EXPLORING THE POTENTIAL OF ENTOMOPATHOGENIC NEMATODES AGAINST WIREWORMS IN MONTANA

by

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ABSTRACT

Wireworms (Coleoptera: Elateridae), are important soil-dwelling pests and have become a serious threat to spring wheat in the Northern Great Plains because of lack of effective control measures, creating a need for alternative control methods such as biological control with entomopathogenic nematodes (EPNs). Ten EPN strains were tested against sugarbeet wireworm, *Limonius californicus* (Mannerheim) in laboratory bioassay. Out of these ten strains, *Steinernema carpocapsae* (Weiser) (All and Cxrd strains) and *S. riobrave* Cabanillas, Poinar, and Raulston (355 and 7-12 strains) were found effective in laboratory and shade house. However, the dose required to kill at least 50% of the treated *L. californicus* larvae was 200 infective juveniles/cm^2. Two Montana native EPN species (*S. feltiae* and *Heterorhabditis bacteriophora*) were extracted and were evaluated against *L. californicus* in laboratory and shade house. Although, 50% mortality was observed due to *S. feltiae* isolates in laboratory conditions, none of the two isolates of *S. feltiae* performed well against *L. californicus* in shade house. *Steinernema carpocapsae* and *S. riobrave* in the form of infected *Galleria mellonella* L. cadavers were evaluated against wireworms (*L. californicus* and *H. bicolor*) in field and greenhouse. In field, none of the four EPN strains were found effective in reducing wireworm populations or protecting crop yield in both spring wheat and barley fields. However, only one infected *Galleria* cadaver of *S. carpocapsae* (All and Cxrd) and *S. riobrave* 355 was able to kill wireworm larvae as well as reduce wheat plant damage in greenhouse. The imidacloprid treatment enhanced the infection and killing ability of EPNs against *L. californicus*.

Steinernema carpocapsae All and Cxrd were able to kill 30-46% of *L. californicus* larvae in all four soil types tested as compared to *S. riobrave* 355 and 7-12 strains, when maintained at field capacity moisture levels. However, *S. carpocapsae* All and Cxrd strains were able to infect 35-50% wireworm larvae in sandy loam and clay loam soil type at standardized moisture (18%). *Steinernema carpocapsae* All and Cxrd killed greater wireworm numbers at 16% moisture level and 25°C as compared to other moisture and temperature levels in sandy clay loam soil. These results indicate that EPN based strategies could be useful for wireworm management but need to be explored further under field conditions. This strategy can at least be part of a large Integrated Pest Management system for wireworm control in Montana.

CHAPTER 1

REVIEW OF LITERATURE

Introduction

Wheat (*Triticum* spp.) is one of the world's most important food crops and is a staple food for much of the world's population. It is now the most widely cultivated cereal in the world with more than 220 million ha planted annually under wide ranges of climatic conditions in many geographic regions (Shiferaw et al. 2013). Global wheat production was estimated to be 735 million metric tons in 2018 (Statista 2019). In 2017, the United States cultivated over 15 million ha of wheat, with a yield of over 47 million tons (FAOSTAT 2017). In USA, Montana is third in wheat production with 2,090,000 ha and 2.56 tons/ha yield (2018 State Agriculture Overview). The Golden Triangle Region (GTR) of North Central Montana is the most important wheat producing region in Montana.

Among the insect pests impacting wheat in the GTR, wireworms are a major problem for growers. Wireworms are polyphagous pests with biting/chewing mouthparts. The larvae can cause serious damage to a wide range of crops (Parker and Howard 2001; Reddy et al. 2014). Larvae feed on the roots of wheat and bore into the stem, causing wilting and dying, resulting in a thin plant stand (Simmons et al. 1998). Cryptic behavior, poorly known taxonomy and biology, long larval stages, and poorly known vertical and horizontal movement patterns make assessment and management of wireworms complicated (Vernon 2010; Esser et al. 2015; Traugott et al. 2015). Currently, there is no completely effective insecticide available for wireworm management. Neonicotinoids

(imidacloprid, clothianidin and thiamethoxam) and the pyrethroid tefluthrin are used in other crops as seed treatment against wireworms (Vernon et al. 2009, 2013, van Herk et al. 2018), but provide only limited protection to crops (Rashed et al. 2015). Labrie et al. (2020) reported only a 5% effectiveness of neonicotinoid seed treatments in corn and soybean fields in Quebec.

Important wireworm species as crop pests in the GTR

Wireworms, the larval stage of click beetles (Coleoptera: Elateridae), are soil-inhabiting pests in a wide variety of field, cereal, and vegetable crops, worldwide (Seal et al. 1992; Kohno and Miyai 1993; Parker and Howard 2001; Traugott et al. 2015; Milosavljević et al. 2017; Rashed et al. 2017). In the Pacific Northwest, *Limonius* (Lanchester) including *Limonius canus* Leconte*, Limonius californicus* (Mannerheim)*, Limonius infuscatus* (Motschulsky)*,* and *Limonius subauratus* LeConte are most frequently genera reported to cause economic damage (Andrews et al. 2008). Wireworms are among the major pests of spring and winter wheat in the northern Great Plains, from Montana to Minnesota (Hermann et al. 2012). Morales Rodriguez et al. (2014) found *L. californicus*, *L. infuscatus*, *Hypnoidus bicolor* (Eschscholtz), and *Aeolus mellillus* (Say) as the most common wireworms in Montana wheat during a three year survey from 2011 to 2013. Of these species, *L. californicus* and *H. bicolor* were the main species damaging spring wheat in the GTR, with the former being the single most important wireworm in the region (Reddy et al. 2014). Therefore, in my study, I concentrated on *L. californicus* as my experimental model, given its importance in the GTR.

Biology and life cycle

Limonius californicus is commonly known as the sugar beet wireworm. In California, Stone (1941) observed oviposition by this species in early summer, and females laid 500 to 1400 eggs on the upper surface of the soil within a week of mating. The larval stage lasted from 3 to 7 years passing through 10 to 13 instars. The larvae pupated in the soil, about 15 cm below the surface. The pupal stage lasts for about three weeks from June to October. Under cold conditions, adults can survive for more than seven years (Stone 1941). As insects go, elaterids tend to have a comparatively long life cycle. Overwintering click beetles emerge from the soil in early spring to late summer. *Limonius californicus* females began laying eggs about eight days after mating (range 3–30 days) and complete oviposition at an average of 31 days post mating (range 7–75 days) (van Herk and Vernon 2014). The females can lay an average of 268 eggs throughout their adult life (van Herk and Vernon 2014). The larvae hatch within 2 to 3 weeks and start feeding immediately. *Limonius californicus* can have life cycles of 2–11 years (Stone 1941). Adults emerge from pupal chambers in early to late spring within the soil and begin mating.



Figure 1. Mature larvae of *Limonius californicus* from the Golden Triangle Region of Montana.

Management

No single control method can be expected to effectively manage wireworms alone. Integrated Pest Management (IPM), combining several different methods such as host plant resistance, cultural control, chemical insecticides, pheromones, and biological control, should all be considered to deal with this pest complex. For my study, I have examined the potential of biological control to reduce the impact of wireworms in the GTR, using *L. californicus* as my model organism.

The Potential of Biological Control for the control of wireworms in the GTR

Biological control includes the use of predators, parasitoids, and pathogens to suppress insect pest population. However, due to the cryptic habitat and soil dwelling behavior of wireworms, biological control can be difficult to develop and implement against this pest. A few studies have addressed biological approaches against wireworms.

For the most part, predators and parasitoids have not been very effective in reducing wireworm populations below economic thresholds. Carabid beetles (Carabidae), rove beetles (Staphylinidae) and the larvae of stiletto flies (Therevidae) are reported to prey on wireworms [e.g., *Agriotes* (Becker), *Athous* (Eschscholtz) and *Limonius* (Knodel and Shrestha 2018)]. Traugott et al. (2015) reported 2-10% wireworm mortality caused by parasitoids in Europe and the United States. In addition, predaceous vertebrates, such as frogs, toads, moles, and birds, were found to feed on wireworms (Traugott et al. 2015) but were unable to reduce wireworm populations below economic levels under field conditions (Parker and Howard 2001).

Efforts to develop the use of entomopathogenic fungi (EPF) against wireworms are very recent, although these agents have been known since early in the 20th century (Barsics et al. 2013). Entomopathogenic fungi have been used successfully to control wireworms in Canada (Kabaluk et al. 2007), the UK (Parker and Howard 2001) and Switzerland (Keller and Schweizer 2001). *Metarhizium anisopliae* (Metschnikoff) Sorokin (Hypocreales: Clavicipitaceae) was found to be pathogenic on wireworms (Ericsson et al. 2007; Ansari et al. 2009). Reddy et al. (2014) reported significant differences among plant stand densities due to *M. brunneum* F52, *Beauveria bassiana* (Bals.-Criv.) Vuill. GHA and *Metarhizium robertsii* (Bisch, Rehner and Humber) DWR 346 granules soil drench. Antwi et al. (2018) have also observed that seed treatment with imidacloprid along with *M. brunneum* F52 protected wheat seedlings from wireworm damage in Montana. Sharma et al. (2019) reported greater spring wheat yields due to *M. robertsii* (strains DWR356 and DWR2009) applied on a millet carrier.

