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Effects of Thiamethoxam Seed Treatments on Bean Leaf Beetles

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by

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The increased use of thiamethoxam seed treatments for controlling target pests such as the bean leaf beetle, *Cerotoma trifurcata* (Forster), suggests the need for methods to measure and monitor the development of resistance to these insecticides. Overwintering and F1 bean leaf beetles were collected from alfalfa and soybean fields and used in early growth stage soybean studies to measure toxicity of thiamethoxam both in greenhouse experiments and laboratory bioassays involving exposure to treated foliage. Lethal and sub-lethal effects were detected in both greenhouse and lab bioassays. Lethal concentrations determined from laboratory assays were compared with residues determined from field grown plants that were sampled through the early vegetative stages.

Results of these studies show that thiamethoxam is highly active against adult bean leaf beetles. Commercial rates and bioassay concentrations of thiamethoxam provide effective control causing lethal and sublethal effects. The quantification of insecticide levels in soybean leaves from new nodes over time indicate that thiamethoxam provides control at early vegetative growth stages, but insecticide concentrations fall off as the plant grows and insecticide available for uptake becomes limited. These results provide a foundation for resistance monitoring and detection.

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CHAPTER 1-INTRODUCTION AND LITERATURE REVIEW

SOYBEAN

The soybean (*Glycine max*) is a legume native to East Asia. Soybean varieties are sensitive to photoperiod which influences plant growth and development (Hartwig 1973). Most varieties grown in the United States are adapted for full-season growth and have a range of maturity groups based on length of growing season required.

Soybean growth and development is classified into two categories with sub-classification of reproductive development. Vegetative growth stages occur and the soybean transitions into reproductive stages later in the growing season. Vegetative growth stages are measured by the expansion of cotyledon, unifoliate, and trifoliate leaves from new nodes. Cotyledon leaves are fleshy leaves that first emerge. Unifoliate leaves are the next set of two leaves that arise from the next node on the plant. At every node following, a leaflet containing 3 leaflets known as a trifoliate emerges. This corresponds to a standardized growth stage. Emerging seedlings are at stage VE, the seedlings with open cotyledons are at stage VC, and after the VC stage, each node is counted starting with the unifoliate node (Fehr and Caviness 1977). The reproductive stages include bloom, pod development, seed development, and maturity sub-classification and are designated as R stages.

There are several insects pests of soybeans. Some seasonal pests include *Heliothis zea, Cerotoma trifurcata, Pseudoplusia includes, Aphis glycines, Acrosternum hilare, Euchistus sternum,* and *Dectes texanus* (Higley and Boethel 1994). Insect pests

that cause severe damage, or insects pests that are present year-round, such as *C. trifurcata,* are of high concern for soybean producers.

BEAN LEAF BEETLE

Origin and Importance

The bean leaf beetle, *Cerotoma trifurcata* (Forster) (Coleoptera: Chrysomelidae), is a recognized pest of legumes, primarily cultivated soybeans, *Glycine max* (L.) Merr., across the United States. *Cerotoma trifurcata* is native to North America and is predominately found east of the Rocky Mountains (Kogan and Herzog 1980). The bean leaf beetle (BLB) was historically more common as a soybean pest in the southern states (Kogan and Herzog 1980) before populations expanded their range into North Central and Midwestern states by milder winter temperatures and the expansion of soybean acres (Hammack et al. 2010).

Bean leaf beetle is one of the most important pest species attacking soybeans, and its management is important to soybean growers across the United States. Adult bean leaf beetles feed on soybean leaves resulting in defoliation. Pod feeding is also a significant concern for soybean growers, which can cause yield loss and increase the risk of disease, as diseases may be transmitted easier through the holes and weakness in the pod created by BLB feeding. Safeguarding soybeans from yield loss may require management of bean leaf beetles from the time they colonize a field to later in the season when they feed on pods.

There are limited options available for bean leaf beetle management, and chemical control has been the primary method of control. Many insecticides have been used to manage bean leaf beetles. Although insecticide resistance has not been

documented, BLB susceptibility to pyrethroid insecticides is lower in the Mississippi and Louisiana Delta regions than any other region in the United States (Musser et al. 2011). Pyrethroids are frequently applied to soybeans in these regions to manage stink bug populations, and bean leaf beetles are not the target of these applications. After reports in Mississippi about unsatisfactory control of bean leaf beetles with pyrethroids, studies to monitor for pyrethroid resistance were initiated and reduced susceptibility was reported (Musser et al. 2011). Today, neonicotinoid insecticides are the most commonly used insecticide in the Midwest for management of bean leaf beetles, primarily through seed treatments, but also through foliar application. Baseline susceptibility of bean leaf beetles to neonicotinoid insecticides has not been determined.

Life History

Bean leaf beetle adults (Coleoptera: Chrysomelidae) are small, sub-oval, and convex (Kogan and Herzog 1980). They range in coloration from red, orange, tan, yellow, or gray. Many beetles have spotted markings on their elytra, but not all bean leaf beetles exhibit these spots. The identifying characteristic of the bean leaf beetle is a black triangle at the base of its forewings (Kogan and Herzog 1980). Teneral adults can be identified by their light gray color.

The bean leaf beetle populates a large portion of the United States east of the Rocky Mountains. In southern states, the bean leaf beetle completes three generations per year (Kogan and Herzog 1980). The number of generations decreases the further north the beetles are found. Two generations are found primarily across the middle of the United States (Witkowski and Echtenkamp 1996). Single generations occur in northern

states including South Dakota and Minnesota (Loughran and Ragsdale 1986). In Nebraska, bean leaf beetles emerge from overwintering sites from mid-April to May. They can typically be found first in various legumes, such as alfalfa, but then colonize and oviposit in emerging soybeans. First generation beetles emerge in mid-July. A second generation emerges in late August to September and overwinters in wooded areas, under vegetation, and in residue left in soybean fields (Carrillo et al. 2005).

Bean leaf beetles rely on survival of overwintering adults to colonize fields the following year (Lam and Pedigo 2000). Adult females that overwinter are characteristically unmated, have small, immature ovaries, and high fat content (Lam and Pedigo 2000). Mating occurs after emergence from overwintering sites in alternative hosts until soybeans plants emerge. Mated females colonize early emerging soybean fields and deposit eggs within a 7.6 cm circumference around a soybean stem and to a maximum depth of 3.8 cm below the soil surface (Waldbauer and Kogan 1973, 1975). Eggs are red-orange, oval in shape, and about 0.08 cm long. There are three instars that take 15-16 days from first instar to third instar at the temperature of 26.2°C (Kogan and Herzog 1980). The larvae are white and cylindrical. Pupae are also white and resemble the adult in size and shape.

Impact on Soybean Production

Bean leaf beetles primarily feed on soybeans in the United States. Before soybeans emerge, native and cultivated legumes serve as alternative host plants. *Desmodium, Lespedeza,* and *Strophostyles* all serve as native hosts (Kogan and Herzog 1980), and cultivated alfalfa, *Medicago sativa*, and clovers, *Trifoliums*, are often

abundant and available in many soybean growing areas. Bean leaf beetles have also been documented infesting other crops including pumpkins, squash, and cucumber in Minnesota (Koch et al. 2004), but serious infestations have not been regularly reported.

Movement into soybeans occurs just as soybeans emerge, or shortly after emergence, and as they continue to grow. Often the injury at early growth stages through the vegetative stages is not high enough to contribute to significant stand loss or yield loss. Simulated injury resembling bean leaf beetle defoliation at a seedling stage resulted in a 12% yield decrease as seedling defoliation reached 68% (Hunt et al. 1994). Economic injury levels are sometimes reached late-season and are associated with pod injury. Pod injury includes removal of the pod-wall down to the endocarp, and peduncle feeding, which may cause pods to dislodge from plants. Both feeding patterns may result in reduced yields (Smelsler and Pedigo 1992). Adult beetle feeding is associated with above ground damage, while larvae feed on roots. Larval damage has not been studied extensively, and the behavior of the subterranean larval stage remains poorly understood (Lundgren and Riedell 2008). Larval injury has not been documented in the extent it may contribute to yield loss.

The bean leaf beetle is a pest of soybeans not only because of defoliation and pod injury, but also through disease transmission. The bean leaf beetle is the main vector of Bean Pod Mottle Virus (BPMV) (Krell et al. 2004). BPMV can cause yield loss and reduced seed quality (Krell et al. 2004). Seed quality is reduced by the purple mottling caused by the disease. This in turn decreases the market value of the soybean grain. The virus affects soybean production regions in the North Central states, as well in the Southeastern states. A major concern about BPMV is that there are no proven

management practices recommended to reduce the BPMV occurrence in soybean (Krell et al. 2004), although management of the bean leaf beetle has been recommended as a method of BPMV management.

