CHARACTERIZATION OF THE NATURAL ENEMY COMMUNITY, WITH EMPHASIS ON ENTOMOPATHOGENS, FOR MANAGEMENT OF WESTERN CORN ROOTWORM (DIABROTICA VIRGIFERA VIRGIFERA) IN WEST CENTRAL NEBRASKA

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CHARACTERIZATION OF THE NATURAL ENEMY COMMUNITY, WITH EMPHASIS ON ENTOMOPATHOGENS, FOR MANAGEMENT OF WESTERN CORN ROOTWORM (DIABROTICA VIRGIFERA VIRGIFERA) IN WEST CENTRAL NEBRASKA

by

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A DISSERTATION

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Major: Entomology

Under the Supervision of Professors Julie A. Peterson and Lance J. Meinke

Lincoln, Nebraska
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CHARACTERIZATION OF THE NATURAL ENEMY COMMUNITY, WITH EMPHASIS ON ENTOMOPATHOGENS, FOR MANAGEMENT OF WESTERN CORN ROOTWORM

(DIABROTICA VIRGIFERA VIRGIFERA)

IN WEST CENTRAL NEBRASKA.

Camila Oliveira-Hofman, Ph.D.

University of Nebraska, 2018

Advisors: Julie A. Peterson and Lance J. Meinke

Diabrotica virgifera virgifera LeConte (Coleoptera: Chrysomelidae), the western corn rootworm (WCR), is a major pest of corn (Zea mays L.) in the United States and Europe. WCR management options comprise mainly transgenic hybrids, insecticide applications and crop rotation. WCR is highly adaptable to management practices and field-evolved resistance to transgenic corn, insecticides and crop rotation in the United States Corn Belt has been reported. Therefore, the motivation for this project was to look into alternative options for WCR management. The goal of this dissertation is to characterize the natural enemies from irrigated commercial cornfields in Nebraska and examine their potential as biological control agents of the WCR.

We surveyed five cornfields to document populations of arthropod predators, entomopathogenic fungi (EPF) and entomopathogenic nematodes (EPN). Yellow sticky cards and dry pitfalls captured a diverse community of above-ground natural enemies but their impact on WCR population dynamics is unlikely. In the laboratory, we isolated EPF and EPN species from soil samples using a baiting technique with Galleria mellonella L. and Tenebrio molitor L. Entomogenous fungi with a variety of ecological roles were
detected in every cornfield. Entomopathogenic fungi made up the majority of isolates, primarily represented by *Metarhizium*, but other genera of known and potential EPF include *Beauveria, Penicillium, Pseudogymnoascus*, and *Purpureocillium*. In the laboratory, forty-eight strains were screened against WCR larvae. Results showed that *Metarhizium anisopliae*, *M. robertsii*, *Pseudogymnoascus* sp. and BotaniGard (*Beauveria bassiana*) caused mortality higher than the control and should be explored further in field studies. Six strains that were tested against the WCR can also infect prepupae of western bean cutworm (*Striacosta albicosta* Smith), another damaging pest of corn in Nebraska.

We also determined that EPN strains of *Heterorhabditis bacteriophora* Poinar and *Steinernema* spp. are present in Western Nebraska cornfields. An inoculation project with commercial and New York strains of EPN did not cause significant mortality in WCR populations, potentially due to native *Steinernema* spp. being present in the control plots. Describing the natural enemy community from WCR-infested fields is a necessary first step in the exploration of biological control as a management tool against this devastating pest.
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CHAPTER 1: LITERATURE REVIEW

Western corn rootworm

The chrysomelid beetle *Diabrotica virgifera virgifera* LeConte, the western corn rootworm (WCR) is widely distributed east of the Rocky Mountains in the United States (Meinke et al. 2009). The WCR is highly invasive and has become one of the most damaging pests of field corn in North America and Europe (Gray et al. 2009). The western corn rootworm is also Nebraska’s most prevalent rootworm species.

Corn rootworm, as the name implies, cause injury to corn (*Zea mays* L.) via root feeding by the pest’s larval stage. Root feeding and pruning cause a reduction in nutrient uptake and can lead to lodging of the plant (Kahler et al. 1985). Rootworm damage can negatively impact plant-water relations, decrease photosynthetic rate, and above ground biomass (Riedell 1990; Godfrey et al. 1993; Urías-López et al. 2000). Reduced nutrients to the plant impact corn yield and lodged plants are difficult to harvest. Lodged plants may exhibit what is called “goosenecking” in which plants grow curved instead of straight up, making it hard to harvest grain (Purdue University, 2009).

Biology and ecology

The life cycle of the WCR is tightly connected to corn phenology since corn is its primary host plant. A number of grasses can serve as alternate hosts for larvae, but only a few are comparable to corn as hosts e.g.: western wheatgrass, *Pascopyrum smithii* (Rydb.); pubescent wheatgrass, *Elytrigia intermedia* (Host); side-oats grama, *Bouteloua curtipendula* Michx; quackgrass, *Elytrigia repens* L.; Rhodes grass, *Chloris gayana* Kunth; and fall panicum, *Panicum dichotomiflorum* Michx (Oyediran et al. 2004; Wilson and
Hibbard 2004). Pubescent wheatgrass produced an adult population size comparable to that obtained from corn (Oyediran et al. 2004). A number of other grasses also allow development to adulthood, but the emerging beetles are usually not comparable to beetles from corn in terms of size and weight (Clark and Hibbard 2004, Oyediran et al. 2004, Wilson and Hibbard 2004, Chege et al. 2005). The importance of these alternative hosts on WCR pest status in the field is uncertain (Clark and Hibbard 2004; Oyediran et al. 2004; Chege et al. 2005).

Corn seedlings exude carbon dioxide (CO$_2$) and other volatiles that attract neonate larvae to start feeding (Hibbard and Bjostad 1988). Younger larvae feed on root hairs and tips, burrowing into the root and crown to continue feeding as they grow. Initial feeding injury can lead to brown root tips, which is usually followed by tunneling marks and root pruning (Purdue University, 2009). Larvae go through three instars and each instar takes about one week to develop. Third instar WCRs create an earthen pupal cell in which they develop into adults. In the Corn Belt, pupation often occurs during the mid-June to late August period with a duration of around five to ten days at a temperature range of 18-30°C (Fisher 1986).

Adult emergence takes place during late June through September. Adults feed on silk and pollen, and on pollen of weeds and other crops after corn has matured (Maredia and Landis 1993; Campbell and Meinke 2006). Males initiate emergence about 2-10 days before females, a phenomenon known as protandry (Mabry and Spencer 2003). Females are generally ready to mate when they emerge, and can mate multiple times but generally only mate once throughout their lifespan (Hill 1975; Branson 1987;
Hammack 1995; Moeser and Hibbard 2005; Marquardt and Krupke 2009). Studies show a wide range of oviposition averages from around 200-1000 eggs/female throughout their lifespan (Spencer et al. 2009). Females don’t oviposit eggs on the corn plants, but place them in the soil adjacent to the plant (Moeser and Hibbard 2005). The majority of eggs are oviposited in the top 10 cm of soil, but eggs can be found up to 30 cm deep (Weiss et al. 1983; Gray and Tollefson 1988; Gray et al. 1992). Females tend to move deeper via soil cracks to oviposit in areas with adequate moisture (Gustin 1979; Kirk 1979; Weiss et al. 1983). In the Corn Belt, oviposition usually occurs from mid-July to early fall.

Eggs go through an extensive, obligate diapause through the winter and then enter into a quiescent stage until the temperature warms up to 11-12.7°C (Krysan 1978; Gustin 1981; Krysan et al. 1984; Levine et al. 1992). After reaching this temperature threshold, post-diapause development begins and neonate larvae hatch after a few weeks depending on temperature conditions (Wilde 1971; Wilde et al. 1972; Gustin 1981; Krysan et al. 1984; Levine et al. 1992; Godfrey et al. 1995). In the U.S and Europe, initial egg hatch occurs approximately between late May to the beginning of June (Meinke et al. 2009).

**Larval mortality factors**

Larvae are the most damaging stage of this pest and for this reason larval mortality factors must be well understood in order to establish effective pest management programs. Besides pest management practices, larvae are subject to abiotic and biotic mortality factors in the soil. Primary abiotic factors include soil
conditions such as moisture, temperature and texture. These factors are interactive and different combinations of these conditions can have different impacts on WCR larval survival. For instance, larvae exposed to high temperatures had increased survival when they were simultaneously exposed to wet conditions, whereas desiccation rapidly occurred when larvae were exposed to high temperatures and low soil moisture (MacDonald and Ellis 1990). Besides direct mortality, abiotic factors can also affect larval movement in the soil, which in turn can disrupt larval establishment on the host, leading to mortality (MacDonald and Ellis 1990). Larvae show reduced movement when soils are dry (12% moisture) or saturated (38% moisture); movement was greater at moderate conditions (24-30% moisture) (MacDonald and Ellis 1990).

WCR tend to prefer soils with higher clay content (Turpin and Peters 1971). Larvae exposed to soil with high clay content (48.2%) had higher adult emergence than larvae from sandy soils that have lower clay content (4.8-11.5%) (Turpin and Peters 1971). Sand particles are thought to physically injure WCR larvae through abrasion, which increases desiccation of larvae (Turpin and Peters 1971), but this hypothesis has never been proven. Larvae in silt-clay and loam soils moved greater than three times the mean distance (>18 cm) of larvae in loamy-sand soil (6.1 cm mean) (MacDonald and Ellis 1990). Clay soils hold water better than sandy soils; this may be a reason why WCR larvae have a tendency to prefer clay soils.

When exposed to severe hypoxia during underwater submersion experiments, WCR larvae took longer to reach mean time mortality (LT$_{50}$) at lower temperatures (10 and 15°C) than at higher temperatures (20 and 25°C) (Hoback et al. 2002), meaning that,
larvae that experience flooding will die quicker in higher temperatures. But not all larval stages tolerate flooding equally. Third instars had approximately half (~26 hrs) the LT$_{50}$ at 25°C in hypoxia than second instars (~56 hrs) exposed to the same conditions (Hoback et al. 2002). This suggests that if flooding occurs when larvae are older (3$^{rd}$ vs. 2$^{nd}$ instars) they will have a decreased chance of survival.

Biotic factors that contribute to WCR larval mortality include density-dependent factors such as resource competition, as well as density-independent factors such as presence of suitable hosts and natural enemies. The role of mortality by natural enemies is discussed in “Biological control of western corn rootworm” section.

Larval density-dependent factors can influence mortality in WCR populations. High larval density is directly correlated with decreased survival to adulthood (Onstad et al. 2006). High WCR infestation rates (1200 and 2400 eggs/30.5 cm of row) resulted in lower percentage survival to adulthood, plus, adults were smaller and exhibited lower egg viability when compared to lower infestation rates (300 eggs and 600 eggs/30.5 cm of row) (Branson and Sutter 1985). In a regression analysis of six infestation levels (25, 50, 100, 300, 600, 1200 and 2400 eggs/30.5 cm of row) it was determined that density-dependent mortality began at approximately 851 eggs/30.5 cm of row (Hibbard et al. 2010). In the same study, a regression analysis with previously published data showed that density-dependent mortality began at approximately 800 eggs/30.5 cm of row (Hibbard et al. 2010). WCR in this study were also subject to unknown density-independent mortality factors, which caused at least 91% mortality, even at lower densities (Hibbard et al. 2010). In low egg densities (25 eggs/plant), larvae develop
faster than at higher densities (75 eggs/plant) (Weiss et al. 1985). At these higher densities, the sex ratio of emerged adults was biased more towards males, probably due to the fact that males inherently develop faster than females and are able to complete development before resources become limiting (Weiss et al. 1985). Females emerging from the high density treatment weighed significantly less than females in the low density treatment (Weiss et al. 1985). Smaller females with head capsule width of 1.15 mm lay fewer eggs (mean 506.6 eggs/female), than bigger females with head capsule widths of 1.17-1.22 mm (mean 735.6-845.5 eggs/female) (Branson and Sutter 1985).

Presence of a suitable host is essential for larval survival. Neonates are thought to have a maximum of 84 h to establish on a host before dying (Strnad and Bergman 1987a; Branson 1989), but this doesn’t mean that larvae can’t die before this time period. Strnad and Bergman (1987) found that larval survival was the same after 12, 24 and 36 h of starvation. However, after 12 h of starvation, only ~75% of larvae were able to burrow into roots, significantly lower than 100% of larvae in the non-starved treatment (Strnad and Bergman 1987). This indicates that, although they can survive starvation for relatively long periods, WCR lose their capability to establish successfully on the host, which contributes to decreased rates of survival to adulthood (Branson 1989).

**Current WCR management practices**

Management strategies for WCR are mostly focused on controlling the larval stage, although adult control can be employed as well to reduce egg laying and egg
density in next season’s corn crop. Current management strategies include crop rotation with non-hosts, insecticides and transgenic hybrids.

**Rotation**

Crop rotation is the most effective way to minimize the WCR population in a field. This cultural management strategy is based on two principles of the WCR life cycle described above: larvae only feed on corn and adults have a strong oviposition fidelity to cornfields (Mabry and Spencer 2003). The switch from corn to a non-host breaks the WCR life cycle and reduces insect pressure in the field. WCR larvae that hatch in a non-host field are unable to feed and will likely die from starvation. Similarly, WCR adult females from neighboring fields will be less likely to oviposit in a non-host crop. If volunteer corn is present in the rotated field some WCR can survive, but populations will likely be low in corn the following year.

Corn rotation can also reduce costs associated with WCR management because first-year corn can often be planted without the use of soil insecticides (Roth, 1996) and without the use of WCR-Bt hybrids. Rotation also provides benefits beyond WCR control. Growers that adopt a rotation schedule instead of continuous corn may see an increase in corn yield (Roth, 1996). Additionally, corn followed by another crop requires less fertilizer and less tillage than continuous corn (Roth, 1996).

A typical rotation in the Midwest is the annual corn-soybean rotation. This rotation works well for corn rootworm management for the majority of growers, but there are some problem regions in East Illinois and West Indiana where WCR have developed resistance to rotation (Levine and Oloomi-Sadeghi 1996; Levine et al. 2002;
Gray et al. 2009). In Nebraska, no cases of this phenotype have been reported to date. These rotation-resistant (RR) WCR adults exhibit less affinity to stay in corn, even when silk and pollen is still available (Levine et al. 2002, Pierce and Gray 2006). Movement out of corn to soybeans by RR-WCR peaks after corn reaches R2 (reproductive stage 2, or blister stage), hence cornfields are still very susceptible to WCR injury if corn is planted in the following year (Pierce and Gray 2006, 2007). The WCR adults that move away from corn can feed on soybean foliage and oviposit in soybean fields (Levine et al. 2002). Soybeans are a sub-optimal source of food when compared to corn and it seems that WCR females under stress may oviposit prematurely in soybean fields due to this physiological stress (Mabry and Spencer 2003). It was also found that RR-WCR carry a different gut microbiota that facilitates its tolerance of antiherbivore defenses produced by soybeans (Chu et al. 2013). This lowered fidelity to oviposit in corn can lead to root injury and yield losses in corn following soybeans. Management of RR-WCR includes using transgenic corn in a 2-year rotation, increasing the rotation schedule to a 3-year rotation and/or sampling for WCR in the soybean year (O’Neal et al. 2001, Onstad et al. 2003). The use of unbaited yellow sticky cards to quantify WCR populations in soybean can indicate whether or not rootworm protection will be needed in first-year corn (O’Neal et al. 2001).

**Chemical control**

Chemical control of the WCR is targeted against the larval or adult stages. To prevent or slow down larval feeding, a grower can opt for high-rate insecticidal seed treatments, planting-time insecticides, or post-planting insecticides. Seed treatments
used are neonicotinoids such as clothianidin and thiamethoxam (Eisley and Hammond 2008). Planting-time insecticides include: organophosphates (chlorethoxyphos, chlorpyrifos, and tebupirimfos) and pyrethroids (bifenthrin, cyfluthrin and tefluthrin) (Eisley and Hammond 2008, Bledsoe and Obermeyer 2010, Krupke et al. 2014). Planting-time and post-emergence insecticides work best when they are applied when larvae are already present or right before larvae are present (Bledsoe and Obermeyer 2010).

Adult control of the WCR can be applied to stop adult feeding, but it is primarily used to reduce egg laying and hence reduce the following year’s WCR population. If silk feeding is intensive during pollen shed, silk clipping can interfere with pollination (Maredia and Landis 1993). A variety of organophosphates and pyrethroids, and one oxadiazine (indoxacarb) can be used to control adult populations of the WCR (DeVries and Wright 2016, Krupke et al. 2014). To prevent pollination interference by the WCR, insecticide application should take place before 50% of pollination has occurred or when pollen is being shed and silks are clipped to ½ inch or less (Krupke et al. 2014).

Numerous cases of WCR insecticide resistance have been reported in the literature. In Nebraska, WCR adults collected from several counties throughout the state showed different levels of susceptibility to methyl parathion (organophosphate) and carbaryl (carbamate) (Meinke et al. 1998). The populations with the highest levels of resistance to these insecticides came from Phelps and York counties in South-Central NE where organophosphates and carbamates were consistently used for both adult and larval control (Meinke et al. 1998). Overuse of these insecticides led to failure of the adult management program (Meinke et al. 1998). Also in Nebraska, field populations
were shown to have aldrin (cyclodiene organochlorine) and bifenthrin (pyrethroid) resistance (Ball and Weekman 1963, Parimi et al. 2006, Pereira et al. 2015). Although resistance to insecticides, especially overused products, has been reported in some regions of the country, the same active ingredients may still be effective in other regions.

**Host plant resistance**

Host plant resistance (HPR) to insects has three main categories: antibiosis, antixenosis and tolerance. Plants exhibiting antibiosis have detrimental effects on the insect’s biology and survival (Painter 1951). Antixenotic plants exhibit adverse effects on the insect’s behavior in a way that the insect exhibits non-preference for the plant (Kogan and Ortman 1978). Lastly, tolerance is a category of HPR that unlike antixenosis and antibiosis does not affect the insect’s biology or behavior. Rather, tolerant plants can withstand and/or recover from insect damage when compared to susceptible plants (Painter 1951). Hence, tolerance is a category that is defined from the plants’ perspective not the insects.

Prior to transgenics, or genetically engineered plants with gene(s) from other species, HPR to rootworm species was done via selection of naturally occurring antibiotic, antixenotic and tolerant traits. Corn plants are fairly susceptible to WCR feeding and since there are not many innate antibiotic and antixenotic characteristics available; WCR-HPR was primarily achieved via tolerance (Wilson and Peters 1973, Riedell and Evenson 1993, Gray and Steffey 1998, Urias and Meinke 2001). Corn hybrids that have rapid root growth and large root systems may be preferred since they are
more tolerant to WCR damage (Ortman et al. 1974; Branson et al. 1982). However, hybrids with over-compensatory root regrowth can exhibit less vegetative biomass including kernel weight and therefore negatively impact yield (Godfrey et al. 1993; Urias-López and Meinke 2001).

Host plant resistance for WCR today is largely achieved via transgenic *Bacillus thuringiensis* Berliner (Bt) corn hybrids. The *B. thuringiensis* bacterium produces delta-endotoxins, crystalline proteins that when ingested by certain, susceptible insects cause septicemia and death. Delta-endotoxin genes are transformed into corn plants that can in turn produce crystalline proteins in the plant’s tissues. Sub-lethal effects due to Bt feeding also occur, and WCR larvae show increased developmental time when they feed on Bt roots (Meissle et al. 2009, Petzold-Maxwell et al. 2013). Crystalline proteins are categorized as Cry proteins and are known for their specificity to target organisms. The available registered Cry toxins against the WCR are: Cry3Bb1, mCry3A, Cry34/35Ab1, and eCry3.1Ab (DiFonzo 2017).

In 2003, Cry3Bb1 was the first plant-incorporated rootworm-active protein registered by the EPA in the U.S. (tradename: YieldGard® Rootworm, USEPA 2010). Since then, there has been a shift over time to incorporate more than one WCR toxin per plant, also known as trait pyramiding. The concept is that if WCR develops resistance to one toxin, but is still susceptible to the other, death will still occur, and this will delay resistance evolution.

Bt corn hybrids require a refuge, or in other words, non-rootworm Bt corn areas where susceptible individuals can survive. Refuge systems proposed for Bt hybrids is the
high-dose refuge, with high-dose being defined as 25x the toxin concentration to kill 99.99% of susceptible insects (USEPA 2001). However, none of the current WCR-Bt hybrids are registered as high-dose, which allowed for some insect survival on those hybrids (Andow et al. 2016). The moderate-dose approach together with the wide use of WCR-Bt hybrids has led to the development of field-evolved resistance. The first resistance case to rootworm-protected Bt corn was reported in Iowa in 2011 for Cry3Bb1 hybrids (Gassmann et al. 2011). There was a strong relationship between the number of years Cry3Bb1 hybrids had been grown and resistance, or in other words, continuous Bt toxin pressure lead to increased WCR survival in problem fields. Cross-resistance between Cry3Bb1 and mCry3A or eCry3.1Ab has been reported but no cross-resistance between these proteins and Cry34/35 (Gassmann et al. 2014, Wangila et al. 2015, Zukoff et al. 2016). Field-evolved resistance to Cry3Bb1 or mCry3A has subsequently been reported from other Corn Belt states (Schrader et al. 2016, Wangila et al. 2015, Zukoff et al. 2016) as well as initial documentation of field-evolved WCR resistance to Cry34/35 (Gassmann et al. 2016, Ludwick et al. 2017).

**Biological control of western corn rootworm**

Biological control utilizes living natural enemies to manage pest populations. Three types of biological control exist: classical, augmentation and conservation biological control. According to (Eilenberg et al. 2001) classical biological control is defined as: “The intentional introduction of an exotic, usually co-evolved, biological control agent for permanent establishment and long-term pest control.” Classical
biological control aims to restore the natural balance present in the pest’s native habitat that prevents pest outbreaks (Hajek, 2004).

Augmentation biological control involves both methods of inoculative and inundative control and is different from classical biological control in that it does not aim for permanent establishment of the biological control agent (Hajek, 2004). Inundative control is often referred to as a “biopesticide” since it is expected to cause high mortality in a short period (Hajek, 2004). These biopesticides are applied in high doses and only the released agents, not subsequent generations, cause mortality. Inundative biological control can be defined as: “The use of living organisms to control pests when control is achieved exclusively by the released organisms themselves” (Eilenberg et al. 2001). Inoculative biological control is different from inundative control in which the progeny from the released biological control organism can also inflict pest mortality (Hajek, 2004). Inoculative biological control is defined as: “The intentional release of a living organism as a biological control agent with the expectation that it will multiply and control the pest for an extended period, but not permanently” (Eilenberg et al. 2001).

The last category is conservation biological control which is defined by Eilenberg et al. (2001) as: “Modification of the environment or existing practices to protect and enhance specific natural enemies or other organisms to reduce the effect of pests”. Conservation biological control aims to enhance natural populations instead of introducing biological control agents. Here, knowledge of the factors that are preventing natural enemies from being effective biological control agents is necessary in order to
establish the program (Hajek, 2004). With this understanding, practitioners can reduce disturbances such as pesticides and lack of hosts/food by manipulating the habitat to benefit natural enemies (Landis et al. 2000).

Biological control of corn rootworm species is an underexplored area when compared to other available management practices. Several natural enemies are found to prey on or infect WCR (Kuhlmann and van der Burgt 1998, Toepfer et al. 2009); however, the extent of their impact on WCR population reduction is often unknown. Biological control of WCR has been investigated in the U.S. and in Europe, but the adoption of biological control on commercial farms has been limited. There are five main groups of natural enemies of the WCR: arthropod natural enemy predators and parasitoids; and entomopathogenic fungi, bacteria, and nematodes. Biological control doesn’t have to be a stand-alone strategy to pest management. Its compatibility with other pest management methods is discussed in section 2.4.

**Arthropod natural enemies: Predators**

A wide variety of arthropods have been observed feeding on WCR eggs and larvae. Main taxa include predatory mites (Mesostigmata), ground beetles (Carabidae), hister beetles (Histeridae), rove beetles (Staphylinidae), carpet beetles (Dermestidae), centipedes (Chilopoda), and spiders (Araneae) (Kuhlmann and van der Burgt 1998). The ant species *Lasius neoniger* Emery has also been linked to consuming WCR larvae (Kirk 1981).