The use of insecticidal formulations of bacteria against wireworm infestations have not been well studied (Wraight et al. 2009). Pons et al. (2005) reported *Bacillus thuringiensis* (Berliner) ineffective against wireworms in field trials. Leclerque et al. (2011) have found a new pathotype of the intracellular bacterium *Rickettsiella agriotidis* (Legionellales: Coxiellaceae) in an *Agriotes* wireworm. Danismazoglu et al. (2012) also studied the insecticidal potential of the bacterial flora of *A. lineatus*. However, these bacteria did not control wireworms under field conditions.

Given the lack of success of most biological control approaches to wireworm problems, I looked for a novel approach. From among these, I have concentrated my

studies on evaluating the potential of entomopathogenic nematodes (EPNs), i.e. Steinernematidae and Heterorhabditidae, for controlling wireworms. EPNs are globally distributed and can be found in virtually every terrestrial ecosystem (Hominick 2002). EPNs are insect parasitic nematodes mutualistically associated with bacterial symbionts; all life-stages of the nematodes, except for the dauer stage, are found inside the insect host; examples are Steinernematidae and Heterorhabditidae (Onstad et al. 2006). However, Dillman et al. (2012) felt this definition to be incomplete and considered rapid death of the host and passage of the associated bacteria to future generations as important missing components in the earlier definitions to differentiate EPNs from phoretic, necromenic, or other less virulent forms of parasitism.

The complete life cycle of EPNs consists of an egg, four juvenile stages, and an adult stage. In *Heterorhabditis* (Poinar) (Rhabditida: Heterorhabditidae), each Infective juvenile (IJ) molts into a hermaphroditic female. In the second generation, both amphimictic and automictic forms can occur (Strauch et al. 1994). In most first-generation *Steinernema* (Travassos) (Rhabditida: Steinernematidae), the IJ molts into either an amphimictic female or male. The exception is *Steinernema hermaphroditum* (Stock, Griffin and Chaenar) which produce hermaphrodites (Griffin et al. 2001), and in the second generation will produce both amphimictic females and males. Both steinernematids and heterorhabditids take about 3-7 days to complete one generation inside the host. Emergence of IJs from the host take about 6–11 days for steinernematids and 12–14 days for heterorhabditids (Kaya and Koppenhöfer 1999).

Xenorhabdus (Thomas and Poinar) spp. and *Photorhabdus* (Boemare et al.) spp. are the mutualistic bacteria for steinernematids and heterorhabditids, respectively (Adams and Nguyen 2002; Lewis and Clarke 2012). The symbiotic bacteria associated with these nematodes are Gram-negative, facultative anaerobic rods in the family Enterobacteriaceae, and found within the intestine of the IJ, the only free-living stage. The symbiotic bacteria require EPNs for dissemination from one host individual to another (Akhurst and Boemare 1990; Boemare 2002), as well as for shelter from unfavorable environmental conditions. In return EPNs rely on their symbionts' production of antibiotic and hydrolytic enzymes that degrade the insect body into a nutrient soup and provides nutrition for growth and reproduction (Goodrich-Blair 2007).

EPNs locate the hosts and enter through natural openings (mouth, anus, and spiracles), or through the cuticle (mainly in heterorhabditids) (Campbell and Gaugler 1991; Hazir et al. 2003). The IJs release their symbiotic bacteria in host's body cavity and develop fourth stage juveniles and adults. These bacteria kill the host within 24–48 h due to septicemia and sometimes septicemia results in bacterial toxaemia (Gaugler 2002) (Figure 2). The nematodes molt and complete up to three generations within the host body after which IJs exit the cadaver to find new hosts (Poinar 1990; Lewis and Clarke 2012).

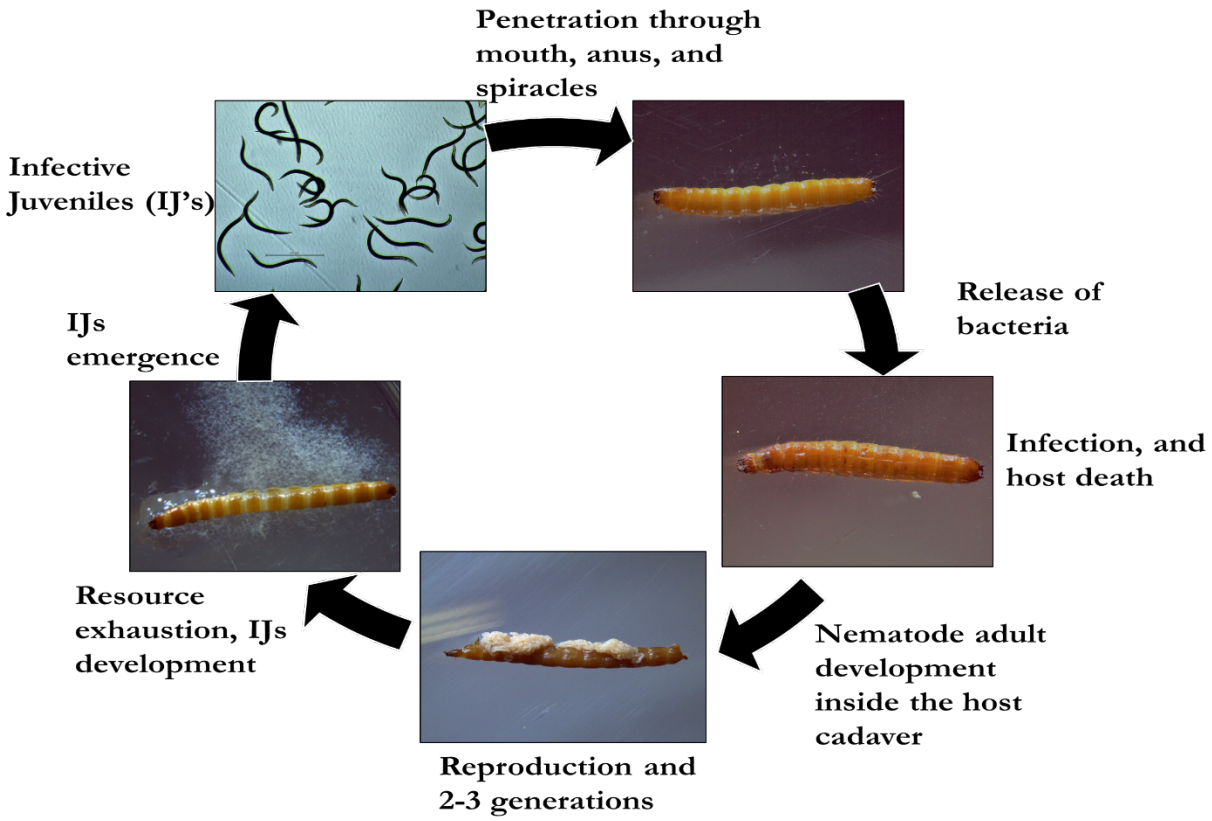


Figure 2. Life cycle of EPNs

Entomopathogenic nematodes in biological control

EPNs are promising biological control agents of many insect pests due to their broad host range, ability to cause rapid death, high reproduction rate, short generation time, suitability for mass-rearing in artificial media, and limited harm to non-target species (Gaugler et al. 1997; Ehlers 2001). EPNs have been found successful as inundative biological control agents for different soil inhabiting insect pests such as such as the black vine weevil, *Otiorhynchus sulcatus* (F.) (Coleoptera: Curculionidae), *Diaprepes* root weevil, *Diaprepes abbreviates* (L.) (Coleoptera: Curculionidae), fungus gnats (Diptera: Sciaridae), various white grubs (Coleoptera: Scarabaeidae), foliar and wood-boring insects,

and fruit-tree pests such as peach-tree borer, *Synanthedon exitiosa* (Say) (Lepidoptera: Sesiidae) and lesser peach-tree borer, *Synanthedon pictipes* (Grote and Robinson) (Lepidoptera: Sesiideae) (Arthur et al. 2004; Grewal et al. 2005; Lacey and Georgis 2012; Shapiro-Ilan et al. 2010; Shapiro-Ilan et al. 2014; Shapiro-Ilan et al. 2015; Shapiro-Ilan et al. 2017). Generally, EPN species possess a broad host range at least in the laboratory, with limited efficacy observed sometimes under field conditions due to various environmental factors.

Efficacy of EPNs against wireworms

As wireworms and EPNs share soil as their habitat, EPNs might be effective against this group of pests and has been investigated in a limited way in the past (see Table 1). Most of the studies were focused on EPN efficacy against different wireworm species in laboratory and greenhouse situations (Campos-Herrera and Gutierrez 2009; Ansari et al. 2009; San-Blas et al. 2012; Morton and Garcia-del-Pino 2016; Ensafi et al. 2018; La Forgia et al. 2020) with some studies conducted in the field (Toba et al. 1983; Schalk et al. 1993; Ester and Huiting 2007; Arrington et al. 2015; Morton and Garcia-del-Pino 2016). Overall, different EPNs have varied in their ability to kill wireworms, and consequently in their efficacy (Table 1). Most of the wireworm species involved in these studies were European species, therefore it would be interesting to evaluate EPN infectivity to North American wireworm species that might differ from those discussed in the available literature.

Advantages and Challenges in EPN use for wireworm management

One of the major advantages of using EPNs against wireworms is the soil as shared habitat. As EPNs live and disperse in the soil, wireworms can be a suitable target for these pathogens. Additionally, EPNs are widely distributed throughout the world and have been reported from natural and managed habitats and a wide variety of soils (Hominic, 2002; Adams et al, 2006, 2007). These indigenous EPNs have been used successfully to suppress various insect pests (Shapiro-Ilan et al. 2002; Lewis et al. 2006). There is a possibility of the occurrence of native EPN species in Montana soils that might infect the wireworms and persist longer in the soil. Therefore, different natural and cultivated areas in Montana need to be surveyed for extracting native EPN species and should be explored against wireworms.

