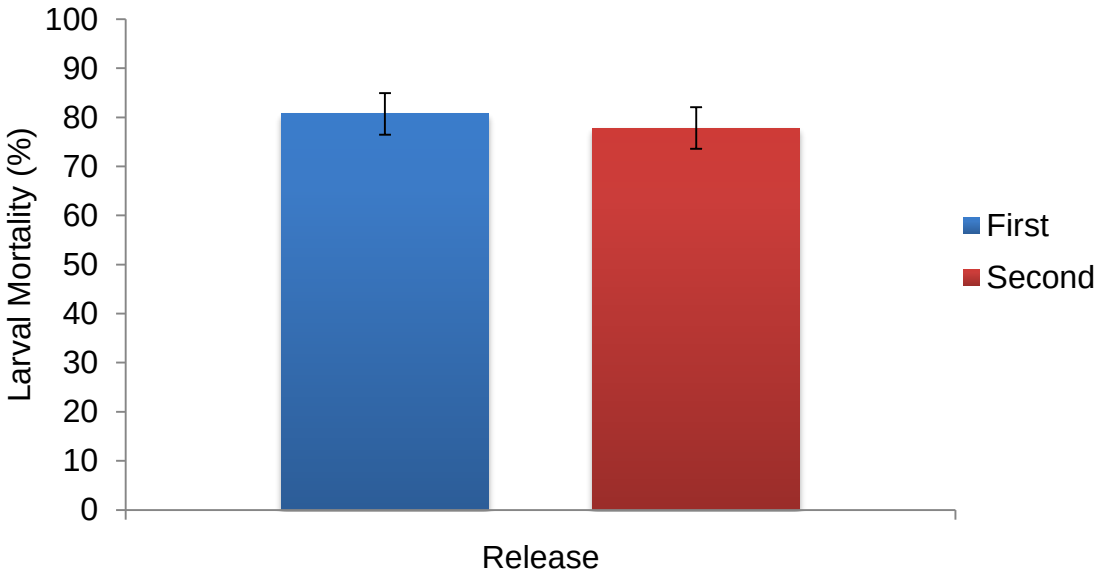

Figure 3-4. Mean (± SE) larval mortality (%) of *L. trifolii* from various treatments of Aza-Direct and NimBioSys neem oil from leafminer adults released 1 and 7 days after foliar application.

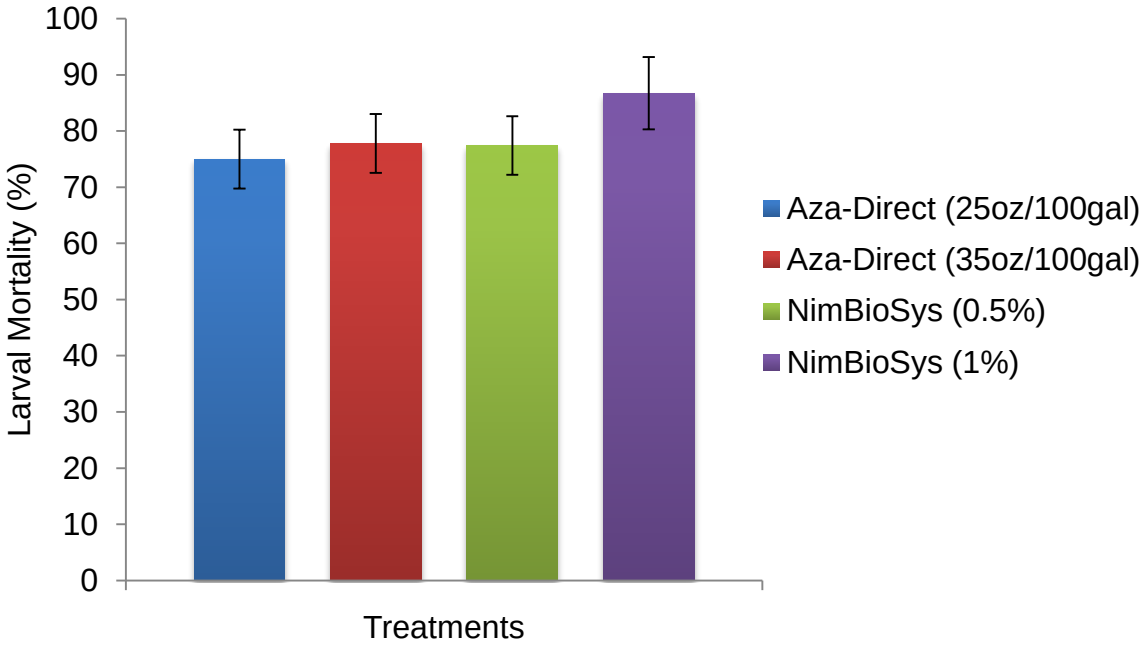

Figure 3-5. Mean (± SE) larval mortality of *L. trifolii* from different treatments of Aza-Direct and NimBioSys using foliar application.

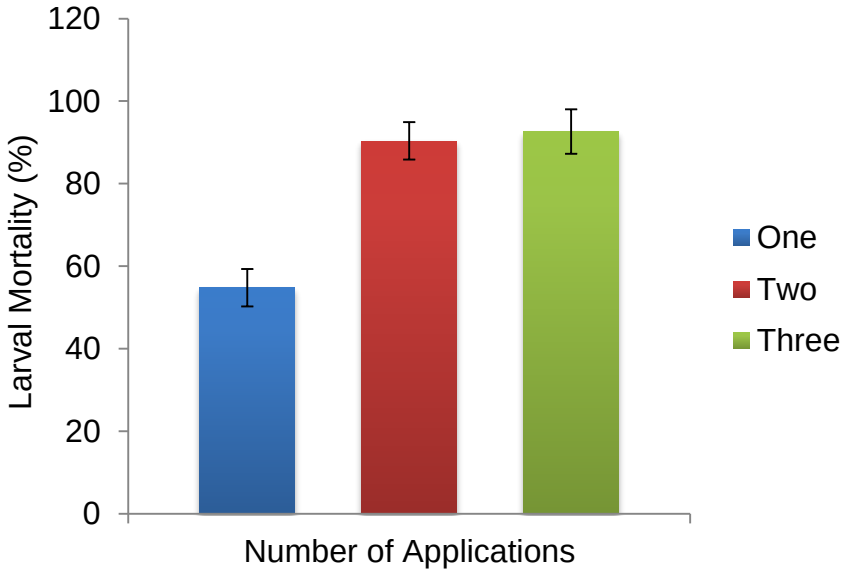

Figure 3-6. Mean (± SE) larval mortality developed from different number of applications (Aza-Direct and NimBioSys data pooled).

