TOXICOLOGICAL PROPERTIES OF NEEM EXTRACTS AND THEIR EFFECTS ON THE SQUASH BUG, *ANASA TRISTIS* DE GEER

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PREFACE

Research was conducted from 1999 to 2001 at the Wes Watkins Agricultural Research and Extension Center (WWAREC) in Lane, Oklahoma to evaluate the effects of neem derived compounds on the squash bug. These studies were done both in the laboratory and in the field with the squash bug *Anasa tristis* De Geer, its host *Cucurbita pepo* 'lemondrop' and its parasite *Gryon pennsylvanicum* Ashmead. The first two chapters are introductory while the last four present results of these studies in complete manuscripts to be submitted to scientific journals following manuscript preparation guidelines established by the Entomological Society of America.

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CHAPTER I

INTRODUCTION

INTRODUCTION

Cucurbits make a significant contribution to agricultural income especially in the southern United States. Watermelon, for example, which is a popular cucurbit, has a narrow profit window in which premium prices may be received. The southern states have the advantage of producing both an early-season and late-season crop. This avoids the mid-season harvest between mid-July and August when market prices tend to be low. Squash, however, is often produced continuously on a relay crop system to supply a regular fresh harvest to the market.

Squash bugs are major pests of cucurbit crops and are most effectively managed by applying insecticides. The use of insecticides, however, is restricted by the Food Quality Protection Act (FQPA), which may limit the use of several insecticides for pest management in vegetables. Further restrictions are expected in the near future, thus raising the need to effectively utilize 'reduced risk' pesticides, especially those that may also be used by 'organic' growers.

This study was conducted to allow us to better understand how one of the 'reduced risk' pesticides, neem (azadirachtin) affects the squash bug. The toxin's effects on different stages of the pest are examined using different methods of application and neem formulations. We hope to further realize ways to optimize the effects of this toxin on the squash bug and other hemipteran pests.

Understanding the toxin and how its activity differs for different formulations also provides a criteria for selecting among the several neem formulations currently available in the market. This study included an investigation into how azadirachtin interacts with other biologically active neem components to cause the overall pesticidal effect that is observed in the field. As a 'reduced risk' pesticide, it is useful to consider the possible effect of the toxin

on beneficial insects, especially those likely to be used as bio-control agents. This study involved observations on interactions between the squash bug and its egg parasite *Gryon pennsylvanicum* Ashmead.

The goal of pest management programs is to maintain squash bug populations below economic injury levels by either directly suppressing early season infestation or reducing the numbers in overwintering populations that successfully emerge to cause early season crop damage the following year.

CHAPTER II

LITERATURE REVIEW

LITERATURE REVIEW

Cucurbits

Cucurbits are frost-sensitive crops most of which are native to the tropics and sub-tropics. They belong to the Cucurbitaceae family, which includes watermelon, squash, pumpkin, cucumber, canteloupe (muskmelon) and honeydew melon. Watermelon is the world's most popular cucurbit crop with an estimated 2.9 million acres grown worldwide producing over 63 million metric tons of fruit (FAO, 2000). Next in tonnage for world production is cucumber, followed by melons, squash and pumpkin (FAO, 1995). The United States is the fifth largest watermelon producer in the world with 1.9 million metric tons after China, Turkey, Iran and Egypt (FAO, 2000). In 1997, Oklahoma was ranked seventh in the United States in watermelon acreage estimated at an annual farm-gate value of 6.2 million dollars (Picha and Motsenbocker, 1997). For the year 2002, watermelon acreage in Oklahoma is estimated at 22,000 acres yielding 18,000 pounds per acre (8160 Kg per acre)

at a farm gate value of 23.8 million dollars (Bolin and Brandenberger, 2002). While in 1997 Oklahoma ranked seventh in total acreage and sixth in earnings per acre, in 1998 the yield per acre was only a tenth of that of Florida (USDA, 1999). This reflects largely on management practices with great opportunities for yield improvement in Oklahoma.

This family of plants is characterized by the presence of cucurbitacins, which are secondary defense compounds protecting the plants against generalist herbivores. Some specialized insects like cucumber beetles and squash bugs have overcome this line of defense and use cucurbitacins as cues to identify their host plants (Robinson and Decker-Walters, 1997).

Environmental conditions, abundance of pathogens and sustained insect pest pressure are some of the challenges facing cucurbit growers (Bolin and Brandenberger, 2002; Picha and Motsenbocker, 1997; Robinson and Decker-Walters, 1997). Cucurbits are grown under diverse management conditions ranging from low external inputs using open pollinated seed to intensive high external input systems using hybrid seed, fertilizer and elaborate irrigation systems. Profitable production of cucurbits, however, demands substantial levels of external inputs such as producer management, quality seed, fertilizer, irrigation and pesticides. The use of several insecticides is restricted by law and many of the effective ones are currently under review by the EPA following enactment of the Food Quality Protection Act (FQPA) of 1996. These pesticides face the risk of de-registration in favor of those under the 'reduced risk' category (Picha and Motsenbocker, 1997).

Cucurbits can be grown under diverse conditions. Watermelon, for example, is commonly grown on light sandy loam soils, and warm weather above 15°C. Other cucurbits like cantaloupe may produce best in soils with higher clay content (Robinson and Decker-Walters, 1997). Warm soil temperatures in early spring as found in the southern United

Walters, 1997). Warm soil temperatures in early spring as found in the southern United States allow for an early crop and an extended production season. More northern production areas, however, have a shorter production season and produce a much later crop. Watermelon grown in northern states, for example, generally do not mature in time for the July 4 market when fruit is bought at a premium price (Picha and Motsenbocker, 1997; Robinson and Decker-Walters, 1997). The short growing season in the northern states does not allow a late crop, which would be harvested in August when market prices improve.

Various approaches have been used to manage insect pests on cucurbits. Those used on squash bugs include chemical, physical and cultural techniques. Some cultural insect pest management strategies used by small-scale producers are; growing resistant varieties, using row covers, hand picking of insect pests, use of boards to trap insects, trap cropping and late season crop residue management (Elliot, 1935; Margolies, 1998). Cultural and physical control alone are rarely sufficient for controlling key pests and thus foliar and soil insecticide applications are often necessary (Robinson and Decker-Walters, 1997).

The Squash Bug

The squash bug *Anasa tristis* (De Geer) is a consistent pest of cucurbit crops through out North America (Bonjour et al., 1991; Elliot, 1935; Foster and Flood, 1995). The genus *Anasa* has 63 described species widely distributed throughout Mexico, Central America, West Indies and South America (Brailovsky, 2001). Common to the United States, however, is *A. tristis* and *A. armigera*, the armored squash bug. *A. tristis* completes two to three generations per year in Oklahoma (Fargo et al., 1988). Multiple overlapping generations result in increased insect densities, which are responsible for large crop losses (Bonjour et al., 1991; Foster and Flood, 1995; Nechols, 1987).

A female squash bug may lay up to 150 eggs in its life (Girault, 1904). In Oklahoma, these eggs hatch after about 7 days and the emergent insects go through five nymphal stages before becoming adults. The first instar is the shortest and lasts 2-5 days with adults emerging in 5-6 weeks at temperatures of about 25°C (Elliot, 1935; Fargo et al., 1988; Nechols, 1987). First instar nymphs stay aggregated around the eggs and are more sensitive to pesticides than later instars (Beard, 1940). In all its life stages, the insect feeds by inserting its piercing-sucking mouthparts through the cuticle into the vascular bundles of its host plant (Bonjour et al., 1991). The feeding has been found to interrupt xylem transport directly, causing the plants to wilt in the process (Neal, 1993). Previous observations led to the conclusion that the insect injects a "poisonous substance" into the plant causing rapid wilting (Britton, 1919). Yellow chlorotic spots develop on the leaf at the point of feeding, which later turn brown leading to secondary infections that result in growth retardation, wilting and even death under high insect densities. Insect feeding on the fruit affects quality largely by reduced shelf life while intense feeding may cause the fruit to rot (Foster and Flood, 1995).

Squash bugs overwinter as adults that move to their host plants early the following spring to mate and lay eggs mostly on the underside of leaves (Foster and Flood, 1995). Nymphs and the older adults do not survive the cold winter temperatures (Wadley, 1920). It is believed that virgin adults and those that have only produced a few eggs may live and go into hibernation under plant debris, weeds, and tree barks to become active the following spring (Beard, 1940; Elliot, 1935). Overwintered squash bugs emerge from hibernation in the spring as soon as temperatures rise above 15°C (Wadley, 1920). Apart from temperature, this emergence is also influenced by photoperiod and food availability (Fielding, 1990; Nechols, 1987). The overwintered squash bugs pose the greatest potential damage because

plants at the seedling stage are most vulnerable to injury. High infestations may result in death of seedlings and a reduced crop stand sometimes requiring re-planting. Intense infestation at flowering may result in yield reduction while infestations that occur during fruiting may result in physical damage to the fruit. The dense late season foliage requires adequate spray penetration for effective pest management. It is these late season squash bug populations moving to overwintering sites that are responsible for initial attacks the following spring.

Successful squash bug management may be achieved by treating plants with insecticide sprays after scouting for eggs. The current treatment threshold recommendation for squash at or before flowering is at four eggs or one egg mass per plant (one egg mass averages 16 eggs). Nymph and adult counts are used to assess the level of control after insecticide treatments have been applied (Foster and Flood, 1995). Early insecticide applications expose emerging nymphs immediately on emergence when they are most sensitive to pesticides (Beard, 1940; Elliot, 1935; Foster and Flood, 1995). The squash bug has been found to adapt rapidly to otherwise resistant squash cultivars, a characteristic that is likely to be maintained with respect to pesticides (Margolies, 1998). Coupled with the fact that most of the effective registered pesticides are under regulatory review, managing this pest will be increasingly challenging as alternative management approaches become necessary. Some of the chemicals recommended for squash bug management are carbofuran, imidacloprid, oxydemeton-methyl, esfenvalerate, endosulfan, methomyl and neem extracts (Edelson et al., 1999b; OSU, 2001).

Azadirachtin is a component of neem oil, which is extracted from seeds of the neem tree *Azadirachta indica* A. Juss (Dorn, 1997). This tropical tree is a member of the Mahogany family (Meliaceae) and its extracts have been used for centuries in human medicine, personal hygiene and protection of stored grains in India and neighboring countries (Schmutterer, 1988). The plant contains many bioactive compounds also present in the oil pressed from its ripened seeds (Ermel et al., 1987b; Jarvis et al., 1998; Kraus et al., 1987). Azadirachtin was first isolated from neem oil by Butterworth and Morgan in 1968 and identified as the "key active ingredient" responsible for insecticidal activity of the oil (Butterworth and Morgan, 1968; Immaraju, 1998). Azadirachtin has since then been extensively studied for use as an insecticide, fungicide, nematicide, bactericide and in dental hygiene, to name a few (Ascher, 1993; Mordue (Luntz) and Blackwell, 1993). Azadirachtin and related neem extracts have been studied for many years and are considered by many as one of the most potent natural pesticides available today (Jarvis et al., 1998).

Azadirachtin is unstable although its breakdown products are believed to maintain some biological activity. Compounds other than azadirachtin, present in the neem oil also have bioactive qualities although they may be less potent insecticides (Ascher, 1993; Kraus et al., 1987). Some of these compounds found in neem oil are salanin, nimbin, azadarachol, and several azadirachtin analogs (Ramji et al., 1998).

Types of neem compounds

Neem oil is obtained from seeds in three ways. First, ripe seeds may be heated to yield the oil, or second, organic solvents may be used to extract it from ground seed kernels. Lastly, seeds may be mechanically pressed to release the oil. Cold-pressed oil provides the most common group of neem products characterized by an array of compounds. The

common group of neem products characterized by an array of compounds. The proportion of azadirachtin in these extracts may range from 300-1600ppm depending on a variety of factors. Seed kernel samples collected from different regions of the world have notably different levels of azadirachtin (Ermel et al., 1987b). The oil is further processed to increase its overall proportion of azadirachtin to yield a technical grade product containing 12-26% of the active ingredient (Lale and Abdulrahman, 1999). It is from this technical grade azadirachtin that most commercial neem products like Margosan®, Neemix 4.5® and Neemazal® are formulated. A third type of neem product recently registered is Trilogy, which has only traces of azadirachtin and is an oil formulated from the hydrophobic extract of neem. This is the product left behind when azadirachtin has been extracted from neem oil.