Apart from these advantages, there are various challenges in utilization of EPNs as a control strategy for wireworms. Wireworms' narrow mouth, obstructing oral filters, septa covering the spiracles, and strong anal musculature can inhibit the EPN infectivity (Lewis et al. 2015). Soil desiccation can reduce nematode survival, dispersal, and persistence (Koppenhöfer and Fuzy 2007) and can affect the commercial use of the nematodes at every stage, from their mass production to field application (Strauch et al. 2004). Survival and pathogenicity of *Steinernema carpocapsae* (Weiser) and *Steinernema glaseri* (Steiner). decreased as relative humidity decreased from 100 to 25% over a month (Kung et al. 1991). Addition of anti-desiccants to the EPN suspension before application can increase their efficacy by increasing their survival time in soil (Glazer 1992). Shapiro-Ilan et al. (2014) observed the highest level of desiccation tolerance in *S. carpocapsae* followed by

Steinernema feltiae (Filipjev) and with heterorhabditids being least tolerant and *Steinernema riobrave* (Cabanillas, Poinar and Raulston) was observed as intermediate desiccation tolerant in the laboratory.

Nematode migration, survival, pathogenicity and host-finding ability decrease in soil with more clay content due to its small pore sizes (Kung et al. 1990a). The EPN species *S. glaseri* and *Heterorhabditis* spp. and *S. carpocapsae* all showed better dispersal in sandy soils (large pore size) (Georgis and Poinar 1983; Molyneux and Bedding 1984; Koppenhöfer and Fuzy 2006). However, some other studies reported high efficacy of EPNs in clay soil than sandier soils (Shapiro et al. 2000; Toledo et al. 2009; Toepfer et al. 2010). In substrates with high pore size like peat, *S. carpocapsae* outcompeted *Heterorhabditis megidis* (Poinar, Jackson, and Klein) for host finding in peat (Kruitbos et al. 2010). Soil pH can also have significant effect on nematode survival and infectivity; pH of 10 or higher are usually detrimental (Kung et al. 1990b).

Temperature also affect the infectivity of nematodes (Hazir et al. 2001); however, optimum temperatures for infection and reproduction vary among nematode species and strains (Grewal et al. 1994). EPN species such as *Heterorhabditis indica* (Poinar, Karunakar and David), *S. glaseri* and *S. riobrave* are relatively heat tolerant and can maintain efficacy at temperatures of 29°C and above. However, *S. feltiae*, *H. megidis* and *Heterorhabditis marelatus* (Liu and Berry), and *Steinernema kraussei* (Steiner) are more cold tolerant species, maintaining efficacy at 15°C and below (Grewal et al. 1994; Kung et al. 1991; Torr et al. 2007; Radová and Trnková 2010). Generally, temperatures higher than

30 °C reduce nematode survival and infectivity, although some species such as *S. riobrave* remain effective at soil temperatures above 35 °C (Lacey and Unruh 1998).

Another important challenge in EPN use against wireworm is the persistence in soil over time. EPNs are usually applied in an inundative manner with high dose of nematodes applied on a short term basis to create a direct impact on the pest population (Shapiro-Ilan et al. 2006; Dillon et al. 2007). However, the long term persistence of the applied EPNs can be helpful in higher efficiency against wireworms for consecutive years. The cadaver application approach has several advantages such as higher nematode dispersal (Shapiro-Ilan and Glazer 1996), infectivity (Shapiro-Ilan and Lewis 1999), survival (Perez et al. 2003), and efficacy (Shapiro-Ilan et al. 2003) as compared to standard application in aqueous suspension. Therefore, this approach should be evaluated for testing different EPN species against wireworms under field conditions considering the dryland farming practices being followed by most of the growers in Montana.

Research approach

The overall aim of this study is to develop an effective and sustainable control method for wireworms. Although significant research has been done in the past few decades to determine the efficacy of EPNs against wireworms, especially with European species, the potential scope of EPN use in wireworm management is still not clear. There is still a need to test different EPN species and strains against different wireworm species in North America. The future use of EPNs for wireworms is very encouraging by considering all the merits EPNs possess, mostly the lack of non-target impacts, environment friendliness, and compatibility with standard spray equipment. As there is

currently no effective control method available for wireworms, it will be interesting to see if EPNs can manage wireworm populations effectively and reduce crops losses. An overview of different studies conducted to achieve the main goal in present study are being discussed as follows:

In Chapter 2, efficacy of ten available entomopathogenic nematode strains was evaluated against, *Limonius californicus* in a concentration response laboratory bioassay and the effective strains were tested against this wireworm species in shade house experiments with two EPN doses. To find the indigenous EPN species, different agricultural sites in GTR of Montana were surveyed and the found species were identified using morphological and molecular techniques (Chapter 3). These Montana native entomopathogenic nematode species were evaluated against *Limonius californicus* in laboratory and shade house by testing different EPN doses (Chapter 4).

As mentioned above, efficacy of EPNs under field conditions is usually less than that implied by laboratory tests because of the limited dispersal and lack of persistence of these nematodes (Lynch and Thomas, 2000). Therefore, four non-native available EPN species found effective under laboratory and shade house experiments in Chapter 1 were tested at two field sites by using EPN infected *Galleria* cadavers and the results are discussed in Chapter 5. Additionally, in Chapter 6, the effect of different soil characteristics (texture, moisture, and temperature) was studied for different EPN species against *L. californicus* under laboratory conditions.

Table 1. Studies testing different EPN species against wireworms.

Wireworm specie	Insect stage	Crop	EPN specie	Lab(L)/ /Field (F) study	Dose	Comments	References
Limonius californicus (Mannerheim)	Larvae (7^{th}-10^{th} instars)	Potato	*Steinernema feltiae* (Filipjev) and *Steinernema glaseri* (Steiner)	L and F	0 to 393 IJs/cm^2 and 0 to 629 IJs/cm^2 in laboratory and 0 to 155 IJs/cm^2 and 0 to 310 IJs/cm^2 in field cage experiment	*S. feltiae* was found effective causing 58 % mortality after 2 weeks in laboratory; *S. feltiae* caused highest mortality i.e. 29 % at 310 IJs/cm^2; *S. glaseri* was not very effective	Toba et al. (1983)
Conoderus spp.	Larvae	Sweet potato	*Steinernema carpocapsae* (Weiser)	F	2.5 and 7.5 billion IJs/ha	Nematodes did not affect the larval count; However, reduced the plant damage in wireworm-*Diabritica* sp.-*Systena* sp. complex	Schalk et al. (1993)

Ctenicera destructor (Brown)	Last instar larva	*S. feltiae* and *Heterorhabditis bacteriophora* (Poinar)	L	250 nematodes/insect	No infection found	Morris (1985)
Agroites sp.	Larvae Potato	*S. feltiae*	F	545,000 nematodes/m^2	No effect on potato tuber damage	Ester and Huiting (2007)
Agriotes sordidus (Illiger)	Larvae	Different strains of *S. feltiae* and *S. carpocapsae*	F	250 IJs/cm^2	In laboratory, only 7-9% mortality caused by *S. feltiae* 38 and Rioja strains.	Campos-Herrera and Gutierrez (2009)
Agriotes lineatus (L.)	Fourth and fifth instars	*H. bacteriophora* UWS1, *Heterorhabditis megidis* (Poinar, Jackson, and Klein), *Heterorhabditis downesi* (Stock, Griffin and Burnell) *S. feltiae*, and *S. carpocapsae*	L	63 IJs/cm^2	*H. bacteriophora* (UWS1) caused 67% mortality and *S. carpocapsae* caused around 50% mortality	Ansari et al. (2009)

Wireworms, *Elaterid* larvae	Alive and dead frozen larvae		*Steinernema affine* (Bovien) and *S. kraussei* (Steiner)	L	200 IJs/larva	Wireworms were resistant to the nematodes; However, frozen larvae treated in laboratory were colonized by the nematode species	Půža and Mráček (2010)
Agriotes sp.	Frozen larvae		*S. carpocapsae*	L	200 IJs/larva	The alive larvae were unaffected by *S. carpocapsae* but 28% of dead larvae were scavenged	San-Blas et al. (2012)
Conoderus vespertinus (Fabricius)	Larvae	Sweet potato	*S. carpocapsae*	F	5 billion IJs/ha (applied in drip irrigation)	No reduction in the proportion of wireworm damage as compared to control	Arrington et al. (2015)
Agriotes obscurus (L.)	Larvae	Potato	F8, F10, F11, and F16 strains of *H. bacteriophora*, *H. bacteriophora* [Larvanem], *S. carpocapsae* B14, *S. carpocapsae* [capsanem], *S.*	L and F	50 and 100 IJs/cm^2 in laboratory and 10^6 IJs/m^2 in field	In laboratory at 100 IJs/cm^2, *S. carpocapsae* B14 caused 76% mortality followed by 3 *H. bacteriophora* strains: larvanem, F10 and F8 with	Morton and Garcia-del-Pino (2016)

		feltiae F4, and *Steinernema* sp. D1 22			36%, 29% and 27% mortality, respectively; In field, *S. carpocapsae* B14 as found effective with 48% mortality.	
Sugar beet wireworm *L. californicus*	Larvae	*S. carpocapsae*	Greenhouse	~52,258 IJs/larva in 22.9 × 22.9 × 24.1 cm pots	20% larval mortality and in sand-dominated medium, S. *carpocapsae* provided relatively better seedling protection.	Ensafi et al. (2018)
Agriotes sordidus	Larvae	*S. carpocapsae* and *H. bacteriophora*	L	250 IJs/larvae	EPNs with potato extracts (attractant and feeding stimulant) in alginate beads showed 17% and 412% wireworm mortality for *S. carpocapsae* and *H. bacteriophora,* respectively.	La Forgia et al. (2020)