Management

Bean leaf beetle management is accomplished through cultural and chemical control. The planting date of soybeans can have a significant impact on the number of beetles that colonize a soybean field. Overwintered beetles are highly attracted to the earliest planted soybean fields (Witkowski and Echtenkamp 1996), and delayed planting of soybeans in an area where other fields have already been planted can reduce the number of colonizing beetles in the later planted field. Seasonal abundance and timing of adult bean leaf beetles has been suggested to be influenced by the soybean planting date (Waldbauer and Kogan 1976). Successive generation emergence will be influenced by the timing of spring colonization in soybean fields.

Insecticides are most often used to manage bean leaf beetle populations. Granular application at planting has been popular in the past, but today foliar insecticide applications and insecticidal seed treatments are the primary methods of bean leaf beetle management (Krell et al. 2004). Neonicotinoids are commonly used insecticides for both application methods (Johnson et al. 2008). Foliar applications are applied after soybean emergence and target the management of adults, as larval impact is not well known. Seed treatments are applied before planting and colonization, resulting in potential control for adults and larvae throughout the growing season. Control of early colonizing

beetles may have an impact on the population of successive generations emerging the same growing season (Lam et al. 2001)..

NEONICOTINOIDS

Neonicotinoids are a class of insecticides that have played a major role in pest management for two decades. The popularity of neonicotinoids has driven this class to global sales from approximately US $1.56 billion in 2006, representing nearly 17% of the global insecticide market, to 24% of the global insecticide market by 2008 (Pandey et al. 2009, Jeschke et al. 2011). Neonicotinoids have provided an important alternative to organophosphates and carbamates for more effective control against resistant populations and because of strong evidence of low mammalian toxicity.

History

The first neonicotinoid prototype, nithiazine, was formed from a lead compound by the former Shell Development Company in Modesto, California in the early 1970's (Tomizawa and Casida 2003). The first prototype was a nitromethylene heterocyclic compound that exhibited high photolability and had high insecticidal activity against houseflies and corn earworm larvae (Tomizawa and Casida 2003, 2005). Derivatives with lower photosensitivity were needed for nithiazine to be an effective crop protection insecticide.

In 1985, imidacloprid was developed by replacement of nitromethylene by nitroimine on the second prototype (Kagabu and Akagi 1997). Imidacloprid was the first commercial product of neonicotinoid insecticides. Imidacloprid was selected out of a

field of possible compounds that rated equally active, because imidacloprid's stability surpassed the other compounds and can be measured through UV absorption wavelengths, bond orders, and bond energies (Kagabu and Akagi 1997). Imidacloprid continues to be by far the leading product in the neonicotinoid insecticide class (Pandey et al. 2009) with sales of US $1.09 billion and 41.5% of the neonicotinoid market (Jeschke et al. 2011). Thiamethoxam, a second generation neonicotinoid, is easily synthesized worldwide and is the second largest neonicotinoid used with sales in the US of $627 million (Maienfisch et al. 2001, Jeschke et al. 2011).

Several other neonicotinoids have entered the market, including thiacloprid in 1985, acyclics nitenpyram in 1988, acetamiprid and clothianidin in 1989, thiamethoxam in 1992, and dinotefuran in 1994 (Tomizawa and Casida 2005). These chemicals vary in chemical structure which influences their toxicity to insects and narrows their selectivity toward non-target insects. Crop protection continues to be the major use for neonicotinoids, but new markets have emerged in recent years for control of urban, turfgrass, and veterinary pests (Tomizawa and Casida 2005). Target insect groups of neonicotinoids include Homoptera, Lepidoptera (Jeschke and Nauen 2008), Hemiptera (aphids, whiteflies, and planthoppers), and some members within the order Coleoptera (Nauen and Denholm 2005).

Chemistry

Neonicotinoids are a class of insecticides that share similar chemistry. First generation neonicotinoids imidacloprid, nitenpyram and acetamiprid have a 6-chloropyrid-3-ylmethyl moiety as a heterocyclic group (Maienfisch et al. 2001b). Second

generation neonicotinoids thiacloprid, thiamethoxam, clothianidin and dinotefuran belong to the subclass of thianicotinyl compounds (Maienfisch et al. 2001b).

Neonicotinoids are effective insecticides because of their chemical and physical properties. Thiamethoxam, like other neonicotinoids, is highly water soluble at 4.1 g litre^{-1} at 25° C and has a low molecular mass (Maienfisch et al. 2001a). Thiamethoxam is also very stable at neutral and more acidic pH values of 7 and 5, respectively, which produces an estimated half life of greater than a year at pH 5 and almost one year (200-300 days) at pH 7 (Maienfisch et al. 2001a). Imidacloprid has a half-life value reported to be higher at a pH of 9 at 41.6 days when tested for persistence in liquid formulation in an aqueous concentration and a half-life of 36.2 days under an acidic condition (Sarkar et al. 1999).

The neonicotinoids are similar to nicotinoids and other alkaloids (Tomizawa and Casida 2003) in chemistry and mode of action. Plant derived nicotinoids were first examined in the 1960's, but the derivatives had no practical insecticidal use (Yamamota and Casida 1999). Highly toxic nicotinoids have a 3-pyridylmethylamine moiety with a highly basic nitrogen atom, a base model for neonicotinoids (Yamamota and Casida 1999). Nicotinoids are very effective, but due to high mammalian toxicity of nicotine, this class was never widely used (Yu 2008). Nicotine has provided a model for several highly effective insecticides in the neonicotinoid class.

Mode of Action

Neonicotinoids are agonists of insect nicotinic acetylcholine receptors (nAChR) and act by mimicking the excitatory neurotransmitter acetylcholine (Yu 2008). The

nAChRs are found in the insect nervous system on post-synaptic nerve terminals, and on the cell bodies of ineterneurons, motor neurons, and sensory neurons (Yu 2008). Neonicotinoids act as neurotoxins by targeting these nAChRs primarily at postsynaptic membranes in insects (Tomizawa and Casida 2005). When neonicotinoids are introduced into the insect body and reach the nAChR, activation occurs and causes an increase in sodium ion conductance and depolarization of the post-synaptic membrane which triggers an action potential to occur (Yu 2008). Activation under normal physiological conditions occurs when acetylcholine (ACh) binds to nAChRs. Regulation of excitatory synaptic transmission occurs through the enzyme acetylcholinesterase, which hydrolyzes the Ach and prevents further stimulation. An insect intoxicated with a neonicotinoid is left in a constantly excitable state from the persistent activation of nAChRs, and overstimulation of the cholinergic synapse leads to hyperexcitation, convulsion, paralysis, and death of the insect (Yu 2008).

Application methods and Efficacy Spectrum

The neonicotinoids' physiochemical properties have played an important role in the success of this insecticide class. Neonicotinoids are very versatile chemicals. They are applied as foliar sprays, soil applications, and seed treatments. Almost 60% of all applications of neonicotinoids are via soil applications and seed treatments (Jeschke et al. 2011). Other application methods include drip or drench systems through irrigation water, seedling box application, soil drenches, direct injection into tree trunks or buds, and applications onto the ground around the base of tree trunks in orchards (Jeschke et al. 2011). A further benefit of neonicotinoids is the broad variety of crops that these insecticides can be applied to. Neonicotinoids target both sucking insects and some

chewing species, and have high efficacy against aphids, whiteflies, leafhoppers and planthoppers (Jeschke et al. 2011). Beetles, weevils, wireworms, and lepidopteran pests can also be targeted in a range of crops (Jeschke et al. 2011).

In addition to agricultural use, neonicotinoids are used in nonagricultural sectors. They can be used for household, lawn, and garden control of insect pests. Bait gels using thiamethoxam as an active ingredient are used to control cockroaches and ants (Jeschke et al. 2011). Use of neonicotinoids also reaches to the animal health sector. Imidacloprid is used to help control pests such as fleas, flies, and lice on pets and livestock (Jeschke et al. 2011).

Biotransformation

The EPA requires studies on metabolism in animals such as rats, lactating goats, and laying hens along with metabolism in crops as part of registration for the insecticides (Tomizawa and Casida 2005). Mammals metabolize imidacloprid, thiacloprid, and clothianidin slowly, and with high water solubility, an oral dose of neonicotinoids is often excreted unchanged in urine (Tomizawa and Casida 2005). Neonicotinoids are metabolized in animals at multiple sites, and degraded to numerous metabolites (Tomizawa and Casida 2003). Photochemical reactions also produce the same metabolic products (Tomizawa and Casida 2003, 2005). Oxidation of nitromethylene carbon in nithianzine is considered a detoxification mechanism (Tomizawa and Casida 2005). Imidacloprid has a nitroguanidine moiety which is a common site for metabolic cleavage to guanidine and reduction to nitrosoguanidine and aminoguanidine derivatives (Kanne et al. 2005).

Bioactivation reactions of neonicotinoids are relative to insect potency and insecticidal activity (Tomizawa and Casida 2003). Bioactivation reactions involving imidacloprid result in active monohydroxy and dehydro metabolites in the imidazolidine moiety (Tomizawa and Casida 2003). Bioactivation resulting in an N-desmethyl derivative with high affinity and potency is common in thiamethoxam and nitenpyram (Tomizawa and Casida 2003). Metabolism of thiamethoxam also produces clothiandian as a primary product by ring methylene hydroxylation, and N-demethylation is needed for a secondary reaction and detoxification (Tomizawa and Casida 2003, 2005).

