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Ecological Risks of the Conventional Insecticide/Fungicide Seed Treatment Mixture of Thiamethoxam and Mefenoxam in Soybean on Beneficial Insects

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ECOLOGICAL RISKS OF THE CONVENTIONAL INSECTICIDE/FUNGICIDE
SEED TREATMENT MIXTURE OF THIAMETHOXAM AND MEFENOXAM IN
SOYBEAN ON BENEFICIAL INSECTS

by

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ECOLOGICAL RISKS OF THE CONVENTIONAL INSECTICIDE/FUNGICIDE
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Carolina Camargo Gil, Ph.D.
University of Nebraska, 2016

Advisors: Blair Siegfried and Thomas Hunt

The impact of neonicotinoid seed treatments on beneficial insects has been a controversial topic during the last years. While neonicotinoids are usually used as mixtures with systemic fungicides, few studies have examined the impact of the mixtures on beneficial insects. Pesticide mixtures can have synergistic, additive, or antagonistic effects on the toxicity of neonicotinoids on non-target species.

Thiamethoxam with mefenoxam is the most used neonicotinoid insecticide/fungicide mixture applied to soybean. Based on the systemic nature of thiamethoxam and mefenoxam, residues of this insecticide/fungicide mixture can be present in soybean vegetative and floral tissue with potential impacts to beneficial insects. This study focuses on the interaction of these compounds, their environmental fate in plants, their toxic effects on honey bees, and lethal and sub-lethal effects on key predatory species in soybean.

Concentrations of neonicotinoids in both floral and vegetative tissues were low or not detected, and the effects on target and non-target insects are more likely to be sub-lethal, if at all. There was a mild antagonist interaction with the fungicide, resulting in
reduced honey bee mortality. In predatory species, there were no significant differences in the abundance of *Orius insidiosus* and *Chrysoperla rufilabris* in soybean treated with thiamethoxam alone or with the mixture. Consumption of soybean aphid by both predators was not affected at evaluated concentrations of thiamethoxam in the insect prey. However, laboratory studies on toxicity of thiamethoxam on *Orius insidiosus* suggest potential toxic effects of this neonicotinoid based on the time of arrival of the predator to the field and the type of exposure to neonicotinoids.

Toxicity studies of mixtures of different classes of pesticides used in seed treatments are rarely available. To our knowledge this is the first study that evaluates the interaction of mefenoxam on acute toxicity of thiamethoxam. Studies of mixture toxicity of seed mixtures are imperative to minimize the risk of pesticides to beneficial insects by a careful selection of products with lower toxicity.
Dedication

To the women who raised me, Sofia and Eloisa and to my sister Laura, for being the best example of dedication, courage and kindness in my life;

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CHAPTER 1

Introduction and Literature Review

Introduction

Plant protection practices include the use of multiple chemical products as a strategy to maintain yields of sufficient quality and quantity in conventional agriculture (Lechenet et al. 2014). Despite regulatory efforts to ensure the safe use of pesticides in agriculture, risk assessment associated with the toxic action of pesticide mixtures has been an enduring challenge for ecotoxicology (Jonker et al. 2005). Within the last few years, increasing efforts have focused on understanding the effects of multiple contaminants in different ecosystems (Faust et al. 2001, Altenburger et al. 2004, Gomez-Eyles et al. 2009, Wang et al. 2013, Chen et al. 2015). In agriculture, pesticide mixtures are frequently used because of their additive benefits in plant growth, yield improvement, low application costs, and integrative pest management approaches (Gaspar et al. 2015). Recognizing the trade-offs between the environmental impacts and crop benefits of pesticide mixtures in agricultural systems is important to assist growers in pesticide application decisions and the improvement of more sustainable practices.

While neonicotinoids usually are used as mixtures with systemic fungicides, few studies have examined the impact of the mixtures on beneficial insects (Blacquiere et al. 2012, Simon-Delso et al. 2015, van der Sluijs et al. 2015). Pesticide mixtures can have synergistic, additive or antagonistic effects, increasing or reducing their toxicity to both target and non-target organisms (Mullin et al. 2015). The potential interaction of multiple
products with multiple modes of action in seed treatments is considered one of the main knowledge gaps in the risk assessment of seed treatments (van der Sluijs et al. 2015).

Thiamethoxam with mefenoxam represent one of the most commonly used neonicotinoid insecticide/fungicide mixtures found in soybean crops (Gaspar et al. 2014, Gaspar et al. 2015). Both, thiamethoxam and mefenoxam have systemic properties and are translocated in the plant xylem after root uptake (Bonmatin et al. 2015). Neonicotinoid residues have been identified in leaves and flowers of different crops including canola, corn, sunflower, and cucumber. Currently, there is no information available on insecticide/fungicide residues in soybean vegetative and floral tissue. Based on the systemic nature of thiamethoxam and mefenoxam, residues of this insecticide/fungicide mixture may be present in vegetative and floral tissue in different concentrations throughout the growing season.

The widespread use of thiamethoxam and mefenoxam for modern crop protection and specifically in soybean crops reflects the importance of evaluating the environmental risks of these products alone and in combination, as well as potential benefits to plant growth and yield (Gaspar et al. 2015). Risk assessment of conventional pesticide mixtures provides a better understanding of what is occurring in real field situations and helps us to recognize if changes in the use of seed treatments are warranted. In soybean, there are many remaining questions on the translocation of thiamethoxam and mefenoxam in plants and the impact of this systemic pesticide mixture on beneficial insects. This study will focus on the interaction of these compounds, evaluating their
environmental fate in plants, their possible toxic effects on honey bees and key predatory species in soybean.

**Literature Review**

**Seed treatments**

For more than 30 years seed treatments have been a widely adopted practice in crop protection worldwide (Munkvold et al. 2014). Seed treatments have evolved from broad-spectrum products with highly negative toxicological profiles to products with more specific activity and lower application rates. Seed treatments are currently used for a wide range of crops including, cereals, corn, soybean, sugar beets, sunflower, oil seed rape, potato, cotton, peanut, fruit, coffee, and others (Jeschke et al. 2011). The top crop markets correspond to cereals, corn, and soybean with more than USD $600 million sales worldwide (Munkvold et al. 2014). The seed treatment market has rapidly grow from USD $1 billion in 2002, to more than $3 billion in 2012, with a predicted growth to more than $7 billion in 2017 (Munkvold et al. 2014). Rapid growth and popularity of seed treatments are associated with reduced cost of application, efficiency in the delivery system, and the protection of the seeds and seedlings during their first critical stages (Nuyttens et al. 2013, Munkvold et al. 2014).

Active ingredients in seed treatments have different types of activity, from products with strictly contact activity, to locally systemic and highly systemic products. Common examples of contact products in seed treatments are the fungicides captan and fludioxynil. The effect of contact active ingredients for target pests can result from the
product diffusion in the soil or by direct interaction with the seeds. Locally systemic products include strobilurin fungicides that can be absorbed by plant tissues through contact, although there translocation does not occur throughout the plant tissues. In contrast, systemic products are translocated because of their high water solubility characteristics. The most common systemic products in seed treatments include neonicotinoid insecticides, as well as phenylamide and some triazole fungicides (Munkvold et al. 2014). Based on the pest profile targeted, different fungicides and insecticides are combined at different application rates (Nuyttens et al. 2013). In addition to the main active ingredients, seed treatments are also combined with other components such as colorants, adhesives, and dispersion substances (Nuyttens et al. 2013, Mullin et al. 2015).

Modern commercial seed treatments consist of mixtures of multiple classes of fungicides, nematicides, and neonicotinoids insecticides, as the main insect control component (Munkvold et al. 2014, Douglas and Tooker 2015). Oomycetes are the universal target for fungicide seed treatments because of their wide diversity and high impacts on seeds and seedlings during early-season conditions (Munkvold et al. 2014). Metalaxyl and mefenoxam are the most important active ingredients used to control oomycete diseases across all crops and are the most widely used fungicides worldwide (Monkiedje et al. 2007).

For insecticides, neonicotinoids have been the most commonly applied insecticides in seed treatments in the last two decades worldwide (Munkvold et al. 2014). Although neonicotinoids seed treatments are applied in several crops, field crops (cotton,
corn, soybean) account for the vast majority neonicotinoid use (Douglas and Tooker 2015). Target insects for neonicotinoids include several soil born insects, as well as early season aboveground insect pests. In addition to pest control, neonicotinoids have been shown to induce physiological changes in plants, giving benefits in plant vigor and yield.

**Seed treatments in soybean**

Soybean production has an important economic significance worldwide as it supplies half of the demand for vegetable oils and proteins (Oerke and Dehne 2004). The United States is the world leader in soybean production, providing 50% of the world’s soybeans and soybean products (Masuda and Goldsmith 2009). The North Central Region of United States is responsible for approximately 90% of all soybeans produced in the U.S. (USDA, 2010). Pest pressure in soybean in this region of the U.S has intensified the use of seed applied pesticides during recent decades (EPA 2014, Douglas and Tooker 2015, Gaspar et al. 2015). Seed applied fungicides and insecticides have become a widely used practice by soybean growers to control a broad spectrum of early and mid season pathogens and insect species. In the north central U.S., more than 70% of soybean seeds are treated with a fungicide, insecticide, or nematicide alone or in combination of two or three products (Douglas and Tooker 2015).

Early planting in northern states has influenced the use of contact and systemic fungicides in seed treatments to prevent losses by soil borne pathogens (Gaspar et al. 2014, Gaspar et al. 2015). Protectant and systemic fungicides are often applied as mixtures to control soil borne diseases such as *Pythium* spp., *Fusarium* spp., *Rhizoctonia* spp. and *Phytophthora* spp. Systemic fungicides in soybean are widely uses as they can be
absorbed into the emerging seedlings and inhibit or kill the fungus inside the plant tissues. The most common systemic fungicides in seed treatment include azoxystrobin, carboxin, mefenoxam, metalaxyl, thiabendazole, trifloxystrobin, and various triazole fungicides, including difenoconazole, ipconazole, tebuconazole, and triticonazole. Mefenoxam and metalaxyl are two of the most commonly used products in soybean targeting *Pythium* spp. and *Phytophthora* spp. Application rates of fungicides in soybean seed treatments range between 0.32 to 3.5 g per seed kg depending on the active ingredient.

At the same time and intensity, neonicotinoid seed treatments are applied in soybean to control different early season above and belowground insect pests, such as wireworm (*Melanotus* spp. Eschscholtz), seed corn maggot (*Delia platura* Meigen), bean leaf beetle (*Cerotoma trifurcate* Foster) and other minor pests (Cox et al. 2008, Gaspar et al. 2015). Although neonicotinoids in soybean have been registered to control one of the main pests in the north central U.S., the soybean aphid (*Aphis glycines* Matsumura), recent studies have suggested a limited bioactivity of neonicotinoid seed treatments at the time of arrival of this pest into soybean crops (EPA 2014). Imidacloprid and thiamethoxam are the two main neonicotinoid active ingredients used in soybean in soybean seed treatments and are applied to approximately 46% of the total soybean acreage in the U.S (EPA 2014, Douglas and Tooker 2015).

The systemic characteristics of metalaxyl, mefenoxam and neonicotinoid insecticides have benefits in pest control because of a more efficient delivery system and a reduction of the insecticide exposure to beneficial insects in comparison to foliar
application (Seagraves and Lundgren 2012). However, the prophylactic use of pesticides as seed treatments has raised many questions concerning the trade-offs between the ecological risks and the benefits of using systemic seed treatment products in several crops, including soybean, corn, canola, sunflowers, and many others (Seagraves and Lundgren 2012, Douglas and Tooker 2015, Smith et al. 2016). In soybean, concerns with the use of neonicotinoids have been related with their extensive use as soybean seed treatments regardless of the presence or absence of the target pest (Stamm et al. 2014).

In 2014, a review on neonicotinoids seed treatments in soybean presented by the Environmental Protection Agency (EPA) suggests that this practice does not show consistent economic benefits for soybean growers, especially for the ones located in the northern regions of the United States where there is no overlap with bioactivity of the compounds and the arrival of the economically important soybean pests. More information is necessary to determine the concentrations of neonicotinoids at different times during the growing season and the bioavailability of these compounds under different environmental conditions throughout soybean growing regions.

Neonicotinoids

History

The history of neonicotinoids started in 1965 when Yamamoto described the insecticidal properties of the natural product nicotine. Nicotinoids were very effective and promising compounds for insect control, but due to their high mammalian toxicity, they were never widely used in pest control (Yu 2008). Industry continued research on
nicotinoids to improve insecticidal activity and to reduce their mammalian toxicity. Their work resulted in the discovery of nithiazine, the lead structure of the actual neonicotinoids (Maenfisch et al. 2001, Tomizawa and Casida 2005). Nithiazine had promising insecticidal activity, but it had low photostability, limiting its use in agricultural settings (Tomizawa and Casida 2003).

In the early 1980’s, Nihon Tokushu Noyaku Seizo working in Bayer improved nithiazine structure and photostability characteristics leading to the discovery of imidacloprid, one of the first neonicotinoid insecticides (Yamamoto et al. 1998). In 1991, imidacloprid was introduced to the market as the lead molecule of the first-generation neonicotinoids. After the launch of imidacloprid, other first generation neonicotinoids were brought to the market including nytenpyram and acetamiprid. In 1998, Novartis launched thiamethoxam, a second-generation neonicotinoid with unique structure and high insecticidal activity (Maenfisch et al. 2001). Currently there are eight neonicotinoids in the market, including two more second-generation compounds clothianidin and thiacloprid (Simon-Delso et al. 2015) a third generation compound, dinotefuran (Wakita et al. 2003), and sulfoxaflor in the fourth generation (Cutler et al. 2013).

Neonicotinoids have become the most widely used compounds in the insecticide market (Jeschke et al. 2011, Simon-Delso et al. 2015). Neonicotinoids are registered in more than 120 countries with a broad range of applications from plant protection, veterinary products, and biocides to invertebrate pests in aquaculture (Simon-Delso et al. 2015). In 2008, imidacloprid became the most sold insecticide worldwide with a global
market value of US $1 billion (Jeschke et al., 2011). In more recent years, thiamethoxam has replaced imidacloprid sales in some countries, reaching approximately US $1.1 billion sales in 2012 (Simon-Delso et al. 2015).

**Physicochemical properties**

The physicochemical properties of neonicotinoids have played an important role in the success of these compounds in the insecticide market (Matsuda et al. 2001, Jeschke and Nauen 2008). Neonicotinoids have greater water solubility than other insecticides, favoring translaminar and acropetal movement in plants through seed treatment, soil drench and foliar applications (Jeschke et al. 2011). The water solubility of neonicotinoids depends on multiple factors such as chemical structure, water temperature, physical state, molecular weight, and pH. In general, solubility of neonicotinoids is between 184 (moderate) and 4100 mg/L (high) at 20 °C and pH 7 (Bonmatin et al. 2015). Water solubility of neonicotinoids can also be altered by commercial formulations of the insecticide. Some surfactants in neonicotinoid commercial formulations have the ability to keep the insecticide soluble for a long period of time facilitating systemic movement in plant vegetative tissue (Gupta et al. 2002, Gupta et al. 2008, Bonmatin et al. 2015).

**Mode of Action**

Neonicotinoids are exogenous agonists of the nicotinic acetylcholine receptors (nAchR) (Tomizawa and Casida 2005). The binding of neonicotinoids to nAchR prolongs the opening of the receptor causing a continuous excitatory response of the nervous system (Tomizawa and Casida 1999, 2005, Jeschke and Nauen 2008). Symptoms of
neonicotinoid intoxication in insects include incoordination, tremors, decreased body temperature, and death (Yu 2008).

The differences in the nAChR between mammals and insects make neonicotinoids a safer compound compared with other classes of insecticides (Tomizawa and Casida, 2003). The nAChR is a neuron transmembrane complex with five subunits: one extracellular domain and four transmembrane domains (Tomizawa and Casida 2005, Honda et al. 2006). The nAChR subunits differ between mammals and insects, making neonicotinoids more specific to invertebrates (Tomizawa and Casida 2003, Casida and Durkin 2013). The selectivity and lower impacts on vertebrates have lead to the rapid adoption of neonicotinoids in both agricultural and urban environments (Casida and Durkin 2013, Simon-Delso et al. 2015).

All neonicotinoids have a strong affinity with insect nAchRs, except for thiamethoxam, which exhibits lower affinity for the target site (Jeschke and Nauen 2008, Jeschke et al. 2011). Low affinities of this compound have been attributed to the proneonicotnoid nature of this compound. Thiamethoxam is activated to clothianidin by hydrolysis of the perhydro-1,3,5-oxidazine ring system in insects and plants (Nauen et al. 2003).

*Environmental Fate*

Neonicotinoids have been extensively criticized from the public and scientific community during the last decade due to their environmental fate (Krupke et al. 2012, Nuyttens et al. 2013, Anderson et al. 2015, Bonmatin et al. 2015). The main concerns are...
related to the widespread use of neonicotinoids seed treatments and the high water solubility of this class of insecticides (Pisa et al. 2015). Neonicotinoids from seed treatments can be transported through air, water, and other natural resources via dust drift (Girolami et al. 2012, Krupke et al. 2012, Nuyttens et al. 2013, Heimbach et al. 2014), surface runoff (Starner and Goh 2012, Hladik et al. 2014), leaching through the soil profile (Miranda et al. 2011, Anderson et al. 2015), translocation to plant guttation (Girolami et al. 2009), and contamination of pollen and nectar in flowers (Stoner and Eitzer 2012).

Translocation of neonicotinoids in plant tissues occurs mainly through the xylem driven by the movement of the water from the root into the upper parts of the plant (Maienfisch et al. 2001). However, phloem mobility of neonicotinoids can also occur, as phloem feeding insects undergo effective mortality from these compounds (Nauen et al. 2003). Compounds with intermediate lipophilicity, such as neonicotinoids ($\log K_{ow}$ between 1 and 3), and weak acidity ($pK_w$) have the ability to move through the phloem and translocate to different reproductive and vegetative tissues. Concentrations in vegetative tissue can vary between approximately 15 and 105 ppb one week after planting, to concentrations between 1 and 5 ppb 40 days after planting (Bonmatin et al. 2005, Magalhaes et al. 2009, Bonmatin et al. 2015).