CHAPTER 4
DRENCH APPLICATION EXPERIMENT

Pre-Drench Release of *Liriomyza trifolii*

To evaluate the systemic effect of neem, commercially available neem products Aza-Direct® (Gowan Company) containing 1.2% azadirachtin and NimBioSys® neem oil (The Ahimsa Alternatives, Inc.) containing 100% neem oil were used for the experiments. Neem products were freshly prepared using the same procedure as in foliar application experiment. Two recommended rates of Aza-Direct (25oz/100 gallons and 35 oz/100 gallons) and two rates of NimBioSys neem oil (0.5% and 1.0%) were used to study the effect of various rates. Additionally, a water treated control was used, adding up to five treatments.

30 snap beans (45 days old) were kept in a large cage and about 200 leafminer adults (1male: 1female) were released into the cage. The plants were exposed to leafminer adults for 48 hours and taken out from the cages. 20 plants were randomly chosen and treatments were assigned to each plant. Each plant represented one treatment and one treatment was replicated four times. The drench application was done after the release of adult leafminers.

FACTEX procedure in SAS was used to randomize the treatments within a block and to randomize blocks as well. Treatments were arranged in a randomized complete block design. Neem treatments were applied by drenching 250 ml of each treatment into the potted plant. 250 ml of each product was used, as it was just enough to meet the saturation capacity of the soil. After drenching, the pots were irrigated based on the water requirement only up to saturation capacity. In most cases, 250 ml of water was used twice a day, usually during late morning and evening.

A single trifoliate leaf was used for data collection. After 72 hours, the number of mines was counted to determine the number of larvae. The total number of mines was recorded again after five days. The larvae were allowed to develop on the trifoliate attached to the plant and a perforated bread bag was placed over the trifoliate leaf and secured. Late third instar larvae and/or prepupae were allowed to pupate in the bread bags. Pupae were then brought in the lab, transferred to a polythene cup and allowed to develop to adults. Data collection was performed by recording number of mines, larvae, pupae and adults. Larval and pupal mortality was assessed using similar method as in the foliar experiment.

Post-Drench Release of *L. trifolii*

To evaluate the systemic and residual effects of Aza-Direct and NimBioSys, similar procedures as in the pre-drench experiment were followed for preparing product dilutions and product application. The treatment rates, treatment numbers and replication were similar to the pre-drench release experiment. However, in this experiment, drench application of neem products was done prior to release of leafminers.

Leafminers were released after drench application of treatment products with a total of 5 releases made 1, 3, 5, 7 and 10 days after drenching. A trifoliate was randomly selected and 5 pairs (1male: 1female) of leafminer adults were released for each trifoliate covered with a perforated bread bag. After exposure to leafminers for 48 hours, the leafminers were killed to obtain synchronous life stages. Data collection was done 72 hours after exposure of trifoliate to leafminer. The total number of mines was recorded after five days; and larvae were left to pupate in the bread bag and reared in

the laboratory to record adult emergence. Larval and pupal mortality were assessed using the formula developed by Leibee (1988) as in other experiments.

Statistical Procedure

Treatments were arranged in randomized block design. PROC FACTEX in SAS was used for randomization of both blocks and treatments within a block. Observations on number of mines, larval and pupal mortality were pooled for analysis. Data with numbers (count values) were transformed using square root and percentage values were transformed using arcsine-square root before running an ANOVA. ANOVA was performed using PROC GLM in SAS version 9.3 (SAS Institute, 2011). In tests where significant F-values ($P < 0.05$) were obtained, means were separated using Duncan's multiple range test.

Results

Larval and Pupal Mortality from Pre-Drench Release of *L. trifolii*

A significant difference in larval mortality was observed between treatments ($F = 11.32$; $df = 4$; $P = 0.0005$). Using contrast analysis, the control was shown to be significantly different than all the neem treatments ($t = 6.28$; $P < 0.0001$). This indicated toxicity of the neem treatments on larval stages of leafminer (Table 4-1). In addition, there was a significant difference in larval mortality between two products- Aza-Direct and NimBioSys ($t = 2.32$; $P = 0.0390$). However, there was no significant difference between rates of Aza-Direct ($t = 0.63$; $P = 0.5386$) and NimBioSys ($t = -0.06$; $P = 0.9509$). Larval mortality ranged from 1 percent to 22 percent (Figure 4-1).

A significant difference in pupal mortality was observed between treatments and control ($F = 183.87$; $df = 4$; $P < 0.0001$) (Figure 4-2). Comparisons using contrast analysis indicated that control and neem treatments were significantly different ($t =$

26.87; P < 0.0001). Pupal mortality ranged from 92 to 100 percent in neem treated population, while in the control treatment, only 3% pupae were dead (Table 4-2). Similarly, comparisons using contrast analysis showed a significant difference in pupal mortality between Aza-Direct and NimBioSys (t = -2.25; P = 0.0441). There was a significant difference between two rates of Aza-Direct (t = -2.91; P = 0.0130) but NimBioSys rates were not significantly different (t = -0.40; P = 0.6962) based on pupal mortality. Adult eclosion was significantly affected by all azadirachtin treatments with very few adults eclosing in the neem treatments.