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CHAPTER TWO

EFFICACY OF AVAILABLE ENTOMOPATHOGENIC NEMATODE STRAINS AGAINST *LIMONIUS CALIFORNICUS* (MANNERHEIM)

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EFFICACY OF AVAILABLE ENTOMOPATHOGENIC NEMATODE STRAINS AGAINST *LIMONIUS CALIFORNICUS* (MANNERHEIM)

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Abstract

Wireworms, the larval stage of click beetles (Coleoptera: Elateridae), are economically important soil-dwelling pests that attack many field crops worldwide. Wireworms have become a serious threat to spring wheat in the Northern Great Plains because of a lack of effective control measures, creating a need for alternative control methods such as biological control with entomopathogenic nematodes (EPNs). A laboratory bioassay was used to test ten EPN strains and identify infective EPN strains against the sugarbeet wireworm, *Limonius californicus* (Mannerheim) (Coleoptera: Elateridae). *Steinernema carpocapsae* (Weiser) (All and Cxrd strains) and *S. riobrave* Cabanillas, Poinar, and Raulston (355 and 7-12 strains) killed 60-70% of *L. californicus* larvae in four weeks when applied at 700 Infective juveniles (IJs) (25 IJs/cm^2), 1400 IJs (50 IJs/cm^2), 2800 IJs (100 IJs/cm^2), and 5600 IJs (200 IJs/cm^2) per larva in the laboratory. Also, *Heterorhabditis bacteriophor*a (Poinar) VS strain and *Steinernema rarum* (Doucet) 17c+e strain caused 50-60% mortality to *L. californicus* larvae after four weeks when applied at 5600 IJs/larva. However, regardless of the concentration applied, the penetration rate of infective juveniles into the host did not exceed 33%. In shade house trials, *S. riobrave* and *S. carpocapsae* strains caused 34-56% *L. californicus* mortality after four weeks with 50 and 56% mortality caused by *S. carpocapsae* All and *S. riobrave* 355 strain, respectively when applied at the rate of 80,000 IJs/pot. These results suggest that *S. carpocapsae* and *S. riobrave* may have significant potential for protecting spring wheat crops from *L. californicus*.

Key words: Wireworm, Biological control, Entomopathogenic nematodes, Sugarbeet wireworm, *Limonius californicus*, Spring wheat

Introduction

Wireworms, the larval stage of click beetles (Coleoptera: Elateridae) are economically important soil-dwelling pests that attack belowground parts of many field crops worldwide (Traugott et al., 2015; Milosavljević et al., 2015). Wireworms are polyphagous pests that feed on seeds, roots, stems, tubers, and belowground plant parts, inhibiting plant growth, causing plant wilting and death, and reducing crop yield (Keiser et al., 2012; Traugott et al., 2015). In general, overwintering click beetles emerge from the soil from early spring to late summer, and become active when air temperatures exceed 10°C (Knodel and Shrestha, 2018). Females lay eggs just below the soil surface up to 15 cm. The eggs hatch within 2–3 weeks and larvae start actively feeding on the plant parts (Knodel and Shrestha, 2018). There can be 8-14 larval instars depending on wireworm species and environmental conditions. Stone (1941) reported 10-13 larval instars of *Limonius californicus* (Mannerheim). However, only six to seven larval instars were reported for *Aeolus mellillus* (Say) in one year (Jewett, 1940). Wireworm larvae show vertical upward and downward movement depending on the environmental conditions, especially temperature. Pupation occurs in the soil and the stage lasts for about a month; adults overwinter and emerge from their soil cocoons the following spring. Immediately after emergence, adults move within fields or disperse to new areas (Knodel and Shrestha, 2018).

All the larval instars are present in the field simultaneously in a non-uniform distribution and the characteristic symptom of wireworm damage is the patchy plant damage in the fields. Crop losses from soil-dwelling wireworms can be up to 25% in North

America and the United Kingdom (Parker et al., 1990; Jansson and Seal, 1994). According to Traugott et al. (2015), the high species diversity, soil habitation, long larval lifespan in the soil, poorly known taxonomy and life history of wireworms make them difficult to control.

For more than 50 years, a few broad-spectrum insecticides such as lindane were used to reduce wireworm damage (Toba and Turner, 1983). However, many of these insecticides have been banned worldwide because of environmental persistence and health concerns (Vernon et al., 2009). Neonicotinoids and other chemicals (e.g. Fipronil) have been registered for some crops as seed treatments for wireworm management in the United States, Canada, and other countries. However, wireworms feeding on neonicotinoid-treated seeds/seedlings become intoxicated but often survive (Vernon et al., 2009; Barsics et al., 2013). Also, the long-term use of conventional insecticides can cause damage to the environment, human health, and beneficial insects such as honey bees (Wright et al., 2015; Nicolopoulou-Stamati et al., 2016). Therefore, there is a need for the development of alternative, environmentally friendly Integrated Pest Management (IPM) techniques to manage wireworm populations effectively. Crop protection and cultural control methods including soil amendments, soil drying, flooding, manipulation of planting or harvesting dates, crop rotation and trap crops have been used to manage wireworms in various crops (Andrews et al., 2008; Landl and Glauninger, 2011; Esser et al., 2015). Entomopathogenic bacteria, fungi, and nematodes are also explored in laboratory and field conditions (Ansari et al., 2009; Morton and Garcia-del-Pino, 2016; Ensafi et al., 2018; Sharma et al., 2019).

Entomopathogenic nematodes (EPNs) (Rhabditida: Steinernematidae and Heterorhabditidae) are obligate soil-inhabiting parasites of many insects. Infective juveniles (IJs) of EPNs penetrate hosts through natural openings (mouth, spiracles, and anus) or, in some instances, directly through the cuticle (Campbell and Gaugler, 1991; Hazir et al., 2003). IJs regurgitate and defecate the symbiotic bacteria (*Xenorhabdus* for Steinernematidae and *Photorhabdus* for Heterorhabditidae) inside the insect's hemocoel to overcome the host defenses and help kill the host and suppress the growth of microbial competitors (Dowds and Peters, 2002; Hazir et al., 2003). EPNs are effective biological control agents of many pests due to their ability to cause rapid death, their high reproduction rate, short generation time, suitability for mass-rearing in artificial media, and limited harm to non-target species (Kaya and Gaugler, 1993; Gaugler et al., 1997; Ehlers, 2001). EPNs can also be integrated with other pest management tactics since they can be applied in standard spray equipment and are compatible with many agrochemicals (Koppenhöfer and Grewal, 2005). EPNs are reported to have a synergistic relationship with certain chemical pesticides (Koppenhöfer et al., 2002). Arthurs et al. (2004) emphasized the potential of EPNs to be used against different foliar and wood boring insect pests. However, different factors such as soil pH, texture, aeration, and temperature can affect the efficacy of EPNs against above ground insect pests (Shapiro et al., 2012). EPNs have been successful as inundative biological control agents for different soil inhabiting insect pests on a variety of crops (Lacey and Georgis, 2012; Shapiro et al., 2012; Shapiro et al., 2017).

As soil-inhabiting pests, wireworms can be a potential target for biological control using EPNs. Various researchers have conducted laboratory and field studies to test the

efficacy of EPN's against wireworms. Some studies have shown high infectivity of different EPN species in various wireworm species in the laboratory (Toba et al., 1983; Ansari et al., 2009; Morton and Garcia-del-Pino, 2016). Toba et al. (1983) reported that *S. feltiae* caused 58% mortality in sugarbeet wireworm larvae (*L. californicus*) in the laboratory but only 29% mortality was observed in field cages. However, Campos-Herrera and Gutierrez (2009) observed only 7-9% *Agriotes sordidus* Illiger mortality was observed when different strains of *S. feltiae* and *S. carpocapsae* were tested in laboratory.

Green house and field studies conducted on the susceptibility of wireworms to EPNs have been less promising so far (Schalk et al., 1993; Ester and Huiting, 2007; Campos-Herrera and Gutierrez, 2009; Půža and Mráček, 2010; Arrington et al., 2015; Ensafi et al., 2018). The non-accessibility of wireworms to EPNs has been related to narrow mouths, hardened cuticle, oral filters, as well as obstructions such as covering spiracles with septa and strong anal musculature (Lewis et al., 2015). Efficacy of EPNs against individual wireworm life stages is difficult to evaluate in the field conditions since almost all life stages of wireworms are present in the soil at the same time. Several untested EPN species need to be tested against wireworms to find an effective EPN species that could be used to at least partially suppress the wireworm population and reduce crop losses.