Vertebrate Toxicity

The diversity in mammalian nAChR's is important in understanding the reduced neonicotinoid mammalian toxicity (Tomizawa and Casida 2005). The vertebrate nAChR is an agonist-gated ion channel consisting of several diverse sub-type combinations of sub-units (Tomizawa and Casida 2005). Neonicotinoids have little effect on vertebrate peripheral nAChR showing differential subtype selectivity in vertebrate nAChRs (Tomizawa and Casida 2005). The difference in target site interaction between vertebrates and insects has attributed the safety and effectiveness of neonicotinoid insecticides (Jeschke and Nauen 2008). There are strong differences in the receptor binding potency in imidacloprid of 100-fold between insect and mammalian acetylcholine receptors selecting for insect sensitivity (Yamamoto and Casida 1999).

Resistance

Imidacloprid was patented in 1985, and resistance has been slow to develop, although the documentation of resistant insect populations suggests a threat to the use of

the neonicotinoid compounds (Jones and Brown 2007). Studies on an important rice pest, *Nilaparvata lugens* Stal, have identified a target site resistance to a neonicotinoid insecticide (Jones and Brown 2007).

Three types of resistance to imidacloprid have been observed under field conditions (Tomizawa and Casida 2003). Insects feeding on tobacco demonstrate a cross-resistance associated with evolutionary selection for nicotine tolerance shown in strains of *Myzus persicae* of 192-fold to imidacloprid and greater than 22-fold resistance to nicotine compared to susceptible populations (Tomizawa and Casida 2003). *M. persicae* also had up to 6-fold resistance to imidacloprid and clothianidin seed treated sugar beets in laboratory bioassays, in which this population also showed an increased survival and reproduction on cabbage and tobacco treated with lower than recommended rates of imidacloprid (Nauen and Denholm 2005). This resistance is likely due to altered nAChR sensitivity to nicotine and imidacloprid (Tomizawa and Casida 2003).

Previous exposure to pyrethroid and organophosphate insecticides can cause resistance in the field. The Colorado potato beetle, *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae), has exhibited resistance of 100-fold in adults developing from pre-existing metabolic mechanisms that were selected by previous exposure to other insecticides (Tomizawa and Casida 2003). In areas of New York, the Colorado potato beetle has been exposed to multiple chemical classes and has developed resistance to those chemicals, as well as resistance to neonicotinoids (Nauen and Denholm 2005). Imidacloprid resistance was studied a few years after imidacloprid was initially used in this area, and results of these studies indicated that adult Colorado potato beetle populations were 100-fold resistant the first year, and 150-fold in adults from a second

study where topical application methods were used in a bioassay (Nauen and Denholm 2005).

Whiteflies are another major pest in many crops world-wide and have developed resistance to several insecticides (Nauen and Denholm 2005). A whitefly neonicotinoid resistant biotype, Q-type, was identified in southern Spain in 1994 where there was overuse of chemicals in a continuous growing season with favorable climatic conditions for whitefly development (Nauen and Denholm 2005). This population demonstrated significant levels of reduced mortality to imidacloprid at a recommended concentration in a systemic bioassay (Nauen and Denholm 2005). This is biotype selective as there is also a B-type strain of whitefly that has not shown a high incidence of resistance. The B-type is still controllable in Arizona and California where neonicotinoids are used on many vegetable crops (Nauen and Denholm 2005).

Cross Resistance

Cross resistance to different neonicotinoids may occur, but if no cross resistance is demonstrated to two neonicotinoids, selection for different resistant traits is the most likely explanation (Jeschke and Nauen 2008). Whiteflies exposed to thiamethoxam under laboratory conditions had almost no cross resistance to acetamiprid, but had a 500-fold cross resistance to thiamethoxam at a second exposure when first being selected by acetamiprid (Jeschke and Nauen 2008). Both neonicotinoids thiamethoxam and acetamiprid are converted to a clothianidin metabolite, but activation and detoxification pathways differ, allowing for different resistance mechanisms (Jeschke and Nauen 2008).

A recently reported case of cross resistance amongst neonicotinoids has occurred in Australia. The cotton or melon aphid, *Aphis gossypii* Glover, is the insect of concern. An increase in the frequency of neonicotinoid resistance occurred from 2007-2008 and an increase from 13% to 82% was observed from 2008-2009 (Herron and Wilson 2011). These strains were found across all of the regions surveyed, indicating widespread resistance. The aphids were resistant to acetamiprid, clothianidin, and thiamethoxam (Herron and Wilson 2011). The development of resistance is suggested to have arisen from the overall neonicotinoid selection pressure without use of alternative chemical classes (Herron and Wilson 2011).

Baseline susceptibility is used to determine an organisms' sensitivity to an insecticide before and after the commercialization of a product (e.g. insecticide). Western corn rootworm, a common pest in the United States, is mainly controlled by transgenic crops, but all Bt protected corn seed is also treated with a low dose of neonicotinoids, which may lend some added help in management of western corn rootworm (Magalhaes et al. 2007). Western corn rootworm populations from Potter Co., SD and York Co., NE in 2005 and 2006 were all very susceptible to exposure to clothianidin treated filter paper (Magalhaes et al. 2007); however, a high selective pressure could lead to neonicotinoid resistant populations (Magalhaes et al. 2007)..

SEED TREATMENT

Treating seed for protection against disease has been practiced for hundreds of years, leading to the current use of fungicides and insecticides. Seed treatments intended

for plant protection from insects are effective when an insecticide possesses the appropriate systemic properties.

The physical and chemical properties of neonicotinoids make this class of insecticides a good option for use as a systemic insecticide through application as a seed treatment. Soil drench application, foliar application, and liquid and granular formulations are also effective methods to use systemic insecticides (Nauen and Denholm 2005). Currently, all commercially available systemic insecticides are one-way systemics being translocated in the plant xylem after root uptake, and they continue to move through the phloem (Nauen et al. 2008). A low molecular mass, relatively high water solubility, and no dissociation in a pH range of 2 to 12 allows rapid and efficient uptake in plants via xylem transport (Maienfisch et al. 2001a). Neonicotinoid uptake in spinach increased for ten days at 100 ppm solutions with thiamethoxam, demonstrating uptake that is slower than acetamiprid, although thiamethoxam and clothiandian showed similar uptake curves (Ford and Casida 2008). Persistence of neonicotinoids in spinach is variable for each compound. Imidacloprid dissipated with a half-life of six days, but this low half-life rate cannot be assumed for all crops, as there is excellent residual activity in some crops, such as rice (Ford and Casida 2008).

Imidacloprid and thiamethoxam applied as seed treatments move within the vascular tubes of soybean plants, which allows for translocation within the plant until the neonicotinoids are taken up at various sites within plant material (Magalhaes et al. 2009). New leaves express lower concentrations of imidacloprid or thiamethoxam, as the older leaves have benefited from the available access to the seed treatment in the soil (Magalhaes et al. 2009). In this particular study, thiamethoxam was detected at higher

levels for a longer period, indicating that imidacloprid may be metabolized in the soybean faster than thiamethoxam (Magalhaes et al. 2009).

Plant Metabolism

The neonicotinoids imidacloprid, thiamethoxam, and clothianidin are used as systemic insecticides (Ford et al. 2010). Hydroxylation, oxidation, reduction, and glutathione conjugation are important biotransformations in plant metabolism of neonicotinoids (Ford and Casida 2008). Imidacloprid and clothianidin undergo oxidative metabolism that cleaves 6-chloropyridinyl-3-carboxylic acid (CPA) and 2-cholorothiazolyl-5-carboxylic acid (CTA) (Ford et al. 2010).

Metabolism of neonicotinoids can sometimes enhance plant growth, vigor, drought tolerance, and protect against abiotic and biotic stress (Ford and Casida 2008, Ford et al. 2011). Enhanced resistance against microbial pathogens has been associated with the imidacloprid metabolite CPA (Ford et al. 2010). Imidacloprid and clothianidin both activate salicylate-associated plant defense responses that are known to increase plant defense against pathogens and modulate abiotic stress responses in *Arabidopsis* (Ford et al. 2010). Corn was documented to have a greater leaf area index and mean growth rate than the control at specific vegetative stages when treated with clothianidin in a study in New York over 2004 and 2005 (Cox et al. 2007).

Neonicotinoids may also alter plant response in a negative way. Soybean seedlings treated hydroponically with the neonicotinoids imidacloprid, acetamiprid, thiamethoxam, thiacloprid, and clothianidin induced peroxidative damage and foliar lesions (Ford et al. 2011). Nitenpyram and dinotefuran did not cause oxidative damage.

Soybeans are reported to be the most sensitive crop examined, compared to spinach, grape, cotton, and corn. Lesions on the latter plants were not induced, and several studies have supported beneficial physiological effects in some of these plants (Ford et al. 2011, Cox et al. 2007, Ford et al. 2010).