Effect of Post-Drench Release of *L. trifolii*

Number of mines

Observation on the number of mines was subjected to ANOVA using GLM procedure in SAS. Data on number of mines observed were pooled for analysis. At 5% level of significance, there was no significant difference in number of mines when leafminers were released at different intervals (F = 1.84; df = 4; P = 0.1290) (Figure 4-3). The mean number of mines from first, second, third, fourth and fifth infestation were similar (Table 4-3). A significant difference in the number of mines was observed between treatments consisting of different neem products with different rates and a water treated control (F = 6.37; df = 4; P = 0.0001) (Figure 4-4). The mean number of mines in neem treatments and water treated control are shown in Table 4-4. A contrast analysis between control and neem treatments showed significant difference in number of mines (t = -4.94; P< 0.0001).

Larval and pupal mortality

Percentage data on larval mortality showed significant differences at different infestation times (F = 4.64; df = 4; P = 0.0022) (Figure 4-5). Leafminer released three

days after drench application showed highest larval mortality compared to other releases (Table 4-5). This could be an indication that systemic effect was prominent 5-7 days after drenching. In addition, a significant difference in larval mortality was observed between treatments ($F = 16.37$; $df = 4$; $P < 0.0001$) (Figure 4-6). All neem treated population had higher larval mortality compared to control (Table 4-6). Larval mortality was observed up to 26 percent in neem treatments while water treated control had lower than 2% larval mortality.

Results on pupal mortality did not show significant difference when leafminers were released at different intervals after drench application of neem ($F = 0.82$; $df = 4$; $P = 0.5133$) (Figure 4-7). However, pupal mortality was high at all different release dates (Table 4-7). This indicated systemic persistency of neem in snap beans when applied as drench. Pupal mortality was also significantly different between treatments (neem products and water treated control) ($F = 296.85$; $df = 4$; $P < 0.0001$). So, contrast analysis was used to compare differences between Aza-Direct and NimBioSys and the rates used. Pupal mortality differed significantly between two neem products- Aza-Direct and NimBioSys ($t = 4.67$; $P < 0.0001$). Similarly, different rates of Aza-Direct showed significant difference in pupal mortality ($t = -3.53$; $P = 0.0007$). A significant difference in pupal mortality was also observed between two rates of NimBioSys ($t = -8.27$; $P < 0.0001$). Contrast analysis showed significant difference in pupal mortality between neem treatments and water treated control ($t = 32.94$; $P < 0.0001$). Higher pupal mortality was seen on neem treated plants and very few, if any, pupae were dead on control treatment (Figure 4-8). Pupal mortality ranged from 71 percent to 100 percent in neem treated plants while it was less than 4 percent on control (Table 4-8).

Discussion

The results from drench application studies clearly demonstrate systemic properties of azadrachtin and the varying levels of toxic effect on different life stages of *L. trifolii.* The studies have shown that different neem-based products (Aza-Direct and NimBioSys) show similar effect on mortality of leafminer. Azadirachtin used as a soil drench had little effect on larval mortality but a strong effect on pupal mortality, as adult eclosion was greatly reduced in all applied treatment.

Effect on Formation of Mines

Neem-derived insecticides used in this experiment resulted in little or no effect on number of larval mines when applied as drench. The number of mines was similar in all treatments including control. Increasing the rate of product used had very little effect on formation of mines compared to lower rates. Number of mines is a direct reflection of the number of eggs laid, as the hatched larvae cause mines by feeding. Thus, it can be inferred that neem products applied as a soil drench have very little, if any, effect on oviposition. Neem products did not possess ovicidal properties as previously seen in the foliar study. Hossain et al. (2007) demonstrated that soil drenching of 0.75, 1.5, 2.25 and 3.0 g/lw Neemazal on tomato did not result significant difference on *L. sativae* oviposition. However, studies evaluating the systemic properties of neem and its effect on oviposition or egg hatch are very limited. The present study does not show differences in number of mines among treatments, except water treated control, which supports Hossain's conclusion on systemic action of neem on oviposition.

Weintraub and Horowitz (1997) used azadirachtin (Neemix-45) at concentrations of 1, 5, 10 or 15 ppm against *L. huidobrensis* but observed no effects on oviposition. Larew et al (1985) also used 0.4% crude neem oil as soil drench against *L. trifolii* and

observed similar results. Besides leafminer, Naumann and Isman (1995) obtained a similar result on noctuid moths using different rates of neem seed oil. These studies conclude that neem products used as soil drench does not deter oviposition significantly but produce toxic effects through systemic action.

In addition to lack of anti-ovipositional properties, neem products do not influence egg hatch. Hossain et al (2007) concluded from his experiment on *L.* sativae that the neem product, NeemAzal did not show any reduction in egg hatch. He used NeemAzal concentrations at 0.21, 0.42, 0.63, and 0.83 g/kg of substrate for drench application but did not observe ovicidal effect. Similarly, Seljasen and Meadow (2005) reported similar results on a study on cabbage moth. Failure of neem products to deter oviposition in leafminer and failure to affect egg hatch are possible explanations for similar number of mines in different neem treatments and control in this study.

Mortality of Larval and Pupal Stages

Azadirachtin possesses systemic properties and is translocated to the larval mining sites. Larval mortality observed from different neem treatments compared to control provides evidence as an effective insecticide for control of leafminer larvae. However, different product and different rates do not seem to produce difference in larval mortality of *L. trifolii*. Drench application of neem was seen to be most effective five days after application, which is similar to results from Meisner et al. (1987). Systemic properties of neem showing toxic effects against leafminer larvae are supported by studies from other authors. Hossain et al. (2007) demonstrated 100% larval mortality of *L.* sativae by using different rates of neem (NeemAzal) as soil drench. Related studies using neem as drench showed toxic effects in pests other than leafminer. Thoeming et al. (2003) and Meadow et al. (2000) demonstrated systemic

action of neem (NeemAzal-T/S) that caused 90 percent larval mortality in western flower thrips and cabbage moth, respectively. Larval mortality due to azadirachtin is primarily attributed to alterations in hormonal levels and interruption of physiological processes of development.