Studies of neonicotinoid uptake in plants from seed treatments have shown that the crop absorbs only 20% of the active ingredients (Goulson 2013). More than 80% of neonicotinoid active ingredients enter the soil after planting (Bonmatin et al. 2015). Reports on the concentration and persistence of neonicotinoids in soil are variable
Half-life in soil ranges from 28-1250, 7-353, and 148-6931 days for imidacloprid, thiamethoxam, and clothianidin, respectively (Sarkar et al. 2001, Rexrode et al. 2003, Gupta et al. 2008). Data on concentration in soil range from 1 ppb to 100 ppb in relation to repeated application or different application rates (Goulson 2013). A small proportion of the active ingredient is lost as aerial dust during planting. The release of dust can be intensified by the addition of talcum and graphite in the planter, which is a common practice during soybean and corn planting in the U.S (Krupke et al. 2012).

Dust particles can be the key route of exposure for neonicotinoids for non-target organisms (Bonmatin et al. 2015). Residues of neonicotinoids have been found near agricultural environments shortly after sowing of treated seeds (Marzaro et al. 2011, Tapparo et al. 2011, Girolami et al. 2012, Krupke et al. 2012, Bonmatin et al. 2015). During planting, planters release pesticide dust and other seed particles that can be moved by air currents to plants or water bodies near agricultural fields (Krupke et al. 2012, Nuyttens et al. 2013). Krupke et al., 2012 reported a range of neonicotinoid concentrations from 1 to 20 ppb in soil and dandelion flowers at field margins shortly after corn and soybean seed planting. Other authors report concentration in water puddles from 0.01 to 63 ppb during planting seasons (Samson-Robert et al. 2014).

Despite the different levels of neonicotinoid residues found in the environment during planting, the overall level of risk of neonicotinoid dust to non-target insects has been difficult to quantify (Marzaro et al. 2011, Krupke et al. 2012). Neonicotinoid concentrations on canola, corn, or sunflowers associated with the translocation from seed treatments are reported at concentrations from 1 to 4 ppb in pollen (Cutler and Scott-
Dupree 2007, Krupke et al. 2012, Stewart et al. 2014) and from 1 to 3 ppb in nectar (Bonmatin et al. 2005, Bonmatin et al. 2015). Similar neonicotinoid concentrations have been reported from plants in field borders after planting (Rundlof et al. 2015). These authors report average concentrations of clothianidin at 1 to 1.2 ppb after planting oilseed treated seeds. Concentrations between 1.1 and 9.4 ppb have been registered in dandelions near agricultural environments after soybean and corn planting in the U.S (Krupke et al. 2012). Higher concentrations of neonicotinoids can be found in flowers through drip or soil applications (Stoner and Eitzer 2012). Neonicotinoid concentrations in nectar and pollen of squash flowers range between 10 and 14 ppb for imidacloprid and 12 to 11 ppb for thiamethoxam at ~48 days after the insecticides were applied (Stoner and Eitzer 2012).

The information on exposure levels of neonicotinoids in field scenarios is still very limited with high variability between studies (Goulson 2013, Bonmatin et al. 2015). Neonicotinoid concentration reported in soil, water, beehives, and particularly, in floral and vegetative plants are quite variable across studies (Goulson 2013). There are no systematic attempts to understand the variability of exposure levels of neonicotinoids between studies. The relation between the initial application rates, environmental conditions and movement of neonicotinoids in the environment is still unclear. The variability between studies makes assessing the risk of neonicotinoids in different environments (Bonmatin et al. 2015) problematic. There is an urgent need of robust and uniform analytical techniques that allow the accurate measurements of neonicotinoids and its toxic metabolites in real field scenarios.
Effects of neonicotinoids on honey bees

For the past 20 years there has been a growing body of research evaluating the effects of pesticides on beneficial arthropods (Desneux and O'Neil 2008, Gentz et al. 2010, Goulson 2013, van der Sluijs et al. 2013, Pisa et al. 2015). Lethal effects from residues in the environment on honey bees and other non-target organisms is still unclear, with the exception of few cases where honey bee colonies were exposed directly to high concentrations through dust emissions (Pisa et al. 2015). Beneficial insects can be exposed through multiple routes, such as neonicotinoid residues in flowers, soil, and vegetation as a result of translocation of the compound in the plant from seed treatment or from dust drift (Krupke et al. 2012, Bonmatin et al. 2015).

The risk of systemic pesticides to pollinators in several crops gained particular attention because of the possible link between honey bee Colony Collapse Disorder (CCD) and the widespread use of neonicotinoids as seed treatments (Blacquiere et al. 2012). After 15 years of active research on the risk of neonicotinoids on honey bees, there are still many gaps in laboratory toxicity results and real field situations (Henry et al. 2015). Laboratory experiments have identified several sub-lethal effects in honey bee behavior, such as effects on mobility, orientation, foraging behavior (Yang et al. 2008, Decourtye and Devillers 2010, Henry et al. 2012, Johnson 2015), and physiology, such as negative impacts on the bee immune system (Mason et al. 2013). However, inconsistency across studies on deleterious effects under field exposure conditions make policy decisions controversial (Henry et al. 2015).
Acute toxicity levels of neonicotinoids show large variability between studies (Lauerno et al. 2011). Based on their social behavior, toxicity bioassays on honey bees are usually measured in groups of bees, which can influence lethal concentration calculations (Decourtye and Devillers 2010). The process of trophallaxis in bees may contribute to the differences in uptake, accumulation and final concentrations of neonicotinoids reaching the target site (Decourtye and Devillers 2010, Blacquiere et al. 2012). Nitroguanidine neonicotinoids, including imidacloprid, thiamethoxam, clothianidin, and dinofeturan, have been shown to be more toxic to honey bees than cyanoguanidine neonicotinoids (acetamiprid and thiacloprid) (Johnson 2015). Acute contact toxicity on honey bees of nitroguanidine neonicotinoids in the literature fluctuates from 4.1 to 7.5 ng/bee; however, toxicity for cyanoguanidine neonicotinoids ranges from 7,100 to 14,600 ng/bee (Nauen et al. 2001, Schmuck et al. 2001, Iwasa et al. 2004). For acute oral toxicity, LD\textsubscript{50} values vary between 4 and 40 ng/bee (Blacquiere et al. 2012).

Reports on chronic lethal toxicity of neonicotinoids to honey bees are also variable (Blacquiere et al. 2012). Under laboratory conditions, chronic exposure to neonicotinoids has not caused observable lethal effects at concentrations below 10 μg/L, with lethal chronic levels at 1760 μg/L for at least 6 days of exposure to neonicotinoids in syrup (Schmuck 2004, Cresswell 2011). Field studies have not shown worker mortality at realistic field concentrations (Schmuck et al. 2001, Cresswell 2011, Blacquiere et al. 2012). Thus, field relevant concentrations in pollen and nectar in flowers may not cause acute or chronic toxicity to honey bees (Blacquiere et al. 2012, Johnson 2015). However, sub-lethal impacts on adult bee performance and foraging behavior can be expected (Johnson 2015). In the meta-analysis developed by Cresswell (2011), performance can be
reduced by 6 and 11% in oilseed rape and between 14 and 16% in sunflower treated with neonicotinoids. However, more studies are necessary to evaluate the correlation between field-realistic doses and the likelihood of sub-lethal effects in different pollinator species.

Effects of neonicotinoids on insect natural enemies

Few efforts have been dedicated to quantify lethal concentrations of neonicotinoids to beneficial insects other than pollinators (Pisa et al. 2015). Insect natural enemies play an important role in pest regulation in agricultural systems (Desneux et al. 2007, Gentz et al. 2010, Prabhaker et al. 2011, Seagraves and Lundgren 2012). Natural enemies can have different routes of exposure in agricultural environments (Pisa et al. 2015). Parasitoids and predators augment their diet with nectar and pollen that can be contaminated by translocation of systemic pesticides into floral tissues (Girolami et al. 2009, Pisa et al. 2015). Consumption of vegetative tissue is also be a common behavior in predatory species, resulting in the potential to ingest neonicotinoids residues translocated in leaves (Moser and Obrycki 2009). Moreover, predators can be exposed to neonicotinoids by consuming residues in phloem feeding insects of treated crops (Moser and Obrycki 2009, Prabhaker et al. 2011). Other routes of neonicotinoid exposure to beneficial insects can be the ingestion of gutation droplets from treated plants or the direct contact with dust or treated surfaces (Pisa et al. 2015).

A few studies have investigated the levels of acute or chronic toxicity of neonicotinoids on parasitic and predatory insects (Prabhaker et al. 2007, Pisa et al. 2015). Neonicotinoid toxicity for natural enemies in agricultural fields have been mainly evaluated on coleopteran species with the majority of studies in the family Coccinellidae.

Coccinellid species have received particular attention because of their ability to control pests in different environments (Pisa et al. 2015). Overall, research on coleopteran species has been conducted with imidacloprid and there is still limited information on contact and oral toxicity of the different neonicotinoid active ingredients on the most agriculturally important coccinelidae species (Cloyd and Bethke 2011). Acute contact toxicity of imidacloprid in *Coleomegilla maculata* (Degeer) has shown an *LD*$_{50}$ of 0.074 ng(AI)/per beetle. Toxicity of imidacloprid residues in leaves under laboratory conditions has reported *LC*$_{50}$’s of 34.2 μg(AI)/ml for *Coccinella undecimpunctata* (L.) (Ahmad et al. 2011), 15.25-23.9 μg(AI)/ml for *Cryptolaemus montrouzieri* (Mulsant), and 364 μg(AI)/ml for *Harmonia axydiris* (Pallas).

Toxicity evaluations for other natural enemies have been mainly focused on hymenopteran parasites and hemipteran predators. For instance, Prabhaker et al. (2011) reported the acute toxicity of thiamethoxam and imidacloprid for parasitoid species such as *Aphytis melinus* (Debach), *Gonatocerus ashmeadi* (Girault), *Eretmocerus eremicus* (Rose & Zolnerowich), and *Encarsia formosa* (Gahan), and in two generalist hemipteran predators species, *Geocoris punctipes* (Say) and *Orius insidiosus* (Say). Using a systemic bioassay, *LC*$_{50}$’s on parasitoid species is between 0.1 and 1 mg (AI)/ml (parts per thousand) for thiamethoxam and 0.2 to 2 mg (AI)/ml for imidacloprid. For adult
predatory species, reported LC$_{50}$ values varies between 1 and 3 mg (AI)/ml for thiamethoxam and 2 and 5 mg (AI)/ml for imidacloprid. These studies used commercial formulations of neonicotinoids which could explain the high lethal values reported. LD$_{50}$ and LC$_{50}$ values for other natural enemies and pollinator species are reported in ppb (parts per billion) and ppm (parts per million) units and not ppt (parts per thousand).

Differences in laboratory methodologies across studies contribute significantly to the variability of estimation of median lethal dose/concentrations and side effects of pesticides on natural enemies (Desneux and O'Neil 2008). Toxicology bioassay methods are developed in function of the evaluated beneficial species and pesticides, usually based on the different guidelines proposed by regulatory agencies in each country (Desneux et al. 2007). This can result in significant differences in the type of exposure, test environmental conditions, insecticide formulation, and insect sources resulting in significant variability in the estimation of median lethal dose/concentrations (Pisa et al. 2015). Moreover, it is also important to develop and standardize methods to evaluate key sub-lethal effects on natural enemies that could affect population establishment and their capacity to control insect pests (Desneux et al. 2007, Cloyd and Bethke 2011).

Regulation of pest populations by natural enemies depends on key physiological and behavioral parameters (Desneux et al. 2007, Cloyd 2012). Some of those key parameters are fecundity, fertility, and prey consumption or parasitism rates (Cloyd and Bethke 2011, Cloyd 2012). Natural enemy feeding behavior can be affected by pesticides through repellency, antifeedancy, and reduction of olfaction abilities to find host or prey (Desneux et al. 2007). Stapel et al. (2000) showed a decrease in the response to host...
associated odors when the parasitoid *Microplitis croceipes* (Cresson) was previously exposed to imidacloprid. Reduced predation capacity has also been observed when insects are exposed to imidicloprid (Poletti et al. 2007, Szczepaniec et al. 2011, He et al. 2012, Malaquias et al. 2013). Despite negative effects on behavior reported in laboratory studies, further investigation is warranted to determine sub-lethal effects of neonicotinoids under realistic concentrations and field conditions (Cloyd and Bethke 2011).

**Mefenoxam**

*Mode of Action*

Mefenoxam is a widely used systemic fungicide for control Oomycete plant pathogenic fungi causing root and stem diseases and damping off of seedlings (Monkiedje et al. 2007, Triantafyllidis et al. 2012a). The Fungicide Resistance Action Committee (FRAC) classifies this fungicide in the acylalanines chemical group and the phenylamides group name. This fungicide targets the RNA polymerase I-template complex causing a disruption of protein synthesis (Hewitt 1998). Mefenoxam acts at a specific developmental stage of oomycete infection beyond the formation of the primary haustorium (Hewitt 1998).

*History*

Mefenoxam, also known as Metalaxyl-M, was introduced in 1996 under different formulation and trade names including Ridomil gold, Fonganil gold, Apron XL, Subdue, and ApronMAXX (Monkiedje et al. 2002). Mefenoxam is 97.5% of the R-enantiomer of
metalaxyl [methyl N-(2,6-dimethylphenyl)-N-(methoxyacetyl)-D-alaninate] and 2.5% of S-isomer methyl N-(2,6-dimethylphenyl)-N-(methoxyacetyl)-DL-alaninate]. Metalaxyl, also commonly used as a systemic fungicide of the phenylamide group, is a 50:50 racemic mixture of R- and S-enantiomers. The R-enantiomeric form gives the fungicidal activity to these products, and therefore, mefenoxam has replaced metalaxyl in the market (Monkiedje et al. 2007). Mefenoxam has the same activity as metalaxyl at half the application rate (Monkiedje and Spiteller 2002, 2005, Monkiedje et al. 2007). Application rates of mefenoxam range from 0.077 to 0.35 g AI kg\(^{-1}\) in seed treatments and 0.075 to 4.485 kg AI ha\(^{-1}\) in foliar applications (Monkiedje et al. 2002).

**Physicochemical properties**

Mefenoxam has higher water solubility (26 g l\(^{-1}\)) than metalaxyl (8.4 g l\(^{-1}\)) and is moderately volatile and weakly absorbed in soil (Monkiedje and Spiteller 2002, 2005, Monkiedje et al. 2007). The broad spectrum activity and wide application range in agriculture is related to its stability to a broad range of pH, temperature, and light conditions (Monkiedje et al. 2002). Although mefenoxam is widely used in different crops and seed treatments, few studies have been performed examining the environmental fate of this compound and its activity as a synergist or antagonist to insecticide toxicity (Triantafyllidis et al., 2012).

**Mixture toxicity of neonicotinoids and fungicides**

Insecticide and fungicide combinations are commonly used in seed treatments that can result in synergistic effects affecting pesticide toxicity (Mullin et al. 2015).
Neonicotinoid toxicity in mixtures with other products on pollinators and other beneficial insects has been poorly studied (Blacquiere et al. 2012, Krupke et al. 2012). To this point, there is only one study available on the interaction of neonicotinoids with fungicides in seed treatments (Iwasa et al. 2004). These authors found that the addition of the fungicides triflumizole and propiconazole increase the acute toxicity of acetamiprid and thiacloprid making them approximately 100 times more toxic in the mixture than applied solely. However, the effect of these fungicides on the toxicity of imidacloprid was minimal (Iwasa et al. 2004). Effects of these fungicides on insecticide toxicity are likely to occur through inhibition of detoxification enzymes, such as cytochrome P450 monooxygenases (Johnson et al. 2013). However, physiological mechanisms on how multiple pesticides affect the toxicity of one compound are not well understood.

Although insecticides and fungicides are being applied at the same time and with the same intensity and have been detected in pollen, honey, and beeswax, ecotoxicological studies on beneficial insects have principally focused on the impact of insecticides alone, with no regard to the effects of these chemicals in combination (Blacquiere et al. 2012). Synergistic effects of triazole fungicides on pesticide toxicity have been documented in other studies (Iwasa et al. 2004, Johnson et al. 2013). However, there is limited information available concerning the effects of other widely used of fungicides in crop protection such as the acylalanines (i.e. mefenoxam) and strobilurins (i.e. azoxystrobin) on insecticide toxicity. The inhibition of the detoxification mechanisms by fungicides in insects could generate synergism or antagonism between two different pesticide classes, increasing or reducing the toxicity of the insecticide (Johnson et al. 2013). I hypothesize that systemic fungicides can have an effect
on the toxicity of neonicotinoid on non-target organisms generating variability in the studies with neonicotinoids under real field scenarios.
OBJECTIVES

General Objective

To evaluate the ecological risks to beneficial insects of thiamethoxam and mefenoxam seed treatments in soybean

Specific Objectives

1. To determine the translocation of thiamethoxam and mefenoxam in leaves and flowers at early reproductive stages of soybean

2. To evaluate the effect that mefenoxam on the acute toxicity of thiamethoxam on worker honey bees.

3. To evaluate the toxicity of thiamethoxam on key predators of soybean aphid
Cited Literature


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CHAPTER 2

Residues of thiamethoxam and mefenoxam in flowers and leaves of early reproductive stage soybean resulting from seed treatments

Introduction

The use of systemic pesticides has gained critical attention due to the risk that they might pose to pollinators, insect natural enemies, and other non-target organisms (Krupke et al. 2012, Pisa et al. 2015). Systemic pesticides must persist in the plant long enough to achieve control of above ground pests; therefore, they may contaminate beneficial insect food sources, such as pollen, nectar, and guttation in leaves (Girolami et al. 2009, Krupke et al. 2012, Seagraves and Lundgren 2012, Bonmatin et al. 2015, Pisa et al. 2015). Thiamethoxam and mefenoxam are two of the most widely used systemic pesticides in soybean seed treatments in the U.S. (Cox et al. 2008, Cox and Cherney 2011, Gaspar et al. 2015). The fate of these and other systemic pesticides in flowers is of extreme importance due to the impact residues could have on several non-target species that use pollen and nectar (Bonmatin et al. 2015).