Lundgren et al. (2009a) used polymerase chain reaction (PCR) to amplify WCR DNA extracted from predatory taxa in plots artificially infested with WCR eggs. Out of
1550 specimens, 166 (~10%) were positive for WCR DNA in their guts. Most common specimens positive for WCR DNA included Acari (*Chausseria* sp. and other mites); Lycosidae and other Aranae; *Phalangium opilio* L. (Opiliones); *Scarites quadriceps* Chaudoi*, Poecilus chalcites* (Say) and other Carabidae species; and Staphylinidae species (Lundgren et al. 2009b). A significantly higher percentage of predators were found positive for WCR DNA when this pest was in the egg stage; however, frequency of consumption and consumption index were comparable between egg and larval stages (Lundgren et al. 2009b). Eggs are probably consumed more because they are in the field for a longer period than larvae (Lundgren et al. 2009b). Predators with chewing mouthparts had a lower consumption index than predators with sucking mouthparts (Lundgren et al. 2009b). This is probably due to the fact that WCR larvae exhibit a hemolymph defense that coagulates quickly in the predators’ mouthparts (Lundgren et al. 2009a). This sticky hemolymph triggers immediate cessation of feeding and intense cleaning of mouthparts (Lundgren 2009a). Predators in the study (*Poecilus cupreus* L. and *Harpalus pensylvanicus* De Geer (Carabidae) spent significantly more time cleaning their mouthparts than eating, and once exposed to the hemolymph defense of WCR, they were hesitant to feed again on WCR (Lundgren et al. 2009a). A diverse community of predators is shown to increase the frequency of WCR egg and larval predation (Lundgren and Fergen 2014). This indicates that in a biological control program against the WCR, multiple predators may enhance mortality.
Arthropod natural enemies: Parasitoids

Insect parasitoids are obligate parasites that develop inside (endoparasitoids) or outside (ectoparasitoids) an individual host and result in the host’s mortality. Hymenoptera and Diptera (Insecta) are the two major orders with parasitoid members. Parasitoids from the genus *Celatoria* spp. (Diptera: Tachinidae) lay eggs containing a fully developed first instar larva on *Diabrotica* spp. adults (Kuhlmann and van der Burgt 1998). *Celatoria* spp. have been studied to control rootworm populations, especially the species *Celatoria compressa* Wulp (Kuhlmann et al. 2005). There are several constraints with the rearing of *Celatoria* spp., including its low fecundity rates, specific temperature requirements, and the univoltine nature of the host (WCR), all of which makes it an impractical biological control agent for augmentation (Toepfer et al. 2005). Moreover, studies are needed to evaluate its potential for conservation biological control (Toepfer et al. 2008).

Entomopathogenic fungi

Entomopathogenic fungi (EPF) are present in ecosystems worldwide and play a role in keeping insect pests regulated. Infective spores can be present above and below ground and as opposed to other entomopathogens, they do not need to be eaten in order to be pathogenic (Hajek, 2004). EPF spores attach to the host’s cuticle, and then they germinate and penetrate the insect host (Hajek, 2004; Wraight et al. 2007). Fungi use both the force of mechanical pressure and digestive enzymes to pierce/digest the insect’s cuticle (Hajek, 2004). The fungus eventually takes over the host’s hemolymph and hemocoel and causes death.
The fungi *Beauveria bassiana* (Bals. -Criv.) Vuill. and *Metarhizium anisopliae* (Metschn.) Sorokin infect WCR larvae and pupae (Kuhlmann and van der Burgt 1998). These species are naturally occurring worldwide and can be used as part of a biological control program. Rudeen et al. (2013) found that *M. anisopliae* and *B. bassiana* were common in commercial cornfields in Iowa and that the WCR mortality caused by field-collected strains was comparable, if not greater, than commercially available strains.

Fungal strains can differ in their virulence to specific stages of WCR. A screen of twenty strains of *M. anisopliae*, *B. bassiana* and *Beauveria brongniartii* (Sacc.) Petch revealed varying levels of mortality (Pilz et al. 2007). In general, most *M. anisopliae* strains were more virulent than *B. bassiana* strains, but no strain induced greater than 50% larval mortality (Pilz et al. 2007). Adults were more susceptible to these strains than larvae, which the authors hypothesize that larvae are better adapted to living in the soil with naturally co-occurring entomopathogens than adults. The most virulent *M. anisopliae* larval strain caused 47% mortality, but in comparison, the most virulent *M. anisopliae* strain for adults achieved 97% mortality (Pilz et al. 2007).

Indigenous *B. bassiana* was also found to infect adult *Diabrotica* spp. at emergence (Bruck and Lewis 2001). This means that emerging WCR adults are exposed to *B. bassiana* from the soil as they emerge, which causes adult mortality. Infection rates were low at 3.2%, but the researchers estimated that with their high level of ~2.6 million WCR beetles/hectare, there is a potential reduction in oviposition of 41 million eggs/hectare (Bruck and Lewis 2001). In addition, *B. bassiana* can also cause non-lethal effects. Infection of *B. bassiana* on mated females causes a 30% or greater reduction of
fecundity in the surviving beetles (Mulock and Chandler 2001a). Oviposition reduction can lessen WCR pressure in the following year’s corn crop.

**Entomopathogenic bacteria**

Entomopathogenic bacteria can enter the host’s body via wounds but the primary entryway is via ingestion (Hajek, 2004). Bacteria proliferate inside the host’s hemocoel and kill the host via septicemia. The most well-known entomopathogenic bacteria, *B. thuringiensis* and *Bacillus sphaericus* Meyer and Neide, are from the spore-forming family Bacillaceae (Hajek, 2004). Non-spore forming entomopathogens include *Serratia entomophila* Grimont and *Serratia marcescens* Bizio (Enterobacteriaceae).

Research on bacteria pathogenic to WCR comprises mainly the study of Bt strains that contain genes that have been incorporated into transgenic plants, but other bacterial species have not been studied in depth. Bacteria symbiotic with entomopathogenic nematodes are discussed in the next section.

**Entomopathogenic nematodes**

Entomopathogenic nematodes (EPN) are natural enemies of soil arthropods that kill hosts within one to four days post-infection (Koppenhöfer 2007). Seven nematode families are considered to be entomopathogenic, but only Heterorhabditidae and Steinernematidae (Nematoda: Rhabditida), which are obligate endoparasites of insect hosts, are consistently researched as biological control agents (Koppenhöfer 2007). The family Heterorhabditidae contains the single genus *Heterorhabditis* and Steinernematidae has two genera: *Steinernema* and *Neosteinernema* (Stock and Goodrich-Blair 2012). The most studied EPN species for biological control of WCR are
**Steinernema feltiae** (Filipvej), **Steinernema carpocapsae** (Weiser) and **Heterorhabditis bacteriophora** Poinar.

Third stage infective juveniles (IJ’s), or dauer juveniles, are the only life stage of the nematodes that can live outside the host in order to search for new ones. IJ’s infect insect hosts by penetrating thin areas of the cuticle or via natural openings such as the mouth, spiracles, genital pores and anus. Once inside the host’s hemocoel, IJ’s release mutualistic bacteria that induce host mortality via septicemia within 48 hours (Kaya and Gaugler 1993). Steinernematidae are associated with bacteria from the genus **Xenorhabdus** and Heterorhabditidae with **Photorhabdus** bacteria. EPN’s develop on the host’s metabolized tissue and the proliferated bacteria for 1-3 generations until host tissues are depleted and IJ’s need to seek a new host (Koppenhöfer 2007).

EPN’s are most effective against WCR in second and third instars (Jackson and Brooks 1989; Journey and Ostlie 2000; Kurtz et al. 2009). The Mexican strain of *S. carpocapsae* infects all stages of the WCR besides eggs. However, based on the mean number of *S. carpocapsae*/instar, they found that third instar rootworms are 5x more susceptible than second instars and 75x times more susceptible than first instars and pupae (Jackson and Brooks 1995). Differences in infectivity are most likely due to bigger orifices/entry points in later instars (Jackson and Brooks 1995). It is important to point out that rootworm larvae only support one generation of nematode reproduction due to the small amount of larval host tissue (Jackson and Brooks 1995).

Different species of EPN’s have different strategies to forage for hosts. These strategies are known as cruise and ambush strategies. “Cruisers” actively search for the
host and because they are mobile they are more prone to encounter sedentary hosts. Alternatively, “ambushers” are likely to encounter moving hosts as they can attach to the passing host. Species can also adapt a foraging strategy that is intermediate between cruising and ambushing (Lewis et al. 2006). _Steinernema carpocapsae_ is an ambusher and _H. bacteriophora_ is a cruiser (Grewal et al. 1993, 1994). However, _S. feltiae_ exhibits both foraging behaviors, as a small subset of the population actively cruise for hosts but the majority of the population acts as ambushers (Gaugler et al. 1989).

IJ’s use chemo-, thermo- and mechanotaxis to locate and recognize insect hosts (Lewis et al. 2006). In the case of the rootworm-EPN system, it was also found that nematodes are attracted to injured corn roots (Rasmann et al. 2005). The sesquiterpene (E)-β-caryophyllene is a secondary compound present in the damaged roots of certain corn varieties (Rasmann et al. 2005). A European corn variety that contained (E)-β-caryophyllene strongly attracted _Heterorhabditis megidis_, which promoted secondary plant defense. (E)-β-caryophyllene treatments had five times the rate of WCR infection by _H. megidis_ than the treatment that didn’t contain (E)-β-caryophyllene (Rasmann et al. 2005). Interestingly, modern corn hybrids in the U.S., seemed to have accidentally lost (E)-β-caryophyllene during breeding programs (Rasmann et al. 2005).

Field studies have shown that EPN applications can add an extra layer of protection to corn roots. Wright et al. (1993) applied a rate of 1.2 and 2.5 x 10⁹ IJ’s of _S. carpocapsae_ per hectare via center-pivot irrigation. In this study, they found that at the higher rate of IJ’s, nematodes could be just as effective as chlorpyrifos in reducing WCR
adult emergence. Similarly, *S. carpocapsae* and *H. bacteriophora* treatments (200,000 IJ/plant) were just as effective as terbufos in suppressing beetle emergence (Jackson 1996). *Heterorhabditis bacteriophora* applied at a rate of $3.8 \times 10^9$ IJ/hectare was comparable to tefluthrin and clothianidin-coated seed treatments in reducing WCR emergence (Pilz et al. 2009). Chlorpyrifos, terbufos (organophosphates), clothianidin (neonicotinoid), and tefluthrin (pyrethroid) are common insecticides utilized for larval WCR treatment. Collectively, these studies indicate that if well timed, EPN treatments can be used as an alternative to WCR pesticides in problem fields. However, improvements in EPN production costs still have to be made to turn that into reality.

In another study, *S. feltiae*, *H. bacteriophora* and *H. megidis* were applied to test plots via two methods: direct spray at sowing time ($2.8 \times 10^9$ IJ/hectare) or spray when WCR larvae were expected to be at second instar ($3.4 \times 10^9$ IJ/hectare) (Toepfer et al. 2008). All three EPN species showed moderate to high levels of WCR control; when they were applied at sowing time, *H. bacteriophora* showed 81% reduction in WCR emergence, while the *S. feltiae* and *H. megidis* only accounted for 36% and 49% reduction, respectively, in adult emergence (Toepfer et al. 2008). Moreover, when EPN’s were applied when second instars of the WCR were present, both *H. bacteriophora* and *H. megidis* were statistically similar in reducing WCR adult emergence (75% and 69%, respectively), but *S. feltiae* only provided 32% emergence reduction (Toepfer et al. 2008a). Even though these species differed in suppressing WCR populations, they were equally successful in protecting roots from damage regardless of which application method was used (Toepfer et al. 2008). In this study they infested 150 WCR eggs/plant,
which is regarded as a low rootworm density; however, it is possible that EPN efficacy is different under high WCR pressure. Europe’s WCR densities are often much lower than U.S. WCR densities and therefore, EPNs may hold greater potential in Europe.

Traditional studies with EPN releases have treated EPN’s as biopesticides (Shields 2015a). Biopesticides act in a similar fashion as pesticides, in which a high-dose application of a biological control agent is applied inundatively in the field and it is expected to cause high mortality of the pest within a short time period (Hajek, 2004). Inundative releases of EPN’s use commercial strains that have lost their ability to persist in the environment (Shields 2015). Few researchers have looked into inoculation, rather than inundation, of EPN in the environment. A recent study showed that it is possible for \textit{S. carpocapsae} and \textit{S. feltiae} to reduce populations of the alfalfa snout beetle \textit{(Otiorhynchus ligustici} (L.) (Coleoptera: Curculionidae) in long-term alfalfa-corn and alfalfa-soybean rotations (Shields and Testa 2015a). This research team has also shown the persistence of these EPN species in vineyards against grubs of the Japanese beetle \textit{Popillia japonica} Newman (Coleoptera: Scarabaeidae) (Shields 2015a). The goal here is to introduce EPN strains that are able to persist in the environment, throughout multiple generations, even in low density host populations and provide long-term pest control (Shields 2015a).

\textbf{Compatibility of biological control with current WCR management options}

The management of WCR requires a complex, multi-strategy approach. Tactics adopted by growers are often over-used and ineffective after less than a decade due to field-evolved resistance. There is a need to develop new technologies against the WCR
that could be efficacious in the long run. More research needs to be done in integrating control strategies so that we have an ecologically and economically sound integrated pest management strategy against this highly destructive pest. Integrating biological control into the scope of management practices against the WCR might help us suppress population growth of this pest.

Some researchers have studied tri-trophic interactions between Bt, WCR and entomopathogens. Growth chamber and laboratory studies determined that a combination of fungal (B. bassiana and Metarhizium brunneum Petch) and nematode (S. carpocapsae, S. glaseri, H. bacteriophora) entomopathogens acted in an additive way with expressing Cry34/35Ab1 to reduce WCR larval densities, and survival to adulthood (Petzold-Maxwell et al. 2012a). Because Bt proteins caused a delay of WCR development and the pathogens increased mortality, the pathogens had a longer window of time to infect the larvae; hence they acted independently but in an additive manner (Petzold-Maxwell et al. 2012a). This additive effect may go away in WCR populations that display complete resistance to Cry3Bb1 and do not exhibit developmental delays (Wangila and Meinke 2017). In the field, the addition of M. brunneum, S. carpocapsae, and H. bacteriophora in plots with Cry34/35Ab1 hybrids increased yield when compared to the Bt only treatment, despite the fact that the entomopathogens did not decrease survival of WCR (Petzold–Maxwell et al. 2013). Moreover, they found that root injury in the Bt + entomopathogens treatment was only reduced when WCR abundance was high. Conversely, root injury in the non-Bt + entomopathogens treatment was reduced when WCR abundance was low (Petzold-Maxwell et al. 2013). The explanation for these
results is that at high WCR abundance, entomopathogens alone cannot protect roots from injury without another management practice, while at low WCR abundance, entomopathogens alone can give roots a good level of protection (Petzold-Maxwell et al. 2013). The addition of entomopathogenic nematodes, such as *S. carpocapsae*, may not increase mortality rates of WCR larvae when used in conjunction with the Cry34/35Ab1 Bt hybrid treatment (Rudeen and Gassmann 2013). *Steinernema carpocapsae* are more infective in later instars of WCR and since larvae feeding on Cry34/35Ab1 had delayed development, the larvae were not in the growth stage most susceptible to the nematode (Rudeen and Gassmann 2013).

A combination of a Cry3Bb1 Bt hybrid and entomopathogenic fungi can also be effective against WCR. *Metarhizium anisopliae* successfully infected WCR larvae and adults that fed on Cry3Bb1 (Meissle et al. 2009). Both WCR larval and adult stages were equally susceptible to fungal infection regardless if they fed on Cry3Bb1 or non-Bt diet (Meissle et al. 2009). Since there is no interaction between *M. anisopliae* and Cry3Bb1 on WCR mortality, this result implies an additive effect of the treatments, rather than synergistic effect (Meissle et al. 2009). Hence, fungi and Bt-hybrids can be used as complementary tactics against rootworms.

Adding a mortality factor, such as entomopathogens to complement a Bt hybrid may delay Bt resistance evolution of herbivores because of associated fitness costs (Gassmann et al. 2008, Gassmann et al. 2009a,b; Hannon et al. 2010, Gassmann et al. 2012). For instance, resistance to Cry1Ac in the pink bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) was delayed in treatments with *Steinernema*
Resistance to Cry1Ac may evolve slower if populations are treated with *S. riobrave* since resistant individuals had higher mortality rates by *S. riobrave* than Cry1Ac-susceptible individuals (Gassmann et al. 2006, 2009b). However, this is not the case with WCR Bt resistance and entomopathogens. There were no fitness costs associated with resistance to Cry3Bb1 when WCR were exposed to entomopathogens (Petzold-Maxwell et al. 2012c; Hoffmann et al. 2014). *Steinernema carpocapsae* and *H. bacteriophora* had no difference in WCR infection rates for Cry3Bb1 susceptible and resistant individuals (Petzold-Maxwell et al. 2012a). Similarly, *S. feltiae*, *H. bacteriophora*, *B. bassiana* and *M. brunneum* did not impose any fitness cost on WCR resistant to Cry3Bb1 (Hoffmann et al. 2014). These entomopathogens increased mortality of both Bt resistant and susceptible individuals when compared to no pathogen treatments but at no resistance-related fitness cost (Hoffmann et al. 2014).

In addition to Bt-entomopathogen interactions, one must also consider the possible interactions of entomopathogens with pesticides (insecticides, fungicides and nematicides). These interactions can be negative, neutral or positive. Negative interactions occur when the pesticides inhibit entomopathogen activity such as reduced reproduction, virulence and altered behavior (Manachini 2002). Positive interactions can create enhanced mortality of the pest in comparison to either product alone (Manachini 2002).

The entomopathogenic nematodes *S. feltiae*, *S. carpocapsae*, *H. bacteriophora* and *Heterorhabditis heliothidis* for the most part can tolerate pesticides even at higher than recommended concentrations (Rovesti et al. 1988; Rovesti and Deseò 1990, 1991).
However, some chemicals were found to have negative effects on movement, infectivity and motility of IJ’s (Rovesti and Deseö 1990, 1991). Alternatively, the insecticide tefluthrin (pyrethroid) in conjunction with S. carpocapsae can be synergistic; together they caused an average of 24% more mortality than the expected additive mortality (Nishimatsu and Jackson 1998). Hence in this case, EPN’s can be used in conjunction with an insecticide to cause greater mortality to WCR (Nishimatsu and Jackson 1998).

The entomopathogenic fungi (B. bassiana and M. anisopliae) are also subject to interactions with pesticides. These species seem to be compatible with neonicotinoids (Neves et al. 2001; Batista Filho et al. 2001). Moreover, at the recommended rate some fungicides decreased B. bassiana and M. anisopliae germination, but virulence seemed to be unaffected for most interactions (Shah et al. 2009). All these studies should be taken with caution as the interactions in the field between chemicals and pathogens are certainly different than in vitro interactions. Also, entomopathogenic nematodes and fungi may be protected in their hosts, in which case they may not come in contact with the agrochemicals in the soil.

Interactions between entomopathogens and crop rotation must be investigated to establish entomopathogen persistence in the environment. Strains of the nematodes S. feltiae and S.carpocapsae were tested to see if they persist in alfalfa-corn and alfalfa-soybean rotations and continue to be infective to the target insect pest, Otiorhynchus ligustici (L.), the alfalfa snout beetle (Shields and Testa 2015a). Data were collected for up to 6 years in some fields and both EPN species were shown to persist in continuous alfalfa as well as in alfalfa-corn and alfalfa-soybean rotations (Shields and Testa 2015). It
was inferred, but not tested, that these nematodes infected rootworms in corn years and hence were able to thrive. In some years, Shield and Testa (2015) saw a spike in nematode detection in the non-alfalfa crop rotations, suggesting that insects of corn and soybeans are hosts for both *S. feltiae* and *S. carpocapsae*.

**Justification**

Western corn rootworm mortality (WCR) is a multifactor issue that must be understood in order to establish a sound management program. Multiple interactions among abiotic and biotic factors occur that influence mortality rates in all stages of the WCR. Nebraska is currently the third largest corn producer in the country and WCR is one of the state’s most important pests. The climate is semiarid in West Central Nebraska and because of that, continuous corn is primarily grown under center-pivot irrigation systems. There is a strong need for new management options for the WCR as populations of this pest in West Central Nebraska and other areas of the Corn Belt have evolved resistance to many currently used management options. Biological control is an understudied area of WCR management and it is unknown what natural enemy predators and entomopathogens are present in West Central Nebraska.

**Research objectives**

The broad goal of this dissertation was to understand the biodiversity of natural enemies present in irrigated commercial cornfields in the context of finding alternative and complementary WCR management options. Specific chapter objectives were:

**Chapter 2:** Identify potential above-ground and epigeal predators of WCR with an emphasis on determining if Carabidae beetles were consuming WCR in the field.
**Chapter 3:** Document the community of entomogenous fungi from the rhizosphere throughout the field season.

**Chapter 4:** Determine the impact of native fungal strains isolated from Chapter 3 on WCR larval mortality in the laboratory.

**Chapter 5:** Document the diversity of entomogenous nematodes in commercial cornfields and evaluate entomopathogenic nematode applications against WCR in the field.

This study, which is novel for Nebraska, can impact future conservation and inoculative biological control programs in the state.
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CHAPTER 2: SURVEY FOR POTENTIAL ABOVE-GROUND PREDATORS OF THE WESTERN CORN ROOTWORM WITH EMPHASIS ON GROUND BEETLES (COLEOPTERA: CARABIDAE).

Introduction

The western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), is the main root-feeding pest of continuous corn production in the United States and Europe. This pest is present in the field all year-long. Eggs typically start hatching in late May (Meinke et al. 2009), going through three larval instars and a pupal stage before adult emergence. Adults emerge from the end of June through September, with delayed mean emergence periods occurring in transgenic *Bacillus thuringiensis* (Bt) hybrid fields (Hitchon et al. 2015). Oviposition occurs from July-September (Meinke et al. 2009). Corn is the primary host of the WCR (Clark and Hibbard 2004) and, therefore, this pest is mainly a problem in continuous corn, except in limited geographic regions where behavioral resistance to rotation has occurred (Gray et al. 2009).

Current management tactics of WCR include crop rotation, seed treatments, soil and foliar insecticides, and transgenic hybrids. Continuous corn production requires constant pest management, and because of the high management pressure and the adaptive nature of this pest (Gray et al. 2009; Miller et al. 2009), populations of the WCR have evolved resistance to a variety of management practices. Resistance or reduced susceptibility has been reported with crop rotation (Levine et al. 2002); chemical insecticides such as organochlorines (Parimi et al. 2006), pyrethroids (Pereira et al. 2015), organophosphates and carbamates (Meinke et al. 1998); and with Bt proteins
such as Cry3Bb1 (Gassmann et al. 2011), with cross resistance to mCry3A (Gassmann et al. 2014) and eCry3.1Ab (Zukoff et al. 2016), and most recently Cry34/35 (Gassmann et al. 2016). Management of WCR populations and resistance issues requires a complex integrated pest management (IPM) approach. Due to the frequency and severity of resistance to current management tactics, investigation of WCR biological control is critical and may lead to an additional management option to integrate with current WCR control practices.

Individual species as well as assemblages of generalist predators have the potential to suppress pest densities and protect yield (Symondson et al., 2002). However, in the WCR system, no keystone predator has been identified (Lundgren and Fergen 2014). A variety of arthropod predators have been reported to prey on the different life stages of the WCR in laboratory and/or field conditions, as reviewed by Kuhlmann et al. (1998) and Toepfer et al. (2009). Taxa reported as predators of one or more life stages of the WCR include ground beetles (Coleoptera: Carabidae), hister beetles (Coleoptera: Histeridae), rove beetles (Coleoptera: Staphylinidae), carpet beetles (Coleoptera: Dermestidae), ants (Hymenoptera: Formicidae), robber flies (Diptera: Asilidae), crickets (Orthoptera: Gryllidae), katydids (Orthoptera: Tettigoniidae), predatory mites (Mesostigmata: Laelapidae), several families of spiders (Araneae), harvestmen (Opiliones: Phalangiidae), centipedes (Chilopoda) and isopods (Isopoda: Armadillidiidae).

Diversity, evenness and abundance of the predator community are driving factors in rootworm consumption in the field (Lundgren and Fergen 2014). Western
corn rootworm is considered sub-optimal prey in cornfields due to behavioral and physiological defenses (Lundgren and Fergen 2014), including hemolymph defense by the larvae that may deter chewing predators (Lundgren et al. 2009b). In addition, larvae exhibit a cryptic lifestyle in which the early instars tunnel and feed within the root system (Strnad and Bergman 1987b) and are therefore protected from many soil antagonists.

Molecular gut content analyses (MGCA) indicate that several species of Carabidae can prey on WCR eggs and larvae (Lundgren et al. 2009a, Lundgren and Fergen 2014). Larvae and adults of Scarites quadriiceps Chaudoir as well as adult Poecilus chalcites (Say) had relatively high consumption rates and predation frequencies of WCR immatures as 20.4% and 17.5% of individuals of each species tested positive for WCR DNA in MGCA (Lundgren et al. 2009a). However, visual observations indicated that Carabidae are incidental predators of rootworms, with predation dependent upon chance encounters instead of searching for prey (Kirk 1982). Therefore, while some carabids have been confirmed as WCR predators, their role in the field might be minimal. But in conjunction with other pest management strategies in place, predators may provide an additional tool to minimize population pressure and resistance issues.