Environmental Impacts

As with any pesticide, certain caution in regard to use of a compound must be taken. Environmental benefits of choosing seed treatment application of neonicotinoids include target specificity and reduction in the amount of insecticides applied in the environment. Seed treatments are taken up within the plant and translocated systemically, allowing specific pests to be targeted. Pests that chew or suck fluids directly from a treated plant are targeted, while beneficial insects that do not feed on the plant avoid exposure. This specific application toward a target pest may also decrease the amount of insecticide applied in a field. With broadcast foliar sprays, less than 1% of the insecticide actually reaches a target pest (Yadav 2010). Foliar canopies, pest position, application method, and drift can be factors in this very low efficiency of exposure. With areal applications under ideal conditions, less than 50% of the pesticide applied reaches the target crops, and this is reduced to less than 25% when ultra-low-volume sprays are used by areal applications under ideal conditions (Yadav 2010). Insecticidal seed treatments are most commonly neonicotinoids that have low residual properties, which is a benefit for the environment as well.

Increased environmental risk is also a possibility with the use of seed treatments. Recently, the increasing occurrence of colony collapse disorder in honey bees prodded

questioning if the cause was related to the use of neonicotinoid seed treatments (Girolami et al. 2009). Guttation, the excretion of xylem fluids at leaf margins in the form of droplets, from neonicotinoid treated plants occurs abundantly for the first 3 weeks of corn plant growth and has been documented to contain amounts of insecticide higher than 10 mg/L, which could cause death of bees after consumption of the droplets (Girolami et al. 2009). The very valuable physical and chemical properties that make neonicotinoids an excellent systemic can also harm non-target bees that feed on guttation drops on corn resulting in paralysis, un-coordination, and death (Girolami et al. 2009). Neonicotinoids have also been recently reported to harm and kill honey bees near agricultural fields. The potential for greatest exposure occurs during planting time, when neonicotinoids in waste talc is exhausted into the environment from the planting equipment (Krupke et al. 2012). Neonicotinoid residues were found in pollen collected from maize planted with a seed treatment which can be transported back to the honey bee hive (Krupke et al. 2012). Residue levels were recorded at levels similar to those that have been shown to impair pollinator health (Krupke et al. 2012), which contributes concern in the environmental risk of using insecticidal seed treatments.

Justification and Objectives

Neonicotinoid insecticides have provided crop producers the options of pest control with seed treatments and follow-up foliar applications targeting leaf and phloem feeding insects. After the registration of seed treatments for soybeans, planting of thiamethoxam treated seeds has increased tremendously. However, the short and long-term effects on insect pest populations from planting thiamethoxam treated soybeans continuously are unknown. Continuous exposure of insect pests to plants systemically

translocating thiamethoxam poses a risk for the development of resistance to neonicotinoids. These risks are even greater with the combined use of foliar sprays. Greenhouse efficacy, reliable bioassay methods, establishing a LC_{50} for natural populations, and determining the concentrations of thiamethoxam and clothianidin, an active metabolite of thiamethoxam, available within a soybean plant as it develops are essential to resistance management programs. The overall objective of this study was to develop methods that could be utilized to monitor bean leaf beetle resistance to neonicotinoids and to quantify thiamethoxam and clothianidin within early growth stage soybean plants. Detailed objectives included:

1. Determine stage-specific effects of neonicotinoid seed treatments for soybeans on the bean leaf beetle.
2. Develop bioassay methods (e.g. sub-lethal, defoliation, mortality) for determining bean leaf beetle susceptibility to neonicotinoids.
3. Quantify the relative concentrations of thiamethoxam and clothianidin in treated soybean plant tissue.

CHAPTER 2-MATERIALS AND METHODS

Efficacy of commercial rates of thiamethoxam soybean seed treatment to control bean leaf beetle.

The study was conducted in **a** greenhouse facility located at the University of Nebraska – Lincoln, Lincoln, NE. Greenhouse conditions were maintained at 24-27°C with a photoperiod of 16:8 h (L:D). Soybean variety NK2752R was planted in 15.2 cm pots containing a soil mixture of peat-perlite-vermicultie-soil mix in 34, 31, 31, 4 percent ratios, respectively. Three seeds were planted per pot, which were thinned to one plant per pot upon soybean emergence. A randomized complete block design was used. There were two planting dates in order to have two seedling stages (VE and VC as reported by Fehr and Caviness 1977). The first planting date was May 10, 2011, and the second planting date was May 14, 2011. At each planting date, 40 pots were planted. There were two treatments; 20 pots of untreated soybean seed with beetles (control) and 20 pots of commercial rate thiamethoxam with fungicides mefenoxam and fludioxonil (3.0 fl oz per 100 lb seed or 0.0762 mg AI per seed) treated seed with beetles.

Bean leaf beetles were collected on May 10, 2011 from an uncut alfalfa field in Gage County, NE. Beetles were collected using the "Lazy 8" sweep net method (Kogan and Herzog 1980) along the edge of the field. Beetles were separated from other insects using an aspirator and placed in Tupperware (modified with two screens glued over 1 inch round holes in the lid for air exchange). Cut alfalfa was placed in the containers to provide a food and moisture source for the beetles. The alfalfa was switched out when containers were cleaned daily to remove frass and moisture build-up. Sweet clover was

used as an alternative forage. Beetles were held in a growth chamber at 14°C to limit eating and reproduction. Beetles were sexed and separated by examining the frons; females have black frons and males have light brown frons (Figure 1.) from John Lundgren, USDA-ARS, North Central Agricultural Research Laboratory, Brookings, SD).

When the soybeans reached VE and VC (first and second planting date, respectively), cages were placed over each soybean plant. Cages were made of cylinders of 0.01 cm gauge plastic to fit into each pot. Organdy fabric was used to cover windows in the cylinders for air movement. The cylinders were held together with polycarbonate cement adhesive.

Bean leaf beetles were removed from the growth chamber and separated into cups, each with three females and two males. After 24 hours of starvation, beetles were released into the caged soybeans. The top of the cages were also covered with organdy fabric and secured with large rubber bands.

At 24 hours after infestation, the first mortality data were recorded. Beetles that were not moving and were on their elytra were recorded as dead. Morbid beetles (on their backs and slight leg or antennae movement) were also reported as dead. Live beetles (walking around, standing upright, or observed feeding) were recorded. If all 5 beetles could not be observed, missing beetles were recorded. Data were also recorded at 48 hours post infestation. At this time, cages were removed and dead beetles were accounted for in cracks and crevices in the soil and along the cage walls (previously missing beetles).

The day after beetles were counted and removed from the soybeans, the plants were harvested with a scalpel at the soil surface. Soybeans were placed in a plastic bag in a cooler with ice for transport back to the laboratory. Soybean images of whole plants were scanned into a computer as TIFF files using a 0.5 x 0.5 cm grid paper positioned behind the soybean plant. Defoliation was analyzed using methods described by O'Neal et al. 2002. ImageJ (Wayne Rasband, National Institutes of Health, USA),was used to digitally measure defoliation.

Mortality and defoliation data were analyzed by one-way analysis of variance (ANOVA) using PROC GLIMMIX procedure (SAS Institute 9.2 2011). If the treatment effect were significantly different ($P<0.05$) by ANOVA, then Fisher's protected least significant difference (LSD) was performed to identify differences among means (PROC GLIMMIX) with confidence intervals.

Bean leaf beetle response to lethal and sublethal doses of thiamethoxam insecticide dilutions.

Untreated soybeans were planted and grown in a greenhouse using the same methods as described in the Greenhouse Efficacy Study. However, soybeans were grown to at least the V1 stage, with one fully expanded trifoliate, and were not thinned to one plant per pot, allowing four soybeans to be grown in one 15.2 cm pot. A single trifoliate with petiole attached was excised from the soybean plant at the node. The trifoliates were placed in a plastic Ziploc bag in a cooler with ice for transport back to the lab.

Preliminary bioassays were performed to determine an appropriate range of insecticide concentrations. Technical grade thiamethoxam (99.5%) was purchased from

Chem Service Inc. (West Chester, PA) and stored at -20° C. A stock solution was prepared (1 µg thiamethoxam/ml acetone) and used to prepare the concentrations for the systemic bioassay: a 0.5 µg/mL dilution, a 0.1 µg/mL dilution, a 0.05 µg/mL dilution, and a 0.01 µg/mL dilution. The insecticide dilutions were used to prepare five treatments and the control: 1000 ng/mL, 500 ng/mL, 100 ng/mL, 50 ng/mL, 10 ng/mL, and 0 ng/mL. The 0 ng/mL was used as the control. For each of the solutions, 100 µL of the preceding dilutions was added to 100 mL of double distilled water in an amber glass vial. Each dilution prep was then shaken on a small centrifuge.