Thiamethoxam is a water-soluble compound (4.1 g l\(^{-1}\) at 20 °C), which allows the uptake and translocation of the active ingredient through the vascular system of the plants (Maienfisch et al. 2001). Mefenoxam (also called R-metalaxyl) is the R-enantiomer of metalaxyl and a commonly used fungicide in seed treatments (Monkiedje et al. 2007). This fungicide is highly systemic and water soluble (26 g l\(^{-1}\) at 20 °C) and one of the most frequently applied fungicides for crop protection worldwide (Triantafyllidis et al. 2012b).
The broad-spectrum activity and highly systemic properties of thiamethoxam and mefenoxam has contributed to the success and widespread use of these compounds in seed treatment applications (Simon-Delso et al. 2015). However, the systemic properties of these pesticides have been a concern during the last decade because they can be translocated and accumulate in flowers that serve as a food source for pollinators and other beneficial insects (van der Sluijs et al. 2015).

Residues of neonicotinoids have been identified in leaves and flowers of seed treated plants for several crops including canola, corn, cotton, and sunflower (Krupke et al. 2012, Stoner and Eitzer 2012, Stewart et al. 2014, Bonmatin et al. 2015, Bredeson and Lundgren 2015, Xu et al. 2016). Residues of thiamethoxam have been identified at ~5 ppb in old leaves at early reproductive stages of soybean (Magalhaes et al. 2009). However, there is limited information on the translocation of neonicotinoids and other systemic pesticides to soybean flowers. Stewart et al. (2014) characterized the translocation of neonicotinoids in soybean in southern states of the U.S., finding very low concentrations in soybean flowers. Information is limited for translocation of systemic products in northern U.S., where more than 80% of the soybean is grown in the U.S. The use of early maturity varieties in northern states versus southern states can increase the probability to find residues in soybean flowers in northernmost regions of the United States (Pedersen and Elbert 2004). Early maturating varieties may exhibit faster development from planting to flowering (Pedersen and Lauer 2004), reducing the time for metabolism of neonicotinoids seed treatment in plant tissue and increasing the probability of translocation to reproductive tissues.
For mefenoxam, information on residues and translocation in the plant is also very limited (Monkiedje and Spiteller 2005). Although several authors report the systemic movement of mefenoxam and metalaxyl in plants, few of those studies indicate the concentrations of the active ingredient in plant tissues and its persistence over time (Singh et al. 1986, Sukul 2000, Wilson et al. 2001, Monkiedje et al. 2007). Mefenoxam and metalaxyl are highly water-soluble compounds, and they have the potential to move to vegetative tissues and pollen and nectar in flowers. Krupke et al. (2012) reported residues of metalaxyl in pollen of seed treated corn at a concentration of 3.1 ppb. Although fungicides in seed treatments are not acutely toxic to insects, they can have synergistic or additive effects with some neonicotinoids and need to be considered when assessing the risk of seed treatments to non-target insects (Krupke et al. 2012).

Quantifying the concentrations of thiamethoxam and mefenoxam in soybean plants at reproductive stages is important to identify the window of activity of these products and the possible risks that these products might have on non-target organisms. The objective of this study was to quantify the concentrations of thiamethoxam and mefenoxam in select stage leaves and flowers of soybean plants after application as seed treatments.
Methodology

_Thiamethoxam and mefenoxam in soybean flowers_

The experiment was conducted during two soybean-growing seasons. In 2013, research plots were located at the University of Nebraska Northeast Research Extension Center Haskell Agricultural Laboratory in Concord, NE (Latitude 42°23'2.38"N; Longitude 96°56'29.14"W). In 2014, research plots were located in two different fields, one at the University of Nebraska-Lincoln Agricultural Research and Development Center at Ithaca, NE (Latitude 41° 9'54.49"N; Longitude 96°24'50.45"W), and the second at the University of Nebraska-Lincoln East Campus field plots maintained by the Department of Agronomy and Horticulture, Lincoln, NE (Latitude 40°50'9.93"N; Longitude, 96°39'44.95"W).

The design was a randomized complete block, with three treatments and three replications in each field. Treatments consisted of: 1) thiamethoxam alone at 0.0756 mg ai/seed, 2) thiamethoxam-mefenoxam at 0.0756 and 0.0113 mg ai/seed, respectively and 3) untreated seeds. Seeds were custom treated by Syngenta Crop Protection, Stanton, MN. Treatment plots consisted of 8 rows planted 76.2 cm between rows and 5.2 m in length, with 1.52 m between replications. Planting density was 140,000 seeds/acre.

Destructive sampling was performed at soybean reproductive stage R1 at 45 days after planting in 2013 and at 38 and 39 days after planting in 2014 (Fehr and Caviness 1977). A total of ~25 g of flowers were randomly collected from plants in the middle four rows and at least 60 cm in from each end of the plot. Flowers were collected from all the
nodes of the plant. Flowers were cut at the calix base and bagged for each plot. Collected flowers were kept in plastic bags in a plastic cooler with ice during transport. Samples were stored at -80°C. Each collected flower included the lateral bract, calix lobe, standard petals, wing petals, keel, ovary, stigma, and stamens.

*Thiamethoxam and mefenoxam in early reproductive stage soybean leaves*

Collection of vegetative tissue was conducted in 2014 and 2015. Fields were located at the University of Nebraska-Lincoln East Campus, Lincoln NE located at 40°50'9.97"N; 96°39'50.20"W in 2014 and at a Latitude 40° 50' 9.93 "N; Longitude, 96° 39' 44.95" W in 2015.

The design was a randomized complete block, with three treatments and three replications in each field. Treatments consisted of: 1) thiamethoxam only, 2) thiamethoxam and mefenoxam-treated, and 3) non-treated seeds. Because neonicotinoids have been reported to occur at low concentrations in foliage 30 and 40 days after planting (Magalhaes et al. 2009), leaves from the entire plant were pooled for further analysis. Plants were randomly selected from R1 stage plants from the two middle rows of each plot at 35 days after planting during 2014 and 37 days after planting during 2015. All the leaves were collected except cotyledons and unopened trifoliates. Samples were kept on ice during transport and transferred to a -20°C freezer for storage.

Although the translocation of metalaxyl has been previously reported for soybean, there are no studies in soybean evaluating the translocation of other phenylamide fungicides, such as mefenoxam. Therefore, to verify the translocation of mefenoxam into
soybean vegetative tissue from the applied rate one sample of leaves from five plants in the mixture treatment (thiamethoxam +mefenoxam) was collected at 18 days after planting (V2).

**Pesticide extraction**

Individual standard stock solutions of thiamethoxam (99.5% A.I), clothianidin (99.4% A.I), mefenoxam (99.9%), internal standards C\textsubscript{3}-thaimethoxam, C\textsubscript{3}-clothianidin, and C\textsubscript{6}-mefenoxam, and the surrogate terbuthilazine were diluted in methanol at 5ug uL\textsuperscript{-1} and stored in amber glass flasks at -20 °C. Calibration spiking solutions were prepared from the stock solutions diluted at 50 ng in 1 μL of methanol.

The sample preparation procedure was based on the modified QuEcChERS methodology (Pohorecka et al 2012). A total of 10g of plant material were used for each extraction. Flower samples included the petals, wing petals, keel, ovary, stigma, and stamens. Plant tissues were ground using a mortar and pestle in liquid nitrogen until a fine powder was obtained. Samples were placed in a 50 ml centrifuge tube with 30 ml of acetonitrile as an extraction reagent. The tube was shaken overnight using a multipurpose rotator and then centrifuged at 5000 rpm for 5 min. A total of 15 ml of the aliquot was transferred to a 15-mL dSPE tube containing 900 mg MgSO\textsubscript{4}, 300 mg PSA and 150 mg ChlороFiltr\textregistered. Samples were then vortexed for 30 seconds and centrifuged at 8000 rpm for 2 mins. An aliquot of 9 ml was diluted in 90 ml of distilled deionized water and passed through a 6 mg HLB cartridge. Cartridges were eluted with 5 ml of methanol and then evaporated at room temperature under a continuous nitrogen flow to 100μL. The
extract was reconstituted to 500μl 80:20 water: methanol and filtered using a 0.45 μm Mini-UniPrep Syringeless Filter. The final extract was analyzed by HPLC/MS/MS.

**HPLC-MS/MS analysis**

For HPLC analysis, a Quattro Micro with APCI (Waters, Milford MA) source system was used. An end-capped BetaBasic C<sub>18</sub> reverse phase HPLC column (250x2 mm) was used for the chromatographic separation. The injected sample volume was 50 μL. The mobile phases consisted in 0.15% formic acid in A) water/methanol (97:3) and B) 0.15% formic acid in methanol/water (97:3); at a constant temperature of 50°C and a flow rate of 0.3 ml/min. The gradient of the mobile phases was formic acid in the methanol/water (97:3) at 5% from 0-1min, at 50% from 1-3 min, at 65% to 75% from 3-10 min, at 100% 10-15 min and back to 5% from 15-20 min. For the mass spectrometry, the ionization of the analytes was performed with a positive ion mode atmospheric pressure chemical ionization (APCI). A pseudo-molecular ion [M+H]<sup>+</sup> was selected as the parent ion for fragmentation, and the corresponding fragment ion(s) were selected for identification and quantitation of the neonicotinoids. Ionization and collision energies were optimized based on procedures described by the instrument manufacturer.

Method recoveries and detection limits were evaluated by spiking untreated soybean leaves from plants maintained under greenhouse conditions. Leaves from plants at V6 were collected and transferred to a -20°C freezer. A total of 10 g of leaves were spiked with 60 ng of the analyte mixture (thiamethoxam, clothianidin and mefenoxam) and 60 ng internal standard (Thiamethoxam d-3, Clothianidin d-3, Metalaxyl d-5).
MDLs for each analyte was analyzed using seven aliquots in soybean plant material fortified with (60 ng/g) of each analyte pipette in 10 gm of uncontaminated plant material with the acetonitrile. MDLs were calculated through the sample standard deviation times of the replicate analysis (S) and the Student’s “t” for the 99% confidence level with n-1 (6) degrees of freedom. Using the averages, non-detection was assumed when the values were equivalent to 0.0 ng/g. “Trace” qualitative detections (non-quantifiable detection <MDL) were also included in the analytical reports.

Statistical Analysis

Flower and leaf residue data were analyzed using an ANOVA with a generalized linear mixed model with a normal distribution to compare the concentration levels between treatments. The model used the effect of the location nested in years as a fixed variable because there were different fields evaluated in each year. The treatment and the level of each analyte (thiamethoxam, clothianidin, and mefenoxam) were also used as a fixed variable in the model taking into account the interaction of the analytes and treatments with the location nested in year. Means between treatments were compared by the Fisher’s least significant difference test. The analysis for this study was generated using SAS/STAT software version 9.1.3 (SAS Institute Inc, Cary NC).
Results

Method recoveries and detection limits

Method of detection limits (MDL) were 1.12 ng/g for Clothianidin, 4.92 ng/g for thiamethoxam and 0.55 ng/g for Mefenoxam (Table 2.1, 2.2). In general, the accuracy of the method (recovery percentage) and its precision (standard deviation) were acceptable based on Environmental Protection Agency Requirements. Recoveries from all analytes ranged from 90 to 110% with relative standard deviations of <25% (Table 2.3). Thiamethoxam showed higher variability in its detection across samples (Table 2.2).

Thiamethoxam and mfenoxam in soybean flowers

The concentration of the analytes in each treatment was not significantly different between locations within the year (Num DF=8, Den DF=57, F-value=1.64, p-value=0.1342). Residues of thiamethoxam and mfenoxam in soybean flowers were very low with mean concentrations below the MDL for all treatments (MDLs: clothianidin: 1.1 ng/g, thiamethoxam: 4.9 ng/g, mfenoxam: 0.5 ng/g) (Table 2.4). However, flowers from plants derived from treated and untreated seeds were significantly different in residues levels (Num DF=2, Den DF=57, F-value=3.81, p-value=0.0279). This difference is probably observed because traces of clothianidin in individual samples in thiamethoxam alone and in the mixture treatments were close or above the MDL values with concentrations at 3.353 and 2.369 ng/g respectively (Figure 2.1). In the control treatment, thiamethoxam concentrations were all zero values, while in seed treatments this analyte show a distribution above zero (Figure 2.1). In general, the control treatment
did not show any samples with concentrations above the MDL (Figure 2.1). Mean concentrations of thiamethoxam were significantly higher in flowers from the seed treatments than from control flowers (Table 2.4). The concentrations of thiamethoxam in flowers from the two seed treatments (thiamethoxam in mixture and alone) were not significantly different from each other. Concentrations of thiamethoxam’s metabolite clothianidin were numerically higher, but not significantly different in flowers from seed treatments compared to the control flowers. There was high variability in the concentration of clothianidan in the flowers from seed treatments, which may have obscured differences among treatments (Figure 2.2). Concentrations of mefenoxam were lower than the neonicotinoid analytes (Figure 2.2), with averages close to zero in all treatments (Table 2.4).

**Thiamethoxam and mefenoxam in early reproductive stage soybean leaves**

Residues of neonicotinoids were present in soybean leaves at 35-37 DAP, with clothianidin concentrations for both seed treatments and the thiamethoxam-only seed treatment above the MDL (Table 2.5). There were no significant differences in the concentration of the analytes in the different treatments across location within years (Num DF=4, Den DF=48, F-value=0.68, p-value=0.6084). The concentrations of neonicotinoids in treated and untreated soybean was significantly different (Num DF=4, Den DF=57, F-value=18.19, p-value<.0001). Significantly higher concentrations of clothianidin and were found in plants with seed treatments compared to the control (Table 2.3). The concentration of thiamethoxam in leaves from the seed treatments thiamethoxam-mefenoxam and thiamethoxam-only was not significantly different from
the control. Clothianidin was the most predominant analyte and was routinely detected at approximately ~5 ppb (Figure 2.2), with concentrations in leaves from the thiamethoxam-
mefenoxam and thiamethoxam-only seed treatments significantly higher than that of the control, but not significantly different from one another. Concentration levels in leaves for the control treatments were below the MDL and close to zero for all the analytes (Figure 2.2). Both, thiamethoxam and clothianidin, were present in leaves at early vegetative stages (V2) at 150.89 ppb and 5.64 ppb respectively, indicating that thiamethoxam is degraded through time with only its metabolite clothianidin present at relatively high levels ~ 37 days after planting.

Concentrations of mefenoxam in leaves were not significantly different between the treatments and the control with values below the MDL for all the treatments (Fig 2.2, Table 2.3). In leaves from V2 soybean, mefenoxam exhibited a concentration of 2.69 ppb proving the translocation of mefenoxam to foliage at very low rates. The concentration found in leaves in this study is relatively low and may not have a significant fungicidal impact, considering that the effective concentration (EC$_{50}$) of this compound against pathogens such as Phytophthora spp is ~500 ppb (Parra and Ristaino 2001).
Discussion

Residues of thiamethoxam and its metabolite clothianidin in soybean flowers were very low to negligible, having mean concentrations close to zero and below the MDL. Similar results have been reported for residues of other neonicotinoids in soybean flowers in the southern United States (Stewart et al. 2014). These authors found either low or no traces of thiamethoxam and clothianidin (<1ng/g) in soybean flowers from plants with a seed treatment at 0.05 mg of a.i. per seed. Although in the current study the seed treatment rate was higher (0.075 mg a.i.), residues of neonicotinoids were also low or not detected in the flowers. Soybean is one of the largest crops in the U.S producing more than a half million flowers per acre that can serve as pollen, nectar, and water resources to pollinators and other beneficial insects (Gill and O’Neal 2015). Thus, the identification of systemic pesticides in soybean flowers is key to understand the level of exposure that beneficial insects can have to these products in floral sources from this crop.

Based on the results of this study, it appears unlikely that residues of neonicotinoid insecticides in soybean flowers that are translocated from treated seeds would cause acute toxic effects to pollinators as in most of the cases values were considerably low with rare cases between 2 and 3 ppb. The neonicotinoid (parent compounds and metabolites) concentrations in flowers associated with the translocation from seed treatments in this and other studies is between 0.1 ppb to 7 ppb (Krupke et al. 2012, Stewart et al. 2014, Xu et al. 2016). These concentrations are below the acute toxic effects (LD50 = 4.5 ng) or chronic toxic effects (LC50 after 6 days of exposure = 1,760
μg/L) in honey bees (Johnson 2015). However, these low concentrations may be related to sub-lethal effects in pollinator and other beneficial insect performance (Desneux and O’Neil 2008, Henry et al. 2012, Johnson 2015). Sub-lethal effects in relation to low concentrations of neonicotinoids in floral tissues require further investigation.

Toxic effects of neonicotinoid residues in flowers on beneficial insects can be associated with the translocation of these insecticides from foliar applications to pollen and nectar in flowers rather than translocation from seed treatments (Stoner and Eitzer 2012, Stewart et al. 2014). In soybean, foliar application of neonicotinoids are used from mid-vegetative and early reproductive stage soybean to control pests such as the soybean aphid and first generation of bean leaf beetle (EPA 2014). Because these applications can occur close to soybean flowering, contamination of soybean reproductive tissue is more likely to occur through translocation from these foliar applications than seed treatments (Dively and Kamel 2012, Stewart et al. 2014). Additional studies evaluating the residues of neonicotinoids in soybean flowers in relation to foliar application practices are necessary to identify exactly how exposure from neonicotinoids occurs.

Despite the trace concentrations of neonicotinoids found in soybean flowers, higher detections were found in seed treated plants than in controls. These results and the translaminar capabilities of these insecticides suggest that the movement of neonicotinoids to soybean flowers is possible (Bredeson and Lundgren 2015). Late planting dates for the region (late May and early June) in this study may have caused higher detection of neonicotinoids in flowers of seed treated plants versus non-seed-treated plants. Neonicotinoids in the plant become less concentrated as plants grow and
metabolism occurs (Jeschke et al. 2011). Soybean planted at later dates has less time for vegetative growth before flowering. In Nebraska, flowering begins in early July as day length decreases (Setiyono et al. 2007). Reduced time for growth before flowering can result in smaller plants and reduced neonicotinoid metabolism, which can increase the probability of translocation of neonicotinoids to soybean flowers. As planting dates in Nebraska are typically earlier (late April through mid-May), expected exposure of pollinators and natural enemies visiting soybean flowers would be likely less than in this study.