Nebraska is the third largest corn producer in the U.S.A. (USDA-NASS 2017) and the WCR is one of the state’s biggest insect pests. Predation of any life stage of this pest can aid plant protection in the long term since this is a continuous corn pest. The objectives of this study were to a) characterize the community of arthropod predators
and to b) identify potential carabid predators of the WCR via molecular gut content analysis (MGCA).

**Materials and Methods**

**Field Sites.** This study was conducted in 2014 in five commercial, irrigated, no-till cornfields located in West Central Nebraska. Four sites had been planted as continuous corn for at least 5 years prior to this study (Fields A-D) and one site had been rotated to winter wheat in 2013 (Field E). Agronomic characteristics of the field sites are listed on Appendix Table 1.

**Collection of Predatory Arthropods and WCR.** Sites were surveyed for arthropods and prey availability on seven dates throughout the corn-growing season (June-September). Four unbaited Pherocon® AM yellow sticky cards (YSC) (Hein and Tollefson 1985) were placed at canopy height at early vegetative stages and then placed at ear-height for the rest of the season. Yellow sticky cards were left out in the field for seven days and then were placed in the refrigerator until processing. In addition, rootworm densities were also monitored in each field with eight single-plant adult emergence cages (Pierce and Gray 2007). Emergence cages were checked weekly during the adult emergence period from July.15 to September.26, for a total of 11 collection dates. Sampling units (YSC and emergence cages) were placed 8 to 15 meters from each other and were located between 60-120 meters from the edge of the irrigated field.

**Carabidae Collection for Molecular Gut Content Analysis.** Five dry pitfall traps were located at each site: four inside the irrigated cornfield and one in the non-irrigated border. Traps were opened for 24-hrs per collection period. On average, traps were
opened around noon and closed at noon the next day. A hardware cloth insert (3.2 mm mesh) was placed inside dry pitfall traps (946 ml vol, 11.5 X 14 cm WXH) (Eskelson et al. 2011) to prevent bigger predators from consuming smaller arthropods. Arthropods trapped in the dry pitfall were individually placed into 1.5 ml microcentrifuge tubes (Fisherbrand™, Pittsburgh, PA, USA) and preserved in 95% EtOH in the field. The samples were then labeled and placed into an Engel 40 portable freezer (Big Frog Mountain, Chattanooga, TN, USA) in the field, and subsequently frozen at -20 °C until MGCA. Morphological identification was carried out for each specimen prior to DNA extraction using dichotomous keys found in Arnett & Thomas (2000) and Lindroth (1961-1969).

**Molecular Gut Content Analysis:** Gut-content DNA extractions were performed using QIAGEN DNeasy Blood & Tissue Kits (QIAGEN Inc., Chatsworth, CA, USA). Specimens under 1.0 cm were extracted whole and specimens larger than 1.0 cm had gut dissections performed prior to DNA extraction. Polymerase chain reactions (PCR) were conducted with a T100 Bio-Rad thermocycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with a WCR-specific primer pair (E-F364 and G-R358) targeting the cytochrome c oxidase 1 gene (Peterson 2012). The primer set was tested against eggs (n=5), larvae (n=2) and adults (n=5) of the WCR to ensure that it could amplify all stages of this pest. Each PCR reaction contained 17.4 μl of PCR-grade water, 2.5 μl 10X Takara buffer (Takara Bio Inc., Shiga, Japan), 2.0 μl Takara dNTPs, 0.5 μl of each primer, 0.125 μl Takara Taq Polymerase, and 2.0 μl of DNA template per sample. PCR cycling protocol included an initial denaturation step at 94 °C for 1 min; then 45 cycles of denaturation at
94 °C for 45 s, annealing at 66 °C for 45 s, and extension at 72 °C for 30 s; and final extension at 72 °C for 10 min. Gel electrophoresis amplification verification was performed on 2% agarose gels in 1× Tris-acetate-EDTA (TAE) buffer stained with GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA). Visualization of gels was carried out on GelDoc™ XR+ Gel Documentation system (Bio-Rad Laboratories Inc.).

**Data Analysis.** Western corn rootworm beetle densities (emergence cage and YSC) were analyzed on SAS (SAS Institute Inc., version 9.4, Cary, NC). Data were fitted to negative-binomial distributions (Tripathi 2006) and analyzed with PROC GLIMMIX. One-way ANOVAs were analyzed to determine field differences on the mean cumulative count of beetles (between beginning and end collection dates) per emergence cage or per YSC. Mean estimates and multiple-mean comparisons were obtained with LSMEANS and t-grouping differences were obtained with the LINES option. Mean differences were considered significant at the P < 0.05 level.

**Results**

**Arthropod survey.** A total of 866 predators were collected using yellow sticky cards. Predators were all adults identified as thirteen taxa in five orders: Araneae, Coleoptera, Hemiptera, Hymenoptera and Neuroptera (Table 2.1). The most abundant taxa were Chrysopidae, followed by Formicidae, *Orius insidiosus*, and Araneae. Chrysopidae were the most reported taxa from each field, although they were most abundant in Fields A and D. Formicidae counts were primarily driven by Field D and and *O. insidiosus* counts driven by Field A. The greatest abundance of predators was found at Field A, followed by Field D, Field B, Field E and lastly, Field C (Table 2.1).
**WCR density.** There was a significant field effect on mean WCR densities per emergence cage ($F_{4,35} = 20.63, Pr > F < 0.0001$) and per YSC ($F_{4,15} = 45.29, Pr > F < 0.0001$). Mean cumulative WCR per emergence cage was higher for field A ($48.9 \pm 12.8$) and field C ($33.2 \pm 8.8$), followed by field B ($15.4 \pm 4.2$), then field D ($4.2 \pm 1.3$), and lastly field E ($1.4 \pm 0.5$) (Fig. 2.1). Mean of cumulative WCR beetles per YSC was higher for field A ($358.7 \pm 83.4$) and field C ($233.0 \pm 54.4$), followed by field B ($31.7 \pm 7.8$) and field D ($20.7 \pm 5.3$), and lastly field E ($2.5 \pm 1.0$) (Fig. 2.2).

**Carabidae and MGCA.** A total of 235 adult carabids were collected, identified, and screened using MGCA to detect WCR DNA (Table 2.3). Thirty-six species were identified belonging to sixteen genera. The most diverse genus was *Harpalus* with ten species, followed by *Anisodactylus, Bembidion* and *Agonum* each with four species. The most abundant species were *Anisodactylus sanctaecrucis* (Fabricius), *Elaphropus aniceps* LeConte, and *Bembidion quadrimaculatum* L.; respectively making up 27.7%, 21.7% and 16.6% of all specimens encountered (Figure 2.3.). All 235 predator extractions were screened with general COI primers that amplify DNA from members of Arthropoda (LCO-1490 and HCO-700dy; Folmer et al. 1994) to ensure that DNA extraction had been completed successfully and eliminate any potential false negative results. The preliminary PCR primer test with WCR eggs (n=5), larvae (n=2) and adults (n=5) all yielded strong positive results, indicating successful prey DNA amplification by this primer set. However, none of the Carabidae specimens tested positive for WCR DNA in our gut-content analysis.
Discussion

The current study provides new knowledge on the above-ground arthropod predator community and epigeal carabids of no-till, continuous, irrigated corn in West Central Nebraska. The role of predators found on the YSC (Table 2.1) in rootworm control in the field is likely to be minimal. However, they may be useful for management of other important pests in Nebraska corn production such as the western bean cutworm (WBC), *Striacosta albicosta* (Smith) (Archibald 2017). Most taxa found on YSC are polyphagous predators but have not been reported in the literature to consume WCR (Kuhlmann et al. 1998, Toepfer et al. 2009). Moreover, three predators commonly found in the present survey, *Coccinella septempunctata* L., *Hippodamia convergens* Guérin-Méneville, and *Orius insidiosus* (Say) were positive for WBC DNA in a MGCA and *Coleomegilla maculata* was visually confirmed as a predator of WBC eggs in the field (Archibald 2017). The results presented here along with Archibald (2017) show the potential of exploiting natural enemy communities for insect pest suppression in continuous corn.

It is also important to notice that all of the field sites in this study expressed Bt traits (below-ground and/or above-ground) (Appendix 1). Perkins County has documented Cry3Bb1 WCR resistance (Wangila et al. 2015), and Field A (Keith Co.) and Field C (Perkins Co.) fields had high densities of WCR (Figures 2.1 and 2.2). Western corn rootworm resistance in the area that we conducted this study stresses even more the need for complementary WCR controls in the region and hence the need for studies like this. All fields had high densities of predators (Table 2.1 and 2.2) which suggests that
predator trends were not adversely affected by Bt traits expressed in the field sites. This finding is supported by analyses that found that Bt fields allow abundant communities of naturally occurring predators to exist because of the reduction in insecticide applications (de la Poza et al. 2005; Ahmad et al. 2006; Marvier et al. 2007; Lu et al. 2012; Svobodová et al. 2017). Increasing the understanding of biological control services in commercial fields may provide complementary pest management tactics as biological control and Bt traits are generally regarded as compatible (Romeis et al. 2006).

Pitfall traps revealed a diverse community of epigeal carabids (Table 2.2). The most abundant species were *A. sanctaecrucis*, *B. quadrimaculatum* and *E. anceps* that collectively made up 66% (155/235) of all Carabidae described (Figure 2.3). *Harpalus* species were also quite abundant and constituted 15% (35/235) of the specimens found (Figure 2.3). The Carabidae data reported herein add to our knowledge of ground beetles in agroecosystems throughout the state. Few studies have focused on investigating the community of ground beetles present in Nebraska. Unique to corn, Hariharan (1988) found seven species in Clay County (south-central Nebraska) that were also found in our study: *Agonum placidum* (Say), *B. quadrimaculatum*, *B. rapidum* (LeConte), *E. anceps*, *Harpalus pensylvanicus* (DeGeer), *P. chalcites* (syn. *Pterostichus chalcites*), and *Poecilus lucublandus* (Say). In addition to the seven species listed above, sugar bee plots in Scotts Bluff County (western Nebraska) revealed an additional six species that were also present in the current study: *Anisodactylus carbonarius* (Say), *Harpalus amputatus* Say, *Harpalus erraticus* Say, *Harpalus herbivagus* Say, *Harpalus somnulentus* Dejean, and *Stenolophus comma* F. The present study together with
Hariharan (1988) and Pretorius et al. (2017) show that ground beetles have a wide geographical distribution in the state and are common members of agroecosystems.

In general, ground beetles have extremely diverse diets which can include insect and weed pests (Holland and Luff 2000). Contrary to our study, the species *A. placidum*, *B. quadrimaculatum*, *B. rapidum*, *H. pensylvanicus*, *P. chalcites* and *S. comma* have all tested positive for WCR DNA in other studies (Lundgren et al. 2009a; Lundgren and Fergen 2011, 2014). One explanation may be the smaller sample size of our study which had a total of 235 ground beetles analyzed in comparison to 432 in South Dakota (Lundgren et al. 2009a). Similarly to our study, Lundgren et al. (2009a) also used pitfall traps to collect their predators but their traps were time-sorting pitfalls in which contents were segregated every 3h (Lundgren et al. 2009b). When comparing Lundgren and Fergen (2014) to our study we find methodology differences that may account for differences in results. Our study used natural levels of infestations in continuous corn, whereas they artificially infested first year corn. It is possible that artificially infested eggs were more readily available at the surface for those predators on the top soil layers. Moreover, Lundgren and Fergen (2014) used 10 cm soil cores for predator sampling, the same depth where the majority of WCR eggs are found (Gray et al. 1992), while our dry pitfalls selected only for surface-dwelling predators. Collecting predators throughout the soil core instead of focusing in the surface-dwellers probably increased the chances of finding predators positive for WCR DNA. However, dry pitfalls were the appropriate method to select for a high abundance of predators to be collected in a cost-efficient and timely manner. Due to time constraints, we decided to focus on
Carabidae taxa only because of their potential as WCR predators; however, the other surface-dwelling arthropod predators that were captured such as spiders are currently being analyzed for their potential as WBC and WCR predators.

Another explanation for the lack of WCR-DNA detection in carabids is the fact that only adult ground beetles were analyzed herein, while some of the beetles that consumed WCR such as *Scarites quadriceps* (not found in this study) did so in their larval stage (Lundgren et al. 2009a). It may also be possible that ground beetles collected were deterred by the sticky hemolymph defense that WCR larvae exhibit when facing predation (Lundgren et al. 2010). However, ground beetles likely also encountered eggs, pupae and adult beetles that do not have the sticky hemolymph defense, and therefore, this defense alone does not explain our negative results. Still, we should consider the possibility of lack of predation. For instance, WCR can be protected from predators while feeding within the roots as larvae (Strnad and Bergman 1987) or as pupae in their earthen pupal cell (Chiang 1973). Absence of WCR prey was expected in Field E (first year cornfield) where 9% of carabids the samples came from (Table 2.2), but it was expected that we would detect some predation in fields A and C, the high rootworm pressure fields (Figures 2.2. and 2.3). Western corn rootworms are also considered sub-optimal prey items for predators as its consumption is correlated with the increase in predator community abundance (Lundgren and Fergen 2014). Hence, it is possible that the abundance of Carabidae species in our fields is low and therefore predators are not being forced into eating non-preferred items. However, the most likely explanation is that there may have been an abundance of other, more preferred prey items or food
items available. As previously stated, carabid beetles have varied diets and many are polyphagous, opportunistic feeders (Holland and Luff 2000). Reviewing the most abundant species in our traps, we notice that *A. sanctaeclerucis* is primarily a granivore although it does have a polyphagous lifestyle (Hagley et al. 1982; Lundgren and Rosentrater 2007), and *E. aneps* and *B. quadrimaculatum* are predators, but the latter feeds on plant tissue as well (Brousseau et al. 2018, Fox et al. 2005, Kamenova et al. 2015). *Anisodactylus sanctaeclerucis* density was primarily driven from Field B (Table 2.3) as 63 out of 65 specimens were found in that site. Field B had a high density of weeds across the field, so it is possible that weed seeds supported *A. sanctaeclerucis* densities.

The different feeding habits of these main species reinforces the notion that WCR are not their primary prey choice. Kirk (1982) anecdotal observations suggest carabids prey on rootworms only on chance encounters. This agrees with the other papers that find a small percentage of Carabidae specimens testing positive for WCR DNA (Lundgren et al. 2009a, Lundgren and Fergen 2014). The negative results from the present study along with other studies support the conclusion that carabids uncommonly use WCR life stages as food in cornfields. This study is the first of this kind in the state of Nebraska and is the first effort to better understand the trophic interactions between WCR and carabids in this region. Future studies should focus on other soil predators with a focus on understanding egg predation as this life stage is present in the field soil for the majority of the year. Documentation of the availability and efficacy of native WCR predators will aid in the development of sound conservation biological control programs in the state of Nebraska.
The trophic web of WCR is still poorly understood. Many predator-prey reports are laboratory experiments, including no-choice experiments or are weak trophic interactions based on MCGA or personal observations. There is a need to describe natural enemies that are effective mortality agents of the WCR that can be used for biological control and IPM programs. Entomopathogens, primarily fungi and nematodes, have shown great promise for WCR control and will be the topic of the next chapters.
References


Lindroth, C.H., 1961. The ground-beetles (Carabidae, excluding Cicindelinae) of Canada and Alaska, parts 1-6. Opuscula Entomologica Supplementa XX, XXIV, XXIX, XXXIII, XXXV.


Meinke, L. J., B. D. Siegfried, R. J. Wright, and L. D. Chandler. 1998. Adult susceptibility of Nebraska western corn rootworm (Coleoptera: Chrysomelidae) populations to selected insecticides. J. Econ. Entomol. 91: 594–600.


### Table 2.1. Total abundance of predator taxa found on yellow sticky cards per field.

<table>
<thead>
<tr>
<th>Identity</th>
<th>Field A</th>
<th>Field B</th>
<th>Field C</th>
<th>Field D</th>
<th>Field E</th>
<th>Total</th>
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<td></td>
<td></td>
<td></td>
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</tr>
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<td>7</td>
<td>13</td>
<td>23</td>
<td>87</td>
</tr>
<tr>
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<td></td>
<td></td>
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<td><strong>Total per Field</strong></td>
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<td>153</td>
<td>119</td>
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<td>135</td>
<td>866</td>
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Table 2.2. Carabidae species found in dry pitfalls per field.

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<th>Species</th>
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<th>C</th>
<th>D</th>
<th>E</th>
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<td>Agonum placidum (Say)</td>
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Figure 2.1. Mean cumulative western corn rootworm (WCR) adults per emergence cage. Upper-case letters represent cornfields A-E. One-way ANOVA from cumulative values on September 26, revealed significant field effects ($F_{4, 35} = 20.63$, Pr $> F < 0.0001$) on mean cumulative WCR beetles per emergence cage ($n = 8$). Means with different lower-case letters are significantly different ($P < 0.05$).
Figure 2.2. Mean cumulative WCR adults per yellow sticky card. Upper-case letters represent cornfields A-E. One-way ANOVA from cumulative counts at September 9 revealed significant field effects ($F_{4,15} = 45.29, Pr > F < 0.0001$) on mean WCR beetles per yellow sticky card ($n = 4$). Means with different lower-case letters are significantly different ($P < 0.05$).
Figure 2.3. Most common Carabidae taxa in dry pitfalls.
CHAPTER 3: CHARACTERIZATION OF POTENTIAL FUNGAL ENTOMOPATHOGENS FROM COMMERCIAL, IRRIGATED, CONTINUOUS CORNFIELDS.

Introduction

The United States is the leading producer of corn (*Zea mays* L.) in the world (USDA-FAS, 2017). Corn production in the U.S. Corn Belt is a high input system, and in some areas, corn is produced as corn-on-corn in a continuous production system involving growing the crop for two or more consecutive years. Continuous crop production can lead to increased disease and insect pressure in the following crop seasons (Tilman et al. 2002). In many areas of the U.S. Corn Belt, the key soil pest of continuous corn is the western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte (Chrysomelidae: Coleoptera) (Gray et al. 2009). The soil also hosts secondary pests that feed on corn seedlings such as white grubs (*Phyllophaga* spp., *Cyclocephala* spp., and *Popillia japonica* Newman; Scarabaeidae: Coleoptera) and wireworms (*Melanotus* spp., *Agriotes* spp., and *Limonius dubitans* LeConte; Elateridae: Coleoptera). Soil pests are very difficult to control because their cryptic feeding and associated damage is difficult to predict (Jackson et al. 2000). Common options for control of soil pests include seed treatments and soil insecticides, however there are often efficacy inconsistencies and environmental issues associated with those methods (Harris 1972; Tooker et al. 2017).

The soil is an important source of natural enemies of insects (Klingen and Haukeland 2006). Fungi that are associated with insects are called entomogenous fungi (EF) and can have important relationships with insects such as commensalism,
parasitism and pathogenesis (Roberts and Humber 1981). The last relationship is of utmost importance for insect pest management. There are many entomopathogenic fungi (EPF) species that have been described and commercialized as biopesticides (Humber 2000; Lacey et al. 2015). Entomopathogenic fungi have evolved complex and diverse life histories (Wang and Wang 2017). Estimates suggest there are 750-1,000 EPF species in 100 genera (Vega et al. 2012). EPF are present in many high fungal taxa but Entomophthoromycota (Entomophthorales) and Ascomycota (Hypocreales) hold the majority of species (Humber 2016). Entomophthorales are responsible for many large-scale epizootics but many species have limited host ranges and are difficult to cultivate in vitro because they are obligate pathogens (Roy et al. 2006). On the other hand, Hypocreales have large host ranges, are easily produced in vitro and are highly explored as biological control agents (BCAs) (Shah and Pell 2003; Vega et al. 2012).

Knowledge of native strains of EPF in the soil can lead to the discovery of novel BCAs and is also a necessary first step in the development of conservation and classical biological control programs (Hajek et al. 2000; Meyling and Eilenberg 2007; Solter et al. 2017). The hypocrealeans Beauveria bassiana (Balsamo) Vuillemin, Metarhizium anisopliae (Metschn.) Sorokin, Lecanicillium and Isaria are commonly produced as commercial strains worldwide (Shah and Pell 2003; de Faria and Wraight 2007; Humber 2016). Entomopathogenic fungi have many environmental benefits in comparison to chemical control such as safety to non-target organisms, including humans, and reduced pesticide residue (Lacey et al. 2001). However, they also possess drawbacks like low persistence, slow killing speed, and higher cost (Humber 2016, Lacey et al. 2001). In
combination with other pest management practices, entomopathogens have the potential to reduce pest population densities including a potential role in delaying or mitigating resistance evolution under certain conditions (Shah and Pell 2003; Meissle et al. 2009; Hannon et al. 2010; Petzold-Maxwell et al. 2012; Gassmann et al. 2012; Hoffmann et al. 2014). Studying the EPF community in continuous, irrigated cornfields can help identify BCA’s that are adapted to this system and that can be further explored for a wide range of insect pests. Therefore, the objectives of this study were to (i) document the biodiversity of insect-associated fungal pathogens from the corn rhizosphere in irrigated cornfields of western Nebraska, and (ii) identify entomopathogens to be further explored as pest management tools.

**Materials and Methods**

**Study Sites.** All five study sites were commercial, irrigated, no-till cornfields located in West Central Nebraska. Details on western corn rootworm (WCR) density, location, soil type, rotational history, hybrids, transgenic traits, planting date, and insecticide and fungicide use are listed in Appendix 1 and Fig. 3.1a. All fields were maintained under standard agronomic practices for the region, including fertilization, irrigation, and weed management.

**Soil Sampling.** In 2014, soil sampling from each of the five sites was conducted on seven dates between June and September. In each field, a total of ten randomly chosen soil samples were taken: eight within the irrigated area and two from dryland corners adjacent to the irrigated field. Randomization was performed using a random number generator with a range of 8 to 15. Then along the pivot tire track (Fig. 3.1b) samples
were taken 8 to 15 meters apart. In 2015, soil sampling from each of the five sites was conducted on five dates between June and August. In each field, a total of eight soil samples were taken from fixed locations that were randomly assigned at the beginning of the season within the irrigated area with no dryland corner sampling (Fig. 3.1b). Soil samples were taken with a hand trowel from the corn-root zone to a depth of approximately 10 cm and 946 ml volume. Tools were sterilized with 90% Ethanol and then flamed between samples.

**Baiting Assays.** In the laboratory, each soil sample was homogenized manually and approximately 200 ml of soil was dispensed into a 13.6 X 11.4 X 5.1 cm (LxWxH) clear plastic dish (Dart®, Mason, Michigan, USA). In 2014, five *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvae were added to each container (Zimmermann 1986). In an attempt to isolate a higher diversity of entomopathogens and to isolate for Coleoptera-specific pathogens, in 2015, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) larvae were also utilized in baiting assays (Pilz et al. 2008). Soil samples were divided into two dishes with approximately 200 ml of soil in each; one received three *G. mellonella* and the other received three *T. molitor* larvae. Both *G. mellonella* and *T. molitor* were obtained from Speedy Worm, Alexandria, MN. Baiting dishes were kept at 20-22°C in the dark and soil was kept moist with double distilled millipore (ddH₂O) water throughout the duration of the baiting period. Larval mortality was assessed after 5, 7 and 10 days. Cadavers were surface-sterilized with 70% ethanol, ddH₂O, and 1% sodium hypochlorite then blot-dried on filter paper (Lacey and Solter 2012). Cadavers were then placed into humid chambers made with petri dishes (Fisherbrand™, Pittsburgh, PA) and moist filter
paper (Fisherbrand™) to allow for sporulation. Condition of sporulation was checked 3-5 days after larvae were placed in humid chambers. Infection status was recorded and specimens that were not consumed by saprophytic fungi were then stored at 4°C for fungal isolation and identification procedures. Larval cadavers with abundant sporulation were not surface sterilized but were saved for fungal isolation procedures.

**Isolation and Identification of Fungi.** Fungal material from *G. mellonella* and *T. molitor* cadavers were transferred to agar plates via direct transfer of spores with sterile wooden toothpicks or by transferring infected small larval pieces (approximately 2 to 3 mm²) that were cut with sterile scalpels. Cadaver pieces were rinsed with distilled water to remove any organic debris, dipped in 95% ethanol for 5 s, rinsed again in sterile distilled water, and allowed to drain on a sterile paper towel. The fungal material were placed on CTC medium containing potato dextrose agar and yeast extract supplemented with chloramphenicol, thiabendazole and cycloheximide (2014 only) and potato dextrose agar amended with 0.01% tetracycline (PDAt) (2014 and 2015) (Fernandes et al. 2010; Adesemoye et al. 2014). In 2015, CTC medium was not used because it was determined in 2014 that PDAt allowed for a greater diversity of potential entomopathogens to grow. Inoculated culture plates were incubated at 25°C and serially transferred until pure cultures were obtained. Fungal isolates were allowed to sporulate for morphological identification using a high-resolution compound microscope mounted with a single-lens reflex (SLR) camera.