General activity of azadirachtin

Azadirachtin acts in a dose-dependent fashion as an insect repellent, antifeedant, nematicide, fungicide, and insect growth disrupter (Ascher, 1993; Kraus et al., 1987). The compound deters leaf chewing insects such as the desert locust *Locusta migratoria* (Orthoptera: Acrididae) and cabbage butterfly *Pieris brassicae* Linnaeus (Lepidoptera: Pieridae) from feeding when topically applied on leaves. Oligophagous insects have been observed to be generally more sensitive to azadirachtin than polyphagous ones (Ascher, 1993). Oviposition by *Pieris rapae* Linnaeus (Lepidoptera: Pieridae) on plant material was disrupted in a dose dependent fashion (Saxena and Rembold, 1984). Such properties are attributed, at least in part, to a general physiological response triggered by the presence of azadirachtin in insect hemolymph at concentrations sometimes as low as 10-40μg per liter for desert locust (Ascher, 1993).

Two levels of feeding deterrence are recognized; primary and secondary. Primary deterrence involves olfactory sensilla where azadirachtin may either stimulate or inhibit feeding related neurons to generally reduce insect feeding as evidenced by studies on *P. brassicae* larvae. Secondary deterrence involves slower physiological processes necessary to stimulate feeding and is often mediated by hormones (Schoonoven, 1990).

Physiological processes including molting, pupation, adult emergence and gametogenesis are significantly disrupted by azadirachtin in several insect species (Ascher, 1993). This is caused by interference of azadirachtin with the neuroendocrine system, which results in decreased ecdysone and juvenile hormone levels. There is a general delay in development at low azadirachtin concentrations that may result in 'permanent larvae' at increased concentrations (Ascher, 1993). A neurological study using *Locusta migratoria* guts *in vitro* found azadirachtin capable of influencing muscular contractions that are responsible for peristalsis (Dorn, 1997).

To date, some studies have been performed to assess the activity of azadirachtin on true bugs (Dorn, 1997; Garcia and Rembold, 1984; Garcia et al., 1989; Isman, 1995; Mordue (Luntz) and Blackwell, 1993) but a limited number of these have specifically investigated the toxicity of the compound on squash bugs. In a study by Edelson et. al., (1999a) neem treatments substantially reduced the numbers of squash bug adults, nymphs and egg masses in summer squash. When treatments were initiated early in the growing season before build-up of squash bug populations, they resulted in control comparable to some common synthetic pesticides like Provado 1.6F®, Meta-systox R®, Warrior SC®, Lannate®, and Thiodan® when applied at rates recommended on their labels. A second study included watermelon at a different location where treatments were started later in the season at more abundant squash bug adult populations (Edelson et al., 1999a; Edelson et al., 1999b).

et al., 1999b). Under these conditions, the neem products performed very poorly in comparison to similar synthetic insecticides. The application of synthetic insecticides, however, resulted in substantial control of the insect. With such varying results, a better understanding of the toxicology of the compound is necessary to ensure its optimal use especially under field conditions.

Effects of azadirachtin on reproduction of insects

Results from studies on *Oncopeltus fasciatus* (Dallas) adults indicate that topical applications of azadirachtin were less active than injections of the same concentration of the compound dissolved in 10% alcohol (Dorn et al., 1986a). Azadirachtin injections inhibited vitellogenesis in females and caused impotence in males. Females treated topically with azadirachtin were able to lay eggs but embryogenesis was impaired. The effect on treated males was not well defined, although their ability to copulate was hindered by failure of the aedeagus to maintain a proper erection. Sub-lethal doses of the compound were associated with reversible reduction in fecundity, egg sterility and a general decline in insect fitness especially when the insect was exposed over extended periods (Meisner et al., 1990; Meisner et al., 1992). Results of studies on *Ceratitis capitata* Wiedemann (Diptera: Tephritidae) indicated that mating behavior can be affected. Males failed to recognize the female sex pheromone and thus did not copulate after azadirachtin was injected directly into their hemolymph (Di Ilio et al., 1999; Dorn et al., 1986b).

Azadirachtin's modes of action in insects

Although the physiological effects of azadirachtin have been extensively studied and well documented, its mode of action is largely unexplained (Mordue (Luntz) and Blackwell,

1993). Effects are clearly dose dependent although azadirachtin also qualitatively affects different processes at various concentrations (Dorn, 1997; Prabhakaran and Kamble, 1996). In *Rhodnius prolixus* Stal (Hemiptera: Reduviidae), for example, normal feeding is maintained while molting is inhibited at low doses. Increasing these doses more than 300 times produces antifeedancy responses (Garcia and Rembold, 1984; Garcia et al., 1989). In *O. fasciatus*, low doses of 0.06 μg/insect were found to suppress ecdysis but not apolysis or cuticulogenesis (Dorn et al., 1986a). Higher doses (above 2.0μg/insect), however, completely inhibited ecdysis. Doses between 0.25 and 2.0μg/insect suppress apolysis but not cuticulogenesis in *O. fasciatus*. *D. koenigii* and *O. faciatus* exhibited inhibited ecdysis with the new cuticle well developed beneath the apolyzed old one. This effect was attributed to a suppressed 'eclosion factor' during the molting process (Koul, 1984).

The developmental stage in which an insect is exposed to azadirachtin greatly determines the insect's sensitivity to the compound. Treatment of third instar nymphs of the cotton stainer *Dysdercus cingulatus* Fabricius (Hemiptera: Pyrrhocoridae) caused it to molt prematurely into adults, skipping the fourth and fifth instars while treatment of the fourth instar with similar amounts did not prevent molting into the fifth instar (Abrahams and Ambika, 1979). The mode of action is therefore dependent on the development stage, which in turn determines sensitivity and overall effect of azadirachtin on the insect. The squash bug has multiple overlapping generations making its population highly stratified with all life stages present in the production fields at most times except a brief portion of the year when only overwintering adults are present. Heterogeneity of this population makes management particularly challenging as the more susceptible nymphal stages live alongside the less sensitive adults. Repeated treatments are, therefore, necessary to achieve any substantial control with neem based compounds. For overall success, insecticide resistance

management techniques will also need to be employed (Fielding, 1990; Margolies, 1998; Nechols, 1987).

It is not clear if azadirachtin inhibits the synthesis or metabolism of ecdysteroids, although it is evident that their peak is both delayed and reduced in a dose dependent manner. This may result in delay or total arrest of the adult molt, giving rise to 'permanent nymphs' (Mordue (Luntz) and Blackwell, 1993). Histological studies on permanent nymphs of *O. fasciatus* indicate that the brain continued to produce prothoracicotrophic hormone (PTTH) but is unable to release it from the corpora cardiaca (Garcia et al., 1990). Some researchers have speculated that azadirachtin blocks the release of PTTH by the corpora cardiaca after it was produced and transferred to the corpora cardiaca for secretion (Schmutterer, 1998). Results of studies using permanent nymphs of *Rhodnius prolixus* Stal, indicated that molt inhibition was reversed by injection of exogenous ecdysteroids. Injecting juvenile hormone analogs into the subjects reversed azadirachtin effects by about 60% (Garcia and Rembold, 1984). These findings were further supported by studies on *Locusta migratoria* in which azadirachtin impaired the secretion of neurohormones by impairing the function of the corpora cardiaca (Rembold et al., 1989). Azadirachtin affects the corpora cardiaca in a rather consistent fashion and seems to be one of its target organs. Since the corpora cardiaca performs the same function in most insects, it is highly probable that azadirachtin will inhibit similar processes in the squash bug, an effect that will be tested in this study using various experiments.

Modes of exposure of insects to azadirachtin

Squash bugs are fluid feeders that spend most of their time on the underside of leaves and in plant debris on the soil. Effective spraying techniques have to be used to

expose them to direct topical sprays, which are required for sufficient cuticular absorption of neem extracts. Such challenges have resulted in limited effectiveness of otherwise active compounds against the pest (Bonjour et al., 1991; Palumbo and Coates, 1996).

We hypothesize that the systemic uptake of azadirachtin by squash plants and its eventual ingestion by insects feeding on these plants offers a longer lasting and effective mode of exposure of insects to the toxin. This is because neem extracts stay active longer when systemically translocated in some plants than when they are exposed to ultra violet light under atmospheric conditions on plant foliage (Naumann et al., 1994; Saxena and Khan, 1985; Schmutterer, 1998). Insecticides derived from neem extracts may be applied as foliar or soil treatments. Soil treatments are taken up by roots and translocated through the plant for systemic accumulation. These systemic concentrations of azadirachtin subsequently affect insects feeding on the treated plants while leaving beneficial insects unharmed. The persistence of systemic concentrations in comparison to topical residues is attributed to rapid breakdown of the residual compound on leaf surfaces by ultra violet light (Ascher, 1993; Naumann et al., 1994; Schmutterer, 1998).

Azadirachtin was first registered in the United States by the Environment Protection Agency (EPA) for use on non-food crops to control insects in 1989. Its rat oral toxicity is estimated at >5g/kg of body weight and based on this, registration for edible produce was granted in 1993 with exemption granted by EPA from residue tolerance limits (Isman, 1995). The use of neem products is allowed under a 'restricted' status by the USDA organic crop production guidelines. This is alongside other non-synthetic biorational pesticides like pyrethrum, rotenone, ryania, and sabadilla. The 'restricted' status allows for the use of such pesticides on certified organic land and crops alongside documentation of efforts in the farm plan to reduce or eliminate their use (USDA, 2001).

Systemic accumulation of azadirachtin in host plants has the potential of exposing herbivores to low doses of the compound over extended periods of time (Schmutterer, 1988). Uptake of azadirachtin by plants is dependent on factors such as penetration through the cuticle, distribution among host plant tissues, phloem mobility, and dilution by plant fluids during transport (Lowery and Isman, 1994; Naumann et al., 1994; Saxena, 1987; Saxena and Khan, 1985). It is, however, not clear how this type of activity could affect hemipteran species such as the squash bug. Sub-lethal levels of about 0.125μg/ insect affect reproduction, mating behavior and normal development of flight muscles (Ascher, 1993). These levels are several times lower than those likely to cause mammalian toxicity and as such, do not pose major regulatory concerns to the EPA. Once absorbed into the insect body cavity, azadirachtin is carried in the hemolymph to its target sites. Its impact on target organs depends on its concentration and persistence over time (Garcia et al., 1990; Garcia et al., 1989).

Hemolymph levels of azadirachtin may vary depending on how fast it penetrates the insect cuticle, sequestration of the compound in specific tissues, possible metabolic conversion and excretion (Garcia et al., 1989). Results from experiments addressing different modes of exposure indicate that these differ in the overall amount of azadirachtin delivered into the insect hemolymph. Delivery systems involving prior accumulation of the toxin in the host plant will certainly be affected by the rate of absorption of the compound by the plant, insect's rate of feeding, and the fate of azadirachtin within the insect hemocoel.

Interaction of azadirachtin with other pesticides

Azadirachtin has the potential to modify the way other insecticides affect their target pests. The experimental insecticide RH-2485 (Rohm and Haas), for example, is an insect growth regulator whose activity is modified when used in concert with azadirachtin. When used alone, RH-2485 mimics ecdysone in insects by binding to its receptor sites (Dhadialla et al., 1998). This process causes immediate lethal molts on *Spodoptera littoralis* Boisd, at 1ppm (Adel and Sehnal, 2000). The same study also found that the insect went through a supernumerary molt with reduced pupal weight and surival at lower concentrations (0.1ppm). Using such concentrations of RH-2485 with azadirachtin at the range of 0.1-10ppm increased by ten the chance of supernumerary molts as well as the number of these molts by up to three.

Comparisons made between two strains of the Colorado potato beetle *Leptinotarsa decemlineata* Say, (Lepidoptera: Chrysomelidae) studied the effects of combining *Bacillus thuringiensis* (Bt) and azadirachtin (Trisyono and Whalon, 1999). Results indicated that the two compounds had improved activity when used on the Bt resistant strain as compared to when these were used on the Bt susceptible strains. Such improved response levels indicate a more beneficial synergistic effect when the two compounds are used at the same time making azadirachtin a Bt resistance-breaking compound. Using azadirachtin in concert with imidacloprid on the tobacco budworm *Heliothis virescens* F. (Lepidoptera: Noctuidae) did not have such clear results (Koppenhofer and Kaya, 2000). While azadirachtin and imidacloprid were found compatible for the management of *H. virescens*, the later did not substantially increase the activity of azadirachtin especially for the second instar. At this development stage, the effects may even be antagonistic. For the first instar, however, additive effects

were observed. In the same study, combinations of azadirachtin with the *Heliothis* single-embeded nucleopolyhedrovirus indicated additive benefits especially in regards to mortality.

Study objective

The true bugs (Hemiptera) were among the first insects to be treated with neem oil and its extracts to determine its efficacy in control. About 32 hemipteran species have been confirmed to be sensitive to azadirachtin (Dorn, 1997). Both phytophagous and hematophagous species have been used to develop models of how azadirachtin is absorbed, distributed, stored and affects this group of insects (Garcia et al., 1989). The sensitivity of true bugs has been observed at concentrations as low as 25ppm. Lepidoptera are generally more susceptible with the lowest active concentration of 0.06ppm recorded for *Spodoptera littoralis* Boisd (Mordue (Luntz) and Blackwell, 1993). Though less than that of Lepidoptera, the susceptibility of Hemiptera to azadirachtin falls within a reasonable range with other groups including Homoptera and Coleoptera (Ascher, 1993).