In this study, we evaluated the efficacy of ten EPN strains against *L. californicus*, one of the dominant wireworm species in the Pacific Northwest (Esser et al., 2015, Milosavljević et al., 2015) and Intermountain regions (Rashed et al., 2015) of the United States. Morales-Rodriguez et al. (2014) in field surveys in 2011 and 2012 reported *Limonius californicus* and *Hypnoides bicolor* Eschscholtz to be the most important

wireworms damaging spring wheat in the Golden Triangle Region of Montana. The objectives of this study were to: 1) screen ten available EPN strains against wireworms in the laboratory and 2) evaluate the efficacy of effective EPN strains selected from the laboratory bioassay against wireworms in indoor conditions (shade house). The information gained from this study should identify the EPN species most likely to control *L. californicus* in spring wheat in the Golden Triangle Region of Montana.

Material and methods

Study site location and wireworm collection methods

All the experiments were conducted at the Western Triangle Agricultural Research Center (WTARC), Conrad, MT in 2018 and 2019. Wireworm larvae were collected in May to August 2018 and from May to August 2019 in Golden Triangle Region of Montana near Pendroy (John Stultz Ranch Inc., Pondera Co. (N48.04130°, W112.16945°)); Conrad (Garrett Grub Ranch Inc, Pondera Co. (N47.56786°, W112.13930°), and Kalispell (Ron Ranch Co. (N48.1945°, W114.2341°)) for different experiments. Three species of wireworms, *L. californicus*, *H. bicolor*, and *A. mellilus* were found, but *L. californicus* was the dominant species and was subsequently the species used in all the experiments. The larvae were collected using stocking traps (Reddy et al., 2014) in which nylon stockings were filled with half a cup of wheat grain or a mixture of wheat and barley grain. These baits were soaked in water for 24 hrs and buried 0.15-0.2 m below the soil surface in wireworm infested fields. The traps were then covered with black plastic sheets to maintain the ambient temperature and moisture for wireworm survival. After 2 weeks, the traps were collected from the field in plastic buckets and brought back to the laboratory for extraction.

The larvae were sorted from the traps by using Berlese funnels. The collected wireworm larvae were stored in an incubator at 10°C in the plastic cups with sterilized sandy loam soil, and soaked wheat seeds were provided as food. The cups were sprayed once a week to keep the soil moist. The collected larvae were of almost all the larval stages. The length of wireworm larvae ranged from 0.5 to 2.2 cm. Medium-sized wireworm larvae (0.8–1.5 cm; more than one year old) were used in different experiments.

Source and culturing of nematodes

Ten strains of *Steinernema* and *Heterorhabditis* species were obtained from the USDA-ARS Entomopathogenic Nematode culture collection (Byron, GA) (Table 1). EPNs were reared in last-instar greater wax moth larvae, *Galleria mellonella* L. (Lepidoptera: Pyralidae), according to the procedure explained by Woodring and Kaya (1988). Wax moth larvae were obtained from Bassett's Cricket Ranch (Visalia, CA, USA). All nematodes were cultured in parallel to avoid differential age effects. However, different EPN strains were always handled separately at different times in the workspace to avoid cross-contamination. For rearing, 10-20 wax moth larvae were placed in 9 cm diameter petri dishes lined with a single layer of Whatman No. 1 filter paper and inoculated with 1000-2000 IJs in 1 ml of water with 100-200 IJs/larva. The petri dishes were incubated at room temperature (~22°C) for 4-5 days. Once all wax moth larvae had died from EPN infection, the cadavers were rinsed in water and transferred to White traps according to the protocol described by Kaya and Stock (1997). White traps were incubated at room temperature (~22°C) and observed daily for IJ emergence. Once IJs emerged from the cadavers, they were collected from the White traps every two days with a pipette, washed 2-3 times and

resuspended in fresh tap water. The collected IJs were stored at 8°C in tissue culture flasks and were used within 10 days of collection.

Efficacy of EPNs against medium sized *L. californicus* larvae (Laboratory concentration response assay)

One oz. (~30 ml) plastic cups were filled with 25 g of soil (surface area: 28 cm^2). The soil used was sandy loam soil (78% sand, 12% silt and 10% clay, pH 7.7, and 1.4% organic matter). The soil is typical of the region and was collected locally. Before use, the soil was sterilized in an autoclave at 121°C and 16 psi for 1hr and left at room temperature for at least two weeks for acclimatization. In each cup, a single wireworm larva was placed with four to five soaked wheat seeds as food. Any larvae that did not enter the soil within 12 hrs were replaced. Four concentrations [5600 IJs (200 IJs/cm^2), 2800 IJs (100 IJs/cm^2), 1400 IJs (50 IJs/cm^2), and 700 IJs (25 IJs/cm^2)] were tested in the bioassay for all ten EPN strains. The concentrations were prepared by counting out the desired number of IJs into 100 µl to 1 ml of tap water (depending on the concentration) in a nematode counting slide (Chalex, LLC, Park City, UT, USA) under a compound microscope by following the formula of Navon and Ascher (2000). Different concentrations of EPNs were collected in tissue culture flasks at 8°C in an incubator.

Before application, EPNs were transferred from 8°C to room temperature for 12 h for acclimatization. The viability of IJs was checked under the microscope before application. The IJs were pipetted onto the soil surface in 1 ml of IJs suspension, and the control cups received one ml of tap water without any IJs. The final soil moisture was adjusted to 15% (v/w) that may be prevalent in Montana soils during EPN application. There were 10 replications for each of the four concentrations for all 10 EPN strains. The

bioassay was repeated five times, on various dates from May to August 2018. The cups were placed in trays with approximately 10 holes in the lids for aeration and then placed in an incubator at 22°C and 75% RH in the dark. 22°C temperature in the bioassay is an average temperature during the wireworm activity in the field and can be targeted for EPN application. Larval mortality was observed daily for a month and cumulative mortality at weekly intervals were analyzed and presented.

From the laboratory bioassay explained above, dead larvae found during daily observations were collected and rinsed with water, and then transferred to individual petri dishes (diameter 5 cm) lined with moist filter paper for 2-3 days to allow the EPNs to reproduce. The penetration rate of different EPNs tested was also determined as the number of IJs found inside each wireworm larva. To measure penetration, dead larvae were dissected with a scalpel in a mixture of water and Ringer's solution (50:50) in a petri dish (9 cm diameter). The contents were poured into a nematode counting slide and the number of adult nematodes was counted. Overall, 20 dead wireworm larvae were observed for each of the four concentrations for all the EPN strains from the laboratory bioassay except there were only 10 infected larvae available in the case of 700 IJs/ml concentration of Sr 17c+e strain.

Efficacy of selected EPN strains against *L. californicus* larvae in shade house trial

Six EPN strains were selected based on their efficacy in the laboratory bioassay and tested against medium- sized wireworm larvae in a shade house experiment. The experiment was conducted in a completely randomized block design. There were 16 replicates (pots) of two concentrations for each of the six EPN strains. Plastic pots (16 cm

diameter) were filled with sterilized sandy loam field soil (depth: 12 cm high) with a surface area of 200 cm^2. The soil was 78% sand, 12% silt, and 10% clay, with pH 7.7 and 1.4% organic matter. Approximately 2.2 kg of soil was added to each pot. Two experiments were conducted, one from July to August 2018 and a second from May to August 2019.

In the 2018 experiment, 8-10 wheat seeds were sown in each pot. The plants were allowed to grow for three weeks and were watered daily to maintain the moisture in the pots. Two concentrations [60,000 IJs/pot (300 IJs/cm^2) and 15,000 IJs/pot (75 IJs/cm^2)] were prepared by following the same procedure as explained in section 2.3. These two concentrations were standardized as 6000 IJs/ml and 1500 IJs/ml of tap water and stored in the tissue culture flasks and used within 10 days. Once the plants were three weeks old, five wireworm larvae were released in each pot. After five hours, larvae that did not enter the soil were replaced. After 24 hours, EPNs IJs were inoculated into the pots with the help of a pipette in 10 ml of water. The pots with the control treatment received 10 ml of plain water. The pots were placed in a shade house and watered daily. Four of the 16 replications were destructively sampled weekly for four weeks and larval mortality was determined. The dead larvae were dissected to confirm nematode infection. The wireworm was counted as dead, if not found because the larvae decompose within three weeks and are difficult to find (Ensafi et al., 2018). Soil temperature and moisture were observed three times each week throughout the experiment using a soil moisture meter (Spectrum Technologies Inc., Illinois, USA) and soil thermometer (Taylor, Illinois, USA). The average daily air temperature in the shade house was 21°C (5-40°C). The average soil temperature and moisture were recorded as 15°C (7-24°C) and 22 ± 5%, respectively. After observing

wireworm mortality each week, soil (300 g) from all the pots was checked for IJs by inoculating with five wax moth larvae in 500 ml deli plastic cups. All the soil samples showed wax moth larval infection, indicating the presence of IJs after four weeks.

The 2019 experiment was conducted in a completely randomized block design, with four EPN strains (*S. carpocapsae* All and Cxrd strains and *S. riobrave* 355 and 7-12 strains) based on their efficacy in the shade house experiment in 2018. The experiment protocol was the same as 2018 with below mentioned minor changes. Plastic pots (16 cm diameter) were filled with approximately 2.4 kg of sterilized field-collected sandy loam soil (depth: 14 cm) with a surface area of 200 cm^2. The source of the soil was the same as that used in the experiment in 2018. Ten wheat seeds were planted in each pot and allowed to grow for 10 days. Ten wireworms were added to each pot after 10 days. After a further 24 hours, any larvae that did not enter the soil were replaced. Two concentrations [80,000 IJs/pot (400 IJs/cm^2) and 10,000 IJs/pot (50 IJs/cm^2)] were used for each of the four strains, following the procedure explained in section 2.3. These two concentrations were standardized as 4000 IJs/ml and 500 IJs/ml of tap water. The IJs, in 20 ml of water were inoculated into the pots with a pipette. The pots with the control treatment received 20 ml of plain water. There were five replicates (pots) for each concentration. The pots were placed in a shade house and watered daily. After 4 weeks, the pots were destructively sampled, and the number of dead wireworm larvae observed. The dead larvae were dissected to confirm nematode infection. If a wireworm was not found, it was recorded as dead. The average air temperature in the shade house was 30°C (26-32°C) with average soil temperature and soil moisture in the pots of 20°C (11-35°C) and 21 ± 5%, respectively.