Genomic DNA from fungal isolates was extracted using DNeasy® Plant Mini Kit (QIAGEN® Inc., Chatsworth, CA, USA). Polymerase chain reaction (PCR) was conducted
using internal transcribed spacer (ITS), ITS4 and ITS5 (White et al. 1990) for the ITS1, ITS2, and 5.8S regions of ribosomal DNA. Additionally, Bt2a and Bt2b (Glass and Donaldson 1995) was used for the β-tubulin gene. Each PCR reaction contained 12.5 μl of GoTaq Green Master Mix (Promega Corp., Fitchburg, WI, USA), 9.3 μl of PCR-grade water, 0.6 μl of 10 μM each primer, and 2 μl of DNA template. The PCR reaction protocol for both primer sets included an initial preheat at 94°C for 2 min; 35 cycles of denaturation at 94°C for 15 s, annealing at 58°C for 15 s, and extension at 72°C for 45 s; and final extension at 72°C for 5 min (Adesemoye et al. 2014). PCR products were verified via gel electrophoresis in 1% agarose gels in 1× Tris-acetate-EDTA (TAE) buffer and photographed with GelDoc™ XR+ Gel Documentation system. PCR products were purified using QIAquick PCR Purification kit (QIAGEN Inc.) and quality-checked with NanoVue Plus UV/Visible Spectrophotometer (GE Healthcare Life Sciences, Marlborough, MA, USA). Sequencing was done at the University of California, Riverside Gencore. Sequences were edited with MEGA 7 and analyzed with DNASTAR Lasergene version 14 software (DNASTAR, Madison, WI, USA) and BLAST search was done on the National Center for Biotechnology Information, United States National Institutes of Health (NCBI) website and sequences were submitted to GenBank.

**Data Analysis.** All statistical analyses were performed using generalized linear mixed models with PROC GLIMMIX (SAS software SAS, v. 9.4; SAS Institute Inc.). All data were analyzed as proportion data fitted to a beta-binomial distribution prior to analyses (Ferrari and Cribari-Neto 2004; Stroup 2015). Two-way ANOVAs of main effects of field and sampling date and their interaction were analyzed for 2014 and 2015. One-way
ANOVA with PROC GLIMMIX was run to determine the effect of insect species (*G. mellonella* and *T. molitor*) on the mean proportion of entomogenous fungi detection across fields and dates. The same procedure was repeated specifying *Metarhizium* spp. detection. The LSMEANS function was used with ILINK option to convert mean estimates, standard errors and confidence limits to the data scale prior to fitting beta-distribution (Schabenberger 2005). Simple effects and interactions at *P* < 0.05 were considered significant.

**Results**

**Frequency of Detection of Entomogenous Fungi.** Entomogenous fungi were detected in 40.6% (138/340) of all soil samples in 2014 and in 34.0% (67/197) in 2015. In 2014, 15.24% (250/1640) of *G. mellonella* larvae were infected. Mean percentage of cadavers infected per arena in 2014 had a significant field by date interaction (*F*<sub>23, 306</sub> = 1.61, *Pr > F* = 0.0394). Least square means comparisons showed varying levels of mean cadaver infection per each interaction of field by date (Fig. 3.2). Field B had higher mean cadavers infected on Sept.3 when compared to all other sampling dates, except June.24 and July.22. All other fields did not show any significant seasonality pattern in mean cadaver infection (Fig 3.2). In 2015, 10.26% of all *G. mellonella* and *T. molitor* larvae (121/1179) were infected. In 2015, there was no significant effect of insect species used for baiting on frequency of fungal detection: 10.7% (63/588) of *G. mellonella* and 9.81% (58/591) of *T. molitor* were infected (*F*<sub>1,48</sub> = 0.46, *Pr > F* = 0.50). There was no significant effect of field by date interaction (*F*<sub>16, 175</sub> = 0.32, *Pr > F*=0.9944) or sampling date effect (*F*<sub>4, 175</sub> = 1.13, *Pr > F* = 0.3462) on mean cadaver infection per arena (Fig. 3.3). However,
there was an impact of field ($F_{4,175} = 4.43, \text{Pr} > F = 0.0020$) on cadaver infection, with Field B being significantly higher than the others (Fig. 3.3).

**Fungal Identification.** In total, 254 pure fungal cultures were obtained from infected cadavers in 2014 and 119 in 2015. From the pure cultures, 132 samples were selected for molecular identification: 64 (25.6% of all infected cadavers) from 2014, which included 18 samples from the non-irrigated borders, and 68 (56.2%) from 2015. Isolate selection was made to represent all morphological groups in the collection.

The isolated fungi were identified as belonging to 11 families, 14 genera and 29 species (Table 3.1). The most diverse family was Trichomaceae: Eurotiales with four genera and nine species, followed by Nectriseae: Hypocreales with four *Fusarium* species, then Myxotrichaceae with three *Pseudogymnoascus* species. Chaetomiaceae: Sordariales, Cladosporiceae: Capnodiales, and Hypocreaceae: Hypocreales all had two species in each family. Clavicipitaceae: Hypocreales was represented by the genus *Metarhizium*. Bionectriaceae, Cordycipitaceae, and Ophiocordycipitaceae (all Hypocreales) were all represented by one species.

*Metarhizium* was the most prevalent genus in both years with 45.3% (29/64) detection of the identified fungi in 2014 and 69.1% (47/68) in 2015. Three species were identified; sixty-six isolates of *M. robertsii* J.F. Bisch., Rehner & Humber, seven of *M. anisopliae* and three remained under *Metarhizium* spp. In 2015, *Metarhizium* spp. detection was not statistically different due to insect species used for baiting: 63.8% (30/47) of *G. mellonella* and 48.9% (23/47) *T. molitor* were positive ($F_{1,48} = 0.66, \text{Pr} > F = \ldots$
0.42). However, some species were identified uniquely in *T. molitor* or *G. mellonella* (Table 3.1). For instance: *B. bassiana*, *Penicillium* spp. and *Trichoderma* spp. were only baited from *G. mellonella*, while *Clonostachys* spp. were only baited from *T. molitor*. The genus *Penicillium* was identified down to the greatest number of species (*n*=4); followed by *Pseudogymnoascus*, *Talaromyces*, (both with 3 species); *Aspergillus*, *Cladosporium*, *Trichoderma* (all with 2 species); and *Beauveria*, *Chaetomium*, *Clonostachys*, *Geotrichum*, *Neosartorya*, *Purpureocillium*, *Taifanglania* all with one species each.

Sequences obtained for the ITS and BT regions were deposited in the GenBank database (Table 3.1).

**Fungal Ecological Classification.** The concept of classifying baited fungi into ecological functions (Table 3.2) was developed from Sun et al. (2008) and Oliveira et al. (2011). While all strains were recovered from insect cadavers, it doesn’t mean they were the causal agents of mortality. Therefore, classification of species was based on available literature, with many species expressing multiple ecological roles. Species described at the genus level were classified on available reports of one or more species.

Entomopathogenic status was confirmed for 62.1% (82/132) of strains with *Metarhizium* spp., *B. bassiana* and *P. lilacinum* being the species most commonly described as insect pathogens. Antagonists of plant pathogens were found in 7.6% (10/132) that may be explored for biological control of plant diseases. Antagonists included *Clonostachys* sp., *Chaetomium* sp., *Trichoderma gamsii*, *T. virens* and *P. lilacinum*. Strains classified as entomogenous/insect antagonists belong to species or genera that have been previously reported from insects or that kill insects via toxins or endophytic properties.
Those placed under this category make up 10.6% (14/132) and are *Clonostachys* sp., *Chaetomium* sp., *Fusarium acuminatum*, *F. oxysporum*, *F. solani*, and *A. flavus*. Potential plant pathogens are those that have been reported with features harmful to plants and were found in 17.6% (24/132) of identified samples (i.e., *Chaetomium* sp., *Cladosporium* spp., *Fusarium* spp., *Aspergillus* spp. and *Penicillium* spp). Species classified under “Others” made up 25.7% (34/132) of samples and included *Taifanglania* sp., *Cladosporium* spp., *Geotrichum candidum*, *Pseudogymnoascus* spp., *Aspergillus* spp., *Neosartorya* sp., *Penicillium* spp., and *Talaromyces* spp. These fungi exhibit saprophytic and/or opportunistic features.

**Discussion**

The present work is the first to characterize entomogenous fungi and identify potential EPF from commercial cornfields in Nebraska. Around the world many studies have evaluated the distribution and abundance of EPF species with insect baits in a variety of cropping systems (Vänninen 1996; Chandler et al. 1997; Ali-Shtayeh et al. 2002; Meyling and Eilenberg 2006a; Quesada-Moraga et al. 2007; Sun et al. 2008; Oliveira et al. 2011; Wakil et al. 2013; Rudeen et al. 2013; Clifton et al. 2015). Soil baiting assay studies are often restricted to searches for EPF genera such as *Beauveria*, *Metarhizium* and *Purpureocillium* (*Paecilomyces*) because of their potential as BCAs. However, we found a wide range of fungi in addition to these known entomopathogens that can be isolated with the insect baiting assay method. The current study is similar to studies from Portugal (Oliveira et al. 2011) and China (Sun and Liu 2008; Sun et al. 2008).
that found secondary colonizers, opportunistic fungi, plant pathogens, and some insect and phytopathogenic antagonistic species from insect cadavers.

There was a significant interaction between field and sampling date (Fig. 3.2) on the mean cadaver infection in 2014 and a field effect in 2015 (Fig. 3.3). This variation between fields and sampling dates is common in agroecosystems and has been reported by other authors (Chandler et al. 1997; Meyling and Eilenberg 2006a; Quesada-Moraga et al. 2007; Pilz et al. 2008; Rudeen et al. 2013; Clifton et al. 2015). Fungal populations are influenced by a variety of dynamic interactions between abiotic (e.g. pH, soil properties, organic matter, temperature, moisture, agronomical practices) and biotic (e.g. resource/host availability, environmental persistence, competition, natural enemies) conditions that can influence their distribution in an area (Quesada-Moraga et al. 2007; Wraight et al. 2007; Meyling and Hajek 2010). For instance, Field B was a particularly weedy field in both years. Weeds can have positive interactions with soil fungi that enables them to compete with crop plants in agroecosystems (Massenssini et al. 2014) but also can have negative interactions with fungi responsible for seed-decay (Müller-Stöver et al. 2016). Field B contained 60% (6/10) of Fusarium spp. isolates (plant pathogens) but also contained 100% (n=3) of the Trichoderma spp. isolates (antagonist of phytopathogens) (Table 3.1). The impact of biotic and abiotic factors on fungal frequency should be studied in the context of creating pest management recommendations for this system.

Insect host species (G. mellonella or T. molitor) used in baiting assays did not impact the abundance of the total EF community or Metarhizium strains isolated during
2015. However, *Clonostachys* spp. were associated only with *T. molitor*; and *B. bassiana*, *Penicillium* spp. and *Trichoderma* spp. only with *G. mellonella* (Table 3.1). The effect of insect species on the detection of EPF has varied between published studies. Pilz et al. (2008) found significantly more *T. molitor* infected with EPF (16.6%) than *G. mellonella* (1.7%), but Rudeen et al. (2013) found more EPF-related mortality on *G. mellonella*, followed by *T. molitor* and then *D. v. virgifera*. Moreover, *Tolypocladium cylindrosporum* W. Gams (Hypocreales: Ophiocordycipitaceae) was isolated only from *Delia floralis* (Fallen) (Diptera: Anthomyiidae), but *G. mellonella* was better at isolating *M. anisopliae* and *B. bassiana* (Klingen et al. 2002). As demonstrated by these studies, the use of multiple insect hosts for fungal baiting can lead to detection of a more diverse community of fungi and therefore can help in the search for taxon-specific strains that can be explored as BCAs against different insect groups.

Two of the most commonly reported genera of EPF (*Beauveria* and *Metarhizium*) were found in this study, even though the frequency of occurrence was highly variable (Table 3.1). *Beauveria bassiana* and *Metarhizium* have a cosmopolitan distribution (Humber 2000; Roberts and St Leger 2004) and have been isolated from cornfields before (Pilz et al. 2008; Rudeen et al. 2013; Clifton et al. 2015). *Metarhizium* was the dominant genus in our survey with 76 identified isolates from all five field sites in both years; while *B. bassiana* had only two isolates, both from the same soil sample from field C in 2015. Both EPF species are found in natural habitats as well as agricultural habitats (Meyling and Eilenberg 2007). However, *M. anisopliae*, the most prominent and widespread member of the genus is regarded as an agricultural species due to its high
prevalence in cultivated/disturbed habitats in comparison to non-disturbed habitats (Vänninen 1996; Bidochka et al. 1998; Quesada-Moraga et al. 2007; Meyling and Eilenberg 2007; Schneider et al. 2012). The generalist *Metarhizium anisopliae* Sorokin was historically considered to have multiple lineages (or varieties) (Driver et al. 2000) but it was later recognized as a species complex of four individual species also known as the PARB clade: *M. pingshaense* Q.T. Chen & H.L. Guo, *M. anisopliae*, *M. robertsii* and *M. brunneum* Petch (Bischoff et al. 2009). Kepler et al. (2014) added an additional six species as part of the *M. anisopliae* species complex: *M. globosum* J.F. Bisch., Rehner & Humber; *M. acridum* and *M. lepidiotae* (Driver & Milner) J.F. Bisch., Rehner & Humber; *M. guizhouense* Q.T. Chen & H.L. Guo; *M. majus* (J.R. Johnst.) J.F. Bisch., Rehner & Humber; and *M. indigoticum* (Kobayasi & Shimizu). Most recently, the *M. anisopliae* species complex received its eleventh species: *M. alvesii* Lopes, Faria, Montalva & Humber (Lopes et al. 2017).

The *Metarhizium* isolates found in the current study, *M. robertsii* and *M. anisopliae*, belong to the PARB clade. *Metarhizium robertsii* was the predominant species in this survey, accounting for 86.8% of all *Metarhizium* isolates. This species has a cosmopolitan distribution and it is morphologically identical to *M. anisopliae*, being only distinguishable with molecular markers (Bischoff et al. 2009; Nishi et al. 2011; Kepler et al. 2014). It is also known that *M. robertsii* is rhizosphere competent and it provides plant protection by acting as an insect pathogen and as a beneficial endophyte that stimulates root development (Barelli et al. 2011; Sasan and Bidochka 2012; Pava-Ripoll 2013). *Metarhizium robertsii* is also the most prevalent strain in other cropping
systems (Bidochka et al. 2001; Wyrebek et al. 2011; Kepler et al. 2015) and it is thought to be the predominant species from the PARB clade in the Holoartic region (Rehner and Kepler 2017). However, studies indicate that *Metarhizium* spp. community structures seem to vary among sampled agricultural locations (Steinwender et al. 2014). In other studies, *M. anisopliae* or *M. brunneum* (Fisher et al. 2011; Steinwender et al. 2014, 2015; Rezende et al. 2015; Brunner-Mendoza et al. 2017) were the most reported species from the PARB clade in the soil. *Metarhizium* species community structures are highly governed by site-specific associations such as insect host distribution, plant root associations, temperature activity profile and soil characteristics (Quesada-Moraga et al. 2007; Schneider et al. 2012; Brunner-Mendoza et al. 2017). The factors governing *Metarhizium* structure in our study system, in the context of increasing pest management services, should be a topic of further studies.

Finding only two strains of *B. bassiana* during this study is not surprising or unusual because the species has been more commonly found in non-disturbed habitats than in agricultural soils (Bidochka et al. 1998; Quesada-Moraga et al. 2007; Meyling and Eilenberg 2007). In Finland, the likelihood for isolating *B. bassiana* declined by 41.5-92.4% from un-managed ecosystems to agricultural fields (Vänninen 1996). *Beauveria bassiana* is regarded as more sensitive to environmental stressors than *M. anisopliae* (Bidochka et al. 1998, Quesada-Moraga et al. 2007). This finding is consistent with Bidochka et al. (2002) who found that only select genetic groups of *B. bassiana* can survive in agricultural soils. In Iowa, a neighboring state to Nebraska, *B. bassiana* was rarely recovered using *G. mellonella* baiting in corn and soybean, *Glycine max* (L.), fields
Moreover, *B. bassiana* is commonly found above-ground, while *M. anisopliae* is more predominant belowground (Meyling and Eilenberg 2006b; Meyling et al. 2011). The bulk of the soil samples in this report came from inside cornfields and all were taken belowground, hence, it may be possible that the sampling method chosen restricted the chances of finding *B. bassiana* strains. However, using similar methodology, but including corn root pieces in the baiting assays with *G. mellonella*, *T. molitor* and *D. v. virgifera*, Rudeen et al. (2013) found *B. bassiana* in 60% of soil samples from corn root masses in Iowa. Endophytic colonization of corn by *B. bassiana* strains may be responsible for the differences between Rudeen et al. (2013) and Clifton et al. 2015 and the present study.

*Metarhizium* and *Beauveria* species are widely used in classical (Hajek and Delalibera 2010) and inundative (de Faria and Wraight 2007; Li et al. 2010) biological control programs worldwide, but not in conservation biological control (Mulock and Chandler 2000a, 2001; Meyling and Eilenberg 2007; Pell et al. 2010). In the irrigated, continuous corn system sampled in this study, a suitable strategy to use the isolated strains would be an inundative approach; however, extensive efficacy and host range tests would need to be performed before large scale field application. In Nebraska, the main pest of continuous corn is the western corn rootworm (WCR) which is also a host for *Metarhizium* and *Beauveria* isolates. Studies show that *M. anisopliae*, *M. brunneum* and *Beauveria bassiana* individually or in conjunction with other entomopathogens or Bt hybrids are able to reduce larval and adult WCR populations (Bruck and Lewis 2001; Mulock and Chandler 2001; Pilz et al. 2009; Meissle et al. 2009; Petzold-Maxwell et al.
Hence our isolates hold promising potential for developing inundative biological control techniques in this system. Strains targeted against insects may also work against phytopathogenic organisms and vice-versa. *Purpureocillium lilacinum* strains have been developed into commercial products to control the eggs cyst and root-knot nematodes. But, *P. lilacinum* also has topical and endophytic entomopathogenic properties against some insects (Fiedler and Sosnowska 2007; Lopez et al. 2014). *Clonostachys* species have been tested as mycoparasites of fungal phytopathogens and against species in Orthoptera, Lepidoptera and Hemiptera (Jensen et al. 2000; Toledo et al. 2006; Sun and Liu 2008; Sönmez et al. 2016). Soil EPF make up only a fraction of all fungi and other microorganisms that can be found in the rhizosphere (Jackson et al. 2000). Although our main goal was to find entomopathogens against key insect pests of corn, the isolates from this study have many ecological functions beyond those associated with insects. Some of the baited fungi are known plant pathogens (*Aspergillus* spp., *Cladosporium* spp., *Fusarium* spp., and *Penicillium* spp.) or are common saprophytic or opportunistic fungi (*G. candidum*, *Pseudogymnoascus* spp., *Taifanglia* sp. and *Talaromyces* spp.) (Table 3). Other strains can have antagonistic properties against insects and/or plant diseases through the production of mycotoxins or endophytic colonization (*A. flavus*, *Chaetomium* sp., *Cladosporium* sp.) (Table 3.2). This study also identified *Trichoderma* sp., a BCA genus widely used against phytopathogens worldwide (Howell 2003). *Trichoderma gamsii* and *T. virens* species are known antagonists of *Fusarium* isolates, including *F. oxysporum* and *F. solani*, which were also identified here and are the causal
agents of plant wilts and rots in many crops (Rinu et al. 2014). The strains found here should be explored in entomological and plant pathology studies in order to understand factors governing plant protection in this corn system. However, trans-kingdom fungi such as *Aspergillus flavus*, *Chaetomium* sp., *Cladosporium* sp., *Fusarium acuminatum*, *Fusarium oxysporum*, *Fusarium solani* and *P. lilacinum* (Table 3.2) should only be explored for use as biological control agents with extreme caution due to the potential risk to crop plants or human health (De Lucca 2007; Luangsa-ard et al. 2011).

This study found an abundant community of entomogenous fungi from commercial fields in Nebraska. Microbial communities and their associated soil processes can have a direct impact on plant health (Garbeva et al. 2004). Beneficial fungi described here may aid in disease and pest suppression in agroecosystems. Entomopathogenic species made up the majority of fungi isolated from the baiting insects but other fungal ecological roles, such as antagonists of phytopathogens, were described. Understanding the soil fungal community can contribute to the exploration of sustainable agriculture practices. In some regions of the US Corn Belt, such as Nebraska, corn production is a high-input system where corn is grown without rotation to other crops for many years, if not decades. The use of EPF in this system should be explored alongside current and future insect management practices such as Bt hybrids, insecticides, tillage and rotation. Pests like the WCR require a multi-tactic approach to successfully reduce populations. The next steps will be to test our isolates against the WCR and other soil-dwelling corn pests to identify strong biological control agents. If a prominent isolate is identified, it could provide an additional or complementary
management practice against this pest that has evolved resistance to so many existing control tactics (Meinke et al. 1998; Levine et al. 2002; Parimi et al. 2006; Gassmann et al. 2011, 2014, 2016; Wangila et al. 2015; Zukoff et al. 2016; Ludwick et al. 2017).
References


De Lucca AJ (2007) Harmful fungi in both agriculture and medicine. Rev Iberoam Micol 24:3


Table 3.1 Fungal isolates from irrigated cornfields identified by the internal transcriber spacer (ITS) and beta-tubulin (BT) regions. Strains were isolated via baiting assays of *G. mellonella* (G.M.) (2014 and 2015) and *T. molitor* (T.M.) (2015 only).

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Table 3.2 Ecological functions associated with baited fungi. The concept for this table was developed from Sun et al. 2008 and Oliveira et al. 2011. Percentage column is the frequency of each species from the 132 samples sent for molecular identification.

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Fig. 3.1. Locations sampled in Keith and Perkins Counties in Nebraska in 2014 and 2015. a) Fields represented by letters, field characteristics can be found in Appendix 1. All fields in Perkins Co. were at least 5 miles apart, fields “a” and “e” were 20 miles apart. and b) Sampling diagram of the soil collection zone from inside each center-pivot irrigated cornfield. Cross-markings represent soil samples along the second and third pivot tire track (60-120 meters from field edge).
Fig. 3.2. Mean percent of cadavers infected with entomogenous fungi per arena in 2014. Borders and irrigated field combined for a total of 10 arenas per sample date by field combination. Field by date interaction was significant ($F_{23,306} = 1.61$, Pr>F=0.0394). Simple effect comparisons of field x date least square means adjusted with Tukey’s adjustment. Letters represent means significantly different at P< 0.05. Field C had no collection date on 22. July because of pesticide sprays.
Fig. 3.3. Mean cadaver infection with entomogenous fungi per arena in 2015. Total of 8 arenas per sample date by field combination. Field by date interaction ($F_{16,175} = 0.32, Pr > F = 0.9944$) and sampling date effect ($F_{4,175} = 1.13, Pr > F = 0.3462$) were not significant. Field simple effect was significant ($F_{4,175} = 4.43, Pr > F = 0.0020$). Letters represent means significantly different at $P< 0.05$ between fields.
CHAPTER 4: SCREENING OF INSECT-ASSOCIATED FUNGI FROM NEBRASKA AGAINST WESTERN CORN ROOTWORM, DIABROTICA VIRGIFERA VIRGIFERA LECONTE.

Introduction

The western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), is the most damaging belowground pest of continuous corn (*Zea mays* L.) in North America (Gray et al. 2009) with annual cost estimates of over US $1 billion associated with control and yield loss (Sappington et al. 2006, Gray et al. 2009, Dun et al. 2010, Tinsley et al. 2012, 2015, Andow et al. 2016). Management strategies are mostly focused on controlling the larval stage of the WCR, although adult control can be employed as well to reduce silk clipping or reduce egg laying, which subsequently decreases larval injury in next season’s corn crop (Chandler 2003). Tactics used to control WCR larvae include soil insecticide applications, seed treatments, corn hybrids expressing *Bacillus thuringiensis* (Bt), and crop rotation. However, there has been a high adaptive rate of the WCR to these tactics when they are used repeatedly in the same location (Gray et al. 2009; Miller et al. 2009). Although these strategies are still effective in many regions of the Corn Belt, some WCR populations have evolved resistance to one or more of the management practices above (Meinke et al. 1998; Levine et al. 2002; Parimi et al. 2006; Gassmann et al. 2011, 2014, 2016; Wangila et al. 2015; Zukoff et al. 2016; Ludwick et al. 2017).