For the first bioassay**,** beetles were collected at the same time intervals using the methods outlined in the Greenhouse Efficacy Study. For the following bioassays, collections were made as the first generation (F1) beetles began to emerge in soybean fields. Collection in soybean fields in southeastern Nebraska in Butler County were made using a single row cross sweep with a net (Kogan and Herzog 1980) in late July. Beetles were provided with soybean leaves upon collection and for transfer back to the laboratory and were held under the same conditions as described in the Greenhouse Efficacy Study until needed for each bioassay.

A complete randomized block design with 10 replications was used for the bioassays. Six mL (16 mm x 50 mm) glass shell vials were glued into place with hot glue in the bottom right corner of each cell in a plastic tray consisting of 8 separate cells (10 cm wide by 8 cm depth) (C-D International, Pitman, NJ). Ten plastic trays were used as replications, with each cell as an experimental unit. The separate insecticide treatments were then dispensed in 5 mL volumes into the vials and covered with either rubber caps with a hole in the middle or Parafilm® with a slit cut in the center. Both were used to

prevent beetles from falling into the solutions and to minimize evaporation. The excised soybean petioles were inserted into the holes so that the end of the petiole was immersed in the treatment solutions. Soybeans were allowed to regain turgidity for at least 15 hours before beetles were released into each treatment cell.

Beetles were sexed, separated, and starved for 24 hours. Using ratios based on the sample population collected, four females and one male were released into each cell of the trays. Adhesive plastic covers were placed on the top of the cells to prevent beetle escape. Mortality data were taken at 24 hour intervals up to 96 hours. Beetles were considered dead when they were on their elytra and no movement was detected. Beetles that exhibited slight leg and antennae movement were considered morbid and recorded as dead. Live beetles were recorded, including beetles that were on their elytra but still thrashing their legs.

Termination of the study occurred when defoliation reached levels that would not support another 24 hour period of beetle feeding, and the leaflets with the highest defoliation were at a point that still preserved enough plant material to make accurate leaf area measurements. Leaf area measurements and defoliation damage was assessed using the ImageJ software and methods explained in the Greenhouse Efficacy Study.

Mortality data were analyzed by probit analysis (Finney 1971) using POLO-Plus (LeOra Software 2002) and corrected using the Abbott's formula (Abott 1925) to determine LC_{50} and LC_{95} values with confidence intervals.

Defoliation data were analyzed by non-linear regression (Marçon et al. 1999) to calculate EC_{50} values (the concentration that caused 50% of defoliation compared to the control) with confidence intervals.

Translocation and detection of thiamethoxam applied as seed treatments in soybean tissue.

Soybeans were planted on May 13, 2011 in 76.2 cm rows and grown under regular agronomic practices at the University of Nebraska Northeast Research and Extension Center Haskell Agricultural Laboratory, Concord, NE. Soil type on plot site is Baltic Silty Clay, which may influence an increase or decrease of insecticide binding to the soil particles. The soybean variety was Garst 25-F2 with the Rag 1 gene (resistant to *Aphis glycines*, the soybean aphid) and commercially seed treated with CruiserMax (3.0 fl oz per 100 lb seed or 0.0762 mg thiamethoxam AI per seed, mefenoxam, and fludioxonil).

Plots were 9.14 mlong by 4 rows wide. Thirty random plant samples were taken from each plot at vegetative growth stages VC, V1, V2, V3, and V4. Whole plants were pulled from the ground, bagged by plot, and taken back to the lab in a cooler with ice. Cotyledon, unifoliate, and trifoliate leaves were removed at the node and separated. Each leaflet sample from the same node was bagged together providing at least a 5 gram sample. Soybean samples were stored in a freezer at -20°C until ready for analysis preparation.

Untreated soybean plants were grown in greenhouse conditions to detect neonicotinoid levels from a calculated LC_{50}. Eighty soybean plants were grown in

greenhouse conditions described in the Greenhouse Efficacy Study. These plants grew to V3, and the second trifoliate was harvested at the node, leaving the petiole intact. Leaves were transported to the lab in a plastic bag in a cooler with ice to reduce wilt. Thirty excised soybean petioles were immersed into 150 mL of a 250 ng/mL thiamethoxam solution in two separate replications (60 total plants) to detect neonicotinoid levels comparable to the calculated LC_{50} (240 ng/ml). After immersion for 48 hours, soybean samples were removed from the solution and frozen until ready for analysis preparation.

Individual stock solutions of all neonicotinoid analytes, the surrogate, and internal standard were prepared at concentrations of 5 μg μL^{-1} in methanol from analytical grade clothianidin (99.4% [AI]), imidacloprid (99.5% [AI]), thiamethoxam (99.5% [AI]) (Chem Service Inc., West Chester, PA), terbutylazine (surrogate, Sigma-Aldrich, Milwaukee, WI), and $^{13}C_3$-labeled atrazine (internal standard, Merck & Co., Whitehouse Station, NJ). Analyte, surrogate, and internal standard calibration spiking solutions were prepared from the stock solutions diluted to 50 ng μL^{-1} in methanol. Calibration standard samples were prepared from the calibration spiking solutions in sample matrix obtained from the method extraction of untreated soybean trifoliates. Analytes and surrogate were added to individual calibration samples in amounts of 250, 1000, and 2500 ng to create a three-point calibration curve. Internal standard (2500 ng) was added to all calibration standards and samples to quantify analyte concentrations on the instrument. Mean percent recovery of the surrogate from the 47 samples was 109% ± 4%, which met the acceptance criteria of the United States Environmental Protection Agency. Analyte detection limits were estimated from instrument signal to noise to be 32 ng mL^{-1} in the final injection matrix, corresponding to an analyte concentration in soybeans of 0.01 μg g^{-1}.

Soybean samples were taken from the freezer to be prepared for extraction and 5 g samples were weighed from the leaflet samples. These samples were frozen with liquid nitrogen and ground with a mortar and pestle. The leaf material was placed in a 10 mL plastic tube with cap. Terbutalyzine was added as a surrogate at 2,500 ng (50 μL of a 50 ng μL^{-1} solution) with 30 mL acetonitrile HPLC grade as an extraction reagent.

After extraction, samples were shaken for 30 min at 4ºC on a multi-purpose rotator (Model #2314, Thermo Scientific, Waltham, MA) and centrifuged at 3500 rpm, 16ºC (IEC Multi-RF, Thermo Electron, Milford, MA) for 20 min. A 10 mL aliquot of the supernatant was mixed with 90 mL of reagent grade water. Aqueous extracts were passed through a 200 mg solid phase extraction (SPE) cartridge (Oasis HLB, Waters, Milford, MA) connected to a vacuum manifold. The Oasis HLB cartridge used for SPE was previously prepared by sequential washing with 5 mL of acetonitrile, methanol, and reagent grade water.

Insecticides were eluted from the SPE cartridge with 2 mL of methanol into a disposable culture tube (13 mm in width by 100 mm in depth) (Fisher Scientific, Pittsburgh, PA) and 2500 ng (50 μL of a 50 ng μL^{-1} solution) of ^{13}C-labeled atrazine was added as an internal standard. The eluant was then evaporated at room temperature under a nitrogen flow to approximately 200-300 μL. The concentrated solution was diluted to a final volume of 500 μL with double distilled water and filtered with a Mini-UniPrep^{TM} Syringeless Filter (0.45 μm, pore size) (Whatman, Florham Park, NJ).

The prepared aliquots (containing analyte, terbutylazine and $^{13}C_3$-labeled atrazine) were analyzed by reversephase HPLC/MS/MS utilizing a Waters 2695 HPLC autosampler/pump coupled to a Finnegan LCQ (Thermo Scientific, Waltham, MA) ion-

trap mass spectrometer as descrbed by Magalhaes et al. (2009) HPLC separation utilized a Luna C8 (5 μm particle size) column (250 mm x 2 mm i.d.) (Phenomenex, Torrance, CA). The mobile phase was a 90:10 ratio of 0.1% (v/v) ammonium formate in water and 0.1% (v/v) ammonium formate in methanol for 2 min, followed by a 8 min linear gradient to a 20:80 mobile phase ratio, held for 12 min, then returned to a 90:10 ratio and held for another 10 min to re-equilibrate the column for a total run time of 30 min. The flow rate was 0.3 mL min^{-1} and sample injection volume was 25 μL. The LCQ mass spectrometer was operated in atmospheric pressure chemical ionization (APCI) mode with the vaporizer temperature at 350ºC, the discharge current at 5.0 μA, the sheath gas as 80 (arbitrary units), the auxiliary gas at 1 (arbitrary units), the tube lens voltage at -5.0 V, the capillary voltage at 3.0 V, the capillary temperature at 150ºC, the lens voltage at -36.0 V, the multipole 1 offset at -3.0 V, the multipole 2 offset at -5.0 V, and the multipole RF amplitude at 500 V_{p-p} . The daughter ion transitions and percent collision energies used in the analysis were for each analyte: imidacloprid (m/z=256->210, 30%), clothianidin (m/z=250->169, 25%), thiamethoxam (m/z=292->211, 25%), terbutylazine (m/z=230->174, 35%), and $^{13}C_3$-labeled atrazine (m/z=219->177, 35%). The isolation width was 3 amu and the activation time was 30 msec for all analytes. The collision gas was helium.