Residues of the thiamethoxam metabolite, clothianidin, in soybean flowers exhibited the highest concentration values. This result is consistent with the residue analyses of neonicotinoids in flower tissues from other crops where neonicotinoids metabolites showed higher concentrations than the parent compounds (Krupke et al. 2012, Stewart et al. 2014). Metabolites of neonicotinoids can have different or higher toxicities compared to the parent compounds (Suchail et al. 2001, Tomizawa and Casida 2005). For instance, clothianidin has greater affinity to insect nAChR than thiamethoxam (Nauen et al. 2003, Tomizawa and Casida 2005); therefore small concentrations of the metabolite may have greater effects than its parent compound. The risk evaluation of lethal and sub-lethal effects on beneficial insects from exposure to neonicotinoids in flowers should take into account not only concentrations of neonicotinoid parent compounds, but also its metabolites.

Furthermore, the evaluation of all floral tissues in this study (lateral bract, calix lobe, standard petals, wing petals, keel, ovary, stigma, and stamens) make it difficult to
identify the levels of neonicotinoids in specific structures of soybean flowers. Previous studies report the concentrations of neonicotinoids in only in pollen and nectar. Based on the systemic nature of neonicotinoids, translocation to all floral structures can be expected (Bredeson and Lundgren 2015). Thus, higher concentrations than the values reported in the literature were expected in this study as the use of all the tissues may increase the final estimated concentrations.

Low concentrations of neonicotinoids and other systemic pesticides from seed treatments in soybean floral tissue could be associated with the mechanisms of water movement in the plant to flowers. Residues of neonicotinoids may arrive to the different flower structures through the movement of water from the xylem, phloem, or both (Bonmatin et al. 2015). In flowers from some early angiosperm species, water potential can be lower than the rest of the plant; therefore, water would move to floral tissues mainly via xylem (Roddy and Dawson 2015). In contrast, on eudicot flowers, such as soybean, water potential in floral structures can be higher than the rest of the plant, and then water will move to flowers mainly via phloem (Roddy and Dawson 2015). Rate of water flux from the phloem is lower compared to xylem (Roddy and Dawson 2015). As neonicotinoids move mainly via xylem (Jeschke et al. 2011), it is expected to have lower concentrations in floral structures if water is coming mainly from the phloem. If soybean flowers are obtaining water mostly from the phloem it is expected to encounter low concentrations of neonicotinoids in these reproductive structures.

The mean concentrations of mefenoxam in soybean flowers were either lower than neonicotinoid concentrations, below the MDL for this compound, or not detected.
Mefenoxam concentrations in flowers were not significantly different than those of the control, which suggests a low risk of translocation of mefenoxam to flowers from treated seeds at the rate used in this study (0.011 mg of a.i per seed). Furthermore, mefenoxam was not detected in soybean leaves at early reproductive stages 37 DAP, and at low concentration levels (2.69 ppb) at early vegetative stages 18 DAP, supporting the hypothesis of reduced movement of mefenoxam from treated seeds into the plant vegetative and floral tissue. (Gupta 1985) found that the stereoisomer of mefenoxam, metalaxyl, remained in the cotyledons, with only a small percentage moving to leaves and stems of soybean plants. The small percentage of mefenoxam recovered in plant tissue in this study indicates that the fungicide likely remained in the root tissue, the cotyledons or diffused into the soil.

Clothianidin remained detectable at 35-37 DAP at ~ 5 ppb in the leaves while thiamethoxam was not detected or found at very low concentrations below its MDL. As the concentration of neonicotinoids decreases with plant growth, it is possible that at early reproductive stages only the metabolites of neonicotinoids parent compounds remain in the plant. Similar results have been reported for thiamethoxam seed treatments in sunflowers where clothianidin remained detectable in leaves during flowering, but not thiamethoxam (Bredeson and Lundgren 2015).

There is a potential effect of using a mixture seed treatment of thiamethoxam and mefenoxam in the concentration levels of thiamethoxam and its metabolite in plant tissue. This effect was not clearly observed in flowers, where there were no significant differences in the concentrations of neonicotinoids between the mixture and the treatment
with thiamethoxam alone. However, in leaves there were significantly lower levels of thiamethoxam on the mixture treatment compared to the insecticide alone. Moreover, the concentrations of clothianidin were numerically higher, although not significantly different between the mixture treatment and the control. Mixture treatments can improve plant growth (Supplemental results), which could affect the final concentration levels of neonicotinoids in the plant. Concentration of neonicotinoids decreases as plant growth increases (Jeschke et al. 2011). As the values of thiamethoxam in this study were below the MDL’s, future studies at mid vegetative stages in soybean can be performed when median concentration levels in the plants are expected to be above the MDL. Analyses comparing the insecticide alone and in the mixture treatment at mid vegetative stages can elucidate the effect on multiple products in seed treatments on the fate of neonicotinoids in plant tissues.

In spite of plant development and days after planting and their effects on the concentration of neonicotinoids in the plant, it is important to consider differences in the detection limits between thiamethoxam and clothianidin. Recovery of thiamethoxam is significantly affected by the matrix components in plant material, while clothianidin exhibited high recovery rates (90-100%) in complex matrices (Xie et al. 2011). The standardization of methods that allow the analysis of neonicotinoids, parent compounds and metabolites, in complex matrixes is crucial for the proper assessment of exposure of neonicotinoids to pollinators and other non-target organisms through residues in plant material.
One of the main difficulties in the accurate estimation of the fate of neonicotinoids in the environment is the variability in the calculation of the limits of detection (LOD), limits of quantification (LOQ), Method detection limit (MDL), and efficiency of the analytical methods used. LOD, LOQ, and MDL are used to describe the smallest concentration of an analyte that can be measured through an analytical procedure. The calculation of these values can be developed through multiple statistical procedures affecting the interpretation of concentrations found through the analytical methods. The information on how the methods of detection limits are calculated is critical to understand the capability and limitations of the information on residues of neonicotinoids reported, and the accuracy of the values obtained through the multiple analytical methods. However, few studies on the environmental fate of neonicotinoids report the methods to estimate these values. Lack of this information compromises the accuracy of the values and the efficiency of the analytical procedure.

MDL’s in this study were between 0.5-5 ppb using QuEChERS methods. Previous studies evaluating neonicotinoids in plant material report limits of detection between 0.5-1 ppb (Krupke et al. 2012, Stoner and Eitzer 2012, Stewart et al. 2014). However it is uncertain how the limits of detection values were calculated in these studies. Other methods such as the limit of detection (LOD), and the approach to calculate it, can affect the conclusions on weather there are quantitative concentrations or only traces (qualitative information) in the analyzed matrix. Statistical methods to calculate MDL is one of the most conservative methods to estimate limits of detection (Snow 2016, personal communication). MDL usually

However, it seems to be one of the least used methods in the quantification of neonicotinoids in the environment.
In conclusion, results of this study provide a better understanding of exposure levels in soybean flowers and leaves at R1 to early R2 (~37 DAP) when the majority of beneficial insects start to arrive to soybean fields. Only 10% of the concentration found in soybean leaves was found in flowers at early reproductive stages. Concentrations of neonicotinoids in both reproductive organs and leaves were low or not detected, and the effects on target and non-target insects from such traces are more likely to be sub-lethal, if at all. Also, it is unlikely to have a significant exposure of non-target insects to residues of mefenoxam in soybean leaf and floral tissue.
Cited Literature  I cannot mark changes in the cited literature but some references are incomplete. Double check for your final version.


**EPA. 2014.** Memorandum on Benefits of Neonicotinoid Seed Treatments to Soybean Production.


**Girolami, V., L. Mazzon, A. Squartini, N. Mori, M. Marzaro, A. Di Bernardo, M. Greatti, C. Giorio, and A. Tapparo. 2009.** Translocation of neonicotinoid


**Xie, W., C. Han, Y. Qian, H. Ding, X. Chen, and J. Xi. 2011.** Determination of neonicotinoid pesticides residues in agricultural samples by solid-phase extraction combined with liquid chromatography–tandem mass spectrometry. Journal of Chromatography A 1218: 4426-4433.

Figure 2.1. Distribution of the concentration data for thiamethoxam, clothianidin, and mefenoxam analytes in soybean flowers. Treatments correspond to: Control: untreated seeds, Mixture: thiamethoxam and mefenoxam seed treatment, Thiamethoxam seed treatment. Mean comparisons were carried out between treatments for each analyte. Whiskers correspond to the maximum and minimum values. Different letters correspond to significant differences at: p-value 0.01.
Figure 2.2. Distribution of the concentration data for thiamethoxam, clothianidin analytes in soybean leaves. Treatments correspond to: Control: untreated seeds, Mixture: thiamethoxam a.i and mefenoxam a.i treatment, Thiamethoxam: insecticide a.i only. Mean comparisons were carried out between treatments for each analyte. Different letters correspond to significant differences at: p-value ≤ 0.01.
Table 2.1. Method recoveries and detection limits for neonicotinoids in soybean plant tissue using QuEcChERS methodology.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>MDL ng/g</th>
<th>Average Recovery %</th>
<th>Std Dev Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clothianidin</td>
<td>1.119</td>
<td>99.96</td>
<td>6.13</td>
</tr>
<tr>
<td>Thiamethoxam</td>
<td>4.915</td>
<td>110.77</td>
<td>23.16</td>
</tr>
<tr>
<td>Mefenoxam</td>
<td>0.551</td>
<td>90.83</td>
<td>3.27</td>
</tr>
</tbody>
</table>
Table 2.2. Method of Detection Limit (MDL) data for chemical extraction from soybean tissue.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MDL #1</th>
<th>MDL#2</th>
<th>MDL #3</th>
<th>MDL #4</th>
<th>MDL #5</th>
<th>MDL #6</th>
<th>MDL #7</th>
<th>Avg</th>
<th>Std Dev</th>
<th>Spike</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clothianidin</td>
<td>54.30</td>
<td>59.32</td>
<td>63.32</td>
<td>65.27</td>
<td>60.71</td>
<td>57.03</td>
<td>59.88</td>
<td>59.98</td>
<td>3.68</td>
<td>60.00</td>
</tr>
<tr>
<td>Thiamethoxam</td>
<td>90.00</td>
<td>66.60</td>
<td>64.43</td>
<td>80.53</td>
<td>65.96</td>
<td>53.63</td>
<td>44.09</td>
<td>66.46</td>
<td>15.39</td>
<td>60.00</td>
</tr>
<tr>
<td>Mefenoxam</td>
<td>51.83</td>
<td>55.99</td>
<td>53.08</td>
<td>53.91</td>
<td>54.21</td>
<td>57.01</td>
<td>55.48</td>
<td>54.50</td>
<td>1.78</td>
<td>60.00</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Compound</th>
<th>MDL #1</th>
<th>MDL#2</th>
<th>MDL #3</th>
<th>MDL #4</th>
<th>MDL #5</th>
<th>MDL #6</th>
<th>MDL #7</th>
<th>Avg</th>
<th>S</th>
<th>MDL (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clothianidin</td>
<td>5.324</td>
<td>5.885</td>
<td>6.054</td>
<td>6.325</td>
<td>5.946</td>
<td>5.426</td>
<td>5.638</td>
<td>5.800</td>
<td>0.3561</td>
<td>1.119</td>
</tr>
<tr>
<td>Mefenoxam</td>
<td>5.081</td>
<td>5.555</td>
<td>5.075</td>
<td>5.224</td>
<td>5.309</td>
<td>5.424</td>
<td>5.224</td>
<td>5.270</td>
<td>0.1753</td>
<td>0.551</td>
</tr>
</tbody>
</table>
Table 2.3. Percent recovery for thiamethoxam, clothianidin and mefenoxam in soybean plant material

<table>
<thead>
<tr>
<th>Compound</th>
<th>MDL #1</th>
<th>MDL#2</th>
<th>MDL #3</th>
<th>MDL #4</th>
<th>MDL #5</th>
<th>MDL #6</th>
<th>MDL #7</th>
<th>AVG</th>
<th>REL % S</th>
<th>STD DEV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clothianidin</td>
<td>90.51</td>
<td>98.87</td>
<td>105.53</td>
<td>108.78</td>
<td>101.18</td>
<td>95.04</td>
<td>99.80</td>
<td>99.96</td>
<td>6.13</td>
<td>6.126</td>
</tr>
<tr>
<td>Thiamethoxam</td>
<td>150.00</td>
<td>111.00</td>
<td>107.38</td>
<td>134.22</td>
<td>109.93</td>
<td>89.38</td>
<td>73.48</td>
<td>110.77</td>
<td>23.16</td>
<td>25.655</td>
</tr>
<tr>
<td>Mefenoxam</td>
<td>86.38</td>
<td>93.32</td>
<td>88.47</td>
<td>89.85</td>
<td>90.35</td>
<td>95.01</td>
<td>92.46</td>
<td>90.83</td>
<td>3.27</td>
<td>2.969</td>
</tr>
</tbody>
</table>
Table 2.4. Mean analyte concentrations and standard errors in soybean flowers for different treatments.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Control</th>
<th>Mefenoxam +</th>
<th>Thiamethoxam</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SE</td>
<td>Mean ± SE</td>
<td>Mean ± SE</td>
</tr>
<tr>
<td>Clothianidin</td>
<td>0.217 ± 0.117a</td>
<td>0.486 ± 0.232</td>
<td>0.504 ± 0.329a</td>
</tr>
<tr>
<td>Thiamethoxam</td>
<td>0.000a</td>
<td>0.323 ± 0.133b</td>
<td>0.462 ± 0.183b</td>
</tr>
<tr>
<td>Mefenoxam</td>
<td>0.104 ± 0.044a</td>
<td>0.073 ± 0.030a</td>
<td>0.051 ± 0.023a</td>
</tr>
</tbody>
</table>

*Different letters within same row indicates significant differences at the 95% CL*
Table 2.5. Mean analyte concentrations and standard errors in soybean leaves for different treatments.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Control Mean ± SE</th>
<th>Mefenoxam + Thiamethoxam Mean ± SE</th>
<th>Thiamethoxam Mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clothianidin</td>
<td>0.324 ± 0.142 a</td>
<td>4.720 ± 0.254b</td>
<td>4.215 ± 0.263b</td>
</tr>
<tr>
<td>Mefenoxam</td>
<td>0.754 ± 0.3175a</td>
<td>0.591 ± 0.094a</td>
<td>0.469 ± 0.105a</td>
</tr>
<tr>
<td>Thiamethoxam</td>
<td>0.034 ± 0.0412a</td>
<td>0.129 ± 0.04a</td>
<td>2.076 ± 0.908 b</td>
</tr>
</tbody>
</table>

*Different letters correspond to significant differences at the 95 % CL.
CHAPTER 3

Toxicity of the conventional insecticide/fungicide seed treatment mixture of thiamethoxam and mefenoxam on *Apis melifera* under laboratory conditions

Introduction

The honey bee, *Apis melifera* L. plays an important ecological and economic role as pollinators of many crops systems and natural environments (Johnson 2015). During recent years honey bee health has received critical attention due to a worldwide decline of honey bees and other pollinators (Johnson et al. 2010, Mullin et al. 2010, Krupke et al. 2012, Biddinger and Rajotte 2015). Losses in honey bee colonies have been attributed to multiple stressors such as pathogens, parasites, malnutrition, and pesticides, including neonicotinoid insecticides (Johnson et al. 2010, Mullin et al. 2015, Pisa et al. 2015). Neonicotinoids are suspected of posing serious risks to honey bees due to the emission of dust particles from treated seed during planting that can cause high concentrations of these insecticides to contaminate pollen and nectar sources in the surrounding area (Krupke et al. 2012, Tapparo et al. 2012, Nuyttens et al. 2013, Bonmatin et al. 2015). Neonicotinoids used as seed treatments are typically applied in combination with a variety of fungicide classes (Biddinger and Rajotte 2015). However, few studies have evaluated the impact of neonicotinoids and fungicide mixtures on honey bees (Blacquiere et al. 2012, Biddinger and Rajotte 2015, Pisa et al. 2015).
The toxicity caused by exposure to a particular neonicotinoid concentration may be affected by simultaneous exposure to other compounds (Johnson et al. 2010, Mullin et al. 2015). Fungicides have been commonly detected in beehives in wax, beebread, and honey at similar levels of contamination with insecticides (Mullin et al. 2010, Simon-Delso et al. 2014, Johnson 2015). Several fungicide classes including phenylamides, strobilurins, and triazoles have been found near agricultural environments, and in beehives (Mullin et al. 2010, Krupke et al. 2012). Although fungicides have generally low acute toxicity to honey bees, they can have synergistic, additive or antagonistic effects, increasing or reducing the toxicity of insecticides to honey bees (Johnson et al. 2013, Zhu et al. 2014, Johnson 2015). The evaluation of toxicity of neonicotinoids coupled with the fungicides used in seed treatments should provide a better understanding of what is occurring in field situations and help to resolve some of the uncertainties in the risk assessment of seed treatments on pollinators (Krupke et al. 2012, Biddinger and Rajotte 2015, Pisa et al. 2015).

The application of both thiamethoxam and mefenoxam to soybean seeds is the one of the most common insecticide/fungicide mixtures used in seed treatments in the United States (EPA 2014, Douglas and Tooker 2015, Gaspar et al. 2015). Mefenoxam, more commonly known as R-metalaxyl, has been the lead product in the phenylamide fungicide class and is the most widely used fungicide in agriculture worldwide (Monkiedje and Spiteller 2002). Mefenoxam was introduced into the market in 1996 (Monkiedje and Spiteller 2002, Monkiedje et al. 2007) and has been widely used in seed treatments in combination with neonicotinoid insecticides (Cox and Cherney 2011). Both thiamethoxam and mefenoxam, have relatively high water solubility, which allows their
uptake by plants and the possible translocation of both compounds to pollen and nectar in flowers. Residues of the phenylamide fungicide, metalaxyl and the neonicotinoid thiamethoxam have been found in pollen from maize anthers at anthesis at 3.1 ppb and 1.7 ppb, respectively (Krupke et al. 2012). Higher concentrations of both compounds have been also found in dust from seed planters ranging from 70 to 13.2 ppm for thiamethoxam and 92 to 263 ppm for metalaxyl.