The increasing incidence of field-evolved resistance to various tactics highlights the need to develop new management strategies against the WCR. New management practices can complement existing tools to mitigate resistance problems and to prolong durability of existing technologies within an integrated pest management framework. In
addition, there is also a need to provide alternatives for low input sustainable corn production, as well as popcorn, white corn, seed corn, and organic production, where Bt hybrids and certain insecticides are not an option. The use of entomopathogens as biological control agents can be a sustainable and selective alternative to pesticides (Lacey et al. 2001; Glare et al. 2012).

Mycoinsecticides, primarily those originating from Beauveria and Metarhizium strains, have been explored in the U.S. and abroad for a wide range of pests (de Faria and Wraight 2007). Entomopathogenic fungi infect through the host’s cuticle, unlike other entomopathogens that need to be ingested to infect the host, and thus, could be considered contact agents. Spores germinate on and penetrate through the insect host’s cuticle, becoming established in the hemolymph, and eventually causing death (Wraight et al. 2007). The fungi Beauveria bassiana (Balsamo) Vuillemin and Metarhizium anisopliae (Metschn.) Sorokin infect all stages of the WCR (Toepfer et al. 2009). The potential for WCR control by EPF alone or in conjunction with other control methods has been demonstrated in lab (Pilz et al. 2007; Meissle et al. 2009; Petzold-Maxwell et al. 2012; Rudeen et al. 2013) and field studies (Krueger and Roberts 1997; Mulock and Chandler 2000, 2001; Bruck and Lewis 2001, 2002; Pilz et al. 2009; Petzold–Maxwell et al. 2013).

A wide range of entomogenous, or insect-associated, fungi were recovered from Galleria mellonella L. and Tenebrio molitor L. baiting assays in a survey of five cornfields in Western Nebraska (see Chapter 3). The fungi recovered included the known entomopathogenic genera Beauveria, Metarhizium and Purpureocillium (Paecilomyces),
as well as 11 other genera, some of which include species that have exhibited antagonistic properties against insects, e.g., Aspergillus, Chaetomium, Cladosporium, and Fusarium (Hajek et al. 1993; Wicklow et al. 1999; Lage et al. 2001; Gange et al. 2011). Therefore, the objective of this study was to screen representatives of the native entomogenous fungal community described in Chapter 3 for their pathogenicity against WCR in soil and immersion-exposure assays.

**Materials and Methods**

**Insect sources and rearing.** Western corn rootworm eggs or third-instar larvae were obtained from non-diapausning colonies maintained at French Agricultural Research Inc. (Lamberton, MN) or Crop Characteristics, Inc. (Farmington, MN). Eggs were received in plastic petri dishes (100 X 15 mm, Fisherbrand™, Pittsburgh, PA) containing pre-sifted, autoclaved soil. Petri dish contents were checked for moisture, then sealed with Parafilm M (Bemis Company, Inc., Neenah, WI) and kept at 25°C until egg hatch. Neonates were moved to non-transgenic corn seedling mats (Reid’s Yellow Dent) with a soft hair paint brush and allowed to develop to third instar. WCR larvae were then recovered from the soil manually or by placing the seedling mats in Berlese funnels employing 40W, 120V bulbs (Philips Lighting Company, Worcester, MA) for 3 hours. Larvae were collected in clear glass jars (Solo Cup Company, Lake Forest, IL) attached to the Berlese funnels that contained moist paper towels and corn seedlings. Larvae were immediately transferred to the laboratory and third instars utilized in experiments within 24 hours.
**Fungal sources and inoculum preparation.** The 48 native fungal strains used in this experiment were isolated from soil samples collected in Nebraska cornfields via *G. mellonella* and *T. molitor* baiting assays (see Chapter 3). Strains were selected to represent the taxa diversity and origin locations from Chapter 3. In addition, BotaniGard® 22WP, being *B. bassiana* strain GHA (Arbico Organics, Oro Valley, AZ) was included as a commercial comparison product in each assay. Native fungal strains were surface-cultured on full strength potato dextrose agar media supplemented with 1 g L⁻¹ yeast extract (PDAY) media, with pH adjusted to 6.7-6.8 prior to autoclaving (Rangel et al. 2004). PDA media was made with fresh homemade potato broth from Russet potatoes. Spore viability was checked on PDAY 1-2 days prior to bioassays. Viability plates were incubated for 16-18 hours at 26.3 ± 0.5°C and then squares of the agar excised, transferred to a microscope slide and stained with lactophenol cotton blue. Spore germination was checked under a phase-contrast microscope at 400X magnification. Spores were considered viable if germ tube length was ≥ 2x the spore diameter (Inglis et al. 2012). Conidia from 14-day-old cultures were gently scraped with cell scrapers into a small volume of 0.1% Tween 80 (Sigma-Aldrich®, St. Louis, MO) and the suspensions were then filtered with Miracloth (22-25µL pore size) (Sigma-Aldrich). Conidia were counted in a hemocytometer and concentrations were adjusted to 1 x 10⁷ viable spores g⁻¹ for soil assays or 1 x 10⁷ viable spores ml⁻¹ for immersion-exposure assays. Sporulation and/or viability was poor for many strains; therefore, it was not always possible to obtain 1 x 10⁷ viable spores ml⁻¹ or g⁻¹. For those strains, the maximum final concentration of viable spores was used. Spore stock suspensions were
made up to 24-hours prior to bioassays due to time constraints, but inoculum was prepared the same day as inoculation.

**Pathogenicity bioassays against WCR larvae.** All 48 strains, BotaniGard and negative controls were initially tested in soil assays against 30 WCR third-instar larvae per treatment. Spore suspensions were mixed thoroughly into 100 grams of autoclaved, pre-sifted (60-mesh) silty clay loam soil at 25% water holding capacity (WHC). This level of soil moisture was chosen to enable good larval survival and good spore germination (Macdonald and Ellis 1990; Jaronski 2007, Hoffmann et al. 2014). The inoculated soil was dispensed into 59 ml plastic soufflé cups containing three 3-day-old corn seedlings (from planting). Ten third instar WCR larvae were then placed into each cup. The cups were subsequently covered with a fine polyester mesh (No-see-um mesh, Quest Outfitters, Sarasota, FL) and vented lid to prevent larval escape. This procedure was replicated three times for a total of 30 larvae per strain. Bioassay cups were placed in between 46 x 36 cm cafeteria-trays (Carlisle, Scottsdale, AZ) lined with moistened paper towels and then all were covered with large trash bags to retain original moisture (100% RH) (Hoffmann et al. 2014). Bioassays were conducted in an incubator at 26.3±0.5°C for 9 days, with 1 ml of water added to each cup on day 5. The number of dead larvae and larvae showing sporulation were recorded at the end of the 9-day bioassay period. In order to confirm mycosis from the tested strains, dead larvae that did not already show external sporulation were placed in a humid chamber at 26.3±0.5°C for another 3-6 days to confirm sporulation. Soil assays were conducted in four batches on separate dates: the first and second each with 15 strains, the third with 5 strains and the fourth
with 13 strains. All batches contained BotaniGard \((1 \times 10^7 \text{ viable spores g}^{-1})\) as the commercial standard and a negative control \((0.1\% \text{ Tween 80})\).

Because of the variability in fungal concentrations of the inocula used in the soil assays and in order to confirm pathogenesis, 14 out of the 48 strains were evaluated using immersion-exposure assays with a constant concentration of \(1 \times 10^7\) viable spores ml\(^{-1}\) (Pilz et al. 2007). Ten third- instar larvae were placed onto fine polyester mesh cloth (No-see-um mesh) and then dipped into 5 ml spore suspension for 5 seconds (Pilz et al. 2007). Control larvae were dipped in 0.1\% Tween 80. Then, larvae were transferred to a 59-ml cup containing three 3-day old corn seedlings and pre-sifted, autoclaved soil moistened to 25\% WHC. This was replicated three times with new inoculum for a total of 30 larvae per strain. Cups were sealed as previously described to prevent larval escape and bioassays were terminated at 7 days.

**Data analysis.** Proportional mortality was determined as the number of dead larvae per replicate/10 at the end of the bioassay. Larvae were considered dead if they did not move in response to prodding by a toothpick. Fungal growth was considered as positive if at least one infected cadaver showed external fungal growth consistent with gross morphology of fungal strain. All proportion data were fitted to a beta-binomial distribution prior to statistical analysis (Ferrari and Cribari-Neto 2004; Stroup 2015). In the soil assays, a preliminary two-way ANOVA of main effects treatment (fungal strain) and batch and their interaction were analyzed using PROC GLIMMIX in SAS (SAS Institute Inc., version 9.4, Cary, NC) to evaluate if there was a significant effect of batch on mortality of larvae from the negative control and BotaniGard treatments \((n = 120\) larvae...
per treatment). Because there was not a significant batch by treatment interaction ($F_{1, 18} = 0.01, Pr > F = 0.9183$), or a significant main effect of batch ($F_{1, 18} = 0.51, Pr > F = 0.4828$) or treatment (BotaniGard and Control only) ($F_{1, 18} = 2.71, Pr > F = 0.1579$) data were pooled across batches and a one-way ANOVA with PROC GLIMMIX was run to determine the effect of treatment on larval mortality. The immersion-exposure assay was also analyzed with a one-way ANOVA in PROC GLIMMIX with treatment as a fixed factor. For both projects, Dunnett’s multiple mean means comparison was used to test the control and the commercial comparison (BotaniGard) against each strain. Means were obtained using the LSMEANS function with the ILINK option to provide mean estimates, standard errors and confidence limits on the probability scale before the beta-distribution (Schabenberger 2005). Comparisons were obtained via the DIFF option and adjusted using DUNNETT adjustment for multiple comparisons. Treatment effects and interactions and mean comparisons at $P < 0.05$ were considered significant.

**Results**

**Soil assays.** A significant treatment effect was detected from the one-way ANOVA of soil assays ($F_{49, 118} = 3.75, Pr > F < 0.0001$). Fourteen strains caused mortality that was significantly higher than the negative control (E1089 through E1016, Table 4.1). These strains were identified as *M. anisopliae* (n=2), *Metarhizium robertsii* J.F. Bisch., Rehner & Humber (n=11), and *Pseudogymnoascus* sp. (n=1). Only one strain (E1089, *M. anisopliae*) caused mortality significantly higher than the commercial standard, BotaniGard (Table 4.1). External sporulation on larval cadavers was present in 92% (23/25) of *Metarhizium* spp. strains. Larval cadavers also showed external sporulation
from BotaniGard, E1060 (Cladosporium sp.), E212 (Penicillium griseofulvum Dierckx), E1035 (Penicillium sp.), E378 (Purpureocillium lilacinum), and E315 (Talaromyces trachyspermus (Shear) Stolk & Samson).

**Immersion-exposure assays.** The one-way ANOVA also revealed a significant treatment effect in this assay ($F_{15, 32} = 2.78$, $Pr > F = 0.0074$, Table 4.1). Eight strains, including BotaniGard, caused mortality that was significantly higher than the negative control. However, the seven field-collected strains were significantly higher than BotaniGard. These strains were identified as *M. anisopliae* (n=1), *M. robertsii* (n=5), and Metarhizium sp. (n=1). BotaniGard (*B. bassiana*) and 61.5% (8/13) of *Metarhizium* spp. tested via immersion-exposure assay exhibited sporulation of cadavers (Table 4.1). A trend of higher mean larval mortality, including controls, was observed for the soil assays (41 ± 10%) in comparison to immersion-exposure assays (19 ± 7%). In both experiments, no fungal growth was detected in larval cadavers from the controls.

**Discussion**

The ability of entomopathogenic fungi to infect the WCR has been tested before (Krueger and Roberts 1997; Mulock and Chandler 2000, 2001; Bruck and Lewis 2001, 2002; Pilz et al. 2007, 2009; Meissle et al. 2009; Rudeen et al. 2013); however, these studies were restricted to studying pathogenesis and virulence of only *Metarhizium* and *Beauveria* spp. The present study is novel in that it tested a wide range of insect-associated fungi from the soil of cornfields (see Chapter 3) against WCR larvae. One strain of *M. anisopliae* (E213) and three strains of *M. robertsii* (E1030, E1056, and E1016) had mean mortality statistically higher than the control for both assay types.
Sporulation of fungi on cadavers confirmed the pathogenic status of 100% and 57% of the *Metarhizium* strains that caused mortality greater than the control in soil and immersion assays, respectively. Sporulation also indicated that *Cladosporium* sp. (E1060), *P. griseofulvum* (E212), *Penicillium* sp. (E1035) and *T. trachyspermus* (E315) are capable of infecting larvae but are weak pathogens since mortality from these strains was comparable to the control.

Mortality significantly higher than the control was found in 52% (13/25) of the *Metarhizium* strains tested (Table 4.1). Tested concentrations varied between $1.4 \times 10^5$ spores gram⁻¹ to $1 \times 10^7$ spores gram⁻¹, but some of the highest mortality (E1000-E1016, Table 4.1) was obtained from strains with concentrations below $10^7$. High mortality from low-concentrations infers superior virulence of those strains compared to high-concentration strains with the same mortality rates. However, a dose-response bioassay would be needed to confirm this hypothesis. Overall, *Metarhizium* strains tested in the soil assays caused higher mortality than reported in other studies using the same small-cup methodology (Rudeen et al. 2013; Hoffmann et al. 2014). Two native strains of *M. anisopliae* from Iowa caused around 10 - 20% corrected mortality at $6.1 \times 10^5$ conidia gram⁻¹ and around 30% mortality at $6.1 \times 10^6$ conidia gram⁻¹ (Rudeen et al. 2013). Moreover, mean mortality of WCR larvae was 9% from *M. brunneum* (F52 strain) inoculations at $10^6$, $10^5$, $10^6$ and $10^7$ spores gram⁻¹ (Hoffmann et al. 2014). It is possible that the isolates collected in Nebraska have superior virulence against WCR larvae, however, variance in soil properties among studies probably played a large role in the mortality differences. Both Rudeen et al. (2013) and Hoffmann et al (2014) used field
collected soil while the soil used here was sterile. Sterilization changes the chemical composition and the microbial community within the soil, which can in turn allow rapid colonization of the fungal inoculum (Wilson et al. 1988; Inglis et al. 2012). In this study, it was unknown whether any strains would cause WCR mortality. Therefore, soil sterilization allowed us to isolate the effect of individual fungal strains on WCR larvae.

This is the first report to describe *M. robertsii* infecting WCR in the literature. *Metarhizium robertsii* is part of the *M. anisopliae* species complex and was just recently described as a new species (Bischoff et al. 2009). The two species are morphologically identical but differ genetically, hence it is possible that other studies have tested *M. robertsii* strains against the WCR but reported it under *M. anisopliae* s.l. *Metarhizium robertsii* is a great target for WCR control because of its multifunctional lifestyle (Barelli et al. 2015). It is rhizosphere competent which means it can survive antimicrobial root exudates and live saprophytically in the absence of a host (Pava-Ripoll 2013).

*Metarhizium robertsii* also promotes plant health by acting as an entomopathogen, and by colonizing the plant endophytically, it also improves root development and aids in translocating insect-derived nitrogen to roots (Sasan and Bidochka 2012; Behie et al. 2012; Barelli et al. 2016). Moreover, *M. robertsii*, like other *Metarhizium* species, is adapted to disturbed environments and is compatible with agroecosystems (Bidochka et al. 2001; Meyling and Eilenberg 2007; Wyrebek et al. 2011; Kepler et al. 2015). An evaluation of the *M. robertsii* strains from this study in the cornfields may not only benefit WCR management, but also promote plant health.
The native *B. bassiana* (E1040) and BotaniGard (*B. bassiana* GHA strain) in the soil assay each had low mortality rates that did not differ from control mortality, but BotaniGard mortality was significantly higher than the control in the immersion exposure assay (Table 4.1). Despite low mortality in the soil assay, BotaniGard infection was confirmed on sporulating cadavers in both assay types meaning that the fungus is able to infect WCR. The WCR mortality from E1040 (21%) is similar to other lab studies that found ≤ 11% corrected mortality from *B. bassiana* in laboratory assays (Pilz et al. 2007; Rudeen et al. 2013; Hoffmann et al. 2014). The low larval susceptibility in the lab is consistent with a field study that showed 0 - 3.2% *B. bassiana* infection at adult emergence (Bruck and Lewis 2001). Moreover, field applications of *B. bassiana* against adult WCR resulted in inconsistent levels of beetle infection in the field (Bruck and Lewis 2002). The results from this study, together with the above lab and field studies suggest that *B. bassiana* is not a good mortality agent of WCR. However, *B. bassiana* can engage in endophytic colonization of plant roots which can increase insect mortality but also promote plant growth (Lopez et al. 2014, Lopez and Sword 2015). Entomopathogenic fungi that are also endophytes have been linked to a variety of plant health roles including disease protection, nutrient acquisition and increased tolerance to abiotic stresses (Bamisile et al. 2018). Testing EPF strains from this paper for endophytic colonization could give insights into their role in the cornfield.

*Pseudogymnoascus* sp. (E376) caused mortality significantly higher than the control in the soil assay, but they did not show cadaver sporulation. *Pseudogymnoascus* spp. are widely found in soils, but their relationship to insects has not been studied.
Fungi that are not regarded as entomopathogenic in the literature can act as insect antagonists via toxin production or endophytic colonization (Chapter 3 and references therein). Beyond direct pathogenicity, *Pseudogymnoascus* sp. can be explored as sources of novel insect resistant genes or other traits that can be beneficial for insect management (Lacey et al. 2015).

Although we cannot make direct mortality comparisons between the assay types, there was a trend of higher mortality in the soil assay versus immersion-exposure assays. It is important to note that WCR larvae in the dipping assay were exposed to the inoculum only for 5 seconds with the bioassay being terminated at 7 days, while the WCR larvae in the soil assay were exposed to inoculated soil for 9 days. Soil assays are thought to simulate field conditions as larvae are exposed to the fungi in the soil (Hoffmann et al. 2014).

The definition of pathogenicity is “the potential ability to produce disease” with disease meaning a “departure from the state of health or normality” (Onstad et al. 2006). If the negative control larvae are considered normal, then 17 strains out of the 48 strains are pathogenic to the WCR. But biological control agents require other characteristics such as virulence, environmental competence and persistence, and host specificity to be successful in reducing pest populations (Glazer 1996, Kaya and Koppenhöfer 1996). Although we cannot directly compare the strains tested in the soil assays because they had varying spore concentrations, the data can provide insight into the potential for using these strains for rootworm control. Under the conditions of this study, *M. robertsii, M. anisopliae, Metarhizium* sp., *Pseudogymnoascus* sp., and
BotaniGard (commercial comparison) were effective mortality agents of the WCR. These species were also distributed in commercial cornfields in Nebraska (see Chapter 3) but their role in WCR mortality in the field is still unknown. Further studies should be conducted to explore the suitability of strains tested here as biological control agents in the field. Sustainable alternatives for WCR pest management could greatly minimize pest-caused yield losses, management costs, insecticide exposure to the environment and growers, and enhance profitability of corn production in the long term.
References:


Lopez, D. C., and G. A. Sword. 2015. The endophytic fungal entomopathogens Beauveria bassiana and Purpureocillium lilacinum enhance the growth of cultivated cotton (Gossypium hirsutum) and negatively affect survival of the cotton bollworm (Helicoverpa zea). Biol. Control. 89: 53-60.
Lopez, D. C., K. Zhu-Salzman, M. J. Ek-Ramos, and G. A. Sword. 2014. The entomopathogenic fungal endophytes Purpureocillium lilacinum (formerly Paecilomyces lilacinus) and Beauveria bassiana negatively affect cotton aphid reproduction under both greenhouse and field conditions. PloS One. 9: e103891.


Meinke, L. J., B. D. Siegfried, R. J. Wright, and L. D. Chandler. 1998. Adult susceptibility of Nebraska western corn rootworm (Coleoptera: Chrysomelidae) populations to selected insecticides. J. Econ. Entomol. 91: 594–600.


Table 4.1 Mean mortality of western corn rootworm larvae in soil and immersion-exposure assays with entomopathogenic fungi collected from cornfields in Western Nebraska.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Species</th>
<th>Spores gram$^{-1}$</th>
<th>Mortality ± SEM (%)</th>
<th>Fungal growth</th>
<th>Mortality ± SEM (%)</th>
<th>Fungal growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1089</td>
<td><em>Metarhizium anisopliae</em></td>
<td>$1.0 \times 10^7$</td>
<td>$75 \pm 9^{**}$</td>
<td>Y</td>
<td>$7 \pm 4$</td>
<td>Y</td>
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<tr>
<td>E1000</td>
<td><em>Metarhizium robertsii</em></td>
<td>$5.8 \times 10^5$</td>
<td>$70 \pm 10^*$</td>
<td>Y</td>
<td>$18 \pm 7$</td>
<td>N</td>
</tr>
<tr>
<td>E645</td>
<td><em>Metarhizium robertsii</em></td>
<td>$2.3 \times 10^6$</td>
<td>$68 \pm 10^*$</td>
<td>Y</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E1026</td>
<td><em>Metarhizium robertsii</em></td>
<td>$1.1 \times 10^6$</td>
<td>$65 \pm 10^*$</td>
<td>Y</td>
<td>$9 \pm 5$</td>
<td>Y</td>
</tr>
<tr>
<td>E653</td>
<td><em>Metarhizium robertsii</em></td>
<td>$8.7 \times 10^5$</td>
<td>$61 \pm 11^*$</td>
<td>Y</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E138</td>
<td><em>Metarhizium robertsii</em></td>
<td>$7.1 \times 10^6$</td>
<td>$61 \pm 11^*$</td>
<td>Y</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E1022</td>
<td><em>Metarhizium robertsii</em></td>
<td>$8.3 \times 10^6$</td>
<td>$60 \pm 11^*$</td>
<td>Y</td>
<td>$7 \pm 4$</td>
<td>Y</td>
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<td>E1030</td>
<td><em>Metarhizium robertsii</em></td>
<td>$1.9 \times 10^6$</td>
<td>$59 \pm 11^*$</td>
<td>Y</td>
<td>$30 \pm 9^*$</td>
<td>Y</td>
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<tr>
<td>E380</td>
<td><em>Metarhizium robertsii</em></td>
<td>$4.2 \times 10^6$</td>
<td>$58 \pm 11^*$</td>
<td>Y</td>
<td>$18 \pm 7$</td>
<td>Y</td>
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<td>E328</td>
<td><em>Metarhizium robertsii</em></td>
<td>$5.6 \times 10^6$</td>
<td>$56 \pm 11^*$</td>
<td>Y</td>
<td>$7 \pm 4$</td>
<td>N</td>
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<tr>
<td>E376</td>
<td><em>Pseudogymnoascus sp.</em></td>
<td>$1.0 \times 10^7$</td>
<td>$56 \pm 11^*$</td>
<td>N</td>
<td>$7 \pm 4$</td>
<td>N</td>
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<tr>
<td>E1056</td>
<td><em>Metarhizium robertsii</em></td>
<td>$2.7 \times 10^6$</td>
<td>$53 \pm 11^*$</td>
<td>Y</td>
<td>$22 \pm 8^*$</td>
<td>N</td>
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<tr>
<td>E213</td>
<td><em>Metarhizium anisopliae</em></td>
<td>$3.3 \times 10^6$</td>
<td>$50 \pm 11^*$</td>
<td>Y</td>
<td>$26 \pm 9^*$</td>
<td>N</td>
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<tr>
<td>E1016</td>
<td><em>Metarhizium robertsii</em></td>
<td>$2.5 \times 10^6$</td>
<td>$50 \pm 11^*$</td>
<td>Y</td>
<td>$38 \pm 10^*$</td>
<td>Y</td>
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<tr>
<td>E161</td>
<td><em>Metarhizium robertsii</em></td>
<td>$8.3 \times 10^6$</td>
<td>$47 \pm 11$</td>
<td>Y</td>
<td>$25 \pm 8^*$</td>
<td>Y</td>
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<tr>
<td>E1038</td>
<td><em>Metarhizium sp.</em></td>
<td>$4.4 \times 10^6$</td>
<td>$46 \pm 11$</td>
<td>Y</td>
<td>$30 \pm 9^*$</td>
<td>Y</td>
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<tr>
<td>E312</td>
<td><em>Chaetomium sp.</em></td>
<td>$2.7 \times 10^5$</td>
<td>$43 \pm 11$</td>
<td>N</td>
<td>-</td>
<td>-</td>
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<tr>
<td>E1093</td>
<td><em>Metarhizium robertsii</em></td>
<td>$1.4 \times 10^5$</td>
<td>$43 \pm 11$</td>
<td>Y</td>
<td>$28 \pm 9^*$</td>
<td>N</td>
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Table 4.1 (Continued)