The objective of this research is to evaluate toxicological properties of azadirachtin on the squash bug. Results will be used to develop methods for using this family of pesticides within integrated pest management programs for squash bug and other hemipteran pests. The results of this study may also serve as a model for managing hemipteran pests using insect growth regulators (IGR's) with useful insights into application methods, rates and timing that optimize the effects of IGR's on similar pests.

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CHAPTER III

FEEDING PREFERENCE AND OVIPOSITION OF SQUASH BUG AS AFFECTED BY INCREASING DOSES OF NEEMIX 4.5 ™ APPLIED EITHER AS FOLIAR OR SOIL TREATMENTS ON SUMMER SQUASH *CUCURBITA PEPO* L.

Abstract

Studies were conducted in the laboratory to determine whether squash bug colonization and oviposition on squash host plants was affected by increasing doses of the neem pesticide applied either as a foliar or soil treatment. A ranking of plant appearance at the end of the experiment was determined to measure feeding intensity.

Foliar applications at increasing concentrations of 0, 1, 10, 100 ppm azadirachtin did not significantly influence colonization or oviposition. Ranking of plant physical appearance, however, showed that high application rates of the toxin were associated with healthier plants – perhaps due to more intense feeding when low levels of toxin were applied. Similar concentrations of toxin applied on soil showed significant dose responses in colonization and oviposition as well as ranking of plant condition. Higher toxin application rates corresponded to fewer insects and eggs as well as healthier plants.

The differences between foliar and soil applications may be due to retained activity of the toxin. Olfactory mechanisms employed by the pest and mere quantities of toxin encountered may have also played an important role. It is believed that the effectiveness of azadirachtin is extended under systemic conditions compared to topical treatments that are rendered short lived due to effects of ultra violet light.

Introduction

Azadirachtin and neem have insect repellent characteristics that have been studied by several scientists (Ascher, 1993; Belmain et al., 2001; Butterworth and Morgan, 1968; Ermel et al., 1987b; Isman et al., 1990; Isman, 1993; Kraus, 1995; Saxena, 1987; Schmutterer, 1998). Systemic accumulation of the ultra violet light sensitive toxins extends their activity while

providing an alternative application method (Naumann et al., 1994; Valladares et al., 1999). The effectiveness of neem compounds as repellents may be improved by being circulated in their active form for longer periods under systemic conditions. In a study on conifers using neem, insecticidal effects of toxins injected in the fall were observed the following spring and even later into the growing season (Helson et al., 2001). With multiple compounds affecting insects in several ways, it is possible that systemic conditions may offer a useful alternative for extending the period of the toxin's activity long after it has been applied.

Several toxicological studies using neem have been based on chewing insects (Mordue (Luntz) and Blackwell, 1993). There are fewer studies involving fluid-feeding hemipterous insects, which might respond differently due to their different feeding habit that might potentially expose them to a different level of the toxin. The dominant mode of exposure of hemipterous insects to neem compounds is not obvious.

Experiments were conducted to evaluate the ability of soil and foliar applications to suppress colonization and oviposition rates of squash bugs on summer squash at four doses of the toxin. The study sought to establish if colonization and oviposition by the pest were preferentially biased towards low application rates of the toxin either as a foliar or soil application. Since neem has antifeedant properties, a ranking of plant physical appearance was used as an indicator of overall intensity of feeding. Squash bugs being fluid feeders would ingest limited amounts of topically applied toxin as compared to chewing insects. The insect is, however, more likely to ingest large amounts of toxin accumulated systemically in plant fluids.

Methods and Materials

Experiments were conducted in the laboratory at the WWAREC (Wes Watkins Agricultural Research and Extension Center) in Lane, Oklahoma to determine effects of azadirachtin on the host plant selection process among squash bugs. Two methods of treatment were used. The first consisted of folliar applications of Neemix 4.5™ on squash *Cucurbita pepo* 'Lemondrop' (Cucurbitaceae) (Agrow Seed Co. Kalamazoo, Michigan) host. The second treatment consisted of neem treatments on the soil media in which the squash plants were grown. In both application methods, potted squash plants were treated with increasing azadirachtin concentrations (0, 1, 10, 100 ppm) obtained by diluting Neemix 4.5™ in water (Certis USA. Columbia, MD).

Squash bug colony

A squash bug, *Anasa tristis* De Geer, colony was established in the spring of 1998 from field collected insects in Lane, OK and maintained at approximately 25°C and 18:6 (L:D) photoperiod. The insects were fed on squash plants and fruit while the colony was maintained over the duration of the experiment. The eggs were deposited on white cotton cloth hung in the cages and emergent first instar nymphs were collected once a week and isolated as a group to produce an individual cohort. Virgin adults of the same age were used for this study. These were obtained after late instars were transferred into separate cages under a similar temperature regime and photoperiod as they were observed for molting. The emergent adults were then collected from this cage once a day and immediately sexed on emergence. Males and females were separated to prevent mating before the experiment was begun. Virgin adults used in the studies were at least a week old to ensure reproductive maturity.

Squash plants

Squash plants, were grown from two seeds planted in a plastic pot 10cm diameter and 10cm deep filled with 500ml of potting soil (Redi-earth®. Scotts. Marysville, Ohio). Slow release 13:13:13 fertilizer (5ml) (Osmocote ®. Scotts. Marysville, Ohio) was added at planting. Seedlings were thinned to one per pot after emergence and pots watered daily as needed and treated with liquid 20:20:20 fertilizer (Peter's ®. Scotts. Marysville, Ohio) once a week at the recommended rate of 1ml fertilizer per liter of water. The plants were used when they were ≈20 centimeters tall with four true leaves.

Foliar applications

Neemix 4.5® (4.5% azadirachtin) was diluted to concentrations of 1, 10, and 100 ppm of azadirachtin in water. The highest concentration (100ppm) was obtained by adding 1ml of Neemix 4.5® to 450ml of water. The less concentrated solutions (1 and 10 ppm) were then obtained by serial dilution. The different solutions were mixed and placed in a hand sprayer (36 Oz Horse Sprayer. Tolco. Toledo, Ohio). Foliar applications were made by squeezing three times on the sprayer to deliver a total of 2.75 ± 0.08ml of liquid. The sprayer was held about 30cm above the plant to ensure complete and uniform spray coverage. Solutions with the lowest concentrations were applied first followed by progressively more concentrated solutions to avoid contamination. The plants were then left to dry (2-3hrs) before being placed in the cage and insects introduced.

Soil applications

Soil treatments were applied to four squash plants at the four-leaf stage. Soil treatments were made by adding 60ml of solution with known concentrations of the toxin onto the soil. Soil treatments were made one day prior to introducing insects to ensure uptake and systemic accumulation in the plant. Prior to introducing insects, a layer of fresh soil 1cm deep was added to each pot to minimize direct contact of the insects with the toxin on wet soil. The potted plants were then placed in a cage and innoculated with insects.

Experimental design

The four treated plants were placed at random on each of the four corners of a rectangular cage measuring 90cm long, 45 cm wide, and 45 cm tall. The cages were placed under fluorescent light (40 Watts. Plant and Aquarium. General Electric) and kept at a 24hr photophase over the duration of the experiment. Twenty virgin adult squash bugs (1:1 of male: female) from the same cohort were placed in the middle of the cage in an open petri dish and allowed to freely move within the cage. The experiment was replicated six times. The number of insects on each of the plants was observed at hourly intervals for the first 3 hours and twice a day for the following five days. At the end of the experiment, the number of insects alive and eggs deposited on each plant were recorded. The number of insects found on each plant was recorded at each time interval. The individual insect counts at each concentration were accumulated over the time of the experiment and expressed as a percentage of the total number of insects in the cage. Insects on the soil and leaves were counted toward their respective treatments while those on the cage walls and floor were not considered. Actual egg counts were recorded against each concentration by counting the number of the eggs on plants. At the end of the experiment, the four plants were ranked by

appearance with the healthiest having a value of one. The least healthy, which was often withered or dead was assigned the highest value with a maximum of four. The relationships between the three variables and the increasing azadirachtin concentration were analyzed using regression methods (SAS, 1989).

Results and discussion

Foliar treatments

None of the variables (number of insects and eggs, and ranking of plant appearance) was dose-dependent at the range of 0-100ppm (Table 2). The means are reported on Table 1. Most insects were found on plants treated with 10ppm azadirachtin (Table 1). Those treated with 1ppm had the fewest although there was not a significant dose-response with respect to insects present (P=0.778)(Table 2)(SAS, 1989). Similarly, the results of regression of insect density (P = 0.6731) plant rank (P = 0.8053) did not show notable dose-dependent relationships with slopes of 0.01 $\pm$ 0.04 and -0.001 $\pm$ 0.005 for insect density and plant ranking respectively (Table 1, 2).

Table 1: Mean responses to foliar sprays in % insect density per plant, number of eggs per plant, and mean rank of plant physical appearance as affected by dose of Neemix 4.5® accumulated over a two week period

Aza. Conc (ppm)	% Mean Insect density ± SE	Mean eggs per plant ± SE	Mean rank of plant ± SE
0	15.0 ± 6.3	18.3 ± 13.5	3.2 ± 1.2
1	11.7± 6.9	16.2 ± 10.7	2.2 ± 1.3
10	18.0 ± 1.9	25.3 ± 23.2	2.5 ± 0.8
100	15.5 ± 12.0	22.8 ± 22.2	2.5 ± 1.0
Slope	0.01 ± 0.04	0.04 ± 0.09	-0.001 ± 0.005

Table 2: Regression analysis of mean percent density of insects, eggs and ranking of plant physical appearance distributed among increasing doses of Neemix 4.5® applied on host plant foliage.

Source	df	SS	MS	F	Pr > F
Insects	1	4.8	4.8	0.08	0.7768
Error	22	1285.1	58.4		
Total	23	1290.0			
Eggs	1	57.3	57.3	0.18	0.6731
Error	22	6890.1	313.2		
Total	23	6947.3			
Rank	1	0.08	0.08	0.06	0.8053
Error	22	27.8	1.26		
Total	23	27.8			

Soil treatments

Increasing dose of toxin in soil treatments resulted in plants with healthier physical appearance. The mean percentages of insects found on the treated plants generally declined with increased levels of the toxin with a significant dose response between these percentages and the concentrations of azadirachtin ($P = 0.0184$, Table 4). Treatments with 0 ppm azadirachtin had most insects (15.8 ± 5.2, Table 3). These proportions declined significantly to a minimum at the highest application rate of 100 ppm azadirachtin. The 100 ppm azadirachtin concentration was associated with a mean of 6.9 ± 2.9 percent of the total number of insects in the arena. This relationship had a negative slope of -0.06 ± 0.02 (Table 3).

Among soil treatments, there were significantly more eggs in the controls (0 ppm) than at the different levels of the toxin (Table 3). Although this distribution had a negative slope of -0.02 ± 0.01, a pattern was not evident as levels of the toxin increased from 1 to 100 ppm azadirachtin. As a result, there was no significant dose-dependent relationship of egg abundance along the concentration gradient after regression analysis ($P = 0.7617$, Table 4).

A ranking of plant appearance had a significant dose-dependent relationship ($P=0.0073$). With a slope of 0.01 ± 0.005, these values generally increased with the concentration of azadirachtin. The ranges overlapped between the concentrations of 1 and 10 ppm at 15.0 ± 2.5 and 14.0 ± 2.3 respectively (Table 3). These two were however distinctly separate from those of 0 ppm and 100 ppm, which were 21.0 ± 3.5 and 10.0 ± 1.7 respectively (Table 3).

Table 3: Mean responses to soil treatments in % insect density per plant, number of eggs per plant, and mean rank of plant physical appearance as affected by dose of Neemix 4.5™ accumulated over a two week period

Aza. Conc (ppm)	% Mean Insect density ± SE	Mean eggs per plant ± SE	Mean rank of plant ± SE
0	15.8 ± 5.2	10.3 ± 16.8	2.1 ± 3.5
1	11.1± 3.8	1.5 ± 3.7	1.5 ± 2.5
10	8.6 ± 3.1	6.5 ± 14.5	1.4 ± 2.3
100	6.9 ± 2.9	4.3 ± 10.6	1.0 ± 1.7
Slope	-0.06 ± 0.02	-0.02 ± 0.01	0.01 ± 0.005

Table 4: Regression analysis of mean percent density of insects, eggs and ranking of plant physical appearance distributed among increasing doses of Neemix 4.5® applied on soil treatments.