Field and laboratory studies attempting to test acute toxicity at realistic exposures of neonicotinoids have shown variable and often conflicting results (Pisa et al. 2015). There are many variables that could be affecting the assessment of acute toxicity of neonicotinoids, such as temperature, honey bee genotype, and the interaction with other stressors, such as fungicides (Krupke et al. 2012, van der Sluijs et al. 2015). Although mefenoxam is one of the most common pesticides used in seed treatments, there are no studies available evaluating possible additive, synergistic or antagonistic effects of this compound in the acute toxicity of neonicotinoid insecticides on honey bees. The objective of this study was to evaluate the effect of mefenoxam on the acute oral and contact toxicity of thiamethoxam on worker honey bees.
Methodology

Chemicals: Technical grade thiamethoxam (99.5%) and mefenoxam (99.9%) were purchased from Chem Services (West Chester, PA). Chemicals were maintained in dark conditions at -4 °C. Stock solution were diluted in acetone at 5 μg μL⁻¹ and stored at 20°C prior the experiments.

Bees: Late stage capped brood frames were collected from healthy colonies at the University of Nebraska-Lincoln at the East campus facilities in July and August of 2014 and 2015. Colonies were maintained using the standard preventative treatments for pest and diseases. Frames with late stage brood were placed in the dark in an environmental chamber (Darwinn Chambers, CO; model M024) at 33-35 °C, with relative humidity between 60% and 70%. Newly emerged bees were brushed daily from the frames and transferred to screened wood cages (1800 cm³) in groups of approximately 200 bees. Each cage was provisioned with 300 ml of sucrose solution at 1:1 (w/v) before the experiments. During the experiments bees were immobilized with carbon dioxide gas (CO₂) for 2 minutes before the exposure to the treatments. If a bee did not move after CO₂ exposure it was discarded from the bioassay.

Interaction of Mefenoxam in the toxicity of Thiamethoxam

To evaluate the interaction between thiamethoxam and mefenoxam, two sub-lethal concentrations of the fungicide were individually added to five different concentrations of thiamethoxam. These two fungicide concentrations were used in both oral and contact toxicity bioassays. To determine the two sub-lethal concentrations of
mefenoxam, worker bees from two different hives were exposed to a sucrose diet solution with four different concentrations of the fungicide (100, 10, 1, 0.1 μg/ml) and an untreated control. The experimental unit consisted of 20 newly emerged bees (3-4 days old) placed in wax coated paper cups covered with cotton cheesecloth and secured with two rubber bands. Two ml of sucrose solution with each fungicide concentration was given to the bees in a 1.5 ml eppendorf tube with two openings at the base of approximately 1 mm of diameter. Mortality of the bees was recorded at 24, 48, and 72 hours after the exposure. Because there was no mortality of the bees for any of the concentrations tested, the high and low doses in this range (100 and 0.1 μg/ml) were chosen as sub-lethal concentrations. These concentrations are also within the range of residues of metalaxyl (0.004 -200 μg/ml) in corn pollen and dust from seeds in planters reported by Krupke et al. (2012). Therefore, these concentrations represent environmentally realistic levels that might be encountered by bees in the environment.

Oral toxicity of A. melifera to thiamethoxam and mefenoxam mixtures was determined in adult workers feeding on a sucrose solution with the mixture of the insecticide and the fungicide. A completely randomized design with a 6 x 3 treatment factorial with 9 replications was used to determine the effect of the mixture. The first factor consisted of five concentrations of thiamethoxam: 100, 10, 1, 0.1, 0.01 μg/ml and untreated control analyzed as a continuous variable. The second factor consisted of the fungicide at 100 and 0.1 μg/ml and a no fungicide treatment. The insecticides were dissolved and mixed with the fungicide first in acetone and then diluted to the appropriate concentration in a 1:1 (w/v) water sucrose solution. The proportion of acetone in each sucrose solution was 1% for all the tested concentrations. For the untreated control, a
sucrose water solution with 1% acetone was used. The experimental unit consisted of 20 newly emerged bees (3-4 days old) placed in paper cups as previously described. Bees were fed with 2 ml of sucrose solution with each insecticide-fungicide concentration mixture in an eppendorf tube using the methodology previously described. Mortality of the bees was recorded at 24, 48 and 72 hours after the exposure.

Contact toxicity of *A. melifera* to both pesticides was determined with newly emerged adult workers (3-4 days old). Bees from three different hives were evaluated using 20, 18, and 10 bees per experimental unit for each one of the three hives, based on availability for a total of 9 replications per treatment combination. Bees were exposed to thiamethoxam/mefenoxam mixtures at 100 μg/ml, 0.1 μg/ml, and no-fungicide combined with a range of five concentrations of thiamethoxam at 5, 0.5, 0.05, 0.005, and 0.0005 μg/ml and untreated control. Mixtures were prepared in acetone previous to the topical application on the bees. Thiamethoxam and mefenoxam were dissolved in acetone and applied to the thorax of worker bees. A volume of 1 μl was applied with a 50 μl syringe mounted to a PB-600 dispenser (Hamilton, Reno NV). Treated bees were transferred to wax coated paper cups covered with cotton cheesecloth and secured with two rubber bands. Bees were fed with 1.5 ml of 1:1 (w/v) water/sucrose solution placed in each experimental unit during the development of the experiment.

*Statistical analysis*

*Toxicity of Mefenoxam only*

Differences between the fungicide treatments and the control in the absence of
Toxicity of Thiamethoxam with mefenoxam

Concentration- and dose-mortality data were fitted to the log-probit scale for each fungicide combination using a generalized linear mixed model for binomial responses using the R statistical package 3.1.2. The lethal concentration for oral toxicity (LC₅₀), lethal dose for contact toxicity (LD₅₀) and 95 % confidence intervals were estimated for each pesticide mixture at 24, 48 and 78 hours using Finney’s method with correction for heterogeneity when necessary. The interaction effects between the two concentrations of mefenoxam and thiamethoxam were determined using the likelihood ratio test to evaluate the “hypothesis of parallelism or equal slopes” and “the hypothesis of equality or equal intercepts” based on the analysis of mixture toxicity developed by Johnson et al. (2013). To have interaction between the compounds, at least one of the hypothesis needs to be rejected (Robertson and Preisler 1992). Slopes and intercepts of the probit regressions of thiamethoxam with each fungicide concentration and without the fungicide were compared by constructing a full model with all the interaction parameters compared to two different simplified models. The full model includes the dose or concentration of the insecticide thiamethoxam as a covariate, the fungicide concentration as a categorical variable and the interaction parameter between the fungicide and the insecticide. For the hypothesis of parallelism analysis, the simplified model lacks the interaction term and is compared with the full model. A significant change in the slope indicates interaction
between mefenoxam and thiamethoxam. Significant interaction values may indicate competitive inhibition between the fungicide and the insecticide. The hypothesis of equality was evaluated using a second simplified model that lacks all the fungicide parameters entirely and is compared to the full model. When a significant difference in the intercepts is observed, the hypothesis of equality is rejected providing evidence of an agonistic or antagonistic interaction (Jeske et al. 2009, Johnson et al. 2013). Statistical significance between the model slopes and intercepts was determined by using pairwise likelihood ratios, corrected for heterogeneity against an F-distribution.
Results

Toxicity of Mefenoxam only

Mefenoxam was not toxic to worker honey bees (Figure 1) under the conditions tested. In general, the mortality of honey bees was significantly lower in both mefenoxam concentrations compared with the control at 24 and 48 hours after oral and contact exposure (Oral data analysis: Num DF=3, DenDF=96, F-value=3.850 p-value=0.0119*; Contact data analysis: Num DF=3, DenDF=70, F-value=5.315 p-value=0.0023**) (Figure 3.1). Mortality in all treatments increased overtime with significant differences between 24, 48 and 72 hours after oral exposure (Num DF=2, DenDF=96, F-value=9.023, p-value=0.0003***). In contrast, contact exposure did not show significant differences in the mortality over time (Num DF=2, DenDF=70, F-value=2.418, p-value=0.0965) (Figure 3.1).

Interaction of Mefenoxam in the toxicity of Thiamethoxam

The response of A. melifera to thiamethoxam alone and in combination with mefenoxam showed a parallel linear regression but unequal intercepts in the oral and contact toxicity bioassays (Table 3.1, Figure 3.2). The results of oral exposure bioassays revealed significant differences in the intercept of thiamethoxam alone compared to thiamethoxam mixed with mefenoxam at 0.1 μg/ml at 24 hours (Equality Deviance= 3.756, NumDF=2, DenDF= 8, p-value=0.04) (Table 3.1, Figure 3.2). At 24 hours, adult honey bees were more susceptible to thiamethoxam alone compared with the mixture of thiamethoxam and mefenoxam at 0.1 μg/ml (Figure 3.1, Table 3.2).
toxicity of thiamethoxam alone and combined with mefenoxam at all evaluated times was in general: thiamethoxam plus 100 μg/ml of mefenoxam > thiamethoxam alone > thiamethoxam plus 0.1 μg/ml (Table 3.2, Figure 3.2). There were no significant differences in the slopes and intercepts between thiamethoxam in combination with a high concentration of mefenoxam (100 μg/ml) compared to thiamethoxam alone (Table 3.1).

The test for the hypothesis of equality for contact bioassays indicated significant differences in the intercepts of both fungicide concentrations with the control (no-fungicide treatment) after 48 hours of exposure (Thiamethoxam + Mefenoxam 100μg/ml: Equality Deviance= 3.404, NumDF=2, DenDF= 8, p-value=0.05; Thiamethoxam + Mefenoxam 0.1μg/ml: Equality Deviance= 4.517, NumDF=2, DenDF= 8, p-value=0.027) (Table 3.3, Figure 3.2). The relative toxicity of thiamethoxam in combination with both mefenoxam concentrations was significantly lower compared with the control treatment at 48 hours (Table 3.2, Figure 3.2). The LD50 values through contact exposure were in general: thiamethoxam alone > thiamethoxam plus 100 μg/ml of mefenoxam ≥ thiamethoxam plus 0.1 μg/ml. The high concentration of mefenoxam had a different effect in the toxicity of thiamethoxam between the contact exposure and oral exposure. In the oral toxicity bioassay LC50s of thiamethoxam with mefenoxam at 100 μg/ml had a slight increase in the relative toxicity of the insecticide, while the contact bioassay showed that the LC50s of this mixture were equal or lower than thiamethoxam alone.
Discussion

Contact and oral administration of mefenoxam alone did not cause acute toxic effects to worker honey bees (Figure 3.1). Dust from seed treatments and contaminated pollen are the most likely routes of exposure to mefenoxam. Reports of residues of the stereoisomer of mefenoxam, Metalaxyl-M, have been reported from 3 ppb to 100 ppm in pollen and seed dust respectively (Mullin et al. 2010, Krupke et al. 2012). Given the high tolerance of honey bees to mefenoxam observed in this study it seems unlikely that adult worker bees will suffer acute mortality in the field from this fungicide. Further studies are necessary to confirm the safety of this compound when combined with other products in seed treatments and possible sub-lethal effects.

When mefenoxam is combined with thiamethoxam, there was a mild interaction with the fungicide, resulting in reduced bee mortality at 24 and 48 hours after oral and contact exposure with the insecticide-fungicide mixture. The effects of mefenoxam on the acute toxicity of thiamethoxam were mainly antagonistic. However, this effect was only found at 24 hours through oral exposure decreasing the toxicity of thiamethoxam by 3-fold and at 48 hours through contact exposure decreasing the toxicity by 2-fold. Both concentrations of mefenoxam caused a slight decrease in the toxicity of thiamethoxam through contact exposure. In contrast, only the lowest concentration of mefenoxam generated an antagonistic effect through oral exposure. Slow movement of mefenoxam through the insect exoskeleton might be related with the final concentration of the fungicide inside the insect internal environment relative to direct oral exposure. As a consequence, a different response may be observed when a high concentration of the
fungicide is applied orally compared to topical application. The epicuticular wax and the integument barriers can affect the movement of highly water soluble compounds such as mefenoxam to the sites of action of the compound. This could cause a later antagonistic response through topical application (effect at 48 hours after treatment) of the fungicide compared to the oral exposure (effect at 24 hours after treatment).

Antagonistic interactions between pesticides on honey bees have been found with other products used to manage bee colonies in North America (Johnson et al. 2013, Zhu et al. 2014). Johnson et al. 2013, reported antagonistic interactions between fumagillin, an antimicrobial product with the acaracide fenpyroximate and the pyrethroid tau-flvalinate on worker honey bees. The mode of action of fumagillin, as well as mefenoxam, is the inhibition of RNA synthesis in fungal pathogens (Jaronski 1972, Georgiev 1997, Hewitt 1998). Nevertheless, the reason for antagonic interactions between molecules with this mode of action and those pesticides is unknown. One possible explanation for antagonistic interaction of these fungicides and the toxicity of pesticides is an effect of RNA synthesis inhibitor products on honey bee fungal pathogens. Fungal pathogens in honey bees include *Nosema apis*, *Nosema ceranae*, *Ascophaera apis*, and *Aspergillus* spp (Evans and Schwarz 2011). Pathogens, such as *Nosema* spp, can cause a suppression of the immune system in bees, making them more susceptible to pesticides (Alaux et al. 2010, Paxton 2010). An effect of mefenoxam on honey bee pathogens could mitigate the toxic effect of thiamethoxam and could also explain the differences in the survivorship of worker bees when exposed to mefenoxam alone compared to the untreated control.
Significant antagonism has been also observed in honey bee larvae exposed to a combination of the fungicide, chlorothalonil, and the pyrethroid, fluvalinate, at low concentrations (Zhu et al. 2014). These authors suggest that antagonism might be associated with detoxification mechanisms becoming overwhelmed with multiple pesticides. The metabolite of an insecticide in some cases can be more toxic than the parent compound. If detoxification enzymes are working on multiple stressors, toxic metabolites can be produced at a slower rate. In the insect haemolymph, thiamethoxam is rapidly converted to clothianidin, a highly active neonicotinoid (Nauen et al. 2003, Jeschke and Nauen 2008). Clothianidin binds to the nictinic acetylcholine receptors with higher affinity than thiamethoxam (Nauen et al. 2003). If thiamethoxam is metabolized to clothianidin more slowly in honey bees, the final toxicity of the compound can be reduced because detoxification enzymes could be functioning in the metabolism of other compounds.

Studies of mixture toxicity between different classes of pesticides used in seed treatments are rarely available for honey bees and other non-target insects. Mefenoxam is a widely used fungicide in combination with neonicotinoid insecticides. Therefore, it is important to elucidate harmful effects that this pesticide mixture could have on honey bee survival. To our knowledge this is the first study that evaluates interaction effects of mefenoxam on acute toxicity of thiamethoxam. Studies of mixture toxicity of seed treatment products are imperative to minimize the risk of pesticides on honey bees by a careful selection of products with lower toxicity (Biddinger and Rajotte 2015).
Cited Literature


Oral Toxicity

Contact Toxicity

Figure 3.1. Toxicity of mefenoxam alone on worker honey bees. Control: Acetone 1%; Mefenoxam High: 100 μg/ml, Mefenoxam Low: 0.1 μg/ml
Figure 3.2. Probit mortality vs. log concentration plots for oral and contact toxicity bioassays at the three time points. The symbols represent the raw mortality data: Thiamethoxam alone “*”, Mixture of thiamethoxam with Mefenoxam at 100 µg/ml “+” and Mixture of thiamethoxam with Mefenoxam at 0.1 µg/ml: “−”. Lines represent the probit regression fitted for each treatment.
Table 3.1. Pairwise comparison of slopes and intercepts of dose-response regressions with the insecticide thiamethoxam alone and in combination with the fungicide mefenoxam at two different concentrations for the oral and contact toxicity bioassay

* Significant differences between the fungicide treatments compared to the control. p-values adjusted for two pairwise comparisons per hour.
Table 3.2. Dose-response parameters and pairwise comparison of thiamethoxam alone and in combination with the fungicide mefenoxam at two different concentrations for the contact and oral bioassay

<table>
<thead>
<tr>
<th>Hours after exposure</th>
<th>Treatment</th>
<th>Oral Toxicity</th>
<th>Contact Toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>LC50 µg/ml</td>
<td>Slope</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lower CL</td>
<td>Upper CL</td>
</tr>
<tr>
<td>24 h</td>
<td>Thiamethoxam alone (Control)</td>
<td>0.934</td>
<td>2.190</td>
</tr>
<tr>
<td></td>
<td>Thiamethoxam+ Mefenoxam_High 100 µg/ml</td>
<td>0.476</td>
<td>0.102</td>
</tr>
<tr>
<td></td>
<td>Thiamethoxam+ Mefenoxam_Low 0.1 µg/ml</td>
<td>3.715</td>
<td>0.649</td>
</tr>
<tr>
<td>48 h</td>
<td>Thiamethoxam alone (Control)</td>
<td>0.451</td>
<td>0.042</td>
</tr>
<tr>
<td></td>
<td>Thiamethoxam+ Mefenoxam_High 100 µg/ml</td>
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</tr>
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<td>Thiamethoxam+ Mefenoxam_Low 0.1 µg/ml</td>
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<tr>
<td>72 h</td>
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<td></td>
<td>Thiamethoxam+ Mefenoxam_Low 0.1 µg/ml</td>
<td>0.418</td>
<td>0.029</td>
</tr>
</tbody>
</table>
CHAPTER 4

Toxicity of thiamethoxam to key predators of soybean aphid Aphis glycines
(Hemiptera: Aphididae)

Introduction

Natural enemy communities (parasitoids, predators, and entomopathogenic fungi) in soybean play an important role in the regulation of soybean aphid populations (Costamagna et al. 2013). Predators are the most important group of natural enemies that provide natural control of the soybean aphid in the United States (Mignault et al. 2006, Schmidt et al. 2008, Costamagna et al. 2013). Studies by Costamagna and Landis (2007) showed that soybean aphid populations could grow from 2 to 7 times faster in absence of predation. The minute pirate bug Orius insidiosus (Say), ladybeetles including Harmonia axyridis and Colleomegilla maculata, and lacewing species Crysoperla spp., are considered key predators of the soybean aphid in the United States (Rutledge et al. 2004, Ragsdale et al. 2007). Given the importance of predators in the control of soybean aphid, there has been an increase interest in the conservation of these species in soybean fields as a key component of integrated management programs of the soybean aphid (Heimpel et al. 2004, Rutledge et al. 2004, Fox et al. 2005, Mignault et al. 2006, Gardiner et al. 2009, Ragsdale et al. 2011, Tilmon et al. 2011, Lundgren et al. 2013).