<table>
<thead>
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<th>Strain</th>
<th>Species</th>
<th>Spores gram (^{-1})</th>
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<th>Mortality ± SEM (%)</th>
<th>Fungal growth</th>
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<tbody>
<tr>
<td>E136</td>
<td><em>Metarhizium robertsii</em></td>
<td>6.6 x 10^6</td>
<td>41 ± 11</td>
<td>Y</td>
<td>-</td>
<td>-</td>
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<tr>
<td>E1033</td>
<td><em>Metarhizium anisopliae</em></td>
<td>7.5 x 10^6</td>
<td>36 ± 11</td>
<td>Y</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E1095</td>
<td><em>Metarhizium sp.</em></td>
<td>7.1 x 10^5</td>
<td>35 ± 11</td>
<td>Y</td>
<td>-</td>
<td>-</td>
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<tr>
<td>E378</td>
<td><em>Purpureocillium lilacinum</em></td>
<td>1.0 x 10^7</td>
<td>35 ± 10</td>
<td>Y</td>
<td>-</td>
<td>-</td>
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<tr>
<td>E648</td>
<td><em>Fusarium oxysporum</em></td>
<td>1.0 x 10^7</td>
<td>30 ± 10</td>
<td>N</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E211</td>
<td><em>Metarhizium robertsii</em></td>
<td>4.7 x 10^6</td>
<td>30 ± 10</td>
<td>N</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E172</td>
<td><em>Penicillium janthinellum</em></td>
<td>9.6 x 10^5</td>
<td>30 ± 10</td>
<td>N</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BotaniGard</td>
<td><em>Beauveria bassiana</em></td>
<td>1.0 x 10^7</td>
<td>29 ± 5</td>
<td>Y</td>
<td>32 ± 10(^*)</td>
<td>Y</td>
</tr>
<tr>
<td>E1090</td>
<td><em>Metarhizium robertsii</em></td>
<td>4.8 x 10^6</td>
<td>28 ± 10</td>
<td>Y</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E212</td>
<td><em>Penicillium griseofulvum</em></td>
<td>1.0 x 10^7</td>
<td>28 ± 10</td>
<td>Y</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E166</td>
<td><em>Penicillium sp.</em></td>
<td>1.0 x 10^7</td>
<td>28 ± 10</td>
<td>N</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E393</td>
<td><em>Pseudogymnoascus pannorum</em></td>
<td>1.0 x 10^7</td>
<td>27 ± 9</td>
<td>N</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E1035</td>
<td><em>Penicillium sp.</em></td>
<td>1.0 x 10^7</td>
<td>26 ± 9</td>
<td>Y</td>
<td>-</td>
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<tr>
<td>E1005</td>
<td><em>Metarhizium robertsii</em></td>
<td>1.0 x 10^7</td>
<td>24 ± 9</td>
<td>Y</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E314</td>
<td><em>Neosartorya sp.</em></td>
<td>1.0 x 10^7</td>
<td>24 ± 9</td>
<td>N</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E1040</td>
<td><em>Beauveria bassiana</em></td>
<td>1.0 x 10^7</td>
<td>21 ± 8</td>
<td>N</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E322</td>
<td><em>Metarhizium robertsii</em></td>
<td>5.8 x 10^6</td>
<td>21 ± 8</td>
<td>N</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E374</td>
<td><em>Metarhizium robertsii</em></td>
<td>1.0 x 10^7</td>
<td>21 ± 8</td>
<td>Y</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E646</td>
<td><em>Talaromyces pinophilus</em></td>
<td>1.0 x 10^7</td>
<td>21 ± 8</td>
<td>N</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E1060</td>
<td><em>Cladosporium sp.</em></td>
<td>6.7 x 10^6</td>
<td>20 ± 8</td>
<td>Y</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E368</td>
<td><em>Penicillium bilaiae</em></td>
<td>1.0 x 10^7</td>
<td>20 ± 8</td>
<td>N</td>
<td>-</td>
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</table>
Table 4.1 (Continued)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Species</th>
<th>Spores gram⁻¹</th>
<th>Mortality ± SEM (%)</th>
<th>Fungal growth</th>
<th>Immersion-exposure Assay b</th>
<th>Mortality ± SEM (%)</th>
<th>Fungal growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>E651</td>
<td><em>Clonostachys</em> sp.</td>
<td>9.6 x 10⁶</td>
<td>19 ± 8</td>
<td>N</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E315</td>
<td><em>Talaromyces trachyspermus</em></td>
<td>5.8 x 10⁵</td>
<td>19 ± 8</td>
<td>Y</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E331</td>
<td><em>Talaromyces trachyspermus</em></td>
<td>7.7 x 10⁶</td>
<td>16 ± 7</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td>0.1 % Tween 80</td>
<td>-</td>
<td>14 ± 3</td>
<td>-</td>
<td>2 ± 1</td>
<td>-</td>
<td>-</td>
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<tr>
<td>E390</td>
<td><em>Talaromyces</em> sp.</td>
<td>1.0 x 10⁷</td>
<td>8 ± 4</td>
<td>N</td>
<td></td>
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<tr>
<td>E1034</td>
<td><em>Metarhizium anisopliae</em></td>
<td>2.7 x 10⁶</td>
<td>8 ± 4</td>
<td>Y</td>
<td></td>
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<tr>
<td>E999</td>
<td><em>Fusarium solani</em></td>
<td>6.9 x 10⁶</td>
<td>7 ± 4</td>
<td>N</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td><em>Pseudogymnoascus destructans</em></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>E370</td>
<td></td>
<td>1.2 x 10⁶</td>
<td>7 ± 4</td>
<td>N</td>
<td></td>
<td>-</td>
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<tr>
<td>E126</td>
<td><em>Cladosporium halotolerans</em></td>
<td>4.2 x 10⁶</td>
<td>7 ± 4</td>
<td>N</td>
<td></td>
<td>-</td>
<td>-</td>
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<tr>
<td>E998</td>
<td><em>Aspergillus flavus</em></td>
<td>1.0 x 10⁷</td>
<td>6 ± 3</td>
<td>-</td>
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<tr>
<td>E325</td>
<td><em>Taifanglania</em> sp.</td>
<td>1.0 x 10⁷</td>
<td>6 ± 3</td>
<td>-</td>
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</tr>
</tbody>
</table>

a: Means with (*) are significantly different from the control and means with (**) are significantly different than the commercial standard (BotaniGard) at P< 0.05 with Dunnet’s adjustment for multiple pairwise comparisons.

b: Immersion-exposure assays: All conducted with 1.0 x 10⁷ spores ml⁻¹.
CHAPTER 5: IDENTITY AND SEASONAL PATTERNS OF NEMATODES ASSOCIATED WITH INSECT CADAVERS FROM CORNFIELDS IN NEBRASKA.

Introduction

Entomopathogenic nematodes (EPN) are common components of insects’ trophic interactions and can function as biocontrol agents of soil insects (Strong et al. 1999; Jackson et al. 2000). These nematodes are distributed globally and show great biological control potential for insect pests (Hominick et al. 1996; Adams et al. 2006). Members of the Steinernematidae Travassos and Heterorhabditidae Poinar families are obligate insect pathogens whose third-stage infective juveniles (IJJs) kill insect hosts through the release of symbiotic bacteria: *Photorhabdus* for Heterorhabditidae and *Xenorhabdus* for Steinernematidae (Koppenhöfer 2007). Steinernematidae contains 2 genera: *Neosteinernema* with one species and *Steinernema* with 70 species; Heterorhabditidae contains a single genus: *Heterorhabditis* with 20 species (Stock and Goodrich-Blair 2012).

Nematodes are identified primarily via morphological, biochemical and molecular tools (Seesao et al. 2017). *Steinernema* and *Heterorhabditis* species identification based on traditional morphological and morphometric analyses is time consuming and requires considerable expertise (Stock 2015). However, molecular approaches have become standard alternatives or complements to morphological identification. In particular, DNA barcoding approaches can be used for species identification and to infer phylogenetic relationships of unknown taxa to known taxa using small genomic sequences from individual nematodes (Bhadury et al. 2006, Powers 2004). Two ribosomal regions are frequently used for EPNs studies: internal transcriber
spacer (ITS1, 5.8S and ITS2) and 28S rDNA (D2/D3 expansion region) (Stock et al. 2001, Spiridonov et al. 2004, Stock 2015). Sequences from these markers can be extremely variable which can prevent them from being used as a universal nematode marker needed for DNA barcoding approaches (Prosser et al. 2013). The mitochondrial gene cytochrome oxidase subunit 1 (CO1) is considered to be a universal barcode for the animal kingdom as it translates into conserved proteins which provide the right specificity to differentiate species and strains (Hebert et al. 2003, Powers 2004, Prosser et al. 2013). Beyond providing biodiversity data, proper EPN identification can aid in biological control efforts. Identifying native persistent EPN strains that can be augmented in the laboratory and inoculated into agricultural fields can help reduce populations of soil pests (Shields et al. 1999; Shields 2015; Shields and Testa 2015).

Since EPN species can co-exist, studies have tested co-inoculation of two or more EPN species for pest suppression with mixed success (Choo et al. 1996, Koppenhöfer et al. 2000, Neumann and Shields 2008, Shields 2015). Successful inoculations occur with the right combination of EPN species that can cover different soil profiles (Shields 2015). Long term EPN persistence can also occur with the introduction of multiple EPN species in crop-rotation systems (Shields 2015, Shields and Testa 2015). Several *Steinernema* and *Heterorhabditis* species have been tested against the western corn rootworm (WCR) (*Diabrotica virgifera virgifera* LeConte) in laboratory and field settings (Geisert et al. 2018, Wright et al. 1993, Journey and Ostlie 2000, Toepfer et al. 2005, 2008, Kurtz et al. 2009). However, *Steinernema feltiae* Filipjev, *Steinernema carpocapsae* Weiser and *Heterorhabditis bacteriophora* Poinar are the species mostly studied under field conditions for WCR control. Field studies have indicated that EPN applications can be just as efficacious as insecticide treatments in killing WCR larvae and providing crop root protection (Wright et al. 1993, Jackson 1996, Toepfer et al. 2008, Pilz et al. 2009).

In an effort to understand how EPNs can be used for WCR management in the continuous corn system in Nebraska, we conducted a study with three objectives: 1) conduct a survey for native EPN that are adapted to irrigated cornfields in western Nebraska; 2) determine efficacy, survival and seasonal distribution of inoculated EPNs in a cornfield artificially infested with WCR; and 3) determine the identity of EPNs from the previous objectives using a DNA barcoding approach.
Materials and Methods

Survey Project

Field sites. Five continuous cornfield sites were the same as described in Chapters 2 and 3. The details on field history, location, hybrids, transgenic traits, and insecticide and fungicide use are listed in Appendix Table 1.

Nematode detection from survey sites. Soil sampling and baiting assay procedures were the same as described in Chapter 3. In 2014, five *Galleria mellonella* (L.) larvae were placed into the baiting assays and in 2015, three *G. mellonella* larvae and three *Tenebrio molitor* L. larvae were used. Dead larvae were placed onto white traps (White 1927) to allow for nematode isolation and detection of infective juveniles. White traps consist of cadavers being placed on a small petri-dish (60 x 15mm) lined with moist filter paper placed inside a larger (100 x 15 mm) “harvest” dish filled with water. Nematodes were then isolated in sterile water in 1.5 ml microcentrifuge tubes (Fisher Scientific™, Lenexa, KS) and stored at 4 °C until DNA extraction.

Inoculation Project

Field Site. This field site was located at the University of Nebraska West Central Water Resources Laboratory near Brule, NE (GPS coordinates at center of plots: N41.09.482', W102.01.452’). The site was first year corn, soybeans *Glycine max* (L.) Merr were planted in 2014. The corn hybrid DeKalb® 52-61 VT Double Pro® that does not express rootworm-active traits (did express Lepidoptera-specific Cry1A.105 and Cry2Ab2 proteins) was planted at 32,000 seeds/acre and 76.2 cm row spacing on May.18.2015. This was a no-tillage field with no at-plant insecticide applications. The field was
maintained with typical agronomic practices for irrigated cornfields in the region including fertilizer, herbicides and fungicides. Each plot measured 24.4 m (32 rows) x 24.4 m. There was a total of 36 plots arranged into a randomized complete block design of 6 blocks (Fig. 5.1). This site was planted to soybeans in 2016.

**Western corn rootworm infestation and nematode inoculation.** Western corn rootworm egg infestation occurred on June.18.2015 when corn plants were at the V1-V2 growth stage (Ritchie et al. 1992). Eggs from French Agricultural Research, Inc. (Lamberton, MN) were suspended in 0.15% agar solution and applied with a syringe at a depth of 10 cm in a single furrow adjacent to the plant along the planted row (Sutter and Branson 1980). The infestation zone within each plot consisted of 42 corn plants from the middle four corn rows (2.5 x 2.5 m) (Fig. 5.1). Each plant received 400 eggs; this infestation rate corresponded to the maximum infestation rate before density-dependent mortality often occurs (Hibbard et al. 2010).

EPN inoculation occurred in the evening of July.8.2015 when corn plants were at the V3-V5 growth stage and WCR larvae were in first and second instars. The three treatments evaluated were: 1) commercial EPN strains NemAttack™ (Steinernema feltiae) and NemaSeek™ (Heterorhabditis bacteriophora) (ARBICO Organics, Tucson, AZ); 2) persistent EPN strains from New York state of S. feltiae “NY 04” strain and H. bacteriophora “Oswego” strain, herein referred as persistent EPNs (Dr. Elson Shields, Cornell University, Ithaca, NY); and 3) non-inoculated, control plots that received water only. Nematodes were applied at a rate of 2.5 x 10⁹ IJ/ha total, representing 1.25 x 10⁹ IJ/ha/species. Hence, each plot (12.5 m²) received approximately 3.12 x 10⁶ IJs total
Commercial EPN products were diluted in batches of four liters of non-chlorinated water and applied over the WCR infestation zone (42 plants) with a watering can. Persistent nematodes were applied via *Galleria mellonella* cadavers, with the calculation that each cadaver releases approximately 100,000 infective juveniles (Shapiro-Ilan et al. 2003, Dolinski et al. 2007, 2015). Cadavers were buried at a 5 cm depth adjacent to corn roots every 122 cm, alternating the cadavers of each species to allow for an even distribution, for a total of 32 cadavers per plot (16 of each nematode species) or four cadavers/row followed by eight liters of water over the treatment area (Fig 5.1).

**Western corn rootworm population and damage assessments.** Western corn rootworm abundance was monitored via single-plant beetle emergence cages (Pierce and Gray 2007). Each plot received 3 cages that were monitored weekly from August 5.2015 until September 24.2015 for a total of eight collection dates. Larval feeding damage assessment was measured on August 12.2015 via the Iowa State Node Injury Scale (Oleson et al. 2005) on five plants/plot. This scale ranges from a 0.00 - 3.00 rating; 0 = no feeding damage; 1 = one node, or the equivalent of an entire node of roots pruned by larval feeding to ≤ 3.8 cm from the stalk (Oleson et al. 2005); 2 = two nodes pruned; 3 = three or more complete nodes pruned.

**Nematode detection and isolation from soil samples.** Soil sampling for nematodes occurred at seven dates: 7 days pre-inoculation, 7 days post-inoculation (dpi), then at 14, 30, 60, 90 dpi and one-year post-inoculation. In an effort to prevent cross-contamination, controls were always sampled first, and different personnel groups...
sampled the commercial and persistent treatments. Soil sampling was obtained from the top 15 cm of the corn root zone with a 2.2 cm x 83.8 cm soil probe (AMS Inc., American Falls, ID). Soil probes were rinsed with water and then sterilized with 70% ethanol between plots. In each block, ten soil samples were obtained per date per treatment. For each sample, the top 5 cm of soil (0-5 cm) was separated from the bottom 10 cm (5-15 cm) into two deli dishes (226.8 ml clear hinged deli container with high dome lid Genpak, Charlotte, NC). Soil cores were broken down with sterile forks prior to receiving *G. mellonella*. Three *G. mellonella* were placed in dishes with 0-5 cm soil samples and six larvae were placed in dishes containing 5-15 cm soil samples (Shields et al. 1999). Deli dishes were kept in the dark and incubated at 22-23°C for 7 days. Larval cadavers were then placed onto white traps or dissected to confirm EPN infection. Isolated IJ from white traps were kept at 4°C inside 1.5 ml microcentrifuge tubes filled with sterile water until DNA extraction.

**Nematode Identification: DNA barcoding.** Single nematodes in 1.5 ml microcentrifuge tubes were placed onto glass coverslips in 18 µl of sterile water. DNA extractions consisted of individual nematodes being macerated with a micropipette tip (Powers et al. 2014). Mashed nematodes in water were then stored at -20°C in 0.25 ml PCR reaction tubes until PCR was conducted. This study used a partial mitochondrial cytochrome oxidase subunit I (COI) gene primer set: COI-F1KF (29bp, 5’-CCTACTATGATTGGTGGTTTTGGTAATTG-3’) and COI-R2KF (23bp, 5’-GTAGCAGCAGTAAAATAAGCACG-3’) (Kanzaki and Futai 2002). Excluding the primers, amplification products yielded 658-bp for sequence analysis. PCR amplification reactions
consisted of 6.4 μl of ddH2O, 1.8 μl of each 20 μM primer, 15 μl of 2XJumpStart RED Taq ReadyMix (Sigma-Aldrich, Inc. St. Louis, MO) and 5 μl DNA template from the macerated nematode, for a total reaction volume of 30 μl. PCR cycling protocol began with a hot-start and an initial denaturation of 5 minutes at 94°C followed by 45 cycles of 15 seconds at 94°C (denaturation), 15 seconds at 55°C (annealing), 60 seconds at 72°C (extension) and a final extension step of 5 minutes at 72°C. All PCR was conducted in a thermal cycler (Techne Equipment, Staffordshire, UK). To confirm successful amplifications, 3 μl of PCR products were loaded into 1% agarose gels stained with ethidium bromide or GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA) in 1× Tris-acetate-EDTA (TAE) buffer. Gels were placed into electrophoresis with 0.5X Tris-Borate-EDTA (TBE) running buffer for 35 minutes at 155V. UV visualized gel images were digitally recorded. Positive PCR reactions were purified with a Gel/PCR DNA Fragment Extraction Kit (IBI Scientific. Dubuque, IA) following the manufacturer’s guidelines. DNA templates were sequenced in both directions by the UCDNA Sequencing Facility at the University of California – Davis. Sequences were edited and aligned on CodonCode Aligner Version 4.2 (CodonCode Corp, Centerville, Massachusetts).

Reference nematode specimens. To provide standards for identification of the isolated nematodes, we obtained a set of nematode strains from Dr. David Shapiro-Ilan (USDA-ARS-SE Fruit and Tree Nut Research Unit, Byron, GA). The isolates used were

*Heterorhabditis bacteriophora* (VS strain), *H. bacteriophora* (Oswego strain), *H. bacteriophora* (HB strain), *H. megidis* UK211, *H. georgiana* (Kesha strain), *H. floridensis* (K22 strain), *Steinernema carpocapsae* (All strain), *S. carpocapsae* (Cxrd strain), *S. feltiae*
(SN strain) and \textit{S. rarum} (17C&E). Live nematodes were received in 250 ml cell culture flasks with vent caps (Corning Incorporated, Corning, NY) in sterile water. These reference nematodes were subjected to the same procedures outlined in the “Nematode identification: DNA barcoding” section.

**Nematode Identification/phylogenetic analysis:** Edited field-collected nematode COI sequences were compared to sequences in the National Center for Biotechnology Information (NCBI) database via Basic Local Alignment Search Tool (BLAST) and sequences from USDA reference species. A Neighbor-joining phylogenetic analysis was also conducted with MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0.6 (Tamura et al. 2013) using Kimura-2-Parameter model, 2,000 bootstrap replications, and treated with pairwise deletion gap treatment. Seventy nematode specimens were used in the construction of a neighbor-joining tree. Fifteen nematode specimens originated from the survey project, twenty from the inoculation project, twenty from USDA reference specimens (representing 2 nematodes for each of the 10 EPN strains) and fifteen nematode COI sequences from GenBank.

**Data analyses.** Survey Project: Soil samples were considered positive for nematode infection if at least one \textit{G. mellonella} or \textit{T. molitor} cadaver detected nematodes. Detection frequency was expressed as proportional number of nematode-infected cadavers per soil sample for each date in all fields. Statistical analyses were performed using generalized linear mixed models with PROC GLIMMIX in SAS (SAS Institute Inc., version 9.4, Cary, NC). Two-way analysis of variance (ANOVA) was used to test for the effect of field site, date and their interaction on detection frequency for 2014 and 2015.
One-way ANOVA was conducted for 2015 data to determine if bait insect species (G. *mellonella* or *T. molitor*) had an impact on nematode detection. Prior to all analyses, all data was converted to beta-binomial distribution (Ferrari and Cribari-Neto 2004, Stroup 2015). Means, standard errors and confidence limits were converted back to the data proportional scale using the ILINK option in LSMEANS (Schabenberger 2005). Multiple comparisons were adjusted with Tukey’s honestly significant difference (HSD) test using the ADJUST option in LSMEANS.

Inoculation project: Nematode detection frequency was expressed as the proportion of infected cadavers at each depth per treatment per block. A three-way ANOVA under generalized linear mixed model was conducted using PROC GLIMMIX in SAS to evaluate the effect of treatment, soil sample depth, date and all interactions on nematode infection rate. Dates were treated as repeated measures with first order autoregressive covariance structure (AR-1). Root injury rates were converted to proportional data by dividing every rating by three (node injury scale 0-3, Oleson et al. 2005). Proportional root injury rates were analyzed in a one-way ANOVA of main effects of EPN treatment. Nematode detection and root injury rates were fitted to a beta binomial distribution and proportional data were converted back to the data scale using the ILINK option in LSMEANS. Emergence cage data were analyzed on PROC GLIMMIX using a one-way ANOVA of main effects of EPN treatment. Emergence cage data was fitted to a negative binomial distribution (Tripathi 2006). Treatment effects and interactions at P< 0.05 were considered significant for both projects.
Results

Survey Project. Nematodes were detected in 15.8% (54/342) of soil samples in 2014 with only 2.3% (8/342) coming from non-irrigated areas. In 2015, 13.2% (26/197) of soil samples were positive for nematodes. In 2014, 5.5% (91/1640) of larvae were infected and in 2015, 3.7% (44/1179) of larvae were infected. There was no significant interaction of field by date or main effects of proportional nematode detection per arena in 2014 or 2015 (Fig. 5.2, Table 5.1). No significant differences in proportional nematode detection between baiting insects (G. mellonella vs. T. molitor) were detected in 2015 ($F_{1,48} = 2.01$, Pr> $F = 0.1624$).

Inoculation Project. Across treatments, only 8% of all G. mellonella larvae (912/11340) detected nematodes; from those, 467/3779 (12.4%) came from the top 0-5 cm soil samples and 445/7561 (5.9%) came from the bottom 5-15 cm soil samples. Nematode detection frequency in pre-inoculation sampling was 0.12% (2/1620). Overall, nematode detection frequency was numerically highest for the persistent treatment (10.2%, 386/3780), followed by the commercial (7.7%, 290/3780) and control treatments (6.2%, 236/3780). Three-way analysis of variance between treatment, soil sample depth and date revealed significant two-way interactions for treatment by date and depth by date were observed (Table 5.2). No other two-way or three-way interactions were significant, but a main effect of date was observed (Table 5.2). Multiple comparisons of interaction least square means between treatment and date, and depth by date are listed in tables 5.3 and 5.4, respectively. The treatment by date interaction was primarily driven by treatment differences at 90 dpi (Fig. 5.3). At this date, the persistent treatment was
significantly higher than the control. Mean differences across the collection period for the control treatment revealed that nematode detection at 7 and 14 dpi was significantly higher than the pre-inoculation sampling and at 90 dpi and 1 yr sampling dates (Fig 5.3). For the depth by date interaction, mean nematode detection frequency per plot was significantly higher in the 0-5 cm layer at 14 dpi than all others (Fig. 5.4).

Western corn rootworm emergence was detected from August.5.2015 until September.24.2015. Mean cumulative WCR emergence per plant was not statistically different among treatments; 8.4 ± 1.15 for the control treatment, 6.2 ± 0.81 for the commercial treatment, and 9.7 ± 1.4 for persistent treatment (F_{2,105} = 2.72, Pr > F = 0.0706) (Fig. 5.6). Root injury ratings were also not significantly different between treatments (F_{2, 177} = 1.38, Pr > F = 0.2534), with mean values (on a 0.00 - 3.00 scale) of, 0.028 ± 0.004, 0.021 ± 0.003 and 0.025 ± 0.004 for the control, commercial and persistent treatments, respectively.