The whole experiment was conducted in two trials with an interval of 10 days in both trials. Plant damage, i.e. number of wheat seedlings damaged by wireworms, was observed in each pot at the end of the experiment and the average percentage of plant damage was recorded. The presence of wilted or dead central leaf and/or seedling death was the main criteria in observing plant damage.

Statistical analysis

All statistical analyses were performed in the R statistical language (version 3.5.2, R Core Team 2020). A Generalized Linear Model (GLM) with a binomial distribution (binary responses) and logit link function was used to analyze the data for the laboratory bioassay. Logit regression analysis was used to calculate LC_{50} values for the laboratory bioassay using the theoretical model: $y_i \sim Binomial\ (\pi_i, 10)$ with

$$logit(\pi_i|Strain, Concentration)=\beta_0 + \beta_1 Concentration_i + \beta_2 I.HbVS_i + \beta_3 I.Hfk22_i + ... + \beta_{11} Concentration_i * I.HbVS_i + \beta_{12} Concentration_i * I.Hfk22_i + ...$$

where y_i is the count of dead wireworms; π_i is the probability of mortality and modeled as a function of concentration and strain; I is the indicator variable for the particular strain; and HbVS is *H. bacteriophora* VS strain. This indicator is 1 when a mortality count is recorded from one specific strain and is 0 otherwise. Indicator variables for all other strains operate similarly. The data on the number of IJs penetrated inside the wireworm larvae in the laboratory bioassay were subjected to ANOVA with the presentation of data on the original scale. GLM with quasibinomial distribution was used for shade house experiments in 2018 and 2019 to avoid the overdispersion problem when wireworm mortality was the response variable and GLM with binomial distribution was used when plant damage was

the response variable. The trial, EPN strains, concentration and interactions among them were the three predictor variables in different models. Data from two trials in 2019 were analyzed separately as significant differences were detected between trials for probability of plant damage. The models were simplified using stepwise removal of predictors with no significant effect (P>0.05) and the lack of fitness test was performed to compare the models. As the model was formulated in terms of transformed probabilities (logit transformation to the log-odds scale), the result was back transformed to an original probability scale after analysis for graphical representation. If significant differences were observed among treatments, Tukey-Kramer test ($\alpha = 0.05$) was used for pairwise comparisons.

Results

Efficacy of EPN species against medium sized *L. californicus* larvae in summer 2018 (Laboratory concentration response assay)

The data from the laboratory bioassay were pooled for trials as no significant interaction was detected between strain, concentration, time and trials (P>0.05). Since the EPN strains, *S. feltiae* SN, *H. bacteriophora* HP88, *H. floridensis* K22, and *H. georgiana* Kesha were the least virulent against wireworms, causing <30% mortality on average (Table 3) even at the highest concentration (5600 IJs/larva) are not being included in the analysis. There was no mortality in the control treatment and therefore was not included in the model. No three-way interaction was found between EPN strain, concentration, and time to mortality ($\chi^2 = 25.18$, df = 45, P=0.99). There were significant differences among EPN strains ($\chi^2 = 157.61$, df = 5, P <0.0001), concentrations ($\chi^2 = 124.75$, df = 3, P

<0.0001), and time (χ^2 = 363.19, df = 3, P <0.0001) with respect to wireworm larval mortality. The interaction between EPN strain and concentration (χ^2 = 73.64, df = 15, P<0.0001) and EPN strain and time (χ^2 = 76.33, df = 15, P<0.0001) on mortality was also significant. However, the interaction between concentration and time on wireworm larval mortality was not significant (χ^2 = 3.60, df = 9, P=0.94).

On average, wireworm larval mortality increased with higher nematode concentrations, with significantly higher mortality caused by strains at 5600 IJs/larva (P<0.006). However, two concentrations (1400 IJs and 2800 IJs) did not differ significantly in terms of wireworm mortality (P=0.99). The wireworm mortality at 7 days after treatment (DAT) was significantly lower than mortality at 14, 21, and 28DAT (P<0.001; Fig. 1). However, no significant differences were detected among 14 and 21DAT (P=0.28) and between 21 and 28DAT (P=0.64) for wireworm mortality (Fig. 1B, C, and D). In addition, 14 and 28DAT also differed significantly (P=0.02) in terms of wireworm mortality. The mortality increased gradually over time with even the best performing species taking at least 14 DAT (days after treatment) to cause 50% mortality or higher. Only *Steinernema riobrave* 355 and 7-12 strains were able to cause 50% larval mortality after 14 DAT at 1400, 2800, and 5600 IJs/larva concentrations (Fig. 1B). However, the mortality reached 50% at 700 IJs/larva for *S. carpocapsae* Cxrd and at 5600 IJs/larva concentration for both *S. carpocapsae* All and *H. bacteriophora* VS strains only at 21DAT (Fig. 1C). For *S. rarum* 17c+e mortality did not reach 50% until 28DAT and that too when applied at the rate of 5600 IJs/larva.

Overall, after 7 days of treatment *S. riobrave* 7-12 and *S. riobrave* 355 caused significantly higher *L. californicus* mortality (21-28±5%) as compared to only 5-7±1% mortality in other tested strains at 1400 IJs/larva concentration (Fig. 1A). However, *H. bacteriophora* VS and *S. rarum* 17c+e strains caused significantly lowest mortality (1-3±0.5%) when applied at the rate of 700 IJs/larva. Similar trend was seen at 2800 IJs/larva concentration, where *S. riobrave* 7-12 and *S. riobrave* 355 strains caused significantly higher mortality (26-31±5%) than *H. bacteriophora* VS strain that caused only 5±1% wireworm mortality. At 7 DAT, only *S. riobrave* 7-12 strain was significantly better at causing 35±7% mortality in *L. californicus* when applied at the rate of 5600 IJs/larvae. However, *S. riobrave* 355 and *H. bacteriophora* VS strain did not differ significantly from *S. riobrave* 7-12 strain (Fig. 1A).

There were no significant differences among all the six tested strains, and they caused 30-58±2% *L. californicus* mortality after 14 days at the rate of 5600 IJs/larva (Fig. 1B). At 1400 and 2800 IJs/larva concentration, *S. riobrave* 355 and 7-12 strains were responsible for the higher mortality depending on the concentration (50-51% mortality at 1400 IJs and 40-57% mortality at 2800 IJs). *Steinernema riobrave* 7-12 caused significantly higher mortality (43±1.6%) than *S. carpocapsae* Cxrd (15±1.5%) and *S. rarum* 17c+e (8±1.3%) at 700 IJs/larva. Similarly, *S. riobrave* 7-12 strain killed significantly higher wireworms (51±0.5%) than *S. carpocapsae* All (17±2%) and *S. rarum* 17c+e (16±1.8%) at 1400 IJs/larva concentration after 14 days of application (Fig. 1B).

Similarly, at 21DAT, all the six EPN strains did not differ significantly and caused up to 48-65±1.0% wireworm mortality when 5600 IJs/larva concentration was tested (Fig.

1C). When EPNs were applied at the rate of 700 IJs/larva, significantly highest *L. californicus* mortality was observed in case of *S. carpocapsae* Cxrd (51±5%), *S. riobrave* 7-12 (50±0%), and *S. carpocapsae* All (40±2%) strains as compared to only 19±0.5% mortality caused by *S. rarum* 17c+e strain. *Heterorhabditis bacteriophora* VS and *S. riobrave* 355 strains were able to cause moderate level of mortality (29-32±1.0%) in *L. californicus* larvae at 700 IJs/larvae concentration (Fig. 1C). *Steinernema riobrave* 355 caused significantly higher mortality (60-64±0.7%) than *S. rarum* 17c+e strain with only 30% mortality but did not differ significantly from *S. carpocapsae* All and Cxrd, *S. riobrave* 7-12 and *H. bacteriophora* VS when tested at 1400 and 2800 IJs/larva (Fig. 1C).

The trend for significant differences among EPN strains at 28DAT was similar to 21DAT (Figs. 1C and D). The only difference was the increase in the mortality in all the EPN strains. The wireworm mortality increased and reached 70-74% in *S. carpocapsae* All and Cxrd, and *S. riobrave* 7-12 strains at 28DAT when applied at the rate of 5600IJs/larva. *Steinernema carpocapsae* Cxrd strain was able to kill 75±0.37% larvae when applied at the rate of 700 IJs/larva as compared to 70±0.9% mortality caused by this strain at the rate of 5600 IJs/larva (Fig. 4).