Seed treatments with neonicotinoid insecticides have been promoted to be relatively non-toxic to natural enemies due to lack of direct exposure to the chemical residues (Seagraves and Lundgren 2012). However, residues of neonicotinoids in the
plant and insect prey can affect the compatibility of these insecticides with biological control in soybean fields by causing direct mortality or negative effects on consumption rates of soybean aphid by key predators (Gentz et al. 2010, Seagraves and Lundgren 2012).

Exposure of predaceous species can occur by ingestion of residues in prey and plant material or through contact with guttation drops or dust particles during planting (Gentz et al. 2010, Pisa et al. 2015). Predators of soybean aphid have plant-feeding habits consuming pollen, nectar, guttation drops, and leaf tissue in the absence of prey, this process is known as zoophytophagy behavior (Albajes and Alomar 1999, Canard et al. 2001, Moser and Obrycki 2009). Zoophytophagy behavior benefits predaceous species by increasing fecundity and reducing developmental time and cannibalism (Albajes and Alomar 1999, Moser and Obrycki 2009). However, consumption of plant material can be detrimental for predatory species if leaf tissues contain lethal concentrations of systemic pesticides (Seagraves and Lundgren 2012).

Predators can also be exposed by contact with pesticide residues from seed dust during planting (Pisa et al. 2015). Vegetation near agricultural crops serves as reservoir of natural enemies species in soybean fields, and this vegetation can receive residues of neonicotinoids from dust drift during planting (Koh and Holland 2015). However, the toxicity risk for predatory insects has been difficult to quantify as few studies have determined the acute and chronic lethal toxicity of neonicotinoids in different beneficial species other than pollinators (Pisa et al. 2015).
Furthermore, neonicotinoid residues in insect prey can affect the consumption behavior of predatory insects by repellent, antifeedant, or reduced olfactory capacity (Desneux et al. 2007). Effects of neonicotinoids on soybean aphid natural enemies have been mainly focused on mortality, and there is no information on how their consumption behavior can be affected by neonicotinoids residues (Varenhorst and O'Neal 2012a). Perturbation of host feeding behavior by neonicotinoids may drastically influence the efficiency of predators in controlling soybean aphid populations (Varenhorst and O'Neal 2012b).

Studies on the effects of neonicotinoids on predatory species in soybean are very limited with some of those studies showing conflicting results. Seagraves and Lundgren (2012) found that populations of the predators *Nabis americoferus* (Carayon) (Hemiptera: Nabidae). *Chrysoperla* spp (Neuroptera: Chrysopidae) adults were reduced in soybean plots treated with imidacloprid. On the other hand, studies by Varenhorst and O'Neal (2012b) and Ohnesorg et al. (2009) report no-observable effects on the abundance of soybean aphid predators in fields treated with imidacloprid or thiamethoxam. Differences between studies may be attributed to different formulation of the seed treatments, differences in translocation of neonicotinoids under different environmental conditions, or effect of pesticides mixtures used on seed treatments. While neonicotinoids in seed treatments are usually applied in combination with multiple pesticides, to this point there is no information on neonicotinoids environmental fate in plants exposed to mixtures nor the effect of such mixtures on beneficial insects in soybean fields.
Due to the limited information on the impact of neonicotinoids on predatory species, this research was conducted to evaluate the toxicity and effects on consumption behavior of thiamethoxam on two of the most representative natural enemies of soybean aphid, *O. insidiosus* and *Chrysoperla rufilabris* (Burmeister), through different exposure routes. From a risk assessment standpoint it is also important to understand if neonicotionids applied solely and in the conventional seed treatment mixture with mefenoxam can affect the mortality of beneficial species. Thus, the abundance of *O. insidiosus* and *Chrysoperla* spp was evaluated in soybean with seed treatments of thiamethoxam applied solely and in the conventional mixture with the fungicide mefenoxam.
Methodology

Toxicity through contact and systemic exposure

*Plant material:* All soybean plants were grown in a greenhouse (24 ± 5°C, 16:8 hour photoperiod) using potting medium comprised of peat moss, perlite, pine bark, and vermiculite (Fafard® 3B Mix). Three plants were grown in plastic pots (15cm diameter × 17cm deep). Plants were watered daily and fertilized weekly with NPK fertilizer of 20:10:20 ratio.

*Insect material:* Predators were obtained from a laboratory colony established by Rincon-Vitova insectaries in California. Larvae of *C. rufilabris* (I and II instar) were shipped overnight in honeycomb hex-cells covered with organdy cells. Hex-cells were placed for 24 hours in plastic containers in a growth chamber (24 ± 3 °C, 70% RH, 16:8 photoperiod). Larvae were fed by sifting eggs of *Ephestia kuehniella* on top of the hex-cells. Only larvae were evaluated in the experiments. *Orius insidiosus* adults of unknown age were shipped overnight from Ventura, California to Lincoln, Nebraska and held at 10°C during shipment. Adults were placed in an environmental chamber at 24 °C and 70 RH% for 24 h before the experiments. To increase genetic diversity of *O. insidiosus* in the toxicity studies experiments, a field population was collected in soybean fields near Lindsay, Nebraska (41°44’22.9”N; 97°41’59.4”). Adults were transported in plastic containers with a mesh panel at 10°C. Populations were maintained for 48h until the development of the experiments by feeding them with *E. kuehniella* eggs.
A colony of soybean aphid was initiated in 2011 from individuals collected from infested fields near the University of Nebraska Northeast Research and Extension Haskell Agricultural laboratory in Concord NE. The colony was maintained under continuous supply of V3 soybean plants of the susceptible variety SD76R. The colony was maintained in an environmental chamber at 24 °C and 70 % RH and a photoperiod of 16:8 light: dark conditions.

*Insecticide* Technical grade thiamethoxam (99.5%) was purchased from Chem Services (West Chester, PA). Chemicals were maintained in dark conditions at -4°C. Stock solution were diluted in acetone at 5 μg μL⁻¹ and stored at 20°C prior the experiments.

*Contact Toxicity bioassay*

For the contact bioassays a glass vial of 5 cm diameter and 3 cm tall was coated with 500 μl of the pesticide solution. The vial was homogeneously coated using a hotdog roller at low temperature for 3 minutes. A range of five concentrations of thiamethoxam was evaluated: 5, 0.5, 0.05, 0.005, and 0.0005 μg/ml. The control treatment was acetone without any insecticide. The number of dead insects was recorded at 6, 12, and 24 hours after the exposure. For each species, a different number of individuals were evaluated per experimental unit. For *O. insidiosus* a total of 10 vials per treatment with five adult predators per vial were evaluated. Due to the cannibalism in lacewings species one individual larva was evaluated per vial. For the lacewing predator, *C. rufilabris*, 20 vials per treatment with one larva predator per vial were evaluated. Mortality was recorded at 6, 12, and 24 hours after exposure.
Systemic Toxicity bioassay

To evaluate the toxicity of thiamethoxam in treated soybean leaves a systemic bioassay was developed following the methodology developed by (Magalhaes et al., 2009). For *O. insidiousus*, the concentration range was 0.5, 0.1, 0.05, 0.01, and 0.005 and 5, 1, 0.1, and 0.01 µg/ml for larvae of *C. rufilabris*. Stock solutions were prepared in acetone and diluted in distilled water. The solution of acetone and the insecticide was 0.01% of the total solution in water. Cut petioles of the first trifoliate of V3 soybean were immersed in each insecticide solution and distilled water. The leaves were exposed to the insecticide solution for 24 hours prior the introduction of the predators to allow the uptake of the insecticide. No alternative food source was introduced in the experiment to guarantee zoophytophagy from the predators. For *O. insidiousus*, 10 insects were introduced per experimental unit. A total of 10 experimental units per treatment were evaluated in two, time blocks. For *C. rufilabris*, one larva of the predator was evaluated per experimental unit. A total of 24 experimental units in three, time blocks were evaluated. The number of living insects was recorded 24 and 48 hours after insecticide exposure.

Effects on Consumption

To evaluate the effect of thiamethoxam on the consumption rate of *O. insidiosus* a completely random design was developed under laboratory conditions. The treatments consisted of four doses of the insecticide and a control: 10, 5, 2.5, 1.25, 0 ng/ml. Based on the consumption behavior of *O. insidiosus* reported by Rutledge & O`Neil (2005) a prey density of 20 aphids was placed in each experimental unit. Seven replicates in two,
time blocks were evaluated per treatment. A systemic bioassay was developed following methods described by (Magalhaes et al. 2008). Cut petioles of soybean leaves were immersed in each insecticide solution and distilled water. After exposing the petiole to the chemical for 24 hours, wingless third instar aphids were placed on the soybean leaves by using a camel hair paintbrush. Aphids were kept on the leaves for 24 hours into the different treatments. One female less than one week old was placed in each experimental unit. The number of consumed aphids was counted after 24 hours after the predator introduction.

For lacewing larvae, the treatments consisted of aphids exposed to four different doses of the insecticide and a control: 100, 50, 10, 5, and 0 ng /ml. An aphid dip bioassay was used to expose aphids to the different concentrations following the methodology developed by Chandrasena et al. (2011). A different method was used to evaluate consumption on this predator, as the sensitivity to this predator was shown to be higher than O. insidious through oral exposure. This method was used to achieve higher concentrations in the aphids without having high mortality on aphids. Solutions were prepared at 0.01% acetone in 200 ml of water. Aphids on V3 leaves were placed in the tea strainer and submerged in the insecticide solution for 3 minutes. After aphid immersion in the insecticide solution, leaves were placed under the microscope to identify aphids that had taken up water into the body. Aphids were then transferred to a moist filter paper in petri dishes of 55 mm diameter. A total of 20 aphids were transferred per petri dish with a total of 4 replications per concentration. One, second instar lacewing larva was transferred per petri dish. The number of consumed aphids was counted 24 hours after the predator introduction.
Predator abundance in the field

To evaluate the toxicity of thiamethoxam and mefenoxam seed treatments in field environments on *O. insidious* and *Crysoperla spp.* an insect collection of these two predators was conducted during 2014 and 2015 in two different fields in Nebraska. Research plots were located in different fields at the UNL Agricultural Research and Development Center (ARDC) near Ithaca NE and the UNL East Campus at Lincoln NE. In 2014, the plots at ARDC were located at a Latitude 41° 9' 54.49 "N; Longitude 96° 24' 50.45 "W and the plots in Lincoln at a Latitude 40° 50' 9.93 "N; Longitude, 96° 39' 44.95" W. In 2015, fields were located in the same research stations with different coordinates, (ARDC) plots were located at the latitude 41° 9' 26.50"N and the longitude 96° 25' 26.04"W, and Lincoln plots were located at the latitude 40°50' 11.40"N and longitude 96° 39' 41.85" W.

The experimental design for all fields and years was a randomized complete block with four replicates of four treatments. Treatments consist of: 1) Thiamethoxam alone (Cruiser™) 0.00756 mg a.i/seed , 2) Mixture: Thiamethoxam-Mefenoxam 0.0075 and 0.0113 mg a.i/seed respectively (Cruiser Maxx), 3) Mefenoxam 0.0113 mg a.i/seed (Apron XL) and 3) Untreated seeds. Plots consisted of 8 rows planted at 76.2 cm rows and 5.18 meters long planted at a density of 350000 seeds/ha. The collection was conducted during soybean flowering (reproductive stages R1-R2) 47 days after planting in 2014 and 50 days after planting in 2015. Insects were collected by using a plastic tray with a white bottom. Sampling length was 80 x 30cm s aking the plants from one row into the tray. The foliage of one row was slowly bent to the tray and beaten vigorously for
5 seconds. After the sample section was pushed aside, predators were counted and removed quickly to avoid recounting. Three tray samples were taken from each plot. Bean leaf beetle were common; therefore, abundance of this pest insect was recorded as an assessment of the effect of the seed treatments on bean leaf beetle in soybean at early reproductive soybean stages.

Statistical Analysis

Laboratory experiments: Differences in the susceptibility of predators between times of evaluation in the contact and systemic toxicity bioassays were evaluated through an ANOVA using a generalized linear model with binomial distribution. The ante-dependence Ante (1) covariance structure was used to take into account the correlation of repeated measurements of the experimental units over time. Lethal concentration (LC) values were calculated for the dose-response curves that display less than 20% mortality in the control. Natural mortality unrelated to the insecticide treatment was corrected through the Abbots formula. Probit analysis for LC estimations was developed using the PROC PROBIT package in SAS/STAT Software version 9.3. The treatment effects on the consumption rate were evaluated by a one-way ANOVA using generalized linear mixed model with a normal distribution in SAS/STAT software version 9.1.3 (SAS Institute Inc, Cary NC).

Field experiment: Data was analyzed using an ANOVA with a generalized linear mixed model with a normal distribution for each insect species. Year, location, and treatment were fixed variables. Random variables include the blocks per location per year. Means between treatments were compared by the Fisher’s least significant
difference test. The analysis was performed using the statistical package SAS/STAT software version 9.1.3 (SAS Institute Inc, Cary NC).
Results

Toxicity through contact exposure

There were significant differences in the mortality of *O. insidiosus* at the different hours in response to the different concentrations of thiamethoxam (Num DF=9; Den DF=162, F-value p-value<0.0001). Mortality of this predator for the control was 52% after 24 hours (Figure 4.1a). Thus, contact lethal concentrations can be more reliability assessed at 6 and 12 hours after exposure through the vial bioassay method for this predator. What about *rufilabris*? There were no significant differences in mortality for lacewing larvae between concentrations at the evaluated hours (Num DF=5; Den DF=56, F-value p-value=0.7328) (Figure 4.1b). Susceptibility through contact was higher for larvae of the lacewing than adult *O. insidiosus* (Table 4.1).

Toxicity through systemic exposure

There was a significant difference in the mortality of both predators between 24 and 48 hours after exposure (*O. insidiosus*: Num DF=1, Den DF=96, F=58.58, p-value<0.0001; *C. rufilabris*: Num DF=1, Den DF=201, F=10.36, p-value=0.0015). Natural mortality of both predators increased over 20% after 48 hours (Figure 4.2). Thus, systemic lethal concentrations can be more reliability assessed before 48 hours for this bioassay technique. Lethal concentrations at 24 hours were higher for *O. insidiosus* than for *C. rufilabris*, suggesting that *O. insidiosus* may be more susceptible to thiamethoxam through systemic exposure than *C. rufilabris*. Include the data from Table 4.2 here and then you can delete that table.
**Effects on Consumption**

There were no significant differences in consumption for either species between thiamethoxam concentrations in soybean aphids and the control (*O. insidiosus*: Num DF=4, Den DF=30, F=1.99, p-value=0.1208; *C. rufilabris*: Num DF=4, Den DF=15, F=0.52, p-value=0.722). However, there was a decrease of the average number of consumed aphids by *O. insidiosus* as the concentration of thiamethoxam increase (Figure 4.3). This decrease in consumption could be related with the mortality of the predator at the higher concentrations (Num DF=4, Den DF=30, F=4.92, p-value=0.0036) (Table 3). For lacewing larvae, the consumption only decreased at the higher concentration of 100 ng/ml (Figure 4.3). However, no mortality was observed for lacewing larvae at 24 hours for any of the concentrations.

**Predator abundance in the field**

There was no significant difference between locations on the effect of treatment in the abundance of predators and bean leaf beetle (*O. insidiosus*: Num DF: 3, Den DF=32, F =0.56, p-value=0.643; *Chrysoperla* spp: Num DF: 3, Den DF=32, F =1.33, p-value=0.2832; bean leaf beetle: Num DF: 3, Den DF=32, F =0.77, p-value=0.517). There were no significant differences in predator or bean leaf beetle abundance between treatments per year (*O. insidiosus*: Num DF: 3, Den DF=32, F =0.19, p-value=0.945; *Chrysoperla* spp: Num DF: 3, Den DF=32, F =1.20, p-value=0.324; bean leaf beetle: Num DF: 3, Den DF=32, F =0.20, p-value=0.897). There were no significant differences
in predator abundance between the seed treatments and the control (*O. insidiousus*: Num DF: 3, Den DF=32, F =0.22, p-value=0.882; *Chrysoperla* spp: Num DF: 3, Den DF=32, F =0.64, p-value=0.598) (Figure 4). There were significant differences in bean leaf beetle abundance between the treatments (Num DF: 3, Den DF=32, F =5.41, p-value=0.0040). Higher numbers of adult bean leaf beetles were observed in the control and fungicide treatments compared to the insecticide alone and the insecticide mixture treatment (Figure 4.4).
Discussion

Little information is available about the toxicity of neonicotinoids to several beneficial species other than pollinators (Pisa et al. 2015). Plant feeding has been considered a direct route of exposure to predators with zoophytophagy habits, such as *O. inisidiosus* and *C. rufilabris* (Moser and Obrycki 2009). Based on the results of these systemic bioassays, residues of thiamethoxam in soybean leaves from seed treatments could cause acute toxic effects on adults of *O. insidiosus* by direct consumption of vegetative plant tissue, primarily at early soybean stages. Direct toxicity can be expected through contact with leaves at early vegetative stages when there are low densities of insect prey and foliar feeding is expected (Seagraves and Lundgren 2012). Concentrations of thiamethoxam at early vegetative soybean stages have been reported at ~0.1 ppm at 17 days after planting, decreasing to ~0.04 ppm after 40 days after planting under field conditions (Magalhaes et al. 2009). In this study, acute LC$_{50}$ of adults of *O. insidiosus* through systemic exposure were close to this range, with values at 0.22 ppm and confidence limits at 0.134 ± 0.387. Thus, planting dates, predator time of arrival to soybean and the availability of different food sources might affect the results on survivorship and abundance of the predator in fields with thiamethoxam seed treatments. However, there are still uncertainties on the level of exposure of this predator in the field and the translation of laboratory studies to real field scenarios.