Among all life stages of leafminer, pupal mortality was highly affected by drench application of neem. The study showed very low adult emergence from pupae developed on neem-treated plants. Hossain et al (2007) studied effects of drench application of NeemAzal on leafminer pupae on tomato and observed 100 percent mortality of *L. sativae* pupae when higher rates were used (2.25 and 3 gram per liter of water). Studies by Weintraub and Horowitz (1997) also demonstrated 100% pupal mortality in *L. huidobrensis* using various concentrations of azadirachtin applied as drench in bean plants. Bean plants drenched with 1, 5, 10 or 15 ppm azadirachtin resulted total pupal mortality when the highest concentration was used. A similar study using drench application of 1.0 and 2.0 ppm neem seed extract resulted 65.4% and 77.3% mortality of *L. trifolii* pupae on chrysanthemum (Parkman and Pienkowskii, 1990). It can thus be concluded that neem-based insecticides possess systemic properties and toxicity resulting in mortality of leafminer pupae. Interference with development, primarily due to change in ecdysone level, resulted in imbalanced hormonal regulation and high pupal mortality.

Neem products are effective as soil drench to control leafminer population and show extended persistency. This study demonstrated extended residual effects of neem products applied as drench for at least two weeks. However, the effect varied greatly based on the days after drenching. Larval mortality appeared to be highest at 5 to 7

days after drench application. Higher pupal mortality was recorded where leafminer adults were released 10 days after drench application of neem. Results on larval mortality also suggest that neem products are effective as soil drench for prolonged time period. Persistency of neem applied as soil drench could be attributed to protection of active ingredients in the soil and plant roots.

Table 4-1. Larval mortality (Mean±SE) from different treatments when leafminer adults were released before drench application of neem.

Treatment	Larval Mortality (Mean±SE)
Aza-Direct (25oz/100gal)	15.78±2.19A
Aza-Direct (35oz/100gal)	13.27±2.19A
NimBiosys (0.5%)	10.17±2.19A
NimBiosys (1%)	9.21±2.19A
Control	0.00±2.19B

Means with the same letter are not significantly different (Duncan's multiple range test)

Table 4-2. Pupal mortality per treatment when leafminer adults were released before drench application of neem.

Treatment	Pupal Mortality (Mean±SE)
Aza-Direct (25oz/100gal)	92.13±1.5B
Aza-Direct (35oz/100gal)	99.31±1.5A
NimBiosys (0.5%)	99.21±1.5A
NimBiosys (1%)	100.00±1.5A
Control	3.02±1.5C

Means with the same letter are not significantly different (Duncan's multiple range test)

Table 4-3. Mean number of mines when leafminer adults were released at different intervals after drench application of Aza-Direct and NimBioSys.

Release	Number of mines (Mean±SE)
Day 1	59.70±3.57A
Day 3	48.50±3.57B
Day 5	49.80±3.57AB
Day 7	47.65±3.57B
Day 10	51.65±3.57AB

Means with same letter are not significantly different (Duncan's multiple range test).

Table 4-4. Mean number of mines from different treatments when leafminer adults were released after drench application of neem at different intervals.

Treatment	Number of mines (Mean±SE)
Aza-Direct (25oz/100gal)	49.20±3.57B
Aza-Direct (35oz/100gal)	48.80±3.57B
NimBiosys (0.5%)	44.35±3.57B
NimBiosys (1%)	47.70±3.57B
Control	67.25±3.57A

Means with same letter are not significantly different (Duncan's multiple range test).

Table 4-5. Mean larval mortality when leafminer adults were released at different intervals after drench application of Aza-Direct and NimBioSys.

Release	Larval Mortality (Mean±SE)
Day 1	8.0±1.9B
Day 3	15.4±1.9A
Day 5	7.1±1.9B
Day 7	7.2±1.9B
Day 10	5.3±1.9B

Means with same letter are not significantly different (Duncan's multiple range test).

Table 4-6. Mean larval mortality from different treatments when leafminer adults were released after drench application of neem at different intervals.

Treatment	Larval Mortality (Mean±SE)
Aza-Direct (25oz/100gal)	14.4±1.9A
Aza-Direct (35oz/100gal)	9.9±1.9AB
NimBiosys (0.5%)	11.3±1.9AB
NimBiosys (1%)	7.3±1.9B
Control	3.0±1.9C

Means with same letter are not significantly different (Duncan's multiple range test).

Table 4-7. Mean pupal mortality from different treatments when leafminer adults were released after drench application of neem at different intervals.

Treatment	Pupal Mortality (Mean±SE)
Aza-Direct (25oz/100gal)	91.06±2.3B
Aza-Direct (35oz/100gal)	99.13±2.3A
NimBiosys (0.5%)	72.38±2.3C
NimBiosys (1%)	97.58±2.3A
Control	2.46±2.3D

Means with same letter are not significantly different (Duncan's multiple range test).

Table 4-8. Mean pupal mortality when leafminer adults were released at different intervals after drench application of Aza-Direct and NimBioSys.

Release	Pupal Mortality (Mean±SE)
Day 1	71.19±5.1A
Day 3	71.06±5.1A
Day 5	75.67±5.1A
Day 7	72.36±5.1A
Day 10	72.34±5.1A

Means with same letter are not significantly different (Duncan's multiple range test).