Source	df	SS	MS	F Value	Pr > F
Insects	1	128.7	128.7	6.48	0.0184
Error	22	436.7	19.9		
Total	23	565.4			
Eggs	1	14.3	14.3	0.09	0.7617
Error	22	3327.1	151.2		
Total	23	3341.3			
Rank	1	8.6	8.5	8.72	0.0073
Error	22	21.5	1.0		
Total	23	30			

Conclusion

There was no significant response to dose of Neemix 4.5® applied on foliage with respect to any of the variables. There was, however, a significant dose response to insect abundance and ranking of plant condition when the toxin was applied to the soil. Oviposition patterns were independent of the dose of toxin used, which may be attributed to the fact that many plants had no eggs deposited on them in the duration of the experiment. Soil applications had a more pronounced effect on oviposition than foliar applications.

A ranking of plant physical appearance was most likely a function of the intensity of insect feeding. Intense feeding rates cause the plant to wither and die even though it did not have the most number of insects compared to other plants receiving different treatment concentrations. This is further confirmed by a similar distribution in number of insects among soil treated plants. This trend is however not obvious among foliar treatments.

Soil treatments had significant responses to dose while foliar treatments did not. The insignificant dose response among foliar treatments may be due to various reasons that may need more studies to isolate effects such as quantity of toxin applied to each plant, feeding intensity at the various toxin concentrations and possible breakdown of the toxin by exposure to ultra violet light. Soil treatments consisted of 60ml of toxin solution while foliar treatments had only 2.75 ± 0.08 ml of the same toxin at a similar concentration. As a result, more actual toxin was applied in soil applications than foliar sprays. Since the soil stayed wet for a large part of the experiment, it is likely that the plant had access to large amounts of toxin in the soil ensuring a continuous supply. It is also known that the biological half-life of azadirachtin is highly extended in plant tissues under systemic conditions (Naumann et al., 1994). This is not the case when foliar applied toxin is exposed

to ultra violet light with a high likelihood for reduced activity from bioconversion (Barnby et al., 1989; Jarvis et al., 1998). The systemic activity of neem compounds has been studied in greater detail among trees (Helson et al., 2001; Naumann et al., 1994), which have been more so noted for extended activity. Studies by Helson et. al. (2001) established that the toxins remained active in the spring after being applied the previous fall. While it is not clear how herbaceous plants will respond to such application methods, the role of systemic accumulation of neem is no doubt of great toxicological significance.

It is not clear what interactions may exist between plants especially under the confined space in the cage used for this study. The micro-environment is important in influencing insect distribution with respect to how they perceive toxin levels among the different plants in the arena. It is, however, evident that soil applications of Neemix 4.5® can offer substantially higher levels of protection of squash plants from squash bug infestation as shown by adults present and number of eggs oviposited on them.

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CHAPTER IV

MATING DISRUPTION AND OVIPOSITION AMONG VIRGIN SQUASH BUGS AND THE ROLE OF EGG FERTILITY ON THE PERFORMANCE OF THE EGG PARASITE *GRYON PENNSYLVANICUM* ASHMEAD

Abstract

A study was conducted to evaluate the effects of azadirachtin on mating behavior and ovipositon among squash bugs. Five microliter injections of 100ppm azadirachtin on either male or female within a mating pair inhibited mating. Virgin females paired with treated males continued laying eggs. Treating male partners was associated with oviposition of unfertilized eggs by untreated female partners. The unfertilized eggs were not viable but served as suitable hosts for the squash bug egg parasite *Gryon pennsylvanicum* Ashmead. Fertilized eggs, however, had higher rates of emergence of the egg parasites than unfertilized eggs.

Introduction

Neem extracts affect insects in several ways (Ascher, 1993; Isman et al., 1990; Isman, 1993; Lowery and Isman, 1986; Mordue (Luntz) and Blackwell, 1993; Schmutterer, 1998). Apart from supressing molting, delaying growth and causing mortality, these products may also affect mating behavior and oviposition among insects. In *Ceratitis capitata* Wiedemann, mating is inhibited by the male failing to recognize females that have been treated with azadirachtin (Casana-Giner et al., 1999; Di Ilio et al., 1999).

Behavioral studies in *Leptoglossus clypealis* Heidemann (Hemiptera: Coreidae) indicate that male secreted hormones mediate the mating process in the Coreid family of insects (Wang and Millar, 2000). Since azadirachtin is known to affect hormone systems in several insects (Feyereisen and Durst, 1978; Lindsay and Kaufman, 1988; Meszaros and Morton, 1997; Mitchell et al., 1997; Rembold et al., 1989; Smith and Mitchell, 1988), we expect similar effects where mating behavior among treated squash bugs is altered. Mediating a processes like mating and oviposition will in turn offer an effective management tool for squash bugs.

Mating and oviposition behavior among squash bugs has been studied for many years although few have studied the consequences of inhibited mating (Beard, 1940; Fielding, 1990; Girault, 1904). We expect that when mating is inhibited, untreated female partners may either experience suppressed levels of oviposition or alternatively proceed to lay unfertilized eggs. Since such eggs are unfertilized and, therefore, not viable, their occurrence will certainly have an influence on the population growth of squash bugs. This phenomenon has a direct influence on management threshold recommendations for the squash bug that currently calls for treatment at four eggs or one egg mass per plant (based on the assumption that all eggs are fertile).

It is not clear from the literature how the egg parasite *G. pennsylvanicum* would be affected by the fertility status of its host's eggs. Having unfertilized eggs in the field, however, is potentially likely to affect the egg parasite in various ways. This will, however, be clarified once it is established if the egg parasite is capable of utilizing non-fertilized squash bug eggs (Girault, 1904; Vogt and Nechols, 1993). In the event that the parasite is independent of the fertility status of host eggs, using it in concert with IGR's will be more effective as a management tool. This is because IGR's will increase the occurrence of unfertilized eggs while *G. pennsylvanicum* as a parasite, constitutes a complimentary approach to a more effective overall pest management strategy.

The egg parasite *G. pennsylvanicum* is a potential bio-control agent for the squash bug (Vogt and Nechols, 1993) and its performance is likely to be affected by quantitative or qualitative changes in squash bug eggs. The overall effect will, however, depend on host selection mechanisms employed by *G. pennsylvanicum* and if they are dependent on the fertility status of squash bug eggs.

This study sought to first establish the effect of neem on mating and oviposition among squash bugs. Secondly, to evaluate the performance of egg parasites on both fertilized and unfertilized eggs.

Methods and materials

A series of experiments were conducted to better understand how azadirachtin affects mating and how this in turn influences oviposition and nymphal emergence from squash bug eggs. Virgin adults were treated with azadirachtin before being paired with non-treated partners and observed for mating and oviposition. Eggs collected from these pairs were incubated for estimation of nymphal emergence rates. On further observation, both fertilized and unfertilized eggs were exposed to the egg parasite *G. pennsylvanicum*, and observed for suitability as hosts by establishing parasite emergence rates.

Squash bug colony

A squash bug colony was established in the spring of 1998 from field collected insects in Lane, Oklahoma and maintained at approximately 25°C and 18:6 (L:D) photoperiod. The insects were fed on squash plants and fruit over the duration of the experiment. Eggs were deposited on white cotton cloth hung in the cages and emergent first instar nymphs were collected once a week. The collected first instar nymphs were isolated as a group to produce insects of the same cohort. Late instars with developed wing buds were isolated from the colony and used to raise adults of uniform age. To obtain newly emerged unmated adults of the same cohort, all emerging adults were collected from the isolated late instars every 2-3 days. The isolated adults were immediately sexed, treated and paired according to treatments to prevent uncontrolled mating.

Injections

Injections with an azadirachtin concentration of 100 ppm were used along with two control treatments. One of the controls was a sham injection of 5µl of the solvent mixture (10% ethanol) without the toxin. The second control treatment was comprised of insects that were not injected at all. Sham injection treatments were made first followed by the azadirachtin treatments to avoid contamination. Needles were changed and the syringe rinsed thoroughly with water between treatments. Injections were made by drawing 5µl of solution into a microsyringe (Gastight® #1701. Hamilton Co. Reno, Nevada.). On eliminating air bubbles, the microsyringe needle was inserted into the intersegmental membrane between the third and fourth abdominal sternite with the insect held still on its back. The solution was slowly introduced into the insect hemolymph before the needle was drawn gently from the insect.

Effect of azadirachtin injections on mating and oviposition

Adult virgin squash bugs, within a week of emergence, were treated with 5µ of 100 ppm azadirachtin solution in water. Each treatment replicate was comprised of two males and two females. Both males and females were treated in the first treatment set (M^+F^+), the second set had only males treated (M^+F^-), and in the third, only females were treated (M^-F^+). The last was a control treatment where neither of the two sexes received the toxin (M^-F^-). The insects were placed in cages with a potted squash plant on the laboratory bench at a temperature of about 25°C and a 24hr photophase. Observations were made twice a day (every 10-12 hrs) on the frequency of mating and the number of mating insect pairs was

recorded. The cumulative total of observed number of mating pairs is here referred to as the mating frequency, which was assigned to each treatment combination.

Oviposition by isolated virgin females

After females were observed to lay eggs while paired with treated males without observed mating, a second experiment was conducted. This was to establish if virgin females would oviposit in the absence of males as it was not positively confirmed that unobserved mating did not occur in the treatments. To confirm these results, four insects were placed in each of six cages, which represented three replicates of two treatments. The first treatment (M^-F^-) comprised of two virgin male and female pairs. The second had four virgin females (F^-F^-). An equal number of insects was maintained on each experimental unit. Each cage had a squash plant and the insects were observed for mating and oviposition over a period of 14 days.

Suitability of unfertilized eggs to the egg parasite *G. pennsylvanicum* Ashmead

Egg parasites were collected from infested eggs in the laboratory colony and exposed to two sets of squash bug eggs for oviposition. The first set of eggs were laid by virgin females while the second was collected from actively mating squash bug adults. The eggs were collected attached to host plant tissue to avoid damage. These were then placed in petri dishes containing 12 adult egg parasites *G. pennsylvanicum*. The egg parasites had been previously collected within a day of emergence and contained in plastic petri dishes sealed shut with Parafilm®. The egg types (fertilized and unfertilized) were the treatments, which were in turn replicated four times. The petri dishes containing the eggs were then maintained at 25°C and a 24 hr photophase. The eggs were observed daily for a period of 30

days after inoculation for emergence of parasites. The final number of adult parasites counted included those initially placed for inoculation. Once the introduced number of adults was subtracted from the total, the number of parasites emerging from the eggs was expressed as a percentage of the total number of eggs in the petri dish.

Results and discussion

Effect of azadirachtin injections on mating and oviposition

Sham injection and non-injection controls were not significantly different at P= 0.05, LSD. These two treatments were, therefore, pooled to get an overall mean assigned to the control treatment (M^-F^-). When one or both partners of a mating pair were treated, mating did not occur during the 14 days that the insects were observed (Table 5).

Oviposition by isolated virgin females

Oviposition occurred when females were not treated regardless of whether their male partners were treated or not. Since the insects were observed for mating only twice a day at 10-12 hr intervals, it was not possible to entirely rule out the possibility of unobserved mating. It was, therefore, necessary to verify these observations by using virgin females that had absolutely no contact with males. This would eliminate the possibility of unobserved mating. The eggs laid by unmated females are unfertilized and, therefore, no emergent squash bug nymphs were expected.

Table 5: Total number of matings observed at 12 hr intervals over a two week period and number of eggs laid among treated and untreated mating partners

Trt	Mating frequency ± SE	Number of Eggs ± SE
M^+F^+	0.00 ± 0.00	0.00 ± 0.00
M^+F^-	0.00 ± 0.00	63.33 ± 17.16
M^-F^+	0.00 ± 0.00	0.00 ± 0.00
M^-F^-	13.67 ± 6.35	86.67 ± 50.12

*(M) Male; (F)Female; (+) treated; (-) untreated

On observing virgin females that had no contact with males over a period of 15 days, it was clear that squash bug females oviposited without mating (Table 6). Incubating the unfertilized eggs under 25°C and a 24 hr photophase yielded no nymphs compared to control treatments that had nymphal emergence rates of 78 ± 10 %. The mean number of eggs oviposited by mating and virgin females was 78 ± 14 and 51 ± 31 percent respectively. With such an overlapping range, there is no sufficient evidence to show that mating influenced female oviposition.

Once it was confirmed that squash bug oviposited irrespective of mating status, it was necessary to determine if these eggs might potentially affect the performance of the squash bug egg parasite.