In this study, abundance of adults of *O. insidiosus* in soybean fields with thiamethoxam seed treatments was not significantly different from untreated plots. This observation is consistent with studies by Ohnesorg et al. (2009) and Seagraves and
Lundgren (2012) where no significant differences of *O. insidiosus* populations were observed between neonicotinoids seed treated and untreated fields. Studies of seasonal occurrence of *O. insidiosus* have shown predator arrival to soybean fields during early and mid vegetative soybean growth (Rutledge et al. 2004). If the arrival of predators occurs at early vegetative stages concentrations of thiamethoxam, direct mortality for this predator could occur by ingestion of contaminated soybean. If arrival occurs at mid vegetative stages, different insect prey present in the field at the time of predator arrival could reduce the exposure of predators to high concentrations in vegetative tissue.

*Chrysoperla* spp larvae show less susceptibility to thiamethoxam through systemic exposure compared to *O. insidious*. Toxicity values for *C. rufilabris* were higher than the maximum concentrations in soybean leaves reported by (Magalhaes et al. 2009). Therefore, acute toxic effects of thiamethoxam on lacewing larvae under field condition would not be expected. Moreover, *Chrysoperla* spp larvae typically first occur in soybean fields during early weeks of July (Rutledge et al. 2004) when soybean is usually entering early reproductive stages at the North Central Region of the U.S (Pedersen and Elbert 2004), and concentrations in the plant would be below ~0.1 ppm (Magalhaes et al. 2009). Field abundance of *Chrysoperla* spp larvae in the field was not significantly different between treated fields and the control, suggesting low impact of thiamethoxam residues in leaves on this species under field conditions. Similarly, Seagraves and Lundgren (2012) did not find differences in the abundance of this species in soybean fields treated with neonicotinoids seed treatments and the control. Zoophytophagy is common in carnivorous species such as *C. rufilabris* (Moser et al. 2008). However, the abundance of food sources during the time of arrival of the predator to the field can make plant feeding
by larvae of these species an uncommon behavior and ingestion of plant material can be mainly accidental (Moser et al. 2008) reducing the likelihood of exposure of lacewing larvae to neonicotinoids in plant residues.

Both predatory species were more susceptible to thiamethoxam through contact exposure compared to the systemic exposure. In the fields, contact exposure to thiamethoxam with respect to seed treatments can occur through residues from dust drift near agricultural environments, guttation drops and direct contact from dust particles (Pisa et al. 2015). Concentrations from dust particles can range between 14.701 ppm in dust residues from planters to 6.9 ppb in vegetation near agricultural environments (Krupke et al. 2012). However, limited knowledge of the concentrations from dust particles in the field and the exposure levels of beneficial insects restrict the field-realistic analysis of the impact of neonicotinoids through contact exposure to predatory species (Pisa et al. 2015).

Moreover, the increased mortality of *O. insidiosus* in the control treatment after 12 hours of exposure restrict the ability of the contact bioassay method used to predict accurate effects of thiamethoxam through contact exposure. Improved methodologies are needed to address contact toxicity to predators to establish safe environmental thresholds for these beneficial species (Pisa et al. 2015). The present study gives a baseline of the concentration response and optimal time of evaluation for adults of *O. insidiosus* and larvae of *C. rufilabris*. However, the lack of an alternative food source during laboratory bioassays using predatory species can be unrealistic to field conditions, resulting in a mortality overestimation in dose-response evaluations.
In terms of consumption, antifeedant and reduction of olfaction abilities to find insect prey are expected due to insecticide residues (Desneux et al. 2007), in this study consumption of soybean aphid by the evaluated predators was not affected at the evaluated concentrations. Other authors have reported negative effects on consumption caused by the neonicotinoid imidacloprid for different predator species including the coccinellid predator *Serangium japonicum* and the predatory mites *Neoseiulus californicus* and *Phytoseiulus macropilis* (Poletti et al. 2007, He et al. 2012). However, concentrations applied to plants in these studies were 100 times higher than the ones used in this study. Therefore, it is possible that effects of neonicotinoids on consumption occurs at higher concentrations than the ones used in this study. Although, consumption was not affected, mortality was observed at the higher doses for *O. insidiosus*. Toxicity of thiamethoxam in predatory species have shown to be 200 times higher through ingestion than through direct contact (Torres and Ruberson 2004). Thus, the ingested concentration can depend on the number of consumed prey and the concentration that each prey can maintain inside the body.

Finally, field studies did not show significant differences between the mixture treatment and thiamethoxam applied solely and thiamethoxam applied in the mixture treatment with mefenoxam. Populations of bean leaf beetle can be a good positive control of this result. No significant differences in abundance were observed between the mixture treatment and thiamethoxam alone in this herbivore species, while significant differences were observed between this treatments and the control. Thus, the mixture of thiamethoxam with mefenoxam might not have significant effects in mortality of insect species in soybean fields exposed to lethal concentrations.
Cited Literature


Moser, S. E., J. D. Harwood, and J. J. Obrycki. 2008. Larval Feeding on Bt Hybrid and Non-Bt Corn Seedlings by Harmonia axyridis (Coleoptera: Coccinellidae) and Coleomegilla maculata (Coleoptera: Coccinellidae). Environmental Entomology 37: 525-533.


**O. insidiosus**

![Graph](image)

**C. rufilabris**

![Graph](image)

**Figure 4.1.** Concentration-response curves for acute contact toxicity of thiamethoxam on predators of soybean aphid at different hours
Table 4.1. Susceptibility of soybean aphid predators to thiamethoxam exposed through contact vial bioassay

<table>
<thead>
<tr>
<th>Predator Species</th>
<th>Hour</th>
<th>LC$_{10}$ μg/ml (95%CL)</th>
<th>LC$_{50}$ μg/ml (95%CL)</th>
<th>Pearson Chi-Square (DF)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>O. insidiodus</em></td>
<td>12 h</td>
<td>0.006 (0.0001 ± 0.32)</td>
<td>0.434 (0.156 ± 0.911)</td>
<td>6.31 (3)</td>
</tr>
<tr>
<td></td>
<td>12 h</td>
<td>0.005 (0.0001± 0.036)</td>
<td>0.287 (0.048± 1.165)</td>
<td>1.43 (2)</td>
</tr>
<tr>
<td><em>C. rufilabris</em></td>
<td>24 h</td>
<td>0.011(0.00002 ± 0.064)</td>
<td>0.211(0.023 ± 0.617)</td>
<td>0.56 (2)</td>
</tr>
</tbody>
</table>
Figure 4.2. Concentration-response curves for acute toxicity through systemic of thiamethoxam on predators of soybean aphid
Table 4.2. Susceptibility of soybean aphid predators to thiamethoxam exposed through systemic bioassay at 24 hours

<table>
<thead>
<tr>
<th>Predator Species</th>
<th>LC\textsubscript{10} µg/ml (95%CL)</th>
<th>LC\textsubscript{50} µg/ml (95%CL)</th>
<th>Pearson Chi-Square (DF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O. insidiodus</td>
<td>0.019 (0.002 ± 0.046)</td>
<td>0.227 (0.134 ± 0.387)</td>
<td>3.394 (2)</td>
</tr>
<tr>
<td>C. rufilabris</td>
<td>0.029 (0.0122 ± 0.119)</td>
<td>1.362 (0.3978 ± 8.178)</td>
<td>4.389 (2)</td>
</tr>
</tbody>
</table>
Figure 4.3. Effects of thiamethoxam on predator consumption of soybean aphid using a prey density of 20 individuals per replication
Table 4.3. Mortality of *O. insidiosus* exposed to aphids feeding on leaves with thiamethoxam. Different letters correspond to significant differences at the 95 % CL.

<table>
<thead>
<tr>
<th>Dose ng/ml</th>
<th>Mean ± SE</th>
<th>Predator mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>0.0 ± 0.0 a</td>
<td>0.0 ± 0.0 a</td>
</tr>
<tr>
<td>1.25</td>
<td>0.0 ± 0.0 a</td>
<td></td>
</tr>
<tr>
<td>2.50</td>
<td>0.29 ± 0.18 a</td>
<td>0.29 ± 0.18 a</td>
</tr>
<tr>
<td>5.00</td>
<td>0.43 ± 0.2 b</td>
<td>0.43 ± 0.2 b</td>
</tr>
<tr>
<td>10.00</td>
<td>0.57 0.21 b</td>
<td></td>
</tr>
</tbody>
</table>
**Figure 4.4.** Abundance of bean leaf beetle and predators of soybean aphid in the field, results were pooled from 4 different fields during 2014 and 2015. Different letters correspond to significant differences at the 95 % CL.
APPENDIX 1. SUPPLEMENTAL RESULTS

Effects of the insecticide fungicide seed treatment mixtures on plant growth and yield of soybean *Glycine max*

Introduction

Seed treatments with both insecticides and fungicides, represents a widely adopted practice by soybean producers in the United States (EPA 2014). In the North Central Region it is estimated that more than 70% of soybean seeds are treated with a fungicide, insecticide or nematicide alone or in combination of two or three products (Douglas and Tooker 2015). The increased adoption of seed treatments in soybean is likely to occur due to early planting, benefits to plant emergence, plant growth and gain in yield as a result of insecticide/fungicide treatment (Gaspar et al. 2015).

Neonicotinoid insecticides, phenylamides (PA) and phenylpyrroles (PP) fungicides are the most common insecticide/fungicide products used in soybean seed treatments (Cox et al. 2008, Gaspar et al. 2014, Gaspar et al. 2015). Insecticide/fungicide treated seeds can provide protection against early season pathogens such as *Pythium* spp., *Phytophthora sojae* (Kaufmann and Gerdemann), *Fusarium* spp., and *Rhizoctonia solani* (Kuhn) (Esker and Conley 2012, Gaspar et al. 2014) and insects such as wireworm (*Melanotus* spp.), seed corn maggot [*Delia platura* (Meigen)], bean leaf beetle [*Cerotoma trifurcata*] and other minor pests (Cox et al. 2008, Gaspar et al. 2015). In the last few years, some studies have suggested that there are few benefits of using neonicotinoid insecticides in soybean fields in northern states of the U.S, because bioactive
concentrations from seed treatments do not overlap with the periods of activity of some key target pests such as the soybean aphid \textit{[Aphis glycines (Matsumura)]} \cite{Ragsdaleetal.2011,EPA2014}. However, neonicotinoid seed treatments are still widely used not only for its benefits in pest control, but also because its benefits on plant growth.

Various studies report different benefits of insecticide/fungicide mixture on seedling emergence, enhancement of growth, vigor and health of soybean plants \cite{Coxetal.2008,CoxandCherney2011,EPA2014,Gasparetal.2015}. However, studies on the effects on seed-applied insecticide/fungicides mixtures on plant growth and yield are still very limited and some have shown opposing results \cite{Gasparetal.2015}. Cox et al. \cite{Coxetal.2008} reported no response for either early soybean establishment or yield increase with the seed-applied insecticide/fungicide mixture of the neonicotinoid imidacloprid and the PP fungicide fludioxonil. In contrast, Gaspar et al. \cite{Gasparetal.2015} showed a yield increase of 12\% in with the mixture of thiamethoxam, fludioxonil and the PA fungicide mefenonaxam.

Sedaxane is a new broad-spectrum fungicide used in seed treatment mixtures with neonicotinoids insecticides, PA and PP fungicides. Sedaxane is a new broad-spectrum fungicide in seed treatments used in mixtures to manage potential resistance development in soybean diseases \cite{Zeunetal.2013}. Increased yield in barley has been reported when sedaxane is used with other fungicides under high disease pressure \cite{Zeunetal.2013}. Because sedaxane has been recently registered for seed treatments in soybean crops, few studies have evaluated its effect on plant growth and soybean yield.

The increased number of pesticides commercially available for seed treatments makes the selection of the appropriate pesticide mixture a difficult decision for soybean
growers annually (Gaspar et al. 2015). Seed treatment costs can increase with the application of more than one active ingredient (Douglas and Tooker 2015). For seed treatments with mixtures of neonicotinoids, PP, PA, fungicides by the supplier, costs are about four times more per 50 pounds of seed, compared to seeds treated only fungicide products (Heatherly 2015). Fungicide seed treatments alone can improve on early planted soybean can improve yield (Lueschen et al. 1991, Bradley et al. 2001). However the information on the effects of the products alone or in combination with other pesticides on plant growth and yield are still very limited, particularly for new active ingredients in seed treatments (Cox and Cherney 2011, Gaspar et al. 2014, Gaspar et al. 2015).

This objectives of this research was to determine the effects of the insecticide thiamethoxam alone and in combination with mefenoxam, fludioxinil and sedaxane on soybean plant height (cm), foliar area (cm²), emergence (%) and yield.

Methodology

Field locations and experiment design

The experiments were conducted during three growing seasons at three locations in eastern Nebraska. In 2013, the research plots were located at the University of Nebraska- Northeast Research Extension Center Haskell Agricultural Laboratory, Concord Nebraska, latitude 42° 23’ 2.38”N; longitude 96° 56’ 29.14” W. In 2014 and 2015, research plots were located at the University of Nebraska Agricultural Research and Development Center, Ithaca, Nebraska and the University of Nebraska-Lincoln East
Campus, Lincoln, NE. In 2014 the plots at Ithaca were located at a latitude 41° 9' 54.49 "N; longitude 96° 24' 50.45 "W and the plots in Lincoln at a latitude 40° 50' 9.93 "N; longitude, 96° 39' 44.95" W. In 2015, Ithaca plots were located at latitude 41° 9' 26.50"N and longitude 96° 25' 26.04"W and Lincoln plots were located at latitude 40°50' 11.40"N and longitude 96° 39' 41.85" W.

The experiment design consisted in a Complete Randomized Block Design with 4 treatments x 4 plots per treatment. Plots consisted of 8 rows of 17 feet long planted 30 inches between rows and 5 feet between plots. Planting density was 140.000 seeds/acre. High pest pressures were not observed at any of the evaluated locations.

Treated seeds were obtained from Syngenta Seeds (Stanton, Minnesota) at the rate applied for available products for the S30-E9 soybean variety. The treatments were: 1) untreated seeds as the control treatment, 2) fungicide: mixture of mefenoxam, fludioxinil and sedaxane at 0.0113 mg ai/seed 3) insecticide/fungicide mixture: mefenoxam, fludioxinil, sedaxane and thiamethoxam at 0.0113, 0.0038, 0.0038 and 0.0756 mg A.I/seed respectively and 4) insecticide alone: thiamethoxam only at 0.0756 mg A.I /seed.

Effects of insecticide/fungicide mixtures in plant growth parameters

Plant height

Plant height was evaluated in the fields located in Concord in 2013 and at Mead and Lincoln in 2014 in the coordinates described above. The average shoot height from the hypocotyl to shoot tip was measured when plants were The average shoot height from
the hypocotyl to shoot tip was measured when plants were at 21 days after planting (DAP) at Concord (V1.2), Ithaca (V 1.4) and Lincoln (V 1.6), 48 DAP in Concord (6.8), and 35 DAP in Lincoln (V 7.5) and Ithaca (V7.8). Five plants were randomly collected from the two middle rows and 2 feet from the border in each plot. Plants were transferred to a plastic bag labeled with the plot number and measured in the laboratory the same day after collection.

Data analysis from plant height was analyzed through an ANOVA using a generalized linear mixed model for a complete randomized block design with a normal distribution. The distribution was evaluated through the variance residual plots. Treatment, plant stage and locations were used as fixed variables to identify significant differences in the treatment effects at early vegetative and early reproductive stages in the three evaluated locations. Means between treatments per growth stage were compared through the Fisher’s least significant difference test. The analysis was performed using the statistical package SAS® version 9.3.

*Foliar area*

Foliar area was measured at 21, 34 and 48 DAP on Concord during 2013, at 21, 35, 48 DAP at both Ithaca and Lincoln in 2014, and 18, 37, 46 DAP at Lincoln in 2015. These dates were selected to represent early vegetative stages, mid vegetative stages and early reproductive stages. For the foliar analysis five different plants were randomly selected from the 2 middle rows of each plot. Selected plants were bagged per plot and held on ice during transport. Samples were analyzed no more than 12 hours after
collections. Fully opened leaves from all the plant were measured. Foliar area measurements were developed the LICOR 3100 (Lincoln, Nebraska) foliar area meter.

Data analysis of foliar area was performed using a generalized linear mixed model with a normal distribution to identify interactions between the treatments, day after planting and locations per year of evaluation. Means between treatments per growth stage were compared through the Fisher’s least significant difference test. Linear regression was fitted for the pooled data and per field using a generalized linear mixed model with normal distribution. Linear regression was fitted in order to estimate the effects of the treatments in plant growth. Treatments in each field where compared through the test of equal slopes using orthogonal contrasts between each regression. The analysis was performed using the statistical package SAS® version 9.3.