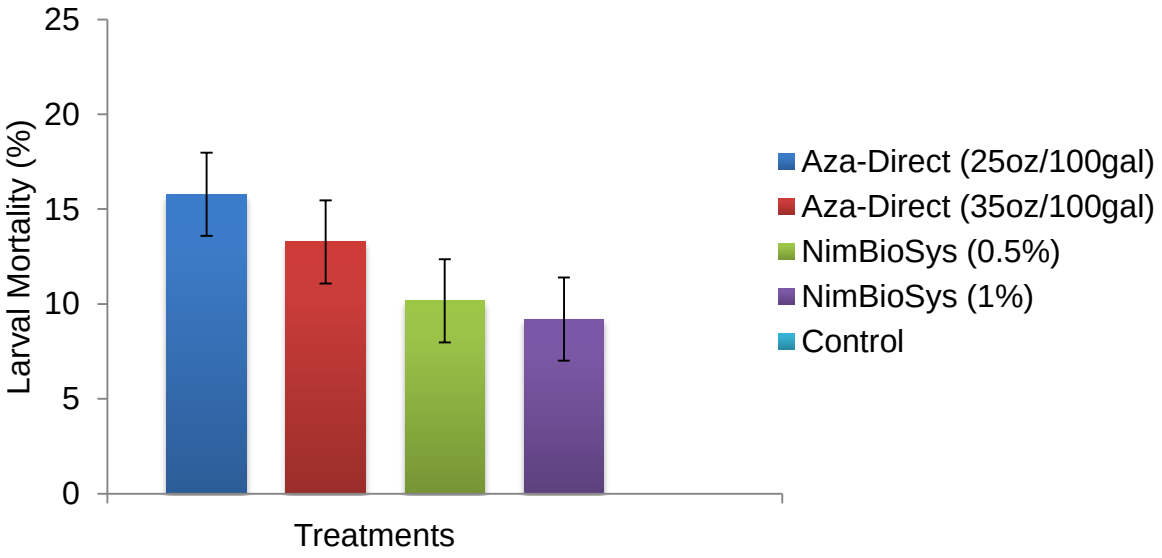

Figure 4-1. Larval Mortality (Mean±SE) of *L. trifolii* from different treatments of Aza-Direct and NimBioSys when leafminer adults were released before drench application.

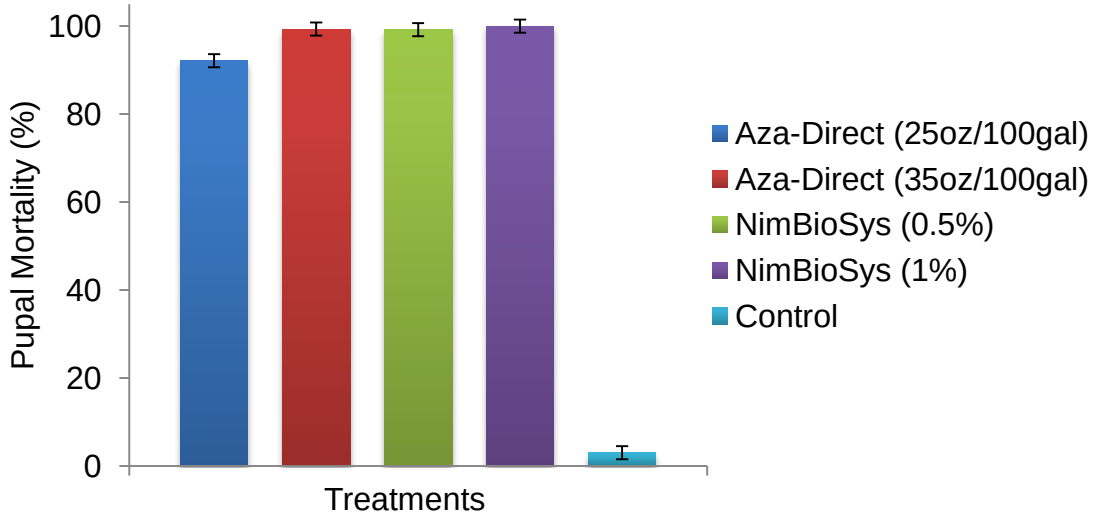

Figure 4-2. Pupal Mortality (Mean±SE) of *L. trifolii* from different treatments of Aza-Direct and NimBioSys when leafminer adults were released before drench application.

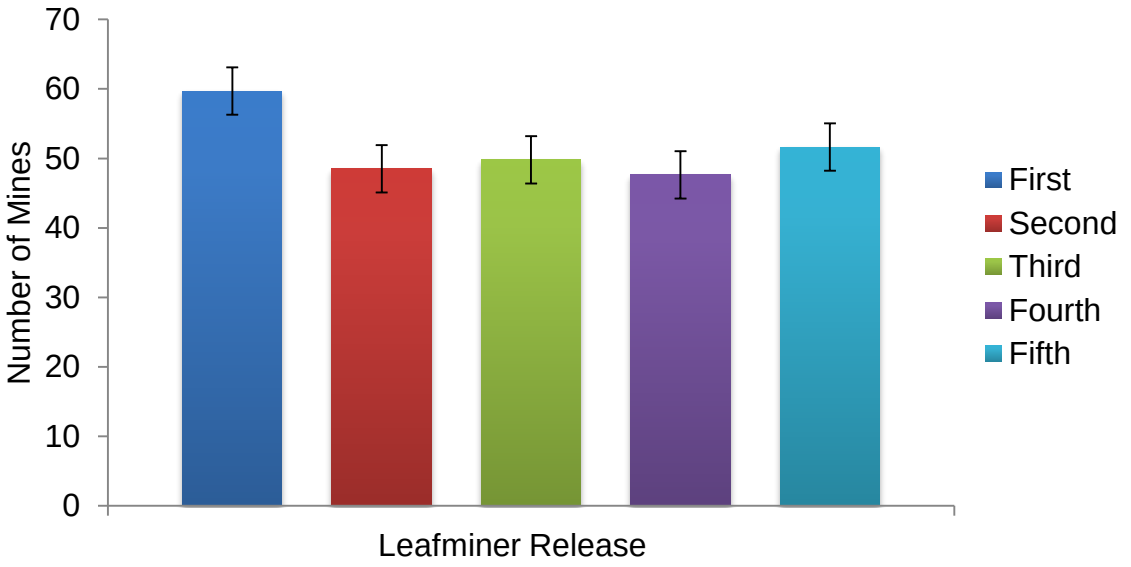

Figure 4-3. Mean number of mines when leafminer adults were released at different intervals after drench application of Aza-Direct and NimBioSys.