Suitability of unfertilized eggs to the egg parasite *Gryon pennsylvanicum* Ashmead

Table 7 is a summary of observed parasite emergence from fertilized and unfertilized squash bug eggs. Both types of eggs (fertilized and unfertilized) supported an appreciable amount of successfully developed egg parasites although the fertilized eggs from non-treated mating pairs had substantially higher rates of emergence. From these results, it is yet to be established if the parasite probes and lays eggs on both fertilized and unfertilized eggs with equal likelihood.

Table 6: Oviposition by untreated mating partners (M^-F^-) compared to untreated female partners (F^-F^-) with their associated egg viability measured by emergence of offspring for the incubated eggs

Trt	Eggs/female	% nymphal emergence
M^-F^-	78.0 ± 14.0	78.0 ± 10.0
F^-F^-	51.0 ± 31.0	0.0 ± 0.0

Table 7: Mean emergence rates of the egg parasites and squash bug nymphs from fertilized (M^-F^-) and unfertilized (M^+F, F^-F^-) eggs

Trt	Eggs	% Parasite emergence	% Nymphal emergence
M^+F^-	63.0 ± 18.0	32 ± 12.7	0.00 ± 0.00
F^-F^-	51.0 ± 31.0	24 ± 11.0	0.00 ± 0.00
M^-F^-	78.0 ± 14.0	88 ± 9.6	0.00 ± 0.00

Conclusions

The percent emergence from eggs laid by females paired with azadirachtin-treated males were not significantly different from those from virgin females. While parasites successfully reproduced on unfertilized squash bug eggs, it was not clear why these rates were lower among unfertilized host eggs. There are, however, two likely possibilities that may cause low emergence rates among unfertilized eggs. First that development of parasite eggs may be improved by qualitative characteristics of the fertilized egg. Secondly, that parasite females may have an oviposition preference for fertilized host eggs after identifying them as such.

The use of neem and other insecticides with similar modes of action is likely to increase in future. It is, therefore, of great concern how these products will affect non-target organisms especially those with a potential for incorporation into IPM programs as biocontrol agents. While the direct effects of neem on *G. pennsylvanicum* have not been addressed here, it is likely that the performance of the parasite will be affected by increased occurrence of unfertilized host eggs in the field. If indeed parasite females indiscriminately oviposit on both fertilized and unfertilized eggs, then the reproduction rates of the parasite are likely to fall. Alternatively, if oviposition by parasite females is biased in favor of fertilized eggs, then the parasite's bio-control potential will be enhanced especially when used in concert with other products that may cause the occurrence of unfertilized eggs. This is because the parasite will further limit the number of viable offspring emerging from oviposited eggs after pesticide effects have initially rendered some of the eggs non-viable due to infertility. This will have an overall reduced reproduction rate of the squash bug.

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CHAPTER V

COMPARING NEEM BASED FORMULATIONS CONTANING DIFFERENT PROPORTIONS OF AZADIRACHTIN AND THEIR ACTIVITY ON THE LATE INSTAR AND ADULT SQUASH BUG *ANASA TRISTIS* DE GEER

Abstract

A study was conducted to establish the role of azadirachtin on the overall biological activity of neem extracts against the squash bug *Anasa tristis* De Geer (Hemiptera: coreidae). Three neem products (neem oil, Neemix 4.5®, and pure azadirachtin) containing different proportions of azadirachtin and Trilogy®, which has no significant levels of azadirachtin, were compared in their ability to suppress molting among late instars and mortality among late instars and adults.

These neem products were diluted to supply 200 ppm of azadirachtin in 5µl of solution, which was injected into the insect hemolymph through the intersternal membrane. Trilogy® was used at a rate equivalent to that of neem oil based on the oil content rather than azadirachtin. The treated insects were observed for molting and mortality.

Azadirachtin significantly inhibited molting among late instars, and caused significant mortality among adults. Neem oil and Neemix 4.5® were more active than azadirachtin in causing mortality in both late instars and adults. Trilogy® was more active than pure azadirachtin in causing adults mortality. While azadirachtin may be the most active ingredient in neem extracts, results here indicate a blend of azadirachtin with other neem oil components is more potent. The activity of Neemix 4.5® is similar in both mortality of late instars and adults. Although likely, there was not enough evidence for increased activity attributed to synthetically increased azadirachtin levels in Neemix 4.5 ® and neem oil. It was however important that a combination of the other components of neem in the absence of azadirachtin is more potent than the purified formulation of azadirachtin.

Introduction

Neem oil contains several compounds including azadirachtin, all known to be biologically active (Isman et al., 1990; Schroeder and Nakanishi, 1987; Ermel et al., 1987; Jarvis et al., 1998; Kraus et al., 1987). Among these are salanin, nimbin, nimbidin, meliantriol, azadarachol, several azadirachtin analogs, and other tetranortitriterpenoids (Ramji et al., 1998). Azadirachtin is, however, believed to be the key active ingredient making the most contribution to the overall activity of neem oil (Butterworth and Morgan, 1968; Immaraju, 1998; Saxena, 1987).

Formulation of neem products through different processes alters the relative proportions of azadirachtin and other neem compounds present. Neem oil is obtained at azadirachtin concentrations of 300-10000 ppm (0.03-1%) (EID Parry India Ltd. Chennai, India), which may occur along with an array of 100 other known compounds (Jarvis et al., 1998; Kraus, 1995; Lale and Abdulrahman, 1999). The 'technical grade' processed product has increased azadirachtin levels (12-26%) (Lale and Abdulrahman, 1999). Commercial formulations like Neemix 4.5®, and Neemazal® are derived from the technical grade product whose azadirachtin levels have been increased during processing (Ermel et al., 1987a; Lale and Abdulrahman, 1999). Several other insect pest management products are formulated from the oil. Pure azadirachtin is available but is largely used for research purposes due to its prohibitive cost. With such a range of neem products in the market formulated through different processes, we sought to identify the role of azadirachtin in the activity of the oil against the squash bug using neem products with progressively declining proportions of azadirachtin in their composition. These ranged from azadirachtin (95% purity) to Trilogy® that contained no substantial amounts of azadirachtin. Results from this study will first directly compare the activity of azadirachtin to that of all other components in the oil put

together. Using these findings, a criteria for grouping neem products will be established based on potential biological activity as derived from the relative composition of the neem product.

Methods and materials

The insects were injected with one of four neem-derived compounds and observed for molting and mortality. Solutions of the four formulations were prepared to deliver the same amount of azadirachtin on dilution (Table 8). The compounds used were azadirachtin (95% azadirachtin. Sigma-Aldrich. USA.), Neemix 4.5® (4.5% azadirachtin. Certis USA. Columbia, MD), Trilogy® (0% azadirachtin. Certis USA. Columbia, MD), and neem oil (1600 ppm azadirachtin. Ahimza Alternative Inc. Oklahoma City, Oklahoma). Trilogy® contains 70% of the hydrophobic extract of neem oil and no substantial amounts of azadirachtin. An amount of Trilogy® equivalent to neem oil was determined using the two proportions (1ml neem oil [100%]≡ 1.4ml Trilogy® [70%]).

Neem oil and azadirachtin were dissolved in 10% ethanol while Neemix 4.5® and Trilogy® were dissolved in water. Responses among these formulations were then directly compared to establish the relative contribution of azadirachtin and other oil components on the overall activity of the neem compounds.

Squash bug colony

A squash bug, *Anasa tristis* De Geer, colony was established in the spring of 1998 from field collected insects in Lane, OK and maintained at approximately 25°C and 18:6 (L:D) photoperiod. The insects were fed on squash plants and fruit and the colony maintained over the duration of the experiment. The eggs were deposited on white cotton cloth hung in the cages and emergent first instar nymphs collected once a week. These were

isolated as a group to produce individuals of the same cohort. Late instars with developed wing buds were collected from the colony for part of this experiment. To obtain adults, some of the isolated late instars were left to molt into adults being collected for the experiment within a week of emergence.

Injections

The four neem compounds were used along with two control treatments. One of the controls was a sham injection composed of 5µl of the solvent mixture (10% ethanol) without the active ingredient azadirachtin. The second control treatment had insects that were not injected at all. The control treatment consisting of a sham injection was administered first followed by the neem treatments. Needles were changed and syringe rinsed throughly between treatments. Injections were made by drawing 5µl of solution into a microsyringe (Gastight® #1701. Hamilton Co. Reno, Nevada.). On eliminating air bubbles, the microsyringe was inserted into the intersegmental membrane between the third and fourth abdominal sternite with the insect held still on its back. The solution was then slowly introduced into the insect hemolymph after which the needle was drawn gently from the insect.

Experimental design

A total of 36 late instar nymphs and an equal number of adults from the same cohort were put in groups of six. Each group represented a single treatment with six replicates. There were six treatments comprised of the four neem compounds (azadirachtin, Neemix 4.5®, Trilogy®, and neem oil) and two controls (a sham injection and the non-injection). Each insect received one of the treatments after which, it was placed in a petri dish and

supplied with a slice of squash for food. The squash was replaced every 2-3 days. The petri dishes were placed on benches at about 25°C under a 18:6 (L:D) photoperiod fluorescent lighting. Daily observations were made over a period of fifteen days to determine mortality and molting.

Binary responses of mortality and molting were recorded and comparisons among the different neem compounds made using ANOVA followed by LSD analysis.

Results and discussion

Late instars were observed for mortality and molting while adults were observed for mortality only. The observed responses were expressed as percentages as shown on Table 9.

Table 8: Proportions of azadirachtin in neem formulations and treatment solutions after dilution expressed as percentages.

Formulation	Percent proportions of components in treatment solutions				
Name	**% Az**	**Az**	**Oil**	**Water**	**Ethanol**
Azadirachtin	95.0	0.0002	0.0000	0.9000	0.1000
Neemix 4.5 ®	4.5	0.0002	0.0042	0.9978	0.0000
Neem oil	0.2	0.0002	0.1250	0.7748	0.1000
Trilogy ®	0.0	0.0000	0.1750	0.8250	0.0000

Table 9: Comparing neem-based formulations with different proportions of azadirachtin in their activity against late instar and adult squash bugs

Treatment	Late Instar Mortality	Late Instar Molting	Adult Mortality
Control	0.0[a]	83.3[a]	0.00[a]
Sham injection	0.0[a]	83.3[a]	10.0[a]
Azadirachtin	16.7[ab]	16.7[b]	40.0[b]
Neemix 4.5 ®	83.3[c]	0.0[b]	100.0[c]
Neem oil	66.7[c]	0.0[b]	90.0[c]
Trilogy ®	50.0[bc]	33.3[b]	80.0[c]

* Means followed by the same letter are not significantly different (LSD P= 0.05)

Mortality

Mortality means for all four neem products were significantly higher than the controls for both late instars and adults. Neemix 4.5® was consistently most potent followed by neem oil, Trilogy® and azadirachtin in that order (Table 9).

Mortality rate associated with Neemix 4.5® was 83% for late instars (Table 9). This was followed by neem oil (67%), Trilogy® (50%), and azadirachtin (17%). Mortality associated with neem oil was significantly higher than that of azadirachtin. The means for Trilogy® were intermediate and not significantly different from those of either neem oil or azadirachtin. The activity associated with neem oil (66.7%) was the sum of those recorded for Trilogy® (50%) and azadirachtin (16.7%)(Table 9).

Patterns of adult mortality took a similar pattern to that of late instars where Neemix 4.5® resulted in the highest rates followed by neem oil, Trilogy® and azadirachtin in the same order. While late instar mortality means associated with azadirachtin were similar to those of Trilogy®, in adults the two treatment means were significantly different.

Molting

Neemix 4.5® and neem oil had lower molting rates than those of the other two treatments although they were not significantly different (Table 9). The percent mean molting rates for all the four products are significantly lower than those of the controls (Table 9). This indicates that the four neem products substantially inhibit molting at an azadirachtin concentration of 200 ppm.

Conclusion

Results from this study show that azadirachtin's potency is enhanced when found in combination with other oil components in neem extract. Azadirachtin has for many years been considered the most active component of neem oil (Isman et al., 1990; Jarvis et al., 1998). While researchers recognize the improved activity of less refined formulations some attribute this to active compounds other than azadirachtin found in neem (Ascher, 1993; Ermel et al., 1987a; Ermel et al., 1987b; Kraus et al., 1987; Xie et al., 1995). Other researchers, however, attribute such increased activity to 'inert' proprietary compounds added during formulation (Williams et al., 1998). In this study, the activity of pure azadirachtin is lowest among the four formulations on both mortality and molting. On comparing Trilogy® with azadirachtin, it is here evident that the other neem oil components combined are more potent than azadirachtin alone. While proprietary compounds may make such contributions to overall activity of the formulation, such is accounted for in this study by compounds found in the crude neem extract.