The LC_{50} limits for different strains are presented in Table 2 (not included in original publication). The LC_{50} values overlapped among different EPN strains at all the time periods. However, LC_{50} limits did not overlap and varied significantly for *S. carpocapsae* All, *S. carpocapsae* Cxrd, and *H. bacteriophora* VS strains when compared between one week and four weeks of treatment. *Steinernema riobrave* 7-12 had the lowest LC_{50} of 4190 IJs/larva at one week after treatment followed by *S. riobrave* 355 strain with

LC_{50} of 4850 IJs/larva. However, *Steinernema carpocapsae* Cxrd and *S. carpocapsae* All were not able to cause 50% *L. californicus* larval mortality within two weeks after treatment, therefore LC_{50} limits were very high. The virulence of all the strains increased with time as shown from the LC_{50} limits. After three weeks of EPN application, *Steinernema carpocapsae* Cxrd strain caused >50% mortality with LC_{50} limit of 2180 IJs/larva followed by *S. riobrave* 7-12 with LC_{50} of 2270 IJs/larva. Similarly, *S. carpocapsae* Cxrd strain had the lowest LC_{50} of 1310 IJs/larva followed by 1840 IIJs/larva and 2070 IJs/larva due to *S. carpocapsae* All strain and *S. riobrave* 7-12 strain, respectively. Meanwhile, the *S. riobrave* 355 strain was also found to be moderately virulent against wireworm larvae with LC_{50} limit of 2880 IJs/larva and 2490 IJs/larva after three and four weeks, respectively (Table 2).

The penetration rate of *S. carpocapsae* All, *S. carpocapsae* Cxrd, *S. riobrave* 7-12, *H. bacteriophora* VS, *S. riobrave* 355, and *S. rarum* 17c+e into the larvae of *L. californicus* was compared at all four concentrations tested in the laboratory bioassay (Fig. 2). The number of IJs that penetrated into the wireworm larvae causing death differed significantly among EPN strains ($F_{5,446}$ = 7.77, P <0.0001) and concentrations ($F_{3,446}$ = 61.45, *P* <0.0001). The interaction between EPN strains and concentration on penetration rate was also significant ($F_{15,446}$ = 4.40, P <0.0001). *Steinernema riobrave* 7-12 strain showed significantly highest penetration (409.45±22.76 IJs) followed by 145-200 IJs penetrated in case of all the other strains when applied at 1400 IJs/larva concentration. *Steinernema carpocapsae* All strain showed significantly lowest IJs penetration (198.90±17.06 IJs) as compared to other five strains tested (263-389 IJs). However, EPN strains did not differ

significantly in terms of penetration rate at 700 and 5600 IJs/larva with average penetration of 168.45 to 231.60 IJs and 361.15 to 522.95 IJs/larva, respectively (Fig. 2). In addition, the penetration rate in all the strains was very low irrespective of the applied concentration. Overall, the penetration rate varied from 24.06-33.09%, 10.39-29.25%, 7.10-13.92%, and 6.40-9.34% for concentrations 700, 1400, 2800, and 5600 IJs, respectively (Fig. 2).

Efficacy of selected EPN strains against *L. californicus* larvae under shade house/potted plant conditions

In 2018 shade house experiment, three-way interaction between EPN strain, concentration, and time to death was not significant (χ^2 = 18.66, df = 18, P = 0.41). No interaction between EPN strains and concentration (χ^2 = 2.58, df = 6, P = 0.86) and between concentration and time (χ^2 = 4.86, df = 3, P = 0.18) on wireworm larval mortality was detected. Similarly, concentrations did not have significant effect on the wireworm mortality (χ^2 = 1.49, df = 1, P = 0. 22), therefore data for two concentrations were combined together for further analysis.

There were significant differences among EPN strains (χ^2 = χ^2 = 32.04, df = 6, *P* <0.0001), and time after treatment (χ^2 = 70.14, df = 3, *P* <0.0001) with respect to wireworm larval mortality (Fig. 3). However, there was no interaction detected between EPN strain and time on mortality (χ^2 = 27.92, df = 18, *P* = 0.06). Wireworm mortality at 7 DAT differed significantly from 14, 21 and 28 DAT (P<0.001). However, the mortality was not significantly different among 14 and 21 days after treatment (P=0.11) and among 21 and 28DAT (P=0.19). In all the treatments, mortality increased gradually over time and virulent species took at least three weeks to cause 50% or more mortality (Fig. 3). There was no mortality observed in the control treatments. *Steinernema carpocapsae* (All and Cxrd

strains), and *S. riobrave* (355 and 7-12 strains) caused significantly higher mortality (45-50% for *S. riobrave* 355 and 7-12 at 21DAT and 40-45% for *S. carpocapsae* All and Cxrd at 28DAT) than control treatments ($P<0.02$). *Heterorhabditis bacteriophora* VS and *S. rarum* 17c+e strains did not differ significantly from control in terms of wireworm mortality ($P>0.05$). These two strains caused up to 27-32% at 28DAT (Fig. 3).

In the 2019 shade house experiment, wireworm mortality proportions differed significantly between the two trials ($\chi^2 = 26.37$, df = 1, $P < 0.0001$), but interaction between trial and strain was not significant ($\chi^2 = 2.795$, df = 4, $P=0.59$) and therefore the data for two trials were pooled for further analysis. EPN strains had a significant impact on wireworm mortality ($\chi^2 = 91.89$, df = 4, $P < 0.0001$). Wireworm mortality also differed significantly among 80,000 IJs and 10,000 IJs/pot concentrations ($\chi^2 = 8.43$, df = 1, $P = 0.004$). However, there was moderate level of interaction between EPN strain and concentration ($\chi^2 = 9.36$, df = 4, $P = 0.052$). Overall, all four EPN strains tested caused significantly higher mortality than control ($P<0.001$) (Fig. 4). The wireworm mortality due to EPN strains ranged from 34 to 56% when applied at 80,000 IJs/pot with 56% and 50% mortality caused by *S. riobrave* 355 and *S. carpocapsae* All strains, respectively. The mortality percentage was 35-40% when EPNs were applied at the rate of 10,000 IJs/pot. However, the wireworm mortality was significantly lower (13%) in control (Fig. 4). When applied at the rate of 80,000 IJs/pot, *Steinernema riobrave* 355 caused significantly higher wireworm mortality (56±4.7%) than only 34±5.9% caused by *S. riobrave* 7-12 strain ($P=0.013$). In addition, significant differences between concentrations were observed especially in case of *S. riobrave* 355 strain ($P=0.022$) that caused 56±4.7%% mortality and

35±4.6% mortality when applied the rate of 80,000 IJs/pot and 10,000 IJs/pot, respectively (Fig. 4).

The two trials in 2019 differed significantly in regards to the rate of plant damage ($\chi^2 = 22.99$, df = 1, P <0.0001), and were therefore analyzed separately. In trial one, plant damage was not significantly affected by EPN strain ($\chi^2 = 7.42$, df = 4, P = 0.12) or concentration ($\chi^2 = 1.02$, df = 1, P = 0.312). No significant interaction was found between EPN strain and concentration ($\chi^2 = 1.04$, df = 4, P = 0.904). None of the four EPN strains tested differed significantly from that of the control, all being in the range of 34-52%, the highest level (52%) being caused when *S. carpocapsae* Cxrd was applied (Fig. 5A). However, in trial two, EPN strain and concentration both had significant effects on plant damage (EPN strain: $\chi^2 = 19.29$, df = 4, P = 0.0007; concentration: $\chi^2 = 4.29$, df = 1, P = 0.038). In addition, a significant interaction was observed between EPN strain and concentration ($\chi^2 = 10.69$, df = 4, P = 0.030). *Steinernema carpocapsae* All strain was associated with significantly lower plant damage (6±4%) at 80,000 IJs/pot as compared to 44±16% damage associated with *S. riobrave* 7-12 at 10,000 IJs/pot concentration (P<0.01) and 40±7% plant damage in control treatment (P<0.013) (Fig. 5B). EPN strains *S. carpocapsae* Cxrd and *S. riobrave* 355 strains did not differ significantly and moderate level of plant damage i.e. 18-22% was observed in these treatments (P>0.05) (Fig. 5B).

Discussion

We found that the wireworm *L. californicus* is susceptible to different EPNs and virulence varied by both EPN species and strain. The steinernematids we tested were more virulent to *L. californicus* than the heterorhabditids. Of the ten EPNs tested, three strains -

S. carpocapsae All, *S. carpocapsae* Cxrd, *S. riobrave* 355, and *S. riobrave* 7–12 strains were the most virulent to *L. californicus*, in both laboratory and shade house studies. However, these ten strains (across various concentrations) showed a wide variation in their infectivity to *L. californicus*, with effective strains causing about 50–70% mortality in the laboratory bioassay but only 30–50% mortality under the shade house conditions.

The high infectivity of the *S. carpocapsae* strains tested in this study agrees with Ansari et al. (2009) and Morton and Garcia-del-Pino (2016) who reported 50 and 50–75% mortality in *Agriotes* spp., respectively due to *S. carpocapsae*. In contrast, Toba et al. (1983) and Ensafi et al. (2018) observed poor control of the sugarbeet wireworm (*L. californicus*) by *S. carpocapsae*. Similar results have been found by different researchers for other wireworm species as well. Morris (1985), Campos- Herrera and Gutiérrez (2009), and Arrington et al. (2015) also observed little to no susceptibility of *S. carpocapsae* against *Ctenicera destructor* Brown, *A. sordidus*, and *Conoderus vespertinus* Fabricius, respectively. Indeed *S. carpocapsae* did not infect living *Agriotes* sp. wireworms (San-Blas et al., 2012). The ability of *S. riobrave* to cause at least 50% *L. californicus* mortality in the laboratory and the shade house in the current study is the first report where this has been observed and suggests that *S. riobrave* can be a potential tool for managing *L. californicus* populations.