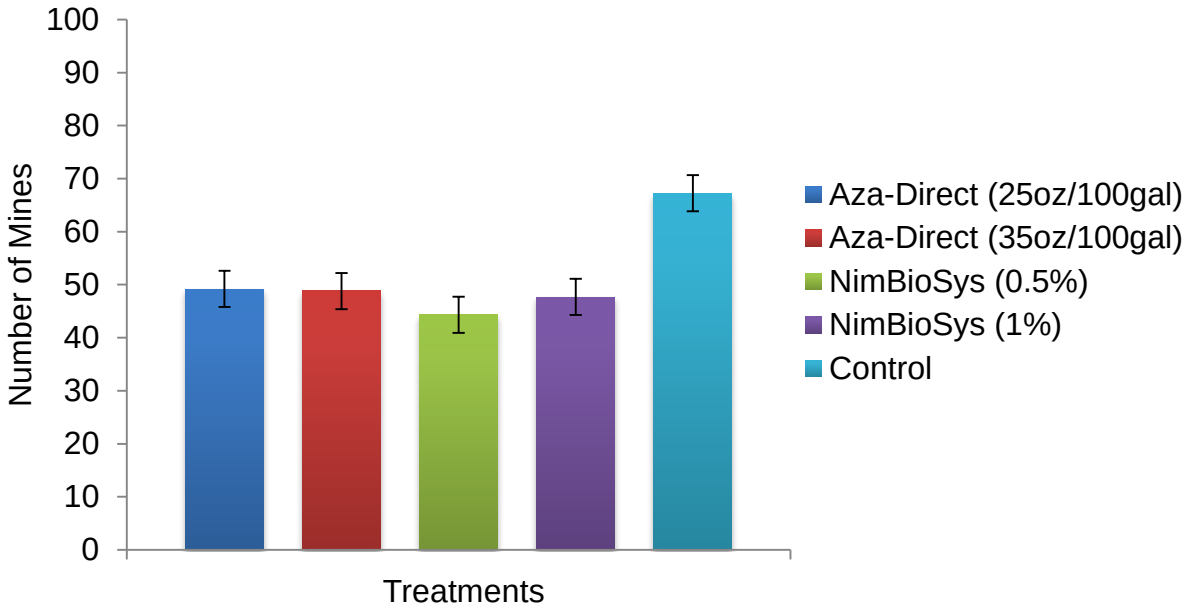

Figure 4-4. Number of mines from different treatments when leafminer adults were released after drench application of neem at different intervals.

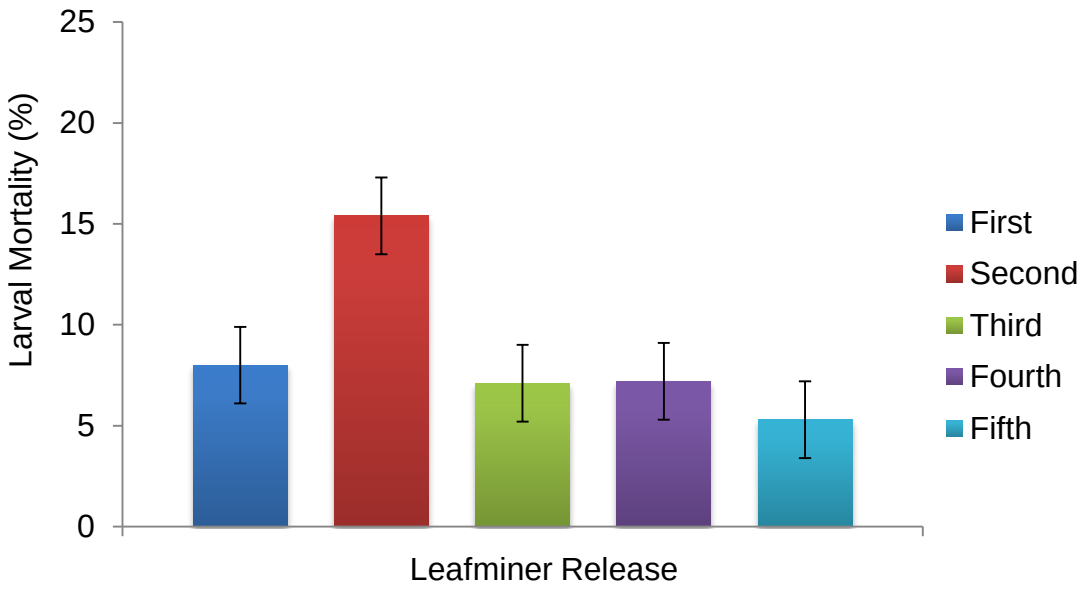

Figure 4-5. Larval mortality when leafminer adults were released at different intervals after drench application of Aza-Direct and NimBioSys.