While high proportions of azadirachtin in formulations are associated with increased potency, the enhancement only occurs when other oil components are initially present. Neemix 4.5® has higher proportions of azadirachtin than neem oil while they have similar proportions of the accompanying neem components. Using neem oil components combined with azadirachtin had an additive effect on the combined potency. The elevated proportions of azadirachtin may be responsible for the observed increase in potency of Neemix 4.5® compared to neem oil. Given that Trilogy® has trace amounts of azadirachtin and is far less active than an equivalent amount of neem oil, similar interactions are likely to be occurring.

From mortality results especially those of late instar nymphs, combining the rate of Trilogy® (50%) with that of azadirachtin (16.7%) equals that of neem oil (66.7%). The pattern is less obvious for adults although similar additive effects are not ruled out. When 'molting rates' on the previous table are converted to 'molting inhibition rates' by subtracting each from 100%, the results are as follows; Control (17%), Sham injection (17%), Neemix 4.5® (100%), Azadirachtin (84%), Trilogy (67%), and Neem oil (100%). Adding the molting inhibition rate of azadirachtin (84%) to that of Trilogy® (67%) gives a total molting inhibition rate above 100%. Since inhibition rates were not measured above 100%, this relationship is not necessarily different from the one observed for late instar mortality rates. Because the activity of azadirachtin consistently remains lower than that of Trilogy®, it implies that azadirachtin is less active than the other neem oil components combined.

These results do not necessarily disqualify azadirachtin as being the most biologically active compound in neem oil, but emphasize the importance of the other neem components in enhancing biological activity of the mixture. As an insect growth regulator (IGR) used for its ability of influence insect molting and cause death. The natural unrefined product (neem oil) is essentially as active as the processed formulations (Neemix 4.5®, azadirachtin, and Trilogy®). Assuming the possibility of similar responses by other insects, some considerations during processing may be necessary in availing these products for more effective insect pest management. The observed marginal increase in potency as a result of elevated azadirachtin proportions might merit the need to seek alternatives to elaborate refining processes of the oil that will certainly have a direct impact on cost.

Azadirachtin is classified as an IGR though the mode of action of neem in general has remained obscure (Mordue (Luntz) and Blackwell, 1993). This might be due to the pronounced effect of oil components other than azadirachtin, which are often regarded as

'impurities' in the refining process. With such a dominant combined effect over that of azadirachtin, extensive studies on the oil components and how they are affected by different processing methods will be necessary to better understand the mode of action of azadirachtin and neem formulations.

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CHAPTER VI

EFFECTS OF AZADIRACHTIN-CONTAINING COMPOUNDS NEEMIX 4.5® AND NEEM OIL ON SQUASH BUGS UNDER FIELD CONDITIONS

Abstract

Increasing doses of two neem formulations (Neemix 4.5® and neem oil) were used in the field to establish their effect on the population structure of the squash bug. One part of the study was based on natural infestations while another was based on confined insects in the field receiving similar treatments. The observed variables were number of eggs, nymphs and adults. Plants were examined weekly while cages were examined once at the end of the season. Natural field populations of the squash bug were observed over three consecutive years beginning in 1999 while the confined populations were observed in the summer of 2001.

There was a decrease in intensity of responses among all variables with increased dose of toxin. This was evidenced by the negative slope and the greatest density was recorded in 1999 with adults showing a significant dose response. Four other treatment combinations had significant responses.

High pest densities showed more distinct patterns across concentrations than overall insect densities were low. The toxin range of 0-600ppm azadirachtin did not result in significant dose effects under low to moderate pest densities. This may, however, be different with greater pest densities, which could be achieved by extending the crop season to include late season populations that have had ample time to develop to greater densities.

Introduction

Neem extracts act as repellents, antifeedants, oviposition deterrents, and insect growth disrupters against several insects orders (Ascher, 1993; Kraus et al., 1987). Neem extracts deter leaf chewing insects such as the desert locust *Locusta migratoria* (Orthoptera: Acrididae) and cabbage butterfly *Pieris brassicae* (Lepidoptera: Pieridae) from feeding when

applied on their host plants. Results from a study of *Pieris rapae* L.(Lepidoptera: Pieridae) conclude that oviposition on host plants is affected in a dose-dependent fashion (Saxena and Rembold, 1984). These extracts significantly disrupt development processes like molting, adult emergence and gametogenesis in several insect species (Ascher, 1993). Generally, there is a delay in development at low azadirachtin concentrations that may result in 'permanent larvae' at increased concentrations (Ascher, 1993).

Sub-lethal doses of the compound were associated with reversibly suppressed fecundity, egg sterility and a general decline in insect fitness especially when the insect was exposed over extended periods (Meisner et al., 1990; Meisner et al., 1992). Results of studies on *Ceratitis capitata* (Diptera: Tephritidae) indicated that mating behavior can be affected where males failed to recognize the female sex pheromone, therefore, disrupting mating (Di Ilio et al., 1999; Dorn et al., 1986b).

Neem extracts contain azadirachtin alongside other active compounds (Ascher, 1993; Kraus et al., 1987; Ramji et al., 1998). The proportion of azadirachtin in cold pressed neem oil may range from 300-1600ppm (0.03-0.16%) (Ermel et al., 1987b). Processing the oil yields a technical grade product containing 12-26% azadirachtin and lower proportions of other oil components (Lale and Abdulrahman, 1999). It is from the technical grade product that Neemix 4.5®, a commercial neem based product is formulated.

Studies on the effect of neem on hemiptera have been conducted (Dorn, 1997; Garcia and Rembold, 1984; Garcia et al., 1989; Isman, 1995; Mordue (Luntz) and Blackwell, 1993) although a limited number have specifically examined its toxicity to the squash bug. In a field study by Edelson et. al., (1999a) using Neemix 4.5®, numbers of squash bug adults, nymphs and egg masses in summer squash were substantially reduced. When treatments were initiated early in the season, neem treatments resulted in control comparable to that of

common synthetic pesticides. A second study using the same insecticides on watermelon at more abundant squash bug adult populations showed an inferior performance of Neemix 4.5® (Edelson et al., 1999a; Edelson et al., 1999b).

This study was conducted to first evaluate the overall effects of neem on field populations of the squash bug as reflected by the density of adults, nymphs and eggs. Part of the experiment evaluated natural insect populations while another used confined insects for close monitoring with controlled movement of insects between treatment plots. Two neem products (Neemix 4.5® and neem oil) were used to represent two types of commercially available neem extracts. Secondly, plant performance under these treatment conditions was evaluated my comparing yield and biomass measurements.

Methods and materials

Field plots

Two crops of summer squash *Cucurbita pepo* 'lemondrop' were planted each year (in May and July) on beds 2m apart with 30cm between plants. Each treatment plot was 7m long with 2m between plots along the row. A pre-emergence herbicide Prefer 4E® was used at planting at the recommended rate of 2.25 kg per acre. After reaching the four-leaf stage, the squash plants were thinned to a spacing of 1.5-2.0m between plants. A drip line was installed at seeding and the plants were irrigated as needed. In 1999 Neemix 4.5® was applied at four rates (0, 150, 300, 600 ppm azadirachtin). This included the recommended rate of about 300ppm based on the active ingredient per acre and the amount of water applied per acre using spray equipment. In the following years, Neemix 4.5 ® and neem oil were applied at the same four rates. The two treatments (Neemix 4.5 ® and neem oil) were applied on separate plots where the four rates and replicates were randomized within each

plot. Sprays were applied using a tractor mounted sprayer set to apply 135lits per acre through three hollow cone nozzles at 2069 mmHg (40psi) pressure. Treatment sprays were applied weekly.

Evaluating the effects of Neemix 4.5® and neem oil on field squash bug populations

Three sample plants per plot were examined for insects. Counts taken during these surveys were number of adults, eggs, and each of four squash bug nymphal instars. Third and fourth instars were pooled together during data collection to facilitate faster counting. At fruit maturity, all fruit from three sample plants per plot were removed and weighed to provide yield data for the same plots. At the end of the growing season, one plant per plot was pulled along with the roots, dried and weighed for biomass.

During statistical analysis, counts of all the nymphal stages were combined. The mean biomass, mean yield and the cumulated totals of eggs, nymphs and adults for the season associated with each replicate and concentration of the toxin were used to compare treatment effects. Regression analysis was applied against the concentration gradient for each compound on all variables (mean yield, mean biomass, and cumulative counts of eggs, nymphs and adults). On all field experiments, significance levels were set at $P = 0.10$. This was to accommodate for natural variations under uncontrolled field conditions.

Effect of Neemix 4.5® and Neem oil on confined squash bugs in the field

Due to high mobility of field populations, an experiment was conducted using insects confined in cages placed in the field. This would in turn be used to quantify treatment effects based on a known starting population that was exposed to regular sprays and natural field conditions. At the beginning of the crop season, a virgin male and female

pair was placed in a screened cage over a single plant in the middle of each treatment plot. The cages measured 45cm on all sides and had a wooden frame covered with aluminum screen to prevent the insects from escaping. Plants in the cages were sprayed along with the rest of the plot on a weekly basis. After five weeks, the cages were opened and visually examined for squash bug eggs, different nymphal instars and adults. These plants were uprooted, dried, and weighed for biomass.

Regression analysis was applied against the concentration gradient for each compound to establish dose-response relationships with respect to yield, biomass, and insect abundance variables like egg, nymph, and adult counts.

Results and discussion

Biomass

The biomass of sample plants collected from plots treated with increasing concentrations of Neemix 4.5® and neem oil was not dose dependent except for one treatment condition where neem oil was applied in the early season of 2000 (R^2 = 0.9206, P = 0.0405)(Table 11). These regression relationships were based on comparisons of biomass means from each toxin concentration. These means were a result of adding individual replicates within each concentration. These relationships were established for each of the two formulations (Neemix 4.5® and neem oil). Except for the early season of 1999 with a negative slope in which Neemix 4.5® was applied, the rest had a positive slope. This indicates that increasing doses of the toxin generally resulted in heavier plants although this was only significant in two cases; early season Neemix 4.5® application in 2000 and neem oil treated cages (R^2 = 0.9778, P=0.0952)(Table 11, 12).

Yield

Squash yields had a positive slope and generally increased with concentration except for the first season of 1999. This early season yield means plotted against concentration had a negative slope though these had no significant regression relationship (Table 13). The second season of 1999 had a significant regression relationship (R^2 = 0.9999, P= 0.0041). Other treatments with marginal dose response effects were the first season 2000 (R^2 = 0.9847, P= 0.0788) and 2001 (R^2 = 0.9778, P= 0.0952) (Table 13) comprised of neem oil treatments.

Insect counts

Natural Populations

There was a general decline in number of adults, nymphs and eggs as toxin concentrations increased on all three years as displayed on Table 14. The slopes for this relationship ranged from -0.01 to –0.45 and five of these constituted a significant regression relationship using regression analysis. A significant dose-response relationship was observed among Neemix 4.5® treated adults in 1999 (R^2 = 0.9132, slope = -0.09, P=0.0444). Others with a marginal response effect were; Neemix 4.5® treated nymphs (R^2=0.8861, slope = 0.45, P = 0.0587) in 1999; neem oil treated eggs (R^2 = 0.8625, slope = -0.40, P = 0.0713) in 2001; neem oil treated nymphs (R^2 = 0.8330, slope = - 0.07, P = 0.0873) in 2001; and Neemix 4.5® treated adults (R^2 = 0.8570, slope = -0.03, P = 0.0743).

Caged insects

There was no significant dose response among caged insects. Caged insects had generally lower means than those of natural field populations as their starting population consisted of only two mating adults, which were restricted to a reproduction season of five weeks. In this short period, the insects would mate, lay eggs and develop.