CHAPTER 3-RESULTS

Efficacy of commercial rates of thiamethoxam soybean seed treatment to control bean leaf beetle.

Significant differences in beetle mortality among treatments were observed ($F = 211.72$, $df = 3, 57$, $P < 0.0001$) (Table 1) with significantly higher mortality on thiamethoxam treated VE soybean (86% ± 3.56) and VC soybean (95.5% ± 3.56) than untreated VE and VC soybean. There were no significant differences in beetle mortality between thiamethoxam treated VE and VC soybean, although mortality on VC soybean was numerically higher.

There were also significant differences among thiamethoxam and control treatments for defoliation ($F = 65.98$, $df = 3, 57$, $P < 0.0001$) (Table 1). Significantly greater defoliation was observed for untreated VE (17.30% ± 1.17) and VC soybean (19.03% ±1.17) relative to thiamethoxam treated VE and VC soybean. However, no significant differences were observed between VE and VC soybean, treated or untreated.

Bean leaf beetle response to lethal and sublethal concentrations of thiamethoxam.

Thiamethoxam had significant lethal effects on bean leaf beetles in bioassays involving treated soybean foliage. However, bioassays were segregated by the overwintering population and F1 generation because overwintering population were more susceptible. An LC_{50} of 250 ng/mL with confidence limits (CL) (95% CL: 161.35 - 327.08) were calculated for the overwintering population at 72 hours after exposure (Figure 2). The calculated LC_{90} was calculated at 950ng/mL (95% CL: 666.07 - 1568.36). An LC_{50} of 750 ng/mL (95% CL: 395.95 - 3279.37) and LC_{90} of 4021 ng/mL

(95% CL: 1450.48 - 294053.51). A lower LC_{50} could occur in overwintering populations that have been subjected to extra stress associated with overwintering, mating, and reproduction. Also, newly emerged F1 beetles would have physiological differences compared to overwintering adults.

Thiamethoxam also caused a significant sublethal response measured by defoliation with an EC_{50} (the concentration that caused 50% of defoliation compared to the control) of 60 ng/mL (95% CL: 37.22 – 93.16) for overwintering beetles (Figure 2). An EC_{50} of 92.2 ng/mL (95% CL: 52.28 – 188.21) was calculated for F1 beetles.

Translocation and detection of thiamethoxam applied as seed treatments in soybean tissue.

Detection of thiamethoxam in soybean plants grown from treated seeds at is shown in Figure 4. The highest concentrations for thiamethoxam per leaflet or structure was detected in the cotyledon of node 1 (N1) of the VC plant (2668 ng/g of tissue), which was the first growth stage sampled 24 days after planting (DAP). Thiamethoxam concentrations decreased in leaves or structures from the same node over time. At V1 (31 DAP), thiamethoxam concentrations in the cotyledon at N1 declined to 1821 ng/g of tissue. At 34 DAP, in growth stage V2 plants, the cotyledon at N1 had a thiamethoxam concentration of 1655 ng/g of tissue. The cotyledons at the first node fell off the plant between 34 and 39 DAP, so no further sample dates contained data for cotyledons.

As the plant matures and more leaves are produced at progressing nodes, less insecticide is apparently available for uptake, therefore less insecticide was detected throughout the plant. Thiamethoxam concentration in the leaf at node 2 (N2) of V1

plants was approximately 10-fold lower (109 ng/g) than the concentration in cotyledons at N1. A similar concentration was detected at N2 leaves of V2 plants (106 ng/g). Leaves at N2 were the earliest leaves sampled 39 and 43 DAP. The thiamethoxam concentration dropped to a concentration of 30 and 26 ng/g of tissue, respectively.

At N3, the first trifoliate leaves were present and thiamethoxam was detected at 77 ng/g of tissue 31 DAP. At 34 DAP, the thiamethoxam concentration at N3 was only 50 ng/g of tissue. This declining trend continued through 39 and 43 DAP with concentrations detected at 20 and 4 ng/g of tissue, respectively.

Leaves at node 4 (N4) were sampled 34, 39, and 43 DAP. The highest concentration was found at the earliest sample date (V2 plant) and had a thiamethoxam concentration of 28 ng/g of tissue. For V3 plants sampled 5 days later, the leaves at N4 thiamethoxam concentration dropped to7 ng/g of tissue, but a slightly higher concentration of 10 ng/g of tissue was detected in V4 plants.

Detection of thiamethoxam within leaves in the newer foliage at node 5 and 6 were below10 ng/g of tissue and just above detection limits. Leaves from V3 plants sampled 39 DAP had thiamethoxam concentration of 6 ng/g of tissue. The leaves at N5 and N6 of V4 plants sampled 43 DAP both had thiamethoxam concentrations of 4 ng/g of tissue. In general, lower concentrations were detected in the newer foliage.

Clothianidin, a thiamethoxam metabolite, was consistently detected in the cotyledons at N1. In VC soybean (24 DAP) the concentration of clothianidin was 72 ng/g of tissue (Figure 5), which is approximately 10-fold lower than thiamethoxam. Clothianidin was also detected in the cotyledons at N1 of V1 soybean (31 DAP) at 44

ng/g of tissue and at 60 ng/g of tissue in V2 plants (34 DAP). The metabolite was not detected with consistent trends in the remaining growth stages. Trace amounts (3 ng/g) were detected in leaves at N2-4 for V3 plants and in N5 and N6 leaves for V4 plants.

Concentration for thiamethoxam and clothianidin were quantified from untreated soybean trifoliates immersed in a 250 ng/mL thiamethoxam solution for 48 hours, comparable to the LC_{50} (240 ng/ml). Thiamethoxam was 844 ng/g of tissue (Table 2). This concentration is lower than thiamethoxam levels detected in cotyledons at N1, but higher than concentrations detected at N2, or any other detection within the soybean leaves. The concentration from the same trifoliates for clothianidin was 19 ng/g of leaflet.

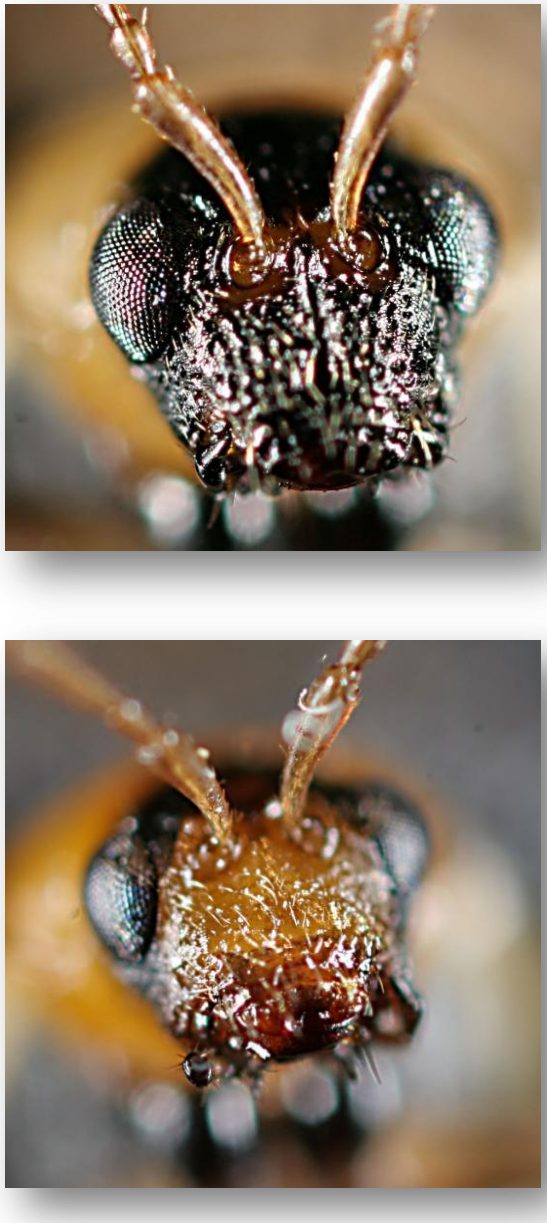

Figure 1. Bean leaf beetles can be sexed by the color of frons. Females (top) have black frons, while males (bottom) have golden brown frons. Courtesy of John Lundgren.

Table 1. Efficacy of thiamethoxam commercial rate seed treatment against bean leaf beetle under greenhouse conditions.

Treatments	**Percent Mortality** (F = 211.72, df = 3, 57, P < .0001)		**Percent Defoliation** (F = 65.98, df = 3, 57, P < .0001)	
	Vegetative Emergence	Vegetative Cotyledon	Vegetative Emergence	Vegetative Cotyledon
Thiamethoxam	86.4 ± 3.56a	95.5 ± 3.56a	2.52 ± 1.17a	1.15 ± 1.17a
Untreated	3.9 ± 3.56b	3.0 ± 3.56b	17.30 ± 1.17 b	19.03 ±1.17 b

Means ± SE. Separate comparisons were made for Percent Mortality and Percent Defoliation. Means with different letters are significantly different (Fisher's protected LSD, $P < 0.05$).