Germination Percentage

Stand counts were taken for each plot per location during 2014 and 2015. Stand count assessment was developed counting the number of plants growing in 10 ft. lengths of the middle two rows (20 ft. total per plot). Germination percentage was calculated as the total number of seedlings that emerged 10 days after planting versus the total number of seeds sown (plants/acre/140.000seeds/acre). The population of plant per acre was calculated through the following equation: Population (plants/A) = (# counted in both rows / 20 ft.) * 17424. Data analysis for germination performed using a generalized linear mixed model using the statistical package SAS® version 9.3 with a normal distribution. Means between treatments were compared through the Fisher’s least significant difference test per year.
Crop Yield

Yield data was collected at Mead and Lincoln in 2014 and 2015 using an Almaco small plot combine equipped with scale. Two rows from the middle plots were harvest per plot. Rows were adjusted to 15 feet long cutting 1 foot at both sides of the plot. Harvest was conducted during October at all the evaluated locations. Statistical analysis was performed per year through an ANOVA using a generalized linear mixed model using the statistical package SAS® version 9.3. A residual plot for the used model was evaluated to verify the normal distribution of the data.
Results

Plant height

There was a significant difference in plant height among treatments (Num DF=3, Den DEF=431, F-value=11.97, p-value<0.0001). The average plant height in the treatments with only fungicides and in the mixture of the fungicides with the insecticide was larger by approximately 3 cm compared to the control (Figure 1.1). This effect had a significant interaction with the growth stage (Num DF=4, Den DEF=431, F-value=4.12, p-value=0.0067). At early vegetative stages there were no significant differences between any of the treatments (Figure 1.1). However, plants at early reproductive stages (V6-V8 vegetative) showed significantly higher plants in the fungicides treatment and the fungicides-insecticide mixture treatment compared to the control (Figure 1.1).

There were no significant differences in the effect of the treatment per stage by location (Num DF=6, Den DF=431, t-value=0.73, p-value=0.6232). The effect for the fungicide and mixture treatments was similar between locations (Figure 2.1) (Num DF=6, Den DF=431, t-value=1.61, p-value=0.1436). However, at Concord this differences are more noticeable compared to the other locations (Figure 1.2). Concord showed significant higher plants in all seed treatments compared to the control at early reproductive stages at V6-V8 vegetative stages (fungicides vs. control: DF=431, t-value=-3.54, p-value=0.0025, mixture vs control: DF=431, t-value=-5.47, p-value<0.0001). In Lincoln, the treatments with the fungicide alone and in the mixture with thiamethoxam also showed significant higher plants versus the control (fungicides vs. control: DF=431, t-value=-2.74, p-value=0.032, mixture vs. control: DF=431, t-
Differences of the insecticide alone and the control were only found in Concord during 2013 (thiamethoxam vs. control DF=431, t-value=-2.77, p-value=0.030). In Mead, the fungicide alone and in the mixture had numerically higher plants although there were not statistically significant differences (Figure 1.2).

**Foliar Area**

Locations did not show significant differences in foliar area in response to the evaluated seed treatments (Num DF=9, Den DF=141, F-value=1.00, p-value=0.439). Treatments did not show a significant interaction with plant stage (Num DF=6, Den DF=141, F-value=1.76, p-value=0.11). Seed treatments show numerically higher foliar areas in all the plant stages. However, significant differences were only detected and mid vegetative and early reproductive stages (Figure 1.3).

Pooled data from locations within the year show that foliar area was significantly different between treatments (Num DF=3, Den DF=141, F-value=6.47, p-value=0.0004). The treatments with the insecticide (mixture and alone) had significantly higher plant growth compared to the control (Figure 2.3, Table 1.4) (control vs mixture: Num DF: 1, Den DF=137, F-value=6.03, p-value=0.015; control vs thiamethoxam alone: Num DF: 1, Den DF=137, F-value=4.16, p-value=0.0434). The fungicide alone show a higher slope compared to the control, although not statistically significant differences were observed between this two treatments (Num DF: 1, Den DF=137, F-value=0.11, p-value=0.745). The treatments with the insecticide show higher growth compared to the fungicide treatment (Fig 2.3). However, statistically significant differences were only observed for the comparison between the mixture treatment and the fungicide (mixture vs. fungicide:
Num DF: 1, Den DF=137, F-value=4.45, p-value=0.036; thiamethoxam alone vs. fungicide: Num DF: 1, Den DF=137, F-value=2.88, p-value=0.092) (Table 1.3).

Data evaluated per location show that the treatments with the insecticide had higher slopes in general in all locations (Figure 1.5). However, this difference was statistically significant only at in Lincoln and Mead (Figure 1.5). At Lincoln there were significant differences between the mixture treatment and the control, however not significant differences were observed between the insecticide and control treatment (Table 1.4). At Mead there were significant differences between the insecticide treatment and control and not between the mixture treatment and the control. No significant differences were observed between treatments at the site in Concord (Table 1.4).

**Germination Percentage**

Soybean germination percentage did not show significant differences between the seed treatments in the evaluated years (Num DF=3, Den DF=42, F-value=0.43, p-value=0.7294). In 2014, germination percentage averages of all treatments were between 90 and 100%, while in 2015 averages were between 80 and 90% (Table 1.5).

**Soybean Yield**

There were no significant differences between the effect of the insecticide per location (Num DF=3, Den DF=33, F-value=0.068, p-value=0.5677) and the effect of the insecticide per year (Num DF=3, Den DF=33, F-value=0.20, p-value=0.893). Soybean yield was not significantly different between the seed treatments and the control at any of
the evaluated locations (Num DF=3, Den DF=39, F-value=0.63, p-value=0.5996) (Table 1.6).
Discussion

In this research, we evaluated the potential effects of thiamethoxam, mefenoxam-fludioxinil-sedaxane alone and in combination of the four products on soybean plant growth. The mixture of the insecticide thiamethoxam, and the fungicides mefenoxam, fludioxinil and sedaxane had an overall positive effect on soybean plant growth, both in terms of plant height and foliar area. The fungicide treatment alone (mefenoxam-fludioxinil-sedaxane) showed a significant positive effect on plant height, but not on foliar area. In contrast, thiamethoxam alone did exhibited plants with slight higher foliar areas compared to the control, but not consistent increase of plant height across the evaluated locations. The individual benefits in plant height and foliar area from the fungicides and insecticide seed treatments can explain the additive effect in soybean plant growth when the pesticides are combined. Although the evaluated parameters for plant growth were positively influenced by thiamethoxam, mefenoxam-fludioxinil and sedaxane alone and in the mixture, soybean yield performance and germination rates were not altered by any of the seed applied products.

The benefits of neonicotinoid and fungicide seed treatments in plant growth parameters such as plant height, emergence percentage, plant stand, plant vigor and yield increases has been widely reported (Bradley et al. 2001, Bradley 2007, Castro et al. 2008, Macedo and Castro 2011, Pynenburg et al. 2011). However, these studies have not always shown consistent results (Bradley 2007, Bredeson and Lundgren 2015). While fungicides and insecticide seed treatments provide protection against early season pests and also can act as a chemical enhancer of plant growth, yield benefits from those robust
plants are only observed under high pest pressure levels and certain environmental conditions (Cox et al. 2007, Wilde et al. 2007, Cox et al. 2008).

In this study, fungicide treatments showed consistently greater height of soybean plants compared to the control in both evaluated years. Soybean plant height is directly correlated with changes in metabolism and vigor of the root system (Cui et al. 2015). Plants can have stunted growth as a consequence of inhibition in root development by soil borne diseases (De Coninck et al. 2015). Mefenoxam, fludioxinil and sedaxane are recommended to mitigate potential negative impacts in soybean seedling germination and establishment, in scenarios where wet and cool conditions can increase the risk of pathogen problems (Bradley 2007, Esker and Conley 2012). In this research, all the evaluated fields were irrigated probably causing an increase of humidity in the soil environment. In spite of the benefits that irrigation have shown in soybean development in the Midwest, plant pathogen incidence can increase in irrigated fields (Hong and Moorman 2005). Therefore, the benefits in plant height in the treatments that contain the fungicides in this study could be related with a decrease in the incidence of root affecting pathogens. However, there is limited information on the density of the pathogens in the evaluated locations and a better correlation between pathogen density, plant height and fungicide seed treatments need to be considered in future studies.

In terms of foliar area, the treatment with the mixture of the fungicides and thiamethoxam showed significant greater plant growth than the control during 2014 and 2015. Although 2013 did not show significant differences between the treatments, higher growth was also observed in the mixture treatment in this year (Fig 1.4, Table 1.4). In
2014 and 2015, we also observed higher foliar areas in the treatments with thiamethoxam alone, although significant differences were not observed when compared to the control treatment. The positive effect of the mixture treatment could be occurring because of additive effects that fungicides and insecticides have individually on plant growth. As previously discussed, soybean growth aboveground can be directly correlated with protection of the root system from soil borne pathogens through seed treatments (Bradley et al. 2001, Bradley 2007). Moreover, positive effects of thiamethoxam on foliar area could be related to the control of defoliator pests and its effect as an enhancer of plant vigor.

Populations of a first generation of bean leaf beetle (BLB) were recorded in the plots evaluated during 2014 and 2015. During those years, significant differences in population levels of this pest were observed between the plots treated with thiamethoxam applied solely and in the mixture compared to the control (Figure 4.4, Results Chapter 4). In contrast, during 2013 there were no significant differences in foliar areas between the treatments with thiamethoxam compared to the control. Populations of BLB were not observed in 2013, suggesting that the effect of thiamethoxam may be related with the control of defoliator pests.

Furthermore, the differences in temperatures between the locations of 2013 and 2014-2015 could be affecting the effect of thiamethoxam as a bioactivator in plant growth. Higher enzymatic activation could happen in warmer temperatures affecting the metabolism of thiamethoxam and its effects in plant growth. Such response could also explain the higher effect of thiamethoxam on foliar area in warmer temperatures such as
the Southeast region in Nebraska in comparison with the Northeast region, which did not show a significant effect of the insecticide on foliar area.

Soybean germination percentage and yield did not show significant differences. Several studies have show that fungicides and insecticides seed treatments did not affect yield or emergence, and only provide benefits when high infestations or incidence of an insect pest or disease is present (Wilde et al. 2007, Bredeson and Lundgren 2015). The lack of differences in soybean yield between the evaluated fungicides seed treatments and untreated seeds, suggests that pest pressures were probably not high in the evaluated years.

Although pathogens causing seedling diseases such as *Fusarium* spp., *Pythium* spp., and *Phytophthora* spp., have been well characterized and reported in Nebraska in the years evaluated. However information of their inoculum densities at the evaluated locations is not available. Bradley (2007) reported that benefits in yield from metalaxyl and mefenoxam occurred only when oomycetes pathogens densities are high and favorable conditions for infection were present. In this study, all plots were planted in late May and early June reducing the time of exposure of the seeds to high soil moisture and the risk of encounter to high pathogenic pressure.

Low insect pest pressure can also explain the lack of yield reduction during the study. Defoliators such as BLB were below the economic threshold (ET) for soybean during 2014-2015. in the control treatment we found and average of 1.8 adults of a first generation of BLB in a 3-foot row; lower pest densities were recorded for the treatments with thiamethoxam (Figure 4.4). Lam et al. (1999) calculated 2 adults /3 foot row as the
lowest economic threshold for the first generation of BLB, using the maximum value of $15/bushel and the minimum management cost of $7/acre. Thus, low densities of insect pests suggest that there was not major defoliation in any of the treatments that could cause significant yield losses. Thus, seed treatments with either compound would be useful only in areas where early season pests are chronic and have high levels of pest pressure (Wilde et al. 2007, EPA 2014).

Seed treatments evaluated in this research caused an increase in height of the plants and foliar areas, but not higher yields. Increased crop growth is often associated with an increase of number of nodes per plant and consequently higher productivity. Egli 2013 reported a positive relation between plant height and number of nodes in soybean. In this study we found a difference of approximately 2.3 cm between the seed treatments and the control. However this difference probably would not increase the node number. Based on the equation that describes the relationship between the number of nodes and height ($y=14.25.7-0.159x + 0.0016x^2$) reported by Egli 2013, a difference of 2.3 cm is not sufficient to increase the unit value of the number of nodes per m².

Another complicating factor in the present study was the generally late planting which may have resulted in a smaller increase in plant height with seed treatments, as there is negative correlation in plant growth and late planting dates. The evaluation of soybean plant height during early planting may be necessary to identify higher changes in plant height and growth, that could potentially affect the number of nodes in and yield. However, it is important to consider that productivity is not only related with an increase
in node number and other factors such as assimilate supply during reproduction, increase of flower and pod production are necessary to increase yield (Egli 2013).

In conclusion, seed treatment benefits in soybean growth could have a positive impact on soybean yield only on scenarios with significant pest pressures. Increase of plant height and foliar area by seed treatments are not always translated to higher yields. Benefits of seed mixture treatments on plant growth need to be evaluated at different planting dates and environmental conditions to elucidate the specific scenarios where seed treatments can have a benefit in soybean production.
Cited Literature


Cox, W. J., E. Shields, and J. H. Cherney. 2007. The Effect of Clothianidin Seed Treatments on Corn Growth following Soybean All rights reserved. No part of this periodical may be reproduced or transmitted in any form or by any means, electronic or mechanical, including photocopying, recording, or any information storage and retrieval system, without permission in writing from the publisher. Permission for printing and for reprinting the material contained herein has been obtained by the publisher. Crop Science 47.


Heatherly, L. 2015. Should Mississippi Soybean Farmers use an Insecticide Seed Treatment - See more at: http://mssoy.org/blog/should-mississippi-soybean-farmers-use-an-insecticide-seed-treatment/ sthash.MWcaol6y.dpuf. MISSISSIPPI SOYBEAN PROMOTION BOARD.


**Figure 1.1.** Means and SE of soybean height for the different seed treatments. Dark bars correspond to the plant heights mean at early vegetative V1-V3 and light bars correspond to early reproductive stages at vegetative V6-V8. Comparisons were developed between treatments in each growth stage. Different letters mean significant difference between treatments at the 95% confidence intervals. Control: Untreated seeds, Fungicides: Mefenoxam + Fludioxinil, Mixture: Mefenoxam + Fludioxinil + Thiamethoxam, Insecticide: Thiamethoxam alone
Figure 1.2. Means and SE of soybean height for the different seed treatments. Dark bars correspond to the plant heights mean at early vegetative V1-V3 and light bars correspond to early reproductive stages at vegetative V6-V8. Comparisons were developed between treatments in each growth stage. Different letters mean significant difference between treatments at the 95% confidence intervals. Control: Untreated seeds, Fungicides: Mefenoxam + Fludioxinil, Mixture: Mefenoxam + Fludioxinil + Thiamethoxam, Insecticide: Thiamethoxam alone.
Fig 1.3. Foliar area medians and CL for pooled data of Concord 2013, Ithaca and Lincoln 2014 and Lincoln 2015. Control: Untreated seeds, Fungicide: Mefenoxam + Fludioxnil, Mixture: Mefenoxam + Fludioxnil + Thiamethoxam, Insecticide: Thiamethoxam alone. Comparisons were developed between treatments during early vegetative (18-21), mid vegetative (34-37), early reproductive (45-48). Significant differences were observed between mixture and insecticide treatments vs control and fungicide treatments (*) ≤ 0.05 (**) p ≤ 0.01, (***) p ≤ 0.001.
Figure 1.4. Fitted linear regressions for the foliar area of soybean in response to four different seed treatments in Nebraska for pooled data of four different fields evaluated during 2013, 2014 and 2015. x-axis correspond to the DAP. Control: Untreated seeds, Fungicide: Mefenoxam + Fludioxinil, Mixture: Mefenoxam + Fludioxinil + Thiamethoxam, Insecticide: Thiamethoxam alone.
Table 1. Linear regressions fitted for foliar area (cm$^2$) for the different seed treatments for pooled information at four locations during 2013, 2014 and 2015. Control: Untreated seeds, Fungicides: Mefenoxam + Fludioxonil, Mixture: Mefenoxam + Fludioxonil + Thiamethoxam, Insecticide: Thiamethoxam alone. Different letters correspond to significant differences: *0.05.

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<th>Treatment</th>
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<td>Control</td>
<td>196.34x -3882.3 $^a$</td>
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<tr>
<td>Fungicide</td>
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<td>Mixture</td>
<td>217.26x -4252.41 $^b$</td>
</tr>
<tr>
<td>Insecticide</td>
<td>222.64 x -4077.35 $^b$</td>
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</table>
Figure 1.5. Fitted linear regressions for the foliar area of soybean in response to four different seed treatments in Nebraska for data per field evaluated during 2013, 2014 and 2015. x-axis correspond to the DAP. Control: Untreated seeds, Fungicide: Mefenoxam + Fludioxinil, Mixture: Mefenoxam + Fludioxinil + Thiamethoxam, Insecticide: Thiamethoxam alone.
Table 1.4. Linear regressions fitted for foliar area (cm\(^2\)) for the different seed treatments for pooled information at four locations during 2013, 2014 and 2015. Control: Untreated seeds, Fungicides: Mefenoxam + Fludioxinil, Mixture: Mefenoxam + Fludioxinil + Thiamethoxam, Insecticide alone: Thiamethoxam only. Different letters correspond to significant differences: *0.05.

<table>
<thead>
<tr>
<th>Location</th>
<th>Treatment</th>
<th>Linear Regression</th>
</tr>
</thead>
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Table 1.5. Average of germination percentage under different seed treatments during the two evaluated years. Control: Untreated seeds, Fungicides: Mefenoxam + Fludioxinil, Mixture: Mefenoxam + Fludioxinil + Thiamethoxam, Thiamethoxam: Insecticide alone

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Table 1.6. Average of soybean yield (kg/ha) under the different seed treatments during the two evaluated years. Control: Untreated seeds, Fungicides: Mefenoxam + Fludioxinil + Sedaxane, Mixture: Mefenoxam + Fludioxinil + Sedaxane + Thiamethoxam, Insecticide: Thiamethoxam alone

<table>
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<tr>
<th></th>
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<th>Average of Yield (kg/ha)</th>
<th>SE (kg/ha)</th>
<th>Average of Yield Bushels/Acre</th>
<th>SE Bushels/Acre</th>
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Table 1.7. Maximum, minimum and average annual temperatures, cumulated annual values of precipitation and reference evapotranspiration (ETr) registered at Concord, Mead and Lincoln at agro-meteorological stations during the years of study.

<table>
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<tr>
<th>YEAR</th>
<th>LOCATION</th>
<th>Temp High (°C)</th>
<th>Temp Low (°C)</th>
<th>Temp Average</th>
<th>%RH</th>
<th>(Precip mm/ Season)</th>
<th>ET (mm/ Season)</th>
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</thead>
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<td>CONCORD</td>
<td>25.75</td>
<td>13.78</td>
<td>19.77</td>
<td>73.18</td>
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<td>846.24</td>
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<td>MEAD</td>
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