Table 10: Summary of squash plant mean biomass among treatments for the year 1999

Year	Season	Trt	Conc (ppm)	Mean	Std Dev
1999	Early	Nx	0	142	47.64
1999	Early	Nx	150	194	59.83
1999	Early	Nx	300	140	58.31
1999	Early	Nx	600	168	21.68
1999	Late	Nx	0	104	38.47
1999	Late	Nx	150	236	211.38
1999	Late	Nx	300	188	57.62
1999	Late	Nx	600	252	78.23

Table 11: Summary of squash plant mean biomass among treatments for the year 2000

Year	Season	Trt	Conc (ppm)	Mean	Std Dev
2000	Early	No	0	110.0	41.83
2000	Early	No	0	115.0	36.89
2000	Early	No	150	153.3	81.65
2000	Early	No	300	195.0	64.55
2000	Early	No	600	220.0	109.54
2000	Early	Nx	0	140.8	33.43
2000	Early	Nx	150	186.7	75.28
2000	Early	Nx	300	136.7	81.65
2000	Early	Nx	600	303.3	201.66
2000	Late	No	0	100.3	38.26
2000	Late	No	150	86.2	28.6
2000	Late	No	300	145.4	93.48
2000	Late	No	600	136.4	53.78
2000	Late	Nx	0	112.1	37.16
2000	Late	Nx	150	88.4	32.59
2000	Late	Nx	300	133.0	61.29
2000	Late	Nx	600	163.4	79.94

Table 12: Summary of squash plant mean biomass among treatments for the year 2001

						Cages	
Year	Season	Trt	Conc (ppm)	Mean	Std Dev	Mean	Std Dev
2001	Early	No	0	166.0	67.22	3827.2	1124.01
2001	Early	No	150	212.5	32.60	4195.8	1463.96
2001	Early	No	300	227.5	77.99	4564.8	1977.98
2001	Early	No	600	245.0	46.37	6095.2	695.19
2001	Early	Nx	0	214.5	71.29	6407.0	2728.63
2001	Early	Nx	150	231.3	96.45	8136.3	1317.16
2001	Early	Nx	300	171.3	57.86	5925.1	1951.80
2001	Early	Nx	600	358.3	23.46	8448.2	1738.36
2001	Late	No	0	78.5	14.18	605.0	230.94
2001	Late	No	150	83.3	16.88	905.0	402.12
2001	Late	No	300	86.8	14.48	1000.0	577.70
2001	Late	No	600	104.5	20.24	907.5	572.97
2001	Late	Nx	0	111.8	18.66	2082.5	1532.94
2001	Late	Nx	150	104.8	18.84	1295.0	556.27
2001	Late	Nx	300	121.5	13.89	1965.0	1919.26
2001	Late	Nx	600	130.5	30.92	1840.0	1692.57

Table 13: Summary of fruit yield from 3 sample squash plants per plot for the years 1999 - 2001

Year	Time	Trt	Conc (ppm)	N	R^2	Slope	*P* Value
1999	Early	Nx	0-600	180	0.145376	-0.37795	0.750966
1999	Late	Nx	0-600	180	0.999958	5.788657	0.00412
2000	Early	No	0-600	216	0.984749	8.211667	0.07882
2000	Early	Nx	0-600	216	0.65506	5.424167	0.399631
2000	Late	No	0-600	216	0.092385	0.380833	0.803389
2000	Late	Nx	0-600	216	0.009208	0.074167	0.938815
2001	Early	No	0-600	162	0.97782	4.346929	0.09517
2001	Early	Nx	0-600	162	0.08944	1.795419	0.806651
2001	Late	No	0-600	162	0.582488	1.487943	0.447245
2001	Late	Nx	0-600	162	0.396093	0.978571	0.566635

Table 14: Summary of Insect population structure under natural squash bug infestations regressed by toxin concentration

Year	Stage	Trt	N	R^2	Slope
1999	Eggs	Nx	60	0.2022	-0.42
1999	Nymphs	Nx	60	0.8861	-0.45*
1999	Adults	Nx	60	0.9132	-0.09*
2000	Eggs	No	72	0.4354	-0.25
2000	Eggs	Nx	72	0.5327	-0.23
2000	Nymphs	No	72	0.7364	-0.11
2000	Nymphs	Nx	72	0.4216	-0.09
2000	Adults	No	72	0.6277	-0.06
2000	Adults	Nx	72	0.2738	-0.02
2001	Eggs	No	54	0.8625	-0.40*
2001	Eggs	Nx	54	0.6222	-0.38
2001	Nymphs	No	54	0.8330	-0.07*
2001	Nymphs	Nx	54	0.6643	-0.03
2001	Adults	No	54	0.5427	-0.02
2001	Adults	Nx	54	0.8570	-0.03*

* significant dose – response by regression analysis at P = 0.10

Table 15: Summary of squash bug population structure under field cages regressed by toxin concentration

Year	Stage	Trt	N	R^2	Slope	P Value
Cages	Eggs	No	32	0.5101	-0.13	0.2858
Cages	Eggs	Nx	32	0.1590	-0.22	0.6013
Cages	Nymphs	No	32	0.3046	-0.05	0.4481
Cages	Nymphs	Nx	32	0.4306	-0.05	0.3438
Cages	Adults	No	32	0.5392	-0.01	0.2657
Cages	Adults	Nx	32	0.3243	-0.02	0.4305

* significant dose – response by regression analysis at P = 0.05

CONCLUSION

While only five significant responses were observed, all treatment combinations had a negative slope indicating an overall decline in means with increased doses of the toxin. Field studies recorded larger insect mean densities and had more distinct patterns than caged insects, which had only two adults at the beginning of a short five-week season. Controlling for migration of insects among treatments by using cages did not reduce variation. Unrestricted field populations showed more distinct patterns for comparison between treatments (Appendix Figs 1-5). This may be a result of low insect numbers in cages or reduced spray penetration through the cage screen. At low insect densities, it was hard to differentiate between the concentrations of the toxin used. To broaden our understanding of such interactions between the toxin and the pest at the population level, it would be necessary to either widen the dose range of the toxin or study higher densities of the pest. Field applications of higher doses of the toxin are impractical due to cost and solubility problems especially with neem oil. A more complete model may be developed using a similar range of toxin over an extended crop season to include high late season densities. This will also allow closer examination of the pest over its entire reproductive cycle from when it emerges in the spring to over-wintering in the early fall.

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SUMMARY

This study that includes various experiments discussed in preceding chapters was designed to evaluate the toxicity of neem products to the squash bug. Both laboratory bioassays and field studies were conducted between the summers of 1998 and 2001. Results led to a better understanding of the interactions between the pest and the toxins found in neem. Such an understanding would in turn be used to develop approaches that specifically optimize the effective use of neem and other similar IGR's on the squash bug and other hemipterous insects.

Behavioral studies considered colonization, mating and oviposition, which were differentially affected by the various treatment methods. Adult squash bugs did not show preferential oviposition or colonization among host plant treated with different rates of foliar sprays. Soil treatments, however, had clear dose-response effects on colonization, oviposition and implied feeding intensity as indicated by plant physical appearance at the end of the experimental period. There are indications from literature that systemically accumulated toxins may last several weeks as opposed to topical application (Helson et al., 2001). Topically applied neem is exposed to ultra violet light and therefore easily broken down to less potent products (Jarvis et al., 1998).

Although only implied by the appearance of plant physical condition, the intensity of insect feeding on host plants might be directly affected by neem. This relationship is, however, not easy to establish without an artificial feeding system that offers a precise quantitative method for measuring feeding rates.

Dose-response effects among soil treatments may be attributed to either the higher quantities of toxin used or a more persistent systemic accumulation of the toxin. Soil treatments, therefore, provide a useful option especially with oil based neem formulations that have phytotoxic effects when used directly on foliage especially at high concentrations.

Apart from confirming the efficacy of systemic neem treatments, a major contribution of this study was to establish the effect of neem-based toxins on oviposition. Oviposition was the most sensitive variable for measuring toxicological effects probably due to the occurrence of large numbers of eggs. Mating was inhibited once a single member of a mating pair was treated. None of the literature known to us addresses the occurrence of unfertilized eggs among virgin squash bug females. The occurrence of unfertilized eggs will in turn influence the establishment of insect economic thresholds, which are currently based on egg counts (assuming all eggs are fertile). Moreover, the performance of the egg parasite *Gryon pennsylvanicum* is likely to be affected by the occurrence of unfertilized host eggs. The magnitude of the impact of neem on *G. pennsylvanicum* will depend on the proportion of unfertilized eggs in treated fields and if other IGR's cause similar effects. Such will only be addressed by further studies on this phenomenon.

Research questions raised by findings on fertility status of squash bug eggs, center around a better understanding the oviposition behavior of the egg parasite *G. pennsylvanicum.* First, to identify any possible direct effects of neem on the parasite. Secondly, to study oviposition behavior and establish if the parasite shows preferential oviposition depending on the fertility status of the host's eggs. It is also necessary to find out if the fertility status of host eggs has an effect on sex ratios of the emerging parasites.

Studies documented on chapter five underscored the importance of distinguishing between the effects of azadirachtin and those of other compounds found in neem oil. Often in the literature and professional interactions, "azadirachtin" and "neem" are used synonymously, thus obscuring the fact that neem oil has several compounds other than azadirachtin that are also biologically active and play a critical role in the overall toxicity of the oil. Many toxicological assays based on analytical grade azadirachtin have been evaluated and deductions made on models that use less refined neem formulations which are essentially different in composition. This is more so with physiological studies that often use pure azadirachtin (>95% purity) compared to field studies that use 8% azadirachtin at their best. The availability of neem oil, Neemix 4.5®, and more recently Trilogy ®, will offer more opportunity to study the various groups of neem based compounds especially at the population level. Results from this study will enable researchers and pest managers to develop a criteria for categorizing neem formulations based on their relative composition of azadirachtin and other neem components. While these categories are in no way absolute, we suggest four general groups of neem formulations currently available namely;

i) pure azadirachtin; a highly purified analytical quality product often >90% purity

ii) cold pressed neem oil with unaltered natural composition of chemical components (300 – 10,000 ppm equivalent to 0.03-1% azadirachtin)

iii) azadirachtin enhanced formulations often prepared from technical grade azadirachtin eg Neemix 4.5 ®, Neemazal ®, and Margosan-O ®.

iv) Azadirachtin-depleted formulations with no substantial amounts of azadirachtin eg. Trilogy ®.

These groups represent formulations with different relative compositions of azadirachtin as well as other biologically active components that may also be associated with different levels of activity depending on the pest and mode of application.

Results from field studies involving natural populations of the squash bug show limited dose responses below 600 ppm azadirachtin. It is useful to know that while doubling application rates might also double the cost of control, it has limited increase in marginal suppression of the pest population at the densities found on our fields. The effects of neem are expected to be more distinct after repeated applications as it is slow acting especially considering such a pest with multiple overlapping generations. These effects may vary at higher squash bug densities or even with different pests. Challenges arise when considering methods for sound insecticide resistance management (IRM) that discourage the repeated use of a single pesticide during the crop season.

Using neem in concert with other toxins may constitute a more effective insecticide resistance management strategy. This is because neem has been associated with breaking of insecticide resistance and enhancing activity of other pesticides like RH 2485, which is also an IGR (as earlier discussed). Since the efficacy of systemic treatments has now been established, an early season high dose application may be sufficient followed by a combination of other pesticides during the crop season.

Using cold-pressed neem oil in its natural composition and avoiding processing into commercial products especially for tropical resource-poor communities will be an economically attractive option. This is because the oil may be easily extracted using on-farm hand operated tools making the oil more readily available and substantially lower in cost while it offers control levels comparable to those of the processed formulations. Research on product development of neem formulations should therefore emphasize more on

managing phytotoxicity of the crude product rather than azadirachtin extraction techniques as an approach to cost reduction. From personal communication with industry representatives, there are indications that research in neem products largely emphasizes on formulation of the product. As such, it will be within industry's interests to focus on formulations that target high systemic accumulations of the toxin within the host plant after it has been applied on soil or otherwise.

Neem was registered for use in food crops in the United States and is exempt from tolerance limits due to its low mammalian toxicity as well as its natural plant origin. While not of immediate concern, it will be useful to develop analytical methods for evaluation of trace residue amounts in the environment as well as the edible harvested crop. To date, an azadirachtin antibody for possible use in immunosorbent assays has been developed although no applications have been documented (Schutz et al., 1997). With increasingly strict pesticide safety standards, it might be necessary to develop analytical tools for residue assessment.

From a purely research perspective, developing an analytical tool for evaluating trace amounts of azadirachtin will help to better evaluate application methods and the fate of this compound in both animal and plant tissues as well as the environment. Such a tool will help develop more efficient formulations aimed at maximizing threshold concentrations of the toxin where its effects are optimized.