**DNA Barcoding.** A full description of all specimens added in the neighbor joining tree can be found in Table 5.5. The neighbor-joining analysis produced eight haplotype groups of EPNs, all strongly supported by bootstrap values of 100 (Figure 5.7). The EPN haplotype groups were structured within two clades, one representing *Heterorhabditis* species and the second comprised of *Steinernema* species. In the *Heterorhabditis* clade there were four haplotype groups. Haplotype group 1 consisted of three *H. bacteriophora* USDA references strains, the *H. georgiana* USDA reference strain, and a separate and distinct subgroup of specimens isolated from the survey fields. Haplotype group 2 in the *Heterorhabditis* clade consisted of two Genbank specimens identified as
*H. bacteriophora*, and three specimens from the inoculation plots that were identical to EF043402.1 from GenBank. The third *Heterorhabditis* haplotype group (#3) consisted of *H. floridensis* USDA reference strain and haplotype group 4 consisted of *H. megidis* UK211 USDA reference strain and a GenBank sequence representing *H. megidis*.

Haplotype groups 5 through 8 belonged to the *Steinernema* clade. Haplotype group 5 was divided into two subgroups and consisted of four USDA reference strains and three GenBank sequences of *S. carpocapsae*. All but two of these strains (N6797 and N6798) represented different haplotypes. Haplotype group 6 consisted of *S. rarum* USDA reference strain. Haplotype group 7 contained *Steinernema* sp. from the inoculation and survey projects and did not match any reference or GenBank sequences. Haplotype group eight consisted of *S. feltiae* strains from the control plots in the inoculation project, USDA reference (SN strain) and GenBank sequences from *S. feltiae*.

A third clade was largely represented by specimens in the family Diplogasteridae isolated from the survey and inoculation projects. An additional four specimens had no close match in GenBank, although one specimen (N6691) was supported by a bootstrap value of 100 with a GenBank sequence from the genus *Oscheius*.

**Discussion**

The nematode detection frequency rates from insect cadavers: 5.5% (2014) and 3.7% (2015) and percentage of positive soil samples: 16% (2014) and 13.2% (2015) fit within the range reported by other studies in cultivated fields (Cabanillas and Raulston 1994, Liu and Berry 1995, Garcia del Pino and Palomo 1996, de Brida et al. 2017) and other ecosystems (Hara et al. 1991, Campbell et al. 1996, Glazer et al. 1996, Hazir et al.
2003, Campos-Herrera et al. 2007, 2008, 2013, Abd-Elbary et al. 2012). Proportional nematode detection rate from the survey field sites did not statistically vary through the season or in between fields in both years (Fig. 5.2, Table 5.1). The proportional nematode detection frequency varied considerably between arenas causing wide standard errors in the data (Fig. 5.2). High standard errors are most likely a reflection of the natural horizontal patchy distribution that nematodes have in the soil (Stuart and Gaugler 1994, Stuart et al. 2015). In 2015, statistically similar numbers of insect cadavers with nematodes were detected in *G. mellonella* vs. *T. molitor* baiting species. While quantitatively, nematode detection for the two baiting species may be the same, qualitatively they may differ as host preferences can vary significantly between entomopathogenic nematode (EPN) species (Simões and Rosa 1996). The small number of samples identified from 2015 (n=10, Table 5.5) did not allow host-EPN species comparisons to be made. Therefore, further studies should investigate whether using multiple host species in baiting assays allow for the isolation of a greater diversity of nematodes.

Days post inoculation (dpi) nematode detection frequency in the inoculation project was comparable to other projects with similar EPN application rates (2.5 x 10^9 IJ/ha) and similar dpi (Shanks and Agudelo-Silva 1990, Klein and Georgis 1992, Shields et al. 1999, Wilson et al. 2003) but was higher than Wright et al. (1993) reported despite similar application rate. Wright et al. (1993) had fewer soil samples per plot and collected the top 10 cm of soil, while we collected the top 15 cm of soil. Hence, taking in account nematode patchy distribution (horizontal and vertical) in the soil, it is possible
that the differences in methodology accounted for the differences in results between studies.

In the inoculation project, it is unclear why significant differences of nematode detection rates occurred at 90 dpi (Tables 5.2-5.3, Fig. 5.3), but it is known that the persistent nematode strains utilized in the inoculation project, *S. feltiae* “NY 04” strain and *H. bacteriophora* “Oswego” strain, are able to survive and remain viable in the laboratory over >300 days without a host (Shields 2015). This long persistence has also been shown in the field, where *S. feltiae* was able to recycle and persist for 3 years in a variety of cropping systems (alfalfa, vineyards, cranberry and apple) and through 7-year alfalfa-corn rotations (Shields 2015, Shields and Testa 2015). In these multi-year alfalfa-corn rotations, there was a large increase in *Steinernema feltiae* “NY04” recovered from soil samples in second-year corn (Shields 2015). The author inferred that this increase occurred because *S. feltiae* was responding to WCR invasion in non-rotated corn but did not test this hypothesis (Shields 2015). However, at the one-year date (July 2016), all treatments recovered comparable low frequencies of nematodes (<2%, Fig. 5.3), and were statistically comparable to pre-inoculation levels (Table 5.3).

The significant interaction between date and depth in the inoculation project, showed that, across treatments at 14 dpi, the mean frequency of nematodes detected were significantly higher for the top 0-5 cm of soil than all others (Fig. 5.4). Nematode vertical distribution in the soil is greatly affected by their foraging and dispersal behaviors (Ferguson et al. 1995, Neumann and Shields 2006, 2008). Both *Steinernema* and *Heterorhabditis* species are present throughout the soil strata (0 – 32.5 cm), but
Steinernema tend to be more dominant in the top layers (<10 cm) and Heterorhabditis on the bottom (>10 cm) (Ferguson et al. 1995, Glazer et al. 1996, Millar and Barbercheck 2001, Neumann and Shields 2006, Salame and Glazer 2015). Current literature that has investigated nematode vertical distribution, has focused on species composition per layer instead of the overall EPN abundance per soil layer (Fig. 5.4). Ferguson et al. (1995) found that the overall percentage of nematode infections decreased as soil depth increased for all nematode isolates, which would support the data from 7-30 dpi in this present study. Moreover, the DNA barcoding approach revealed native Steinernema sp. (haplotype group 7) in control, commercial and persistent plots and S. feltiae (haplotype group 8) in the control plots (Fig. 5.7, Table 5.5). Therefore, one can hypothesize that the superior detection of nematodes in the 0 - 5 cm layer (Fig 5.4) was facilitated by the naturally occurring Steinernema spp. in the experimental plots.

Pre-inoculation baiting assays from the inoculation project, revealed 0.12% (n=1620) nematode detection frequency. A background population of native nematodes throughout the season was expected based on the survey project findings (Fig. 5.1). However, non-inoculated control plots revealed a relatively high level of nematode presence when compared to the inoculated plots throughout the collection dates (Fig. 5.3). Mean nematode detection frequency in the control plots varied significantly between dates (Fig. 5.3). Soil samples from July.15.2015 (7 dpi) and July.22.2015 (14 dpi), recovered significantly more nematodes than July.1.2015 (Pre-inococulation), October.12.2015 (90 dpi) and July.26.2016 (1 year) (Fig. 5.3). Western corn rootworm (WCR) egg infestation occurred on June.18.2015; by mid-July, larvae were in the second
or third larval instars, and by late July WCR pupae were also present. The entomopathogenic nematode *S. carpocapsae*, has similar life cycle to *S. feltiae*, and it completes a full lifecycle in WCR second and third instars, and pupae within 120 – 144 hours (Jackson and Brooks 1995). Western corn rootworm biology, together with the presence of native *Steinernema* sp. and *S. feltiae* (haplotype groups 7 and 8, Fig 5.6) in the control plots suggest that the increase in nematode detection in July may be from native IJs emerging from WCR larval and pupal cadavers.

It is also important to consider the possibility of cross-contamination from the inoculated plots to the control plots. Entomopathogenic nematodes are capable of moving long distances through the movement of newly infected hosts or via farming equipment (Shields et al. 2009, Shields 2015). In our system, however, the potential for nematode movement was reduced, as western corn rootworm larvae have limited movement (up to 46 cm) (Hibbard et al. 2003), farming equipment did not travel through the plots after planting, and the center-pivot irrigation system ran parallel to the plots (Fig. 5.1). Procedures such as tool sterilization, sampling control plots before treated plots and having different personnel sampling controls were also taken to prevent cross-contamination. Evidence of native *Steinernema* spp. from the DNA barcoding analysis, and all the procedures taken against cross contamination, makes the possibility of cross-contamination unlikely.

of *S. feltiae* and *H. bacteriophora* in WCR-infested plots did not generate expected treatment effects (Fig. 5.5). Rootworm emergence was similar in all three treatments (Fig. 5.5) and mean root injury ratings were lower than 0.1 in all treatments. Root injury rates under 0.25 are considered minor feeding and does not cause impactful yield loss (Oleson et al. 2005). Low establishment of WCR infestation is the most likely explanation why minor impacts of WCR feeding were detected across plots. Another explanation for the absence of EPN treatment differences is the presence of endemic EPN populations in the plots (Figs. 5.3 and 5.6), which would’ve kept the WCR populations in the control plots lower than expected.

Through the use of the COI DNA barcoding approach we were able to identify native EPN in both the survey and inoculation projects. The phylogenetic tree showed a native *H. bacteriophora* population was present in fields A and D in 2014 and in field C in 2015 (haplotype group 1, Fig. 5.6) and to the best of our knowledge these fields have not received EPN applications in the past. Four out of the five *H. bacteriophora* from the survey are identical and form a subgroup within Haplotype group 1. This subgroup is most likely *H. bacteriophora* given that strains obtained from the USDA and identified as *H. bacteriophora* (VS, Oswego, HB strains) and *H. georgiana* (Kesha strain) formed another sub-group. *Heterorhabditis georgiana* and *H. bacteriophora* vary slightly in morphology but are genetically identical and form a monophyletic group based on the internal transcriber spacer (ITS) gene and the LSU D2-D3 expansion region of 28S rDNA (Nguyen et al. 2008). Haplotype group 2 contains two GenBank *H. bacteriophora* strains and three identical *H. bacteriophora* haplotypes from persistent EPN-treated plots and a
commercial EPN-treated plot. Having two haplotype groups to be considered *H.*
*bacteriophora* is problematic. The USDA references obtained support *H. bacteriophora*
allocation in haplotype group 1 while the GenBank database support haplotype group 2
as *H. bacteriophora*. A question of validity of species nomenclature is then raised. Is *H.*
georgiana truly a different species than *H. bacteriophora*? Moreover, what defines *H.*
bacteriophora? Heterorhabditids are divided into 3 monophyletic groups:
“*bacteriophora*-group”, “*indica*-group”, and “*megidis*-group” (Andaló et al. 2006,
Nguyen et al. 2008). Haplotype groups 1 and 2 are both considered a part of the
*bacteriophora*-group (Maneesakorn et al. 2011, Spiridonov and Subbotin 2016). A multi-
gene approach together with morphological analyses may help solve species placement
of the strains in this study (Andaló et al. 2006, Nguyen et al. 2008, Spiridonov and
Subbotin 2016).

The diversity of Heterorhabditis is highlighted by haplotype groups 3 and 4.
Haplotype group 3, a monospecies clade of *H. floridensis* is the only member of the
*indica*-group in this phylogenetic tree (Andaló et al. 2006). Haplotype group 4 represents
the *megidis*-group with identical matches of *H. megidis* from the GenBank database and
the USDA reference *H. megidis* UK211 (Andaló et al. 2006, Nguyen et al. 2008).

The Steinernema group was represented in four haplotype groups. Haplotype
group 5 consists of *S. carpocapsae* specimens from the USDA references and GenBank
sequences. Contrary to *H. bacteriophora* classification, the *S. carpocapsae* classification
was supported by both USDA references and Genbank sequences. Haplotype group 6, *S.*
rarum is a monospecies clade, also consistent with previous reports (Nadler et al. 2006,
Haplotype group 7 remained under *Steinernema* sp. and was composed of specimens from the inoculation project, treated and control plots, and one specimen from the survey project (Fig. 5.6). Two identical haplotypes (N6741 and N6762) originated from the persistent and commercial inoculated plots, respectively. Those plots received *S. feltiae* inoculations, yet those strains do not match haplotype group 8, the *S. feltiae* group. Haplotype group 8 consisted of sequences from the control plots from the inoculation project, USDA reference and GenBank. Similar to *S. carpocapsae*, *S. feltiae* classification was supported by GenBank sequences and USDA reference strains.

*Steinernema* phylogenetic classification is also heavily based on ITS and LSU D2-D3 expansion region of 28S rDNA (Spiridonov and Subbotin 2016). Based on those gene sequences, species are currently classified under three monospecies clades and twelve multi-species clades, with ten of those clades forming three super-clades named: Superclade 1: “Glaseri-Karii-Longicaudatum-Khoisanae”, Superclade 2: “Feltiae-Kushidai-Monticolum” and Superclade 3: “Carpocapsae-Bicornutum” (Spiridonov and Subbotin 2016). According to this classification, haplotype group 5 belong to Superclade 1 and haplotype group 8 belong to Superclade 2 (Nadler et al. 2006, Spiridonov and Subbotin 2016). The COI DNA barcoding approach herein highlights the importance of this work in contributing to understanding the phylogenetic diversity of EPNs. The COI phylogenetic tree was able to group Heterorhabdits and Steinernematids in similar patterns to ITS and LSU D2/D3 based phylogeny (Andaló et al. 2006, Nadler et al. 2006, Nguyen et al. 2008, Spiridonov and Subbotin 2016). This approach also enabled the identification of
nematodes from the control plots of the inoculation project and helped determine that resident EPN populations were present in treated and control plots, hence ruling out cross-contamination.

Several nematodes recovered from both projects were not entomopathogenic but were rather free-living nematodes belonging to the Diplogasteridae clade (Fig. 5.6). Diplogasterids have diverse life-histories, they are primarily decomposers but can also be nematophagous or facultative parasites of insects and have also been shown to participate in insect phoresis (Poinar 1969, 1975, Colagiero et al. 2012). Biological control agents face a wide range of obstacles after application in the field, and one of them is their interaction with biotic factors including direct (natural enemies) and indirect antagonism (competition) predation and competition (Kaya and Koppenhöfer 1996). Hence, it can be speculated that it is possible that some of the *S. feltiae* and *H. bacteriophora* species applied in the inoculation project were eaten or displaced by the native diplogasterids in the plots, but we did not gather data to support this claim.

To our knowledge, this is the first description of naturally occurring EPNs in agroecosystems of the Midwest of the United States (Hominick 2002). Prior to human settlements, Nebraska was primarily composed of prairie, with mixed-prairie being predominant in Keith and Perkins Counties (Kaul and Rolfsmeier 1993, UNL Conservation & Survey Division). The nematodes recovered from the cornfields in this study are most likely remnants of pre-agriculture ecosystems (Shields 2015). Nematodes have a wide range of survival mechanisms that allows them to persist in the environment (Glazer 1996). Adams (1998) found native *S. feltiae* and *H. bacteriophora* in
native and grazed prairie of Western Nebraska in Arthur Co. and Keith Co. Both EPN species were also found in this present study in cornfields in Keith and Perkins Counties. Finding native EPN strains that can survive the intensive agricultural practices adopted in local fields may increase EPN efficacy against target pests (Glazer 1996, Shields 2015). Local \textit{H. bacteriophora}, \textit{Steinernema} sp. and \textit{S. feltiae} should be re-isolated from the field sites in this study and tested against WCR larvae and other soil pests. This work provides a foundation for future ecological and pest management studies with EPN in irrigated corn systems.
References


Spiridonov, S. E., A. P. Reid, K. Podrucka, S. A. Subbotin, and M. Moens. 2004. Phylogenetic relationships within the genus Steinernema (Nematoda: Rhabditida) as inferred from analyses of sequences of the ITS1-5.8 S-ITS2 region of rDNA and morphological features. Nematology. 6: 547–566.


### Tables

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Statistical analysis was conducted with PROC GLIMMIX in SAS using two-way generalized linear mixed model ANOVA. Nematode detection was expressed as the mean proportion of nematode-infected cadavers per arena for each sampling date in each field. Mean proportion was fitted to a beta-binomial distribution prior to analysis. Main effects and interactions at $P < 0.05$ were considered significant.
Table 5.2. General linear mixed model analysis of nematode detection in the inoculation project.

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Statistical analysis was conducted with PROC GLIMMIX in SAS using a three-way generalized linear mixed model ANOVA. Nematode detection frequency was expressed as the mean proportion of infected baiting insects per sampling depth per replicate (30 larvae per replicate for 5 cm soil depth, and 60 larvae per replicate for 10 cm soil depths). Mean proportion was fitted to a beta-binomial distribution prior to analysis. Sampling dates were treated as repeated measures with first order autoregressive covariance structure (AR-1). Main effects and interactions at P < 0.05 were considered significant.
Table 5.3. Differences of treatment by sampling date least square means with Tukey adjustment for multiple comparisons. All values were considered non-significant (NS) if adjusted P > 0.05.

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Table 5.4. Differences of depth by sampling date least square means with Tukey adjustment for multiple comparisons. All values were considered non-significant (NS) if adjusted $P > 0.05$.

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Table 5.5. Specimen origins for samples used in phylogenetic tree (Fig 5.6). Nematode Identification Number (NID), strains are in parenthesis. HG= Haplotype group.

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Fig 5.1. Diagram of EPN inoculation project. Top: Plots were arranged into a randomized complete block design of 6 blocks, for a total of 6 replicates per treatment. Blocks are shown outlined in black. Each plot measured 24.4 m (32 rows) x 24.4 m. Bottom: Shown in shadow is the inoculation zone (2.5 x 2.5 meters) of the persistent treatment. Infected *G. mellonella* cadavers of *S. feltiae* (circle) and *H. bacteriophora* (triangle) were placed every 122 cm. Inoculation of commercial treatment consisted of equal concentration of each EPN species mixed together and applied over the inoculation zone with a watering can.
Figure 5.2. Mean nematode detection per arena in survey sites in 2014 (a) and 2015 (b). Nematode detection was analyzed as proportion data and multiplied by 100 to obtain percentages. There were no significant interactions or main effects of field or date in both years (Table 5.1).
Figure 5.3. Mean nematode detection per treatment over sampling dates. Treatment by time interaction was significant ($F_{12,195} = 2.04$, Pr $>$ F = 0.0229). Different letters represent mean detection differences at the P < 0.05 level.
Figure 5.4 Mean nematode detection (averaged across treatments) by sampling depth over collection periods. Depth by time interaction significant ($F_{6,195} = 2.61$, $Pr > F < 0.0185$). Different letters represent mean detection differences at the $P < 0.05$ level. Overall interaction least square mean differences shown in table 5.4.
Figure 5.5. Cumulative WCR emergence distribution among treatments. Treatments received three cages per plot for a total of 36 cages per treatment. Black line on box-plot represent emergence median. Mean WCR emergence per cage was not significantly different among treatments ($F_{2,105} = 2.72, Pr > F = 0.0706$).
Table 5. Tips. NID (Nematode Identification number) sampling characteristics are provided in specimens.

Figure 5. Neighbor-joining tree of COI nucleotide sequence from 70 nematode specimens. Haplotype groups are enumerated in circles adjacent to terminal branch tips. NID (Nematode Identification number) sampling characteristics are provided in Table 5.5.
CHAPTER 6: SUMMARY AND CONCLUSIONS

Management of western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte (Chrysomelidae: Coleoptera) is a complex, multivariate issue. There are many tactics used to control WCR populations to prevent or reduce yield losses in corn. Currently used tactics to manage WCR are crop rotation, *Bacillus thuringiensis* (Bt) hybrids and chemical control. Crop rotation breaks the pest cycle by removing the host for larval feeding. However, there are many reasons why farmers keep their fields as continuous corn, including livestock needs, economics of corn production especially under irrigation, soil properties, and contractual obligations of rented land (Andow et al. 2017). All three controls may be effective but because they are widely used, WCR has adapted to one or more management practices in several areas of the Corn Belt (Meinke et al. 1998, Levine et al. 2002, Parimi et al. 2006, Gassmann et al. 2011, 2014, 2016, Wangila et al. 2015, Zukoff et al. 2016, Ludwick et al. 2017). It is necessary to examine new tools for WCR control to provide new management practices as well as to help extend the lifetime of existing technologies.

The overall goal of this dissertation was to characterize the communities of natural enemies in commercial cornfields in the context of examining their potential as WCR biological control agents (BCAs). Specifically, objectives were developed to look at arthropod predators (Chapter 2), entomopathogenic fungi (Chapters 3 and 4) and entomopathogenic nematodes (Chapter 5) in lab and field studies.

The survey for above ground arthropods (Chapter 2) revealed that commercial cornfields support an abundant and diverse community of predators. The predators
caught on the yellow-sticky cards are not known predators of the WCR (Kuhlmann and Van der Burgt 1998, Toepfer et al. 2009). Also, none of the Carabid beetles from dry pitfalls tested positive for WCR DNA in molecular gut-content analyses. Western corn rootworm prey was available in all fields but especially abundant in fields A and C, even though these fields contained WCR-Bt traits. These results expand on findings that arthropod predators are likely having minimal impacts on WCR mortality in the fields (Kirk 1982, Lundgren and Fergen 2014).

Looking at the below-ground microbial natural enemies, a diverse assemblage of entomogenous fungi were isolated from the soil of the same cornfields from Chapter 2 via *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) and *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) baiting assays (Chapter 3). A total of 373 strains were isolated and 132 of those were selected for molecular identification. Entomogenous fungi were detected in every field site and the recovered strains had a variety of ecological roles such as phytopathogens, antagonists of phytopathogens, insect antagonists, and saprophytes. Entomopathogenic fungi (EPF) made up the majority of fungi isolated with the most prevalent genus being *Metarhizium*, represented by *M. robertsii* J.F. Bisch., Rehner & Humber and *M. anisopliae* (Metschn.). Other genera of potential and confirmed EPF isolates included *Beauveria*, *Penicillium*, *Pseudogymnoascus*, and *Purpureocillium* (*Paecilomyces*). This study was similar to others that reported diverse fungal communities with multiple ecological functions from insect cadavers (Sun and Liu 2008, Sun et al. 2008, Oliveira et al. 2011), but it was a unique study as it focused on commercial cornfields.
Forty-eight fungal strains from Chapter 3 and a commercial strain, Botanigard 22WP®, *Beauveria bassiana* (Bals.-Criv.) Vuill. strain GHA, were tested against the WCR in the laboratory (Chapter 4). Those strains were selected to represent the diversity found in the soil and to determine their impact on mortality of WCR third-instar larvae. In soil assays, fourteen strains caused mortality higher than the negative control: *M. anisopliae* (n=2), *M. robertsii* (n=11), *Pseudogymnoascus* sp. (n=1). Only one strain (E1089, *M. anisopliae*) caused mortality significantly higher than BotaniGard, the commercial standard. In the immersion assay, eight strains caused mortality higher than the control: *M. anisopliae* (n=1), *M. robertsii* (n=5), *Metarhizium* sp. (n=1). BotaniGard (*B. bassiana*). This study was novel as it tested EPF beyond *B. bassiana* and *M. anisopliae* and was able to determine that other pathogens can potentially be explored as BCAs of the WCR.

From the same soil samples of Chapter (3) we also examined the native entomopathogenic nematode (EPN) community (Chapter 5). EPN community showed no seasonal or field variance in the survey project. In 2015, there were no differences in the rate of nematodes or fungi recovered from *G. mellonella* or *T. molitor* indicating that both species are suitable baiting hosts for a general community of entomopathogens. Through the use of a DNA barcoding approach it was determined that *Heterorhabditis bacteriophora* Poinar and *Steinernema* spp. are present in the commercial cornfields sampled. Both *Heterorhabditis* and *Steinernema* contain species that can infect the WCR (Geisert et al. 2018, Wright et al. 1993, Journey and Ostlie 2000, Toepfer et al. 2005, 2008, Kurtz et al. 2009). Strains from the commercial fields have not yet been tested
against WCR larvae, but an inoculation project was set-up to determine the impacts of commercial and persistent *H. bacteriophora* and *S. feltiae* co-inoculation on WCR mortality in the field. Data from the inoculation project didn’t show any EPN treatment effects, potentially due to a high background of native nematodes in the control plots and relatively low infestation level of WCR. Some of the nematodes in the control plots were identified as resident strains of *Steinernema feltiae* and *Steinernema* spp. One hypothesis derived from these results is that the *Steinernema* spp. strains in the control plots were causing WCR mortality and therefore we didn’t see treatment effects.

Application of EPF and EPN individually, as species assemblages or in conjunction with Bt hybrids are able to reduce larval and adult WCR populations (Bruck and Lewis 2001, Journey and Ostlie 2000, Toepfer et al. 2005, 2008, Hoffmann et al. 2014, Kurtz et al. 2009, Meissle et al. 2009, Mulock and Chandler 2001, Petzold-Maxwell et al. 2012a,b, Pilz et al. 2009, Rudeen et al. 2013, Geisert et al. 2018, Wright et al. 1993). Hence our isolates hold promising potential to be incorporated into inundative or conservation biological control programs in this system. Future studies are needed to determine if the strains isolated here have the requirements needed in a good BCA such as virulence, host specificity, compatibility with agrochemicals, and environmental persistence (Glazer 1996, Kaya and Koppenhöfer 1996). If BCA(s) meet field inundation or conservation requirements, then biological control can become a reality within the WCR integrated pest management framework.