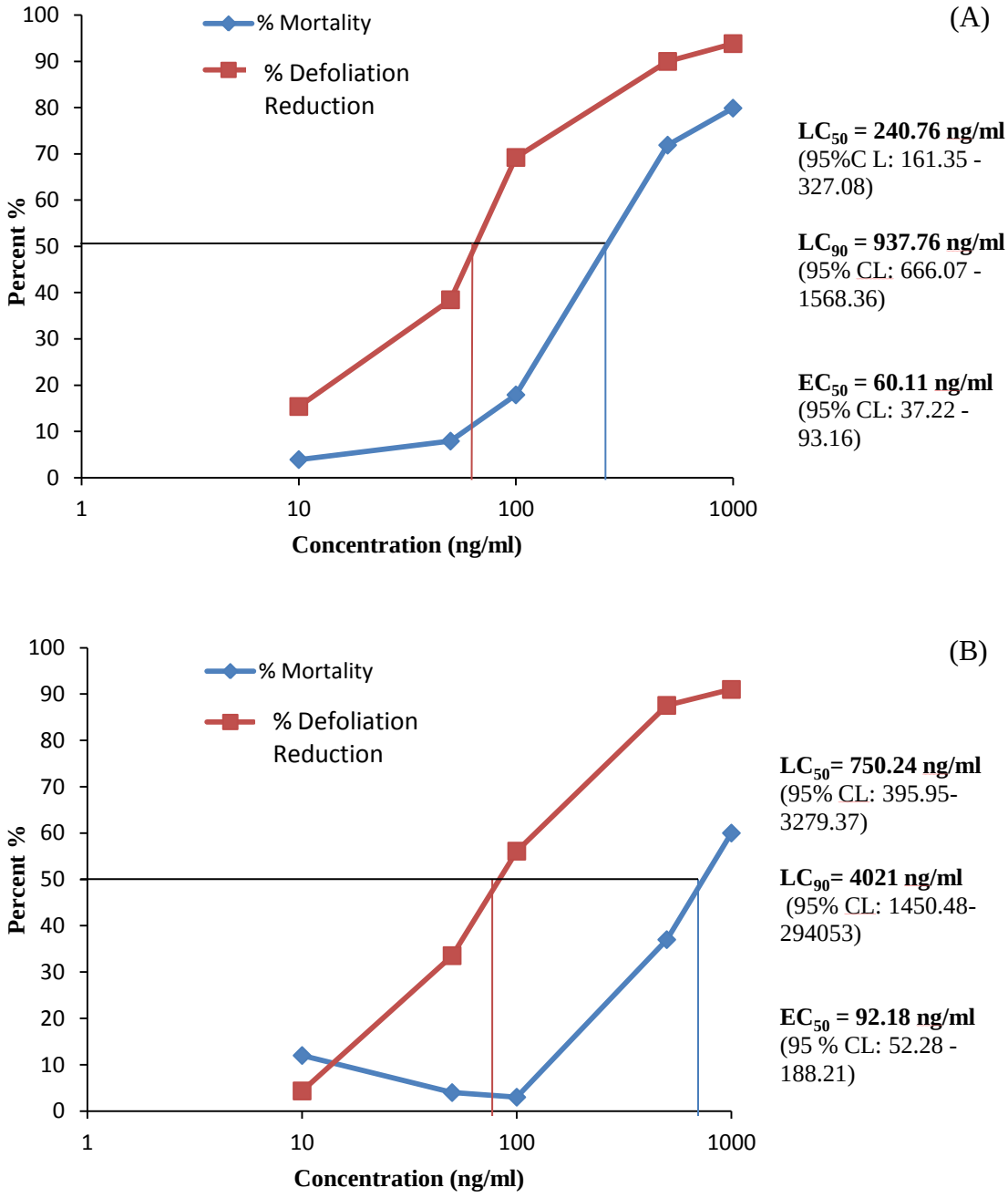

Figure 2. Overwintering (A) and F1 (B) BLB percent mortality response (blue) and percent defoliation reduction compared to the control response (red) with calculated $LC_{50, 90}$ and EC_{50} with 95% Confidence Limits.

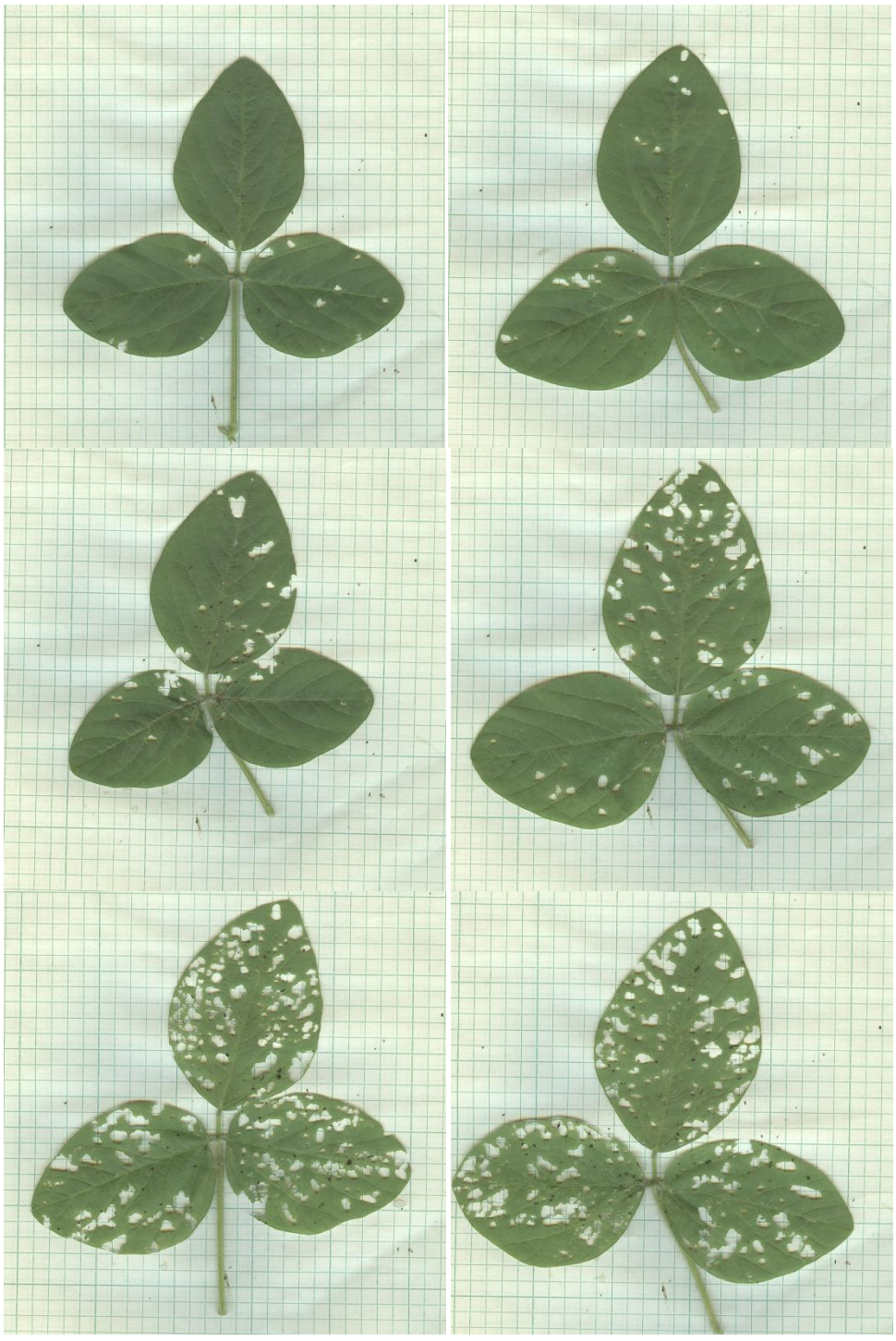

Figure 3. Visual differences amongst thiamethoxam concentration treatments in bioassay. Top left, across, to bottom right: 1000, 500, 100, 50, 10, 0 ng/mL concentrations.