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APPENDIX

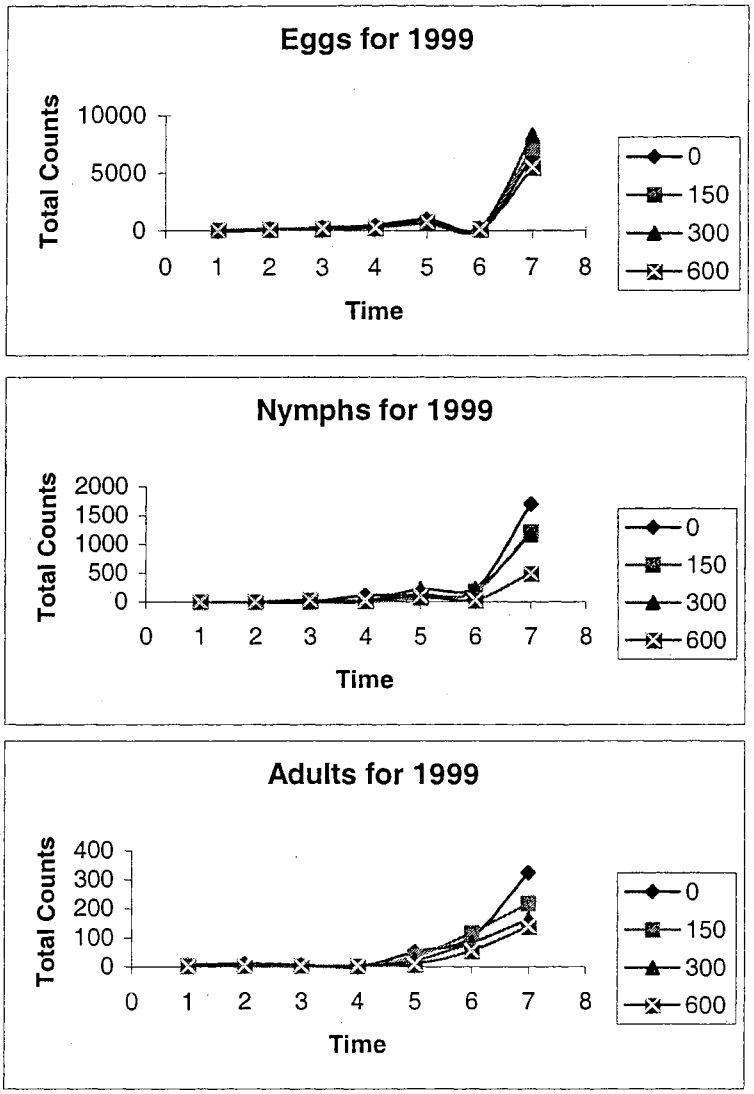

Figure 1: General squash bug distribution over a seven week period under increasing toxin concentrations (0-600ppm) from field applied Neemix 4.5 ® during 1999

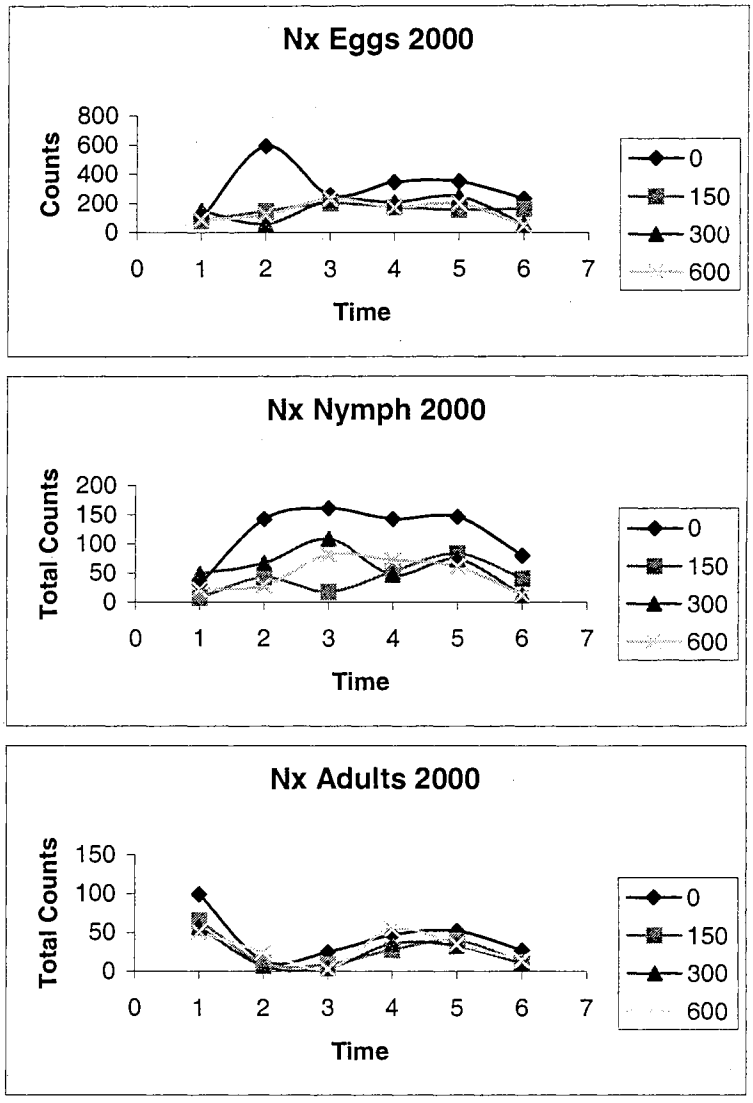

Figure 2: General squash bug distribution over a seven week period under increasing toxin concentrations (0-600ppm) from field applied Neemix 4.5 ® during 2000

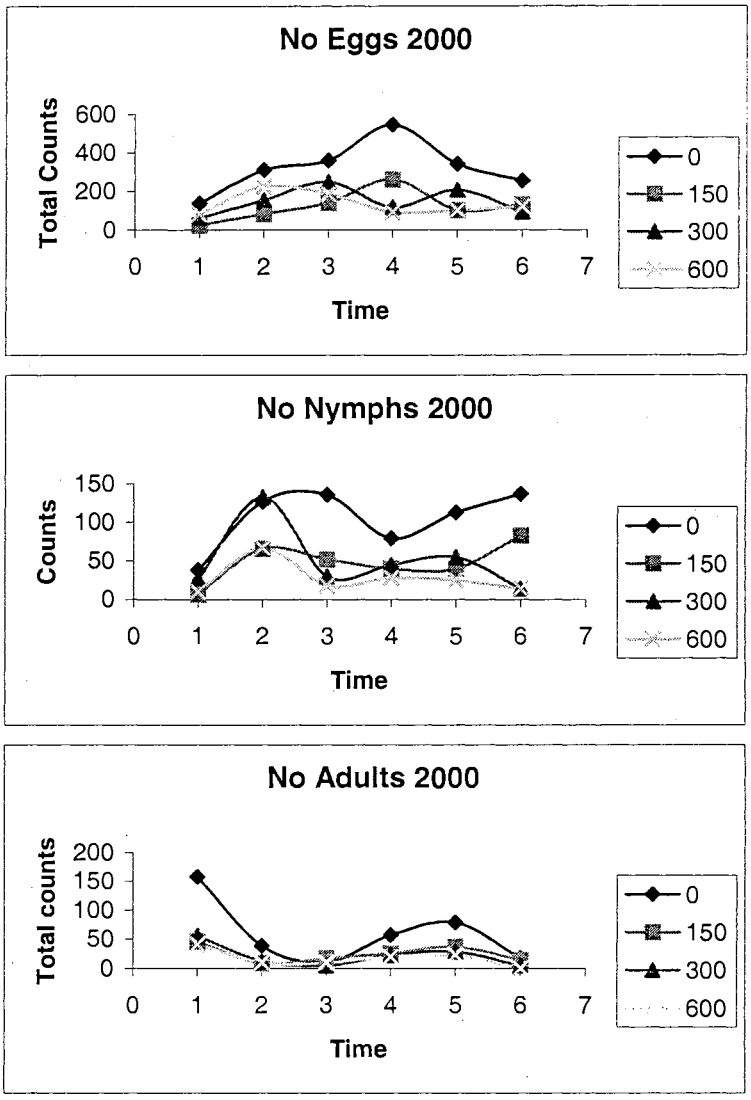

Figure 3: General squash bug distribution over a six week period under increasing toxin concentrations (0-600ppm) from field applied neem oil during 2000

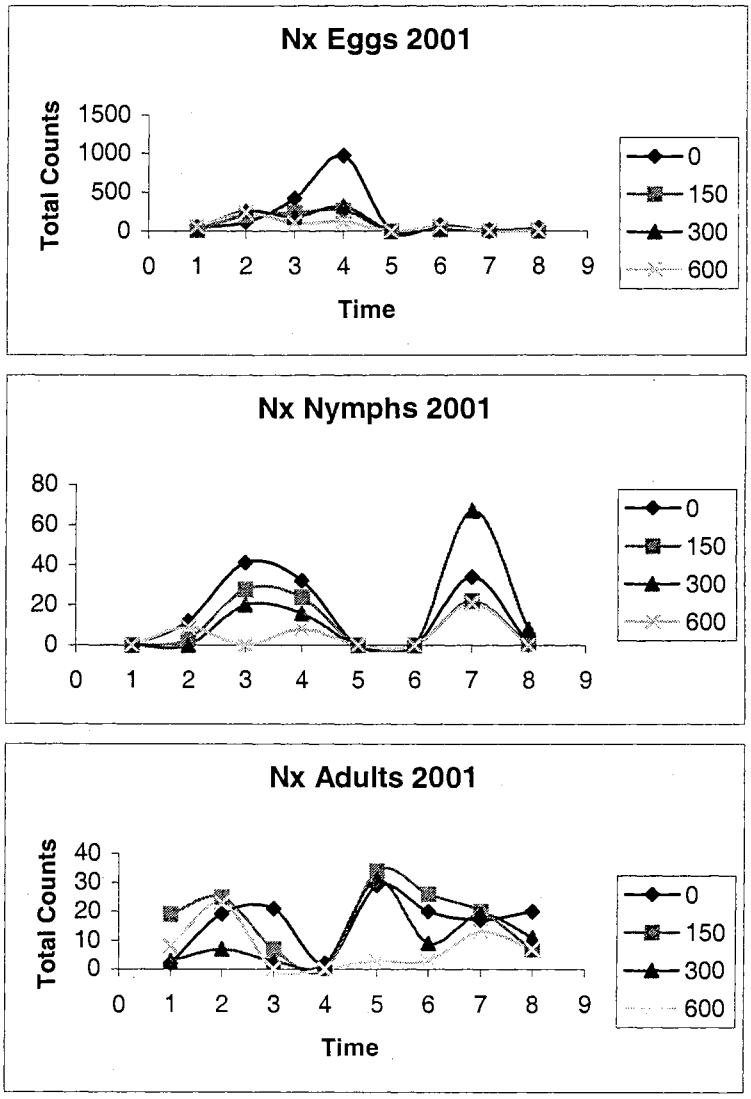

Figure 4: General squash bug distribution over a six week period under increasing toxin concentrations (0-600ppm) from field applied neem oil during 2000

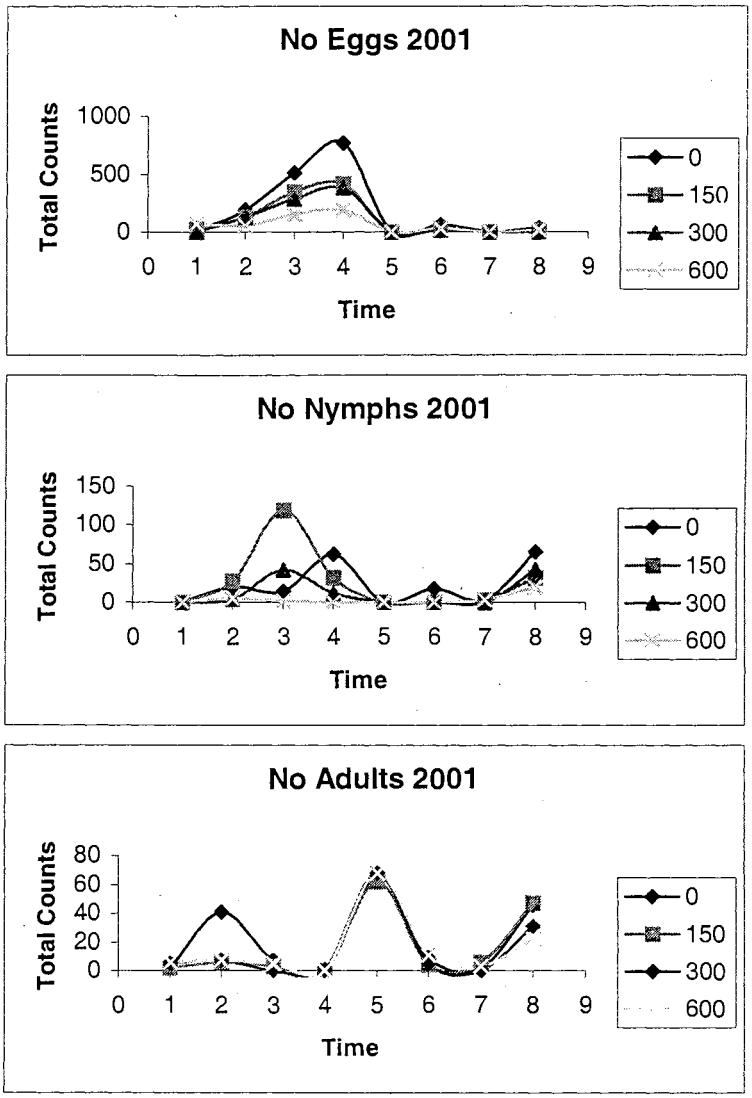

Figure 5: General squash bug distribution over an eight week period under increasing toxin concentrations (0-600ppm) from field applied neem oil during 2001

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