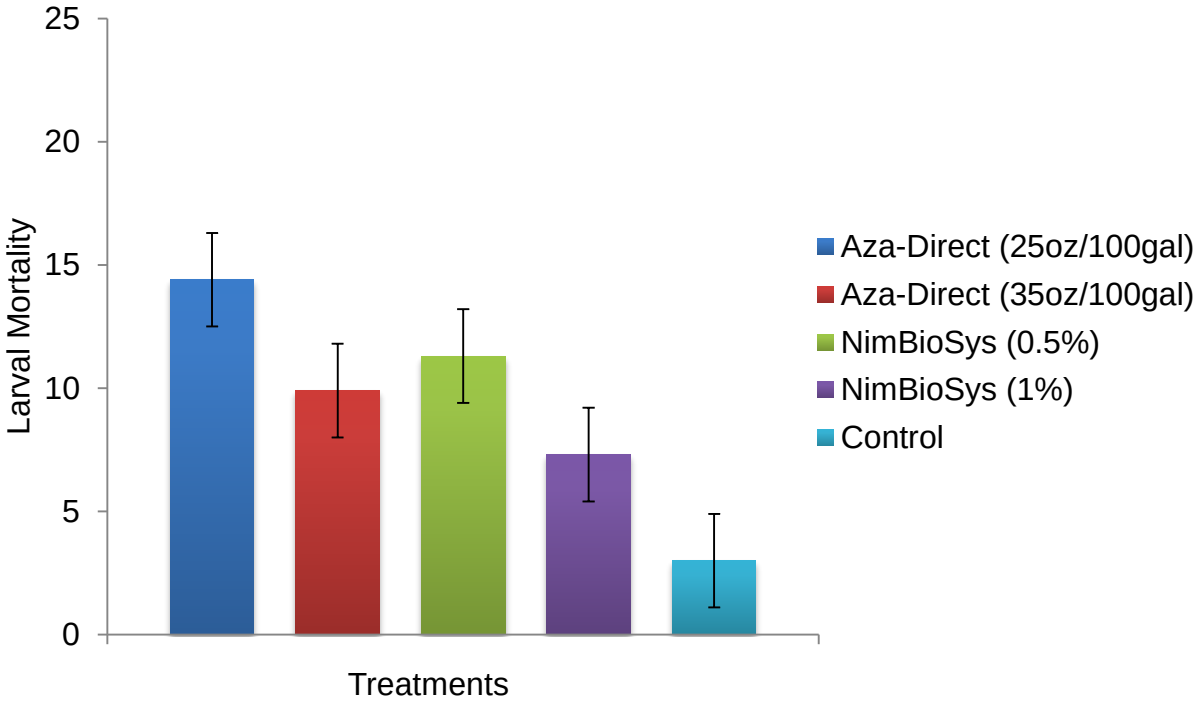

Figure 4-6. Larval Mortality from different treatments when leafminer adults were released after drench application of neem at different intervals.

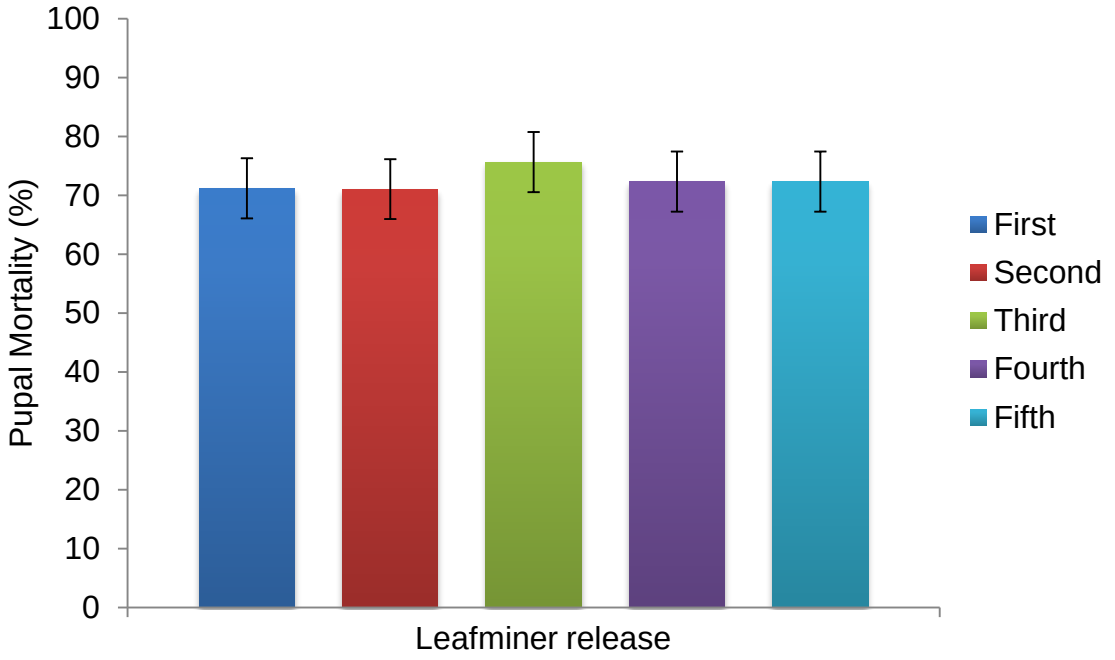

Figure 4-7. Pupal mortality when leafminer adults were released at different intervals after drench application of Aza-Direct and NimBioSys.