Cornfields sampled in this study are high-input systems intensely managed with Bt hybrids, insecticides, nematicides, fungicides, herbicides, and fertilizers. Despite
these practices, natural enemies above ground (arthropods) and below-ground (entomopathogens) are present in our system. Moreover, Cry3Bb1 WCR resistance has been documented in the counties where we conducted our studies (Wangila et al. 2015, Wangila and Meinke 2016, Reinders 2017). In 2014, Field A (Keith Co.) and Field C (Perkins Co.) had single trait Cry3Bb1 hybrids and high densities of WCR in those fields suggest some level of resistance may occur at those sites.

These findings beg the question: if all these natural enemies exist in cornfields, and a lot of them have been shown to kill WCR, then why is the WCR such a problem in Nebraska?

The first contributing factor is the wide adoption of corn monocultures. Keith and Perkins Counties, Nebraska are high yielding counties in the state (USDA-NASS 2018). High densities of WCR are able to build up in these areas as continuous corn is grown on a regional level, not just in isolated fields (USDA-NASS, 2017). In addition, WCR have evolved to suppress corn defenses and have high reproductive capabilities (Robert et al. 2012). All these factors together, greatly favor large WCR populations in the area even though natural enemies are present in the same habitats. Hence, the sheer magnitude of WCR density enables the WCR to overcome both biotic and abiotic mortality factors and therefore maintain economically challenging densities over time in many continuous cornfields.

Western corn rootworms spend the majority of their life cycle in the soil and are most likely adapted to the overall soil environment, including environmental stressors and natural enemies. Natural enemies and hosts are constantly co-evolving in a way to
cause or evade mortality. For instance, although not present in North American corn hybrids, corn roots can emit the insect-induced volatile, \((E)\)-\(\beta\)-caryophyllene, to recruit EPNs to attack WCR larvae (Rasmann et al. 2005), but, WCR larvae sequester benzoxazinoids from plant roots and activate it upon EPN attack, killing the nematodes and its associated symbiotic bacteria required to kill the insect (Robert et al. 2017). Moreover, physical protection from natural enemies can happen while WCR larvae feed within the roots (Strnad and Bergman 1987) or as they pupate in earthen cells (Chiang 1973). Some predators are also deterred by a sticky hemolymph defense that larvae possess when facing predation (Lundgren et al. 2010).

Western corn rootworm trophic interactions are not well understood, and so far, no keystone predator or pathogen has been identified. Nevertheless, studies that targeted WCR larvae with entomopathogens (single or multi-species compositions) together with Bt hybrids have shown the potential for corn plant protection (Meissle et al. 2009, Petzold-Maxwell et al. 2012a,b; Petzold–Maxwell et al. 2013, Rudeen and Gassmann 2013, Hoffmann et al. 2014). In general, these papers found that entomopathogens can act in additive and complementary ways to Bt to increase WCR mortality and/or increase plant protection. Coupling entomopathogens with existing practices can lead to a more sustainable pest management program over time.

In summary, natural enemies are natural components of agroecosystems and a thorough understanding of factors that influence their success as BCAs is necessary. Understanding the soil microbial community and arthropod predators can contribute to the exploration of sustainable agriculture practices. This dissertation generated
foundation work for biological control in irrigated corn in Nebraska. We determined that a wide range of natural enemies are present in fields including pathogens of the WCR such as *M. anisopliae*, *B. bassiana*, *H. bacteriophora* and *Steinernema* spp. We also determined that other pathogens that have not been reported before against the WCR, *M. robertsi*, *Pseudogymnoascus* sp., *Chaetomium* sp., and *P. lilacinum* can cause WCR mortality. Future research should focus on understanding WCR-pathogen relationships and the suitability of biological control programs to increase the efficacy of native EPF and EPN strains. The results of this dissertation generated questions that should be explored in future studies:

1) Investigate pathogenicity and virulence of native *H. bacteriophora* and *Steinernema* spp. against WCR larvae. This study would help us understand whether the native EPN are capable of utilizing WCR as a host.

2) Seed treatments of endophytic EPF have reduced insect densities but also promoted plant health (Sasan and Bidochka 2012, Lopez et al. 2014, Lopez and Sword 2015, Bamisile et al. 2018). All the EPF from this study came from the rhizosphere, hence, it would be interesting to characterize root-EPF relationships to understand how the strains described here can help plant protection beyond insect protection.

3) Determine modes of action of *Pseudogymnoascus* sp., *Chaetomium* sp., and *P. lilacinum* against the WCR. Understanding pathogenic and virulence factors of these strains, and others found in this work, can lead to a better
understanding of factors governing insect susceptibility to EPF, and provide potential sources of insect-resistance genes (Lacey et al. 2015).

4) Study the impacts of standard agronomical practices (fertilizers, herbicides, nematicides, insecticides, fungicides, tillage, and rotation) on the entomopathogenic community found from this dissertation. Studies like this are necessary to make biological control an existing management option for WCR and other pests.
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Chrysomelidae) and fitness costs of resistance to Cry3Bb1 maize. J. Econ. Entomol. 107: 352–360.


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Reinders, J.D. 2017. Spatial variation in western corn rootworm (Coleoptera: Chrysomelidae) susceptibility to Bacillus thuringiensis corn events in Nebraska. M.S. Thesis. University of Nebraska-Lincoln.


## APPENDIX 1. FIELD SITES LOCATION AND AGRONOMIC CHARACTERISTICS.

<table>
<thead>
<tr>
<th>Field</th>
<th>GPS Coordinates at Center of Field</th>
<th>Soil Typea</th>
<th>Years in Continuous Corn</th>
<th>Year</th>
<th>Hybridb</th>
<th>Bt Proteins Expressed for Rootworm</th>
<th>Mean WCR emergence/plantc</th>
<th>Planting Date</th>
<th>Seed Treatmentd</th>
<th>Foliar Insecticides &amp; Fungicides</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>41°06'57.64&quot;N 101°38'55.35&quot;W</td>
<td>Lex loam</td>
<td>5</td>
<td>2014</td>
<td>DeKalb DKC52-04</td>
<td>Cry3Bb1</td>
<td>48.9</td>
<td>07 May</td>
<td>1</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2015</td>
<td>DeKalb DKC52-84</td>
<td>Cry3Bb1 + Cry34/35Ab1</td>
<td>47.5</td>
<td>03 May</td>
<td>1</td>
<td>none</td>
</tr>
<tr>
<td>B</td>
<td>40°45'12.04&quot;N 101°47'09.25&quot;W</td>
<td>Woody fine sandy loam</td>
<td>&gt;30</td>
<td>2014</td>
<td>DeKalb DKC51-19</td>
<td>Cry3Bb1</td>
<td>15.4</td>
<td>28 April</td>
<td>2</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2015</td>
<td>Pioneer 35F50AM1</td>
<td>Cry34/35Ab1</td>
<td>6.5</td>
<td>30 April</td>
<td>3</td>
<td>none</td>
</tr>
<tr>
<td>C</td>
<td>40°47'46.03&quot;N 101°45'27.63&quot;W</td>
<td>Haxtun fine sandy loam</td>
<td>&gt;30</td>
<td>2014</td>
<td>DeKalb DKC51-19</td>
<td>Cry3Bb1</td>
<td>33.2</td>
<td>29 April</td>
<td>2</td>
<td>Chemigation: Bifenthrin (6.4 fl oz/ac) and dimethoate (1 pt/ac) on 22 July</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2015</td>
<td>Pioneer P0419AMX</td>
<td>Cry34/35Ab1</td>
<td>72.7</td>
<td>01 May</td>
<td>3</td>
<td>none</td>
</tr>
<tr>
<td>D</td>
<td>40°46'55.53&quot;N 101°48'57.68&quot;W</td>
<td>Altvan loam</td>
<td>13</td>
<td>2014</td>
<td>DeKalb DKC54-38</td>
<td>Cry3Bb1 + Cry34/35Ab1</td>
<td>4.2</td>
<td>26 April</td>
<td>1</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2015</td>
<td>DeKalb DKC54-38</td>
<td>Cry3Bb1 + Cry34/35Ab1</td>
<td>4.2</td>
<td>30 April</td>
<td>1</td>
<td>none</td>
</tr>
<tr>
<td>E</td>
<td>40°51'12.52&quot;N 101°42'4.78&quot;W</td>
<td>Haxtun fine sandy loam</td>
<td>0</td>
<td>2014</td>
<td>Pioneer 1151AM</td>
<td>none</td>
<td>1.4</td>
<td>03 May</td>
<td>3</td>
<td>Aerial application: Bifenthrin (6.4 fl oz/ac) on 26 July</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2015</td>
<td>Pioneer 1151AMX</td>
<td>Cry34/35Ab1</td>
<td>5.6</td>
<td>17 May</td>
<td>3</td>
<td>none</td>
</tr>
</tbody>
</table>
As determined by the United States Department of Agriculture-Natural Resources Conservation Service Web Soil Survey.  

DKC=DeKalb; P=Pioneer  

Mean WCR/plant: Eight plants/field were monitored with single-plant emergence cages throughout beetle emergence period (July-September). Mean emergence is the cumulative emergence per cage for 11 dates each year.  

Seed treatments of Insecticides and Fungicides: 1: Clothianidin (0.50 mg/seed); metalaxyl, prothioconazole, and fluoxastrobin. 2: Clothianidin (0.25 mg/seed); metalaxyl, prothioconazole, and fluoxastrobin. 3: Thiamethoxam (0.25 mg/seed); thiabendazole, fludioxonil, mefenoxam, azoxystrobin, and ethaboxam
APPENDIX 2: PRELIMINARY TEST OF E

The western bean cutworm (WBC), Striacosta albicosta Smith, is a significant pest of corn, Zea mays L., and dry beans, Phaseolus spp., in the United States (Blickenstaff and Jolley 1982, Seymour et al. 2004, Michel et al. 2010). In corn, larval feeding on ears reduces yield by direct consumption and by providing entry points for quality-reducing fungal infections (Seymour et al. 2004, Michel et al. 2010). Sixth-instar larvae drop to the soil and construct earthen chambers in which they overwinter as prepupae in a quiescent state (Michel et al. 2010). Pupation occurs in late May and adult emergence starts in the beginning of July (Seymour et al. 2004). Any application for larval control of the western corn rootworm (WCR), Diabrotica virgifera virgifera LeConte, in the soil can potentially impact secondary targets that spend part of their lifecycle within the soil matrix, such as S. albicosta. Thus, the objective of this experiment was to screen selected fungal strains that were previously tested against the WCR (see Chapter 4) to determine their pathogenicity to WBC prepupae in soil cup assays.

Materials and Methods

Western bean cutworm source. Western bean cutworm prepupae were obtained from the laboratory colony of the Agroecosystems Entomology Laboratory (University of Nebraska-Lincoln, North Platte, NE). Colony was initiated in 2017 from field-collected egg masses from the Nebraska cities: Benkelman, Grant, Brule, North Platte, Kearney,
Grand Island, O'Neill, and Scottsbluff. Prepupae (the quiescent stage of the 6th larval instar) utilized in the experiment had been in the prepupal stage for 10-15 days (50-55 days since egg hatch). Individual prepupae were placed into moist play sand (ca. 2 ml/cup) (QUIKRETE® Premium Play Sand®, # 1113) in 59 ml plastic soufflé cups (Solo Cup Company, Highland Park, IL) with small holes on the lids to allow for ventilation.

**Fungal sources and inoculum preparation.** The 10 native fungal strains used in this experiment were isolated via *Galleria mellonella* F. and *Tenebrio molitor* L. baiting assays of Nebraska soil samples from cornfields (see Chapter 3). Eight *Metarhizium robertsii* J.F. Bisch. strains, one *Metarhizium anisopliae* (Metschn.) Sorokin strain, and one *Pseudogymnnoascus* sp. strain were selected from strains previously tested against the WCR in soil cup assays (see Chapter 4). Nine strains were selected because they caused mortality higher than the control for WCR and one strain (E211) was a poor performer for WCR but was selected to check for WBC-specific mortality. Fungal culture and spore suspensions were prepared as described in Chapter 5. In addition to the native EPF strains, Botanigard® 22WP, *Beauveria bassiana* (Bals. -Criv.) Vuill. strain GHA (Arbico Organics, Oro Valley, AZ) was included as a commercial comparison product. All strains, including Botanigard, had inoculum concentrations of $1 \times 10^7$ viable spores ml$^{-1}$, with the exception of strains E1000 and E1034, which showed poor germination or sporulation. For those strains, the maximum obtained concentration of viable spores was used ($4.2 \times 10^6$ spores ml$^{-1}$ for E1000 and $2.7 \times 10^6$ spores ml$^{-1}$ for E1034).

**Pathogenicity screening.** Bioassays were conducted in 59 ml cups containing approximately 37 g of moist sand (ca. 2ml /cup) and one prepupa per cup. Each cup
received a total of 3 ml of spore suspension topically applied evenly across the cup. Bioassay cups were sandwiched between café-trays lined with moist paper towels (100% RH) and kept in an incubator set at 26.3 ± 0.5°C for 9 days. There was only one replication per strain and each strain was tested against 16 insects (15 insects for strains E1026, E211, and E376 due to low availability of insects). Control insects received 3 ml of 0.1% Tween-80.

**Data analysis.** Western bean cutworm mortality was analyzed using the FREQ procedure in SAS (SAS Institute Inc., version 9.4, Cary, NC) (O’Rourke and Hatcher 2013). A contingency table between number of dead insects and treatment was created with the TABLE function. Differences between treatment mortality frequencies were analyzed via two-tailed Fisher’s exact test (McDonald 2014). If one or more cadavers showed fungal growth consistent with gross morphology of genera tested, then fungal growth was considered positive.

**Results**

Nine strains of *Metarhizium* spp., *Pseudogymnoascus* sp. and Botanigard 22 WP were tested against WBC prepupae. Negative control mortality was 6.25%, Botanigard mortality was 31.3%, *Metarhizium* spp. strains mortality ranged from 18.8 to 60 %, and *Pseudogymnoascus* sp. had 33.3% mortality (Fig. A1). Out of all the strains tested (including Botanigard), six showed external sporulation: E1000, E1022, E1030, E161, E328, and E380. Treatments, including the control, were not significantly different based on Fisher’s exact test (Pr < P = 0.22).
Discussion

Biological control is an understudied area of WBC management, but studies have explored the efficacy of predators and parasitoids of WBC egg masses (Archibald 2017, Ostdiek 2012). To our knowledge this is the first study to have tested non-microsporidia entomopathogenic fungi against the WBC (Dorhout 2007, Helms and Wedberg 1976). The relationship between *Nosema* and WBC is still unclear; however, it is known that *Nosema* spp. are frequently found in WBC moths and its infection has been implicated to be one of the causes of cyclical fluctuations of WBC populations (Dorhout 2007; Hutchison et al. 2011).

The data herein are preliminary, and strain derived mortality were not statistically different from the control. Additional studies with a larger quantity of test insects are needed to determine the impact of those strains on WBC mortality. However, fungal growth on cadavers confirmed that the WBC is a host for six EPF strains (E1000, E1022, E1030, E161, E328, and E380) and five of those strains inflicted significant mortality to WCR larvae (Chapter 4). The WBC prepupal larval stage overwinters in the soil and pupates in May, with adult emergence beginning in July (Seymour et al. 2004). Therefore, there is an overlap in which prepupae and pupae of the WBC and larvae of the WCR are present in the soil at the same time. A strain that can be used for both species simultaneously may benefit fields in which both pests are a problem. Based on the WCR soil assays (see Chapter 4) and the WBC preliminary assays, it would be worth exploring the feasibility of *M. robertsii* strains E380 and E1022 for the control of both pests in future studies.
References:


Figure A1. Western bean cutworm mortality from entomopathogenic fungi treatments.

Means not significantly different based on two-tailed Fisher’s exact test (Pr < P = 0.22).
APPENDIX 3: CURRICULUM VITAE CAMILA OLIVEIRA HOFMAN

Camila Oliveira Hofman, Ph.D.
Entomology Department, University of Nebraska-Lincoln
103 Entomology Hall, Lincoln, NE, USA 68583-0816
E-mail: camila.o.hofman@gmail.com

Education

Ph.D. Entomology, University of Nebraska-Lincoln. 2014-2018
M.S. Entomology, University of Nebraska-Lincoln. 2011-2013
B.S. Biology, University of Missouri-Columbia. 2006-2010
B.A. Anthropology, University of Missouri-Columbia. 2006-2010

Academic Research Experience

Graduate Research Assistant, Agroecosystems Entomology Lab, North Platte, NE
Jan.2014-Present
University of Nebraska-Lincoln, Dept. of Entomology
Mentors: Drs. Julie A. Peterson and Lance J. Meinke
Research goal: Characterization of the natural enemy community, with emphasis on entomopathogens, in pest management of western corn rootworm (Diabrotica virgifera virgifera) in West Central Nebraska.
Skills: Experimental design (field and lab); entomopathogen (fungi and nematodes) isolation, identification and inoculation; insect pathology bioassays; mycology laboratory techniques; western corn rootworm rearing and infestation techniques; root feeding damage rating; DNA extraction, polymerase chain reaction (PCR), gel electrophoresis, DNA editing, BLAST and phylogenetic tree analysis.

Graduate Research Assistant, Vector Ecology Lab, Lincoln, NE
University of Nebraska-Lincoln, Dept. of Entomology
Mentor: Dr. Gary L. Hein
Research goal: Determine the impact of Wheat streak mosaic virus and Triticum mosaic virus on transmission by Aceria tosichella and virus epidemiology.
Skills: Wheat curl mite colony maintenance; virus inoculations; enzyme-linked immunosorbent assay (ELISA); data collection and analysis of laboratory, greenhouse and field experiments.

Undergraduate Research Technician
May.2009–July.2011
University of Missouri- Columbia, Dept. of Plant Science

Mentor: Dr. Deborah L. Finke

Research goal: Investigate interactions among the plant pathogen Cereal Yellow Dwarf virus, its aphid vector, *Rhopalosiphum padi*, and the aphid’s parasitoid *Aphidius colemani*.

Skills: Greenhouse and insect colony maintenance; virus inoculations; collection, sorting and counting of insects; insect behavior experiments, data collection and analysis.

**Plant Phylogenetics Lab**

University of Missouri- Columbia, Biological Sciences Dept.

Mentor: Dr. Maria Alejandra Jaramillo

Responsibilities: DNA extraction of plants in the genus *Piper*; PCR and gel electrophoresis; data analysis, input and organization; greenhouse maintenance.

**Department of BioChemistry, Proteomics Lab**
Dec.2006-May.2007

University of Missouri-Columbia, Biochemistry Dept.

Mentor: Dr. Jay J. Thelen

Responsibilities: Cleaning duties; *Arabidopsis* seedling counting; data input in Excel; greenhouse maintenance.

**Publications**

**Peer-reviewed**


Editor-Reviewed


Thesis

de Oliveira, C.F. 2013. Impact of Wheat streak mosaic virus and Triticum mosaic virus on transmission by Aceria tosichella Keifer (Eriophyidae) and virus epidemiology in wheat. M.S. Thesis. University of Nebraska- Lincoln

Published abstracts

Suárez Victor V, C Oliveira-Hofman, KA Mollet & JA Peterson. 2016. Identification of potential predators of the western corn rootworm in maize fields of West Central


**Oral Presentations**

**Invited**


**Extension**


**Student Competitions**


de Oliveira, Camila F., S. N. Wegulo and G.L. Hein. 2014. Impact of co-infection of *wheat streak mosaic virus* and *Triticum mosaic virus* on virus transmission rates and wheat curl mite reproduction in the field. NCB-ESA. Des Moines, IA.

de Oliveira, Camila F. and G.L. Hein. 2013. Impact of co-infection of *Wheat streak mosaic* and *Triticum mosaic* viruses on transmission rates by the wheat curl mite. Annual Meeting ESA. Austin, TX.

**Poster Presentations**

Oliveira-Hofman, C., Meinke, L.J., Adesemoye, A.O. and **J.A. Peterson.** 2018. Screen of entomopathogenic fungi from west central Nebraska against key pests of corn. 9<sup>th</sup> International IPM symposium. Baltimore, MD.


**de Oliveira, Camila F.** and G.L. Hein. 2013. Impact of *wheat streak mosaic* and *Triticum mosaic* viruses on transmission by *Aceria tosichella* and virus epidemiology. NCB-ESA. Rapid City, SD.

Awards

3rd Place, Graduate Student Poster Competition. 2017. Kansas Ent. Soc.

1st Place, Ph.D. Student Poster Competition. 2016. NCB-ESA Cleveland, OH ($300)


3rd Place, M.S. Student 10-minute Presentations Competition. 2014. NCB-ESA Des Moines, IA ($100)

3rd Place, M.S. Student Poster Competition. 2013. NCB-ESA Rapid City, SD ($100)

Travel Grants ($1766 total)

Myron H. Swenk Memorial Fund. Dept. of Entomology - UNL.
- September.2016 ($185)
- October.2015 ($145)
- October.2014 ($96)
- October.2013 ($164)
- June.2013 ($226)

NCB Student Travel Scholarship. North Central Branch-ESA.
- June.2016 ($250)
- March.2014 ($500)

Dept. of Entomology - UNL Funds to attend Insect Path. Course. June.2015. ($200)

Research Grants ($18,169 total)


Scholarships and Fellowships ($1000 total)

Milton E. Mohr Graduate Fellowship. 2016. ($1000)

Professional Development Workshops

2017
Write Winning Grant Proposals Workshop. University of Nebraska-Lincoln. Lincoln, Nebraska.

Soft Skills workshop: Skills you need to succeed. Offered by the Graduate Student Assembly. University of Nebraska-Lincoln. Lincoln, Nebraska.

2016
Campuswide Workshops for Graduate Teaching Assistants. Sponsored by the Office of Graduate Studies. University of Nebraska-Lincoln. Lincoln, Nebraska.

2015
Insect Pathology Course. Offered by Cornell University and International Organisation for Biological Control. Ithaca, NY.

Teaching Experience

Teaching Assistant, Entomology 806: Insect Ecology. Online Course.
Fall 2017
Duties: Content development for homeworks and assignments.

Teaching Assistant, Entomology 116: Insect Identification
Fall 2016
Duties: Deliver lectures, engage students in in-class exercises and demonstrations. Answer students’ questions; help with insect collection, specimen identification and preparation techniques.

Teaching Assistant’s Assistant, Entomology 116: Insect Identification
Spring 2014
Duties: Assist teaching assistant with class exercises and demonstrations. Answer students’ questions; help with insect collection, specimen identification and preparation techniques.
**Outreach**

2017
- Dept. of Entomology-UNL. Annual BugFest.

2016
- Dept. of Entomology-UNL. Annual BugFest.
- Women in Agriculture Conference. West Central Research Station, UNL. Grant, NE
- Women in Science UNL Workshops for High School Students. Insects: The good and the bad workshop.

2015
- Women in Science UNL Workshops for High School Students. Insect Pollinators Workshop.
- Lawrence Bruner Club Educational Entomology Booth at Lincoln’s Farmer’s Market.

2014
- Dept. of Entomology-UNL. Annual BugFest.
- College of Agricultural Sciences and Natural Resources. Weatherfest. Educational Entomology Booth.
- Lawrence Bruner Club Educational Entomology Booth at Lincoln’s Farmer’s Market.

2013
- Dept. of Entomology-UNL. Annual BugFest.

**Professional Memberships**

*Society for Invertebrate Pathology* (July.2015-Present)

*International Organization for Biological Control (Nearctic Regional section) IOBC-NRS* (Sept.2014-Present)

*Lawrence Bruner Club*. University of Nebraska-Lincoln (Aug.2011-Present)
- Secretary (May.2015- May.2016)
- Library Committee Member (Aug.2012- Present)
- Education Committee Member (Aug.2012- Present)
  - Chair 2017-2018
- President (Aug.2012- May.2013)
- Student Representative to Faculty (Aug.2013- May.2014)
- Fundraising Committee Member (Aug.2012- May.2015)
• Outreach Committee Member (Aug.2011-Aug.2012)

**Entomological Society of America (ESA) Member** (Nov.2010- Present)
• North Central Branch Student Affairs Committee (NCB-SAC) (Oct.2015-June.2017)

**C.V. Riley Entomology Club.** University of Missouri-Columbia (May.2009- May.2011)

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**Service**

Symposium moderator at Urban Pest Management Conference. Feb.2014. Lincoln, NE.

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**Additional Information**

Portuguese: Native language  |  English: Fluent  |  Spanish: reading knowledge