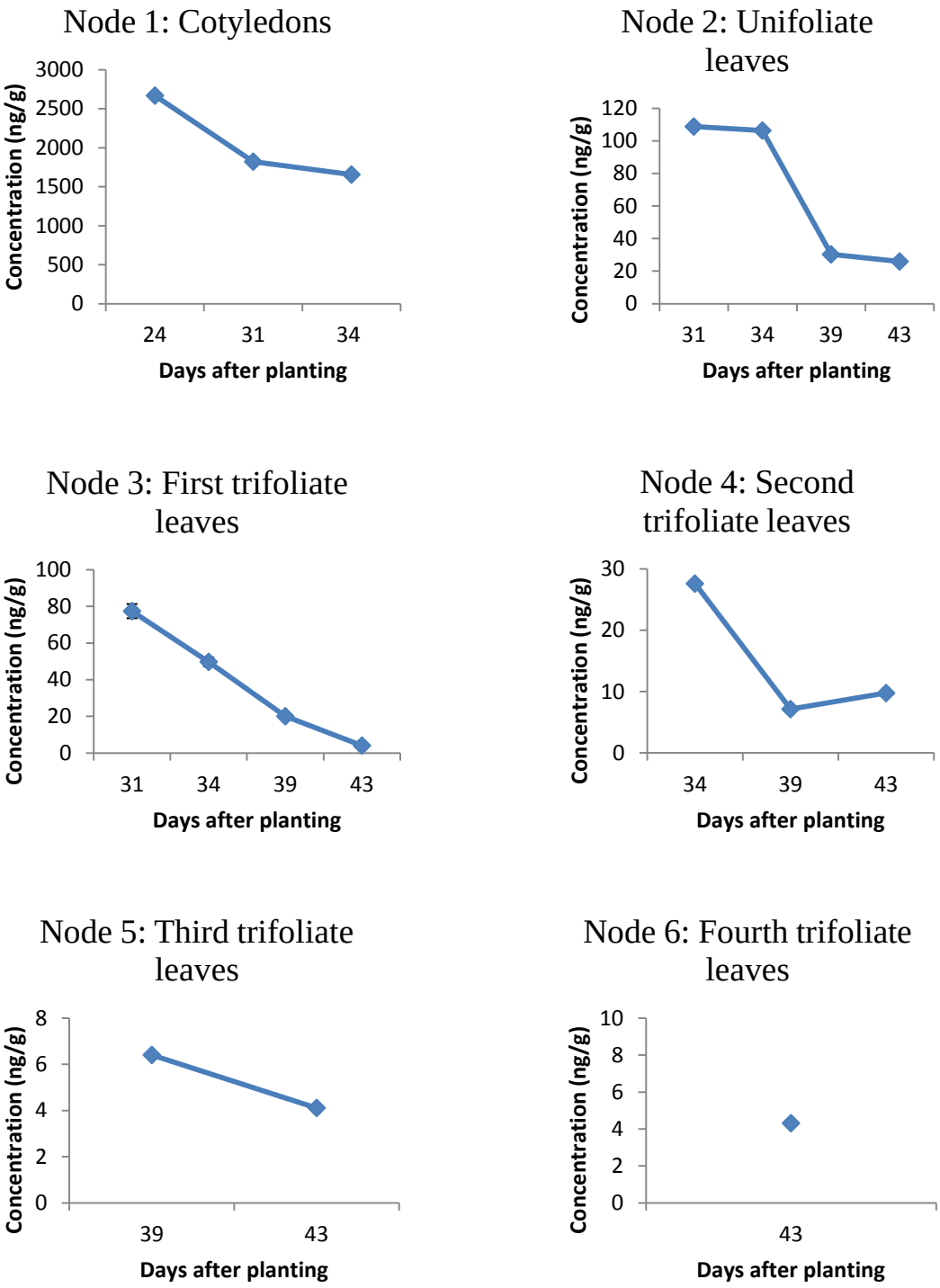

Figure 4. Concentration of thiamethoxam in soybean coteledons and leaves from specific nodes sampled over time at specific vegetative growth stages (VC, V1, etc.). Cotyledons at N1 fell off before sampling of V3 plants (34-39 DAP), sowere not included with the last two samples.

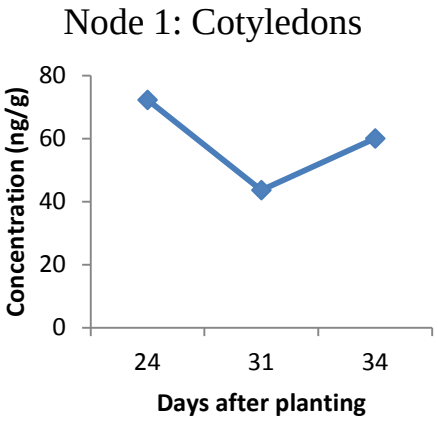

Figure 5. Concentration of clothianidin in soybean cotyledons sampled over time. Values detected as 0 were not plotted.

Table 2. Concentrations of neonicotinoids detected in soybean leaves treated with 250 ng/mL solution.

Insecticide	Concentration (ng/g of leaf)
Thiamethoxam	844 ng/g
Clothianidin	19 ng/g

CHAPTER 4-DISCUSSION

Results of this investigation indicate that thiamethoxam insecticidal seed treatments are highly active against adult bean leaf beetles during early stages of plant development. At commercial rates, high mortality (86-95%) occurs on early growth stage soybean treated with thiamethoxam when beetles feed on cotyledons and new vegetative growth under greenhouse conditions.

Thiamethoxam was highly active against bean leaf beetles in bioassays with an LC_{50} = 250 ng/mL which corresponds to 844 ng/g of leaf tissue under laboratory bioassays. The LC_{50} is much lower than the concentrations of thiamethoxam quantified in cotyledons of soybean plants throughout the first four vegetative growth stages before cotyledons senesced and fell from the plant. However, the LC_{50} was higher than any concentrations of thiamethoxam detected in different leaflets during later stages of plant development. This suggests beetles would have to colonize a soybean field before V1 to be subjected to a thiamethoxam concentration equivalent to or above the LC_{50}. In addition, exposure after V1 may not cause high mortality, but could still play a role in reducing defoliation, as the EC_{50} was much lower than the LC_{50}. Comparable levels of thiamethoxam were found in growth stages up to V2 and leaves at the third node. Thiamethoxam at sublethal concentrations affected feeding and reduced defoliation. This suggests that soybeans are protected from bean leaf beetle defoliation at lower concentrations of insecticides that may not kill adults.

Concentrations of thiamethoxam and its active metabolite, clothianidin, rapidly decrease in each new soybean leaf under field conditions. The concentration of

insecticides is high in cotyledons, but is much lower in other soybean structures. Both compounds also decrease in concentration over time. As the plant matures, less insecticide is available for uptake and translocation within the plant and concentrations in plant material rapidly decline and are not evenly distributed among the plant structures. At the end of the sampling period, 41 DAP, the thiamethoxam concentration was just above the detection limit, and significant control would not be provided in new trifoliates arising from node 4 and after. These results support those reported by Magalhaes et al. (2009) who also reported a rapid decline in neonicotinoid concentrations in soybean foliage from seed treatments. These results indicate that the level of bean leaf beetle control is likely to decrease over time after soybean emergence and control of F1 adults from seed treatment insecticide in leaf tissue is unlikely. This supports results reported by McCornack and Ragsdale (2006) who concluded mortality of soybean aphids was significantly higher in older leaves than in new trifoliates.

The present studies have established methodologies that will support the development of baseline susceptibility for monitoring bean leaf beetle resistance to widely used neonicotinoids. This is extremely important as the number of planted acres to neonicotinoid treated soybeans increases. The implications of these results when choosing a pest management strategy are important, especially when considering resistance management. Integrated pest management (IPM) strategies call for preventative methods such as crop rotation, crop refuse destruction, tillage, and plant spatial arrangements (Quisenberry and Schotzko 1994) and for monitoring pest populations that should reach economic levels before employing curative (often chemical) methods. Choosing seed treatments with this high level of control before bean

leaf beetle populations can be assessed is not a recommended preventative practice and does not follow IPM strategies because the decision is made before populations are present. Monitoring bean leaf beetle response to thiamethoxam seed treatments and supplementary neonicotinoid foliar applications to detect the development of resistance is therefore probably advisable. In addition, as beetles colonize a newly emerged soybean field, there should be adequate amounts of insecticide available to cause significant mortality, but as soybeans grow new vegetative material, the amount of insecticide available to target the bean leaf beetle decreases. This suggests that beetles may have exposure to neonicotinoids below lethal levels, which could also contribute to the development of resistance, or increased tolerance of insecticides such as the response to pyrethroids seen in the Mississippi Delta (Musser et al. 2011)

Other considerations for future study include variability of adult beetle response to treatments across generations. My research suggested overwintering generations have a lower LC_{50} versus F1 generations. It may also be important to look for this difference between generations from year to year. Also, knowing that there is little insecticide available for control of the F1 generation, I would suggest looking if there is any control of the F1 generation at the larval stages which may be subjected to higher concentrations of insecticide and result in less emerging F1 adults. In addition, for quantification of insecticides in plant tissue, water availability and aspects of soil composition and chemistry could also be analyzed. Our field study was conducted at one location, with adequate moisture, but the availability of neonicotinoids could vary depending on soil moisture, since these compounds are water soluble, and soil composition and chemistry

that may affect how the insecticide binds to soil particles and are available for uptake by the plant.

Continued integration of neonicotinoid seed treatments into soybean pest management is expected for the control of various pests, while improving environmental and worker safety. Bioassay methods developed through this research are an efficient way for measuring bean leaf beetle mortality and dose-response. This information will help support the development of baseline data for monitoring bean leaf beetle resistance to neonicotinoids as seed treatments continue to be a growing trend U.S. agriculture.

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