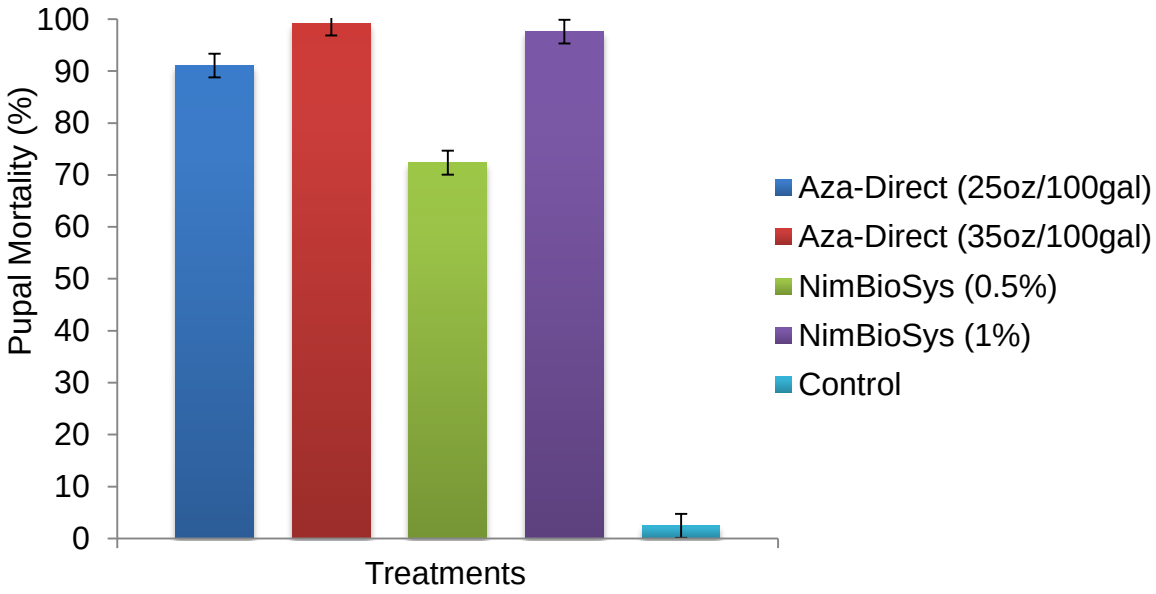

Figure 4-8. Pupal Mortality from different treatments when leafminer adults were released after drench application of neem at different intervals.

CHAPTER 5
CONCLUSION

Two neem-derived insecticides, Aza-Direct and NimBioSys were evaluated for control of leafminer, *Liriomyza trifolii*, on snap beans under greenhouse conditions. Two different recommended rates were used in foliar and drench experiments. Foliar study consisted of one, two and three applications of two products on a weekly interval. Similarly drench experiments was conducted by evaluating systemic and residual effect of two products, two rates of each product and a water treated control, applied before and after infestation.

From the experiments conducted in this study, it can be deduced that neem-derived products are effective in management of leafminer population. This is particularly more important in organic production of vegetable and other crops under greenhouse conditions. Both neem-derived insecticides produced significant toxic effect on different stages of leafminer. Mortality effects were not immediate but ultimately resulted in high mortality in larvae and pupae.

Mortality during immature stages of leafminer is primarily due to growth regulatory effect of neem derivatives resulting in molting disruption. Various insects have shown such sensitivity to neem products. Neem-treated insects that developed into adults had malformed body parts such as crippled wings, absence of proboscis and abnormal legs. Such responses were studied extensively in Orthoptera (*Locusta migratoria migratoriodes*), Hemiptera (*Dysdercus* spp., *Rhodnius prolixus, Sogatella frucifera, Nephotettix virescens* and *Oncopeltus fasciatus*), Coleoptera (*Epilachna varivestis*) and Lepidoptera (*Manduca sexta, Lymantria dispar, Helicoverpa zea* and *Heliothes virescens*).

In dipterans, sensitivity to growth regulatory effect of neem derivatives was more pronounced during the pupal stage compared to larval stage, resulting in inhibition of adult emergence. Similar results were obtained from this study where pupal mortality was higher compared to larval stages in both foliar and drench application methods. Physiological processes involving growth regulatory effect of neem derivatives on immature insects are not fully understood, but are attributed primarily to hormonal control of molting. Studies have demonstrated that azadirachtin inhibits the release of ecdysone from hormone-producing brain/ring gland complex of insects and also delays the production of juvenile hormones.

Foliar application of neem provides effective control of leafminer population compared to drench application. There was no difference between larval mortality and number of mines in leafminers released at weekly interval. This clearly demonstrated the extended residual effect of the neem products applied. Increasing the number of applications resulted in higher mortality of leafminer larvae compared to single application. However, there was no marked difference in formation of mines and larval mortality when two or three foliar applications were made. So, two foliar applications of neem-derived insecticides at a week interval can be recommended for effective management of leafminer in snap beans grown under greenhouse conditions. Different products such as Aza-Direct and NimBioSys and their different rates produced similar toxic effect on leafminer pupae. All pupae developed were dead regardless of number of applications and different rates used in foliar application. Hence, foliar application of neem can be an effective measure for leafminer management under greenhouse conditions with extended residual effects.

Drench application of neem products, on the other hand, resulted in lower larval mortality compared to foliar application. Pupal mortality, however, was greater from different neem treatments compared to control. One limitation on the use of botanicals like neem under field conditions is restricted residual effect due to effects of temperature, ultraviolet light and rainfall that exert negative influence on active ingredients. In addition, delayed effects of neem derivatives may lead to several repeated application of neem in a single cropping season and grower's switching to synthetic insecticides for quicker knockdown of pests. It is thus important that growers be well informed about mode of action and delayed effect of neem to avoid grower's dissatisfaction and wrong conclusions.

Hence we can conclude that neem products are a good fit in integrated pest management programs and their use must be encouraged to reduce dependency on synthetic chemicals. They can play a significant role in management of pests where insecticide resistance is a problem. However, shorter residual persistency of active ingredients pose limitations but such products can be mixed with other bio-products to obtain higher efficacy. Further studies should be aimed at integrating bio-insecticides such as neem with biological control for increasing efficacy in pest management. Possible impacts of bio-insecticides on natural enemies could be another area of study that needs to be further explored.

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BIOGRAPHICAL SKETCH

Manish Poudel was born in 1983 in Chitwan Nepal. He graduated from Institute of Agriculture and Animal Sciences, Tribhuvan University, Nepal with a Bachelor of Science in Agriculture in 2008. He worked as a project coordinator for Tevel b'Tzedek in Nepal for a brief period in the field of agriculture, women empowerment and education. In August 2010, he joined a doctorate degree in Plant Medicine at the University of Florida and later switched to M.S in Entomology in Dr. Gary L. Leibee's lab. His area of interest is in integrated pest management of vegetables and fruits. He graduated with a Master of Science in 2013. His long-term career is to continue research and extension in crop protection towards development and dissemination of novel pest control strategies.