Four EPN strains (*S. feltiae* SN, *H. bacteriophora* HP88, *H. georgiana* Kesha, and *H. floridensis* K22) were unable to cause more than 30% average larval mortality in the laboratory bioassay. The non-infectivity we observed of *S. feltiae* against *L. californicus* is in agreement with Ansari et al. (2009) who observed no mortality of *A. lineatus* with a

commercial strain of *S. feltiae*. Other studies also reported little to no mortality due to *S. feltiae* in various species of wireworms, such as the barley wireworm (*Agriotes fuscicollis* Miwa) (Zhao et al., 1996), the lined click beetle (*A. sordidus* Illiger) (Campos-Herrera and Gutiérrez, 2009), and other *Agriotes* spp. (Ester and Huiting, 2007). However, in contrast, Toba et al. (1983) reported almost 50% mortality of *L. californicus* by *S. feltiae* in a laboratory bioassay. Similarly, the low level of infectivity by *H. bacteriophora* strains in *L. californicus* that we found in the present study is in agreement with Morton and Garcia-del-Pino (2016), who reported only 26–35% mortality in *A. obscurus* in the laboratory. However, Ansari et al. (2009) found 67% *A. lineatus* larval mortality after *H. bacteriophora* application in laboratory. Interestingly, most of the wireworm species involved in these studies were European wireworm species, and EPN infectivity to North American species need to be explored that might differ from the available literature. These variations in results might be due to the different wireworm species tested or the difference in the size of wireworm larvae used in different studies. Late instar wireworm larvae are hard-bodied as compared to early instars, making them less susceptible to EPN penetration and infection. This has been observed in *Diaprepes abbreviatus* L. (Coleoptera: Curculionidae) where older larvae were less susceptible to EPNs as compared to young larvae (Shapiro et al., 1999).

EPN concentrations (per *L. californicus* larva) used in our study were intentionally very high to achieve high wireworm mortality. The main reason for the use of relatively high nematode concentrations was the low mortality of wireworms when lower concentrations were used in preliminary bioassays. Some previous studies have reported

high concentrations to attain significant control of wireworm populations (Danilov, 1974; Toba et al., 1983; Ensafi et al., 2018). These results indicate that both the concentration used and time taken by EPNs to infect hosts are the principal factors affecting EPN efficacy against different insects. Several other factors affecting the efficacy of EPNs are already reported such as developmental and immature stages of insect, nematode species or strain, and environmental variables such as temperature, aeration, moisture, soil type, food availability, and exposure time (Lewis et al., 2006; Kabaluk and Ericsson, 2007; Shapiro-Ilan et al., 2012; Shapiro-Ilan et al., 2017). Soil characteristics such as texture, moisture, and pH plays an important role in EPN efficacy. In general, EPN has been observed to have higher efficacy in sandy soils (El-Borai et al., 2012). However, some other studies reported high efficacy of EPNs in clay soil than sandier soils (Shapiro-Ilan et al., 2000; Toledo et al., 2009; Toepfer et al., 2010). Ensafi et al. (2018) reported more wheat seedling protection from *L. californicus* in sand dominant media than peat dominant media after application of *S. carpocapsae*.

There was no significant variation among different EPN strains in regards to plant damage due to wireworm feeding. In the first trial, the percentage of plant wireworm damage in EPN-treated pots was not significantly different from the control. In contrast, the highest plant damage was observed in control pots in the second trial. As mentioned earlier, EPNs take at least two weeks to infect wireworms, so it is possible that wireworms damaged the plants in the weeks before EPNs could infect them. After two weeks, the wireworms stopped feeding because of infection by EPNs. Insects have been observed using the behavioral defense of avoiding EPN contaminated areas (Wilson-Rich et al.,

2007). The high plant damage in EPN treated pots may also be due to the size and age of the medium-sized larvae used in the study, as old larvae are difficult for IJs to penetrate.

Although, use of different EPN strains against *L. californicus* in laboratory and shade house trials seems promising, further study of these nematodes under field conditions is required as there might be differences in the virulence of these EPNs depending on different biotic and abiotic factors present in the field. According to Gaugler (1988), even though nematodes often infect a broad range of insects in laboratory tests, but they usually have a more restricted host range in nature. In Montana, both dryland and irrigated farming practices are followed which can affect the EPN efficacy against wireworms. We are conducting further field studies under both dryland and irrigated spring wheat and barley fields to determine the actual potential of EPN species against wireworms.

In conclusion, *S. carpocapsae* and *S. riobrave* have potential to be used as biological control agents against *L. californicus* in spring wheat when a high concentration is used (> 200 IJs/cm2). However, it is logical to presume that the efficacy of these EPN strains will vary when used for other wireworm species. Different soil types and with different species of wireworms should be examined to understand the impact of EPNs in spring wheat in the Northern Great Plains. In addition, while the focus of EPN infection is to attain wireworm mortality over time, it would be interesting to study if EPNs have any sublethal effect(s) on wireworm physiology, behavior, and feeding. This will enhance our understanding of entomopathogenic nematodes ecology and their overall efficacy against wireworms.

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Table 1. List of entomopathogenic nematodes strains used in experiments.

Entomopathogenic nematode species	**Strain**
Steinernema carpocapsae	All and Cxrd
Steinernema feltiae	SN strain
Steinernema riobrave	355 and 7-12
Steinernema rarum	17c+e
Heterorhabditis bacteriophora	HP88 and HbVS
Heterorhabditis floridensis	K22
Heterorhabditis georgiana	Kesha

Table 2. Lethal dose (LC_{50}/larva) with confidence limits for EPN strains on *Limonius californicus* in laboratory bioassay

EPN strain	**LC_{50} (95% CI)**			
	7 DAT	**14 DAT**	**21 DAT**	**28 DAT**
ScAll	9080 (3810-14350)	5470 (3250-7690)	2920 (1840-3990)	1840 (880-2810)
ScCxrd	8770 (4310-13220)	5030 (3240-6820)	2180 (820-3530)	1310 (60-2560)
Sr 7-12	4190 (2640-5740)	3150 (1790-4510)	2270 (1210-3320)	2070 (1030-3110)
Sr 355	4850 (2870-6820)	3600 (2080-5120)	2880 (1440-4310)	2490 (1190-3790)
HbVS	6250 (4780-7772)	4280 (2990-5570)	3290 (2220-4360)	2990 (1940-4030)
Sr 17c+e	8930 (4540-13330)	6050 (3810-8290)	4840 (3080-6610)	3960 (2660-5260)

Where ScAll = *Steinernema carpocapsae* All; ScCxrd = *Steinernema carpocapsae* Cxrd; Sr17c+e = *Steinernema rarum* 17c+e; HbVS = *Heterorhabditis bacteriophora* VS; Sr355 = *Steinernema riobrave* 355; Sr7-12 = *Steinernema riobrave* 7-12. DAT=Days after treatment.

Table 3. Average percentage *Limonius californicus* mortality caused by least effective EPN strains in laboratory bioassay

EPN strain	700 IJs (25 IJs/cm^2)				1400 IJs (50 IJs/cm^2)				2100 IJs (100 IJs/cm^2)				2800 IJs (200 IJs/cm^2)			
	7 DAT	14 DAT	21 DAT	28 DAT	7 DAT	14 DAT	21 DAT	28 DAT	7 DAT	14 DAT	21 DAT	28 DAT	7 DAT	14 DAT	21 DAT	28 DAT
Kesha	2.86 ± 0.98	16.00 ± 1.14	23.14 ± 1.06	26.00 ± 0.00	1.43 ± 0.53	7.43 ± 0.88	18.29 ± 1.24	22.00 ± 0.00	2.00 ± 1.07	20.00 ± 2.80	34.00 ± 0.00	34.00 ± 0.00	8.57 ± 2.64	26.29 ± 1.78	37.14 ± 0.79	45.43 ± 0.78
HP88	2.00 ± 0.40	4.57 ± 0.34	7.43 ± 0.34	8.00 ± 0.00	3.71 ± 0.26	8.00 ± 1.07	18.57 ± 1.32	23.14 ± 0.37	0.29 ± 0.26	8.00 ± 0.40	12.86 ± 0.68	14.00 ± 0.00	6.86 ± 1.45	17.14 ± 1.20	31.43 ± 1.79	36.00 ± 0.00
K22	0.57 ± 0.34	3.71 ± 0.63	6.57 ± 0.34	8.00 ± 0.00	6.00 ± 1.62	14.86 ± 0.79	19.71 ± 0.63	22.29 ± 0.26	0.29 ± 0.26	6.57 ± 0.97	10.57 ± 0.34	12.86 ± 0.37	11.43 ± 1.84	20.86 ± 0.55	27.71 ± 0.75	32.86 ± 0.55
SN	1.43 ± 0.34	3.14 ± 0.68	6.00 ± 0.00	8.00 ± 0.00	6.00 ± 0.81	11.14 ± 0.55	21.71 ± 1.02	28.57 ± 0.67	3.43 ± 1.12	10.00 ± 1.07	22.86 ± 1.10	28.00 ± 0.70	5.71 ± 1.24	14.00 ± 0.81	22.00 ± 0.57	24.00 ± 0.00

Where, Kesha = *Heterorhabditis georgiana* Kesha All; HP88 = *Heterorhabditis bacteriophora* HP88; K22 = *Heterorhabditis floridensis* K22; SN = *Steinernema feltiae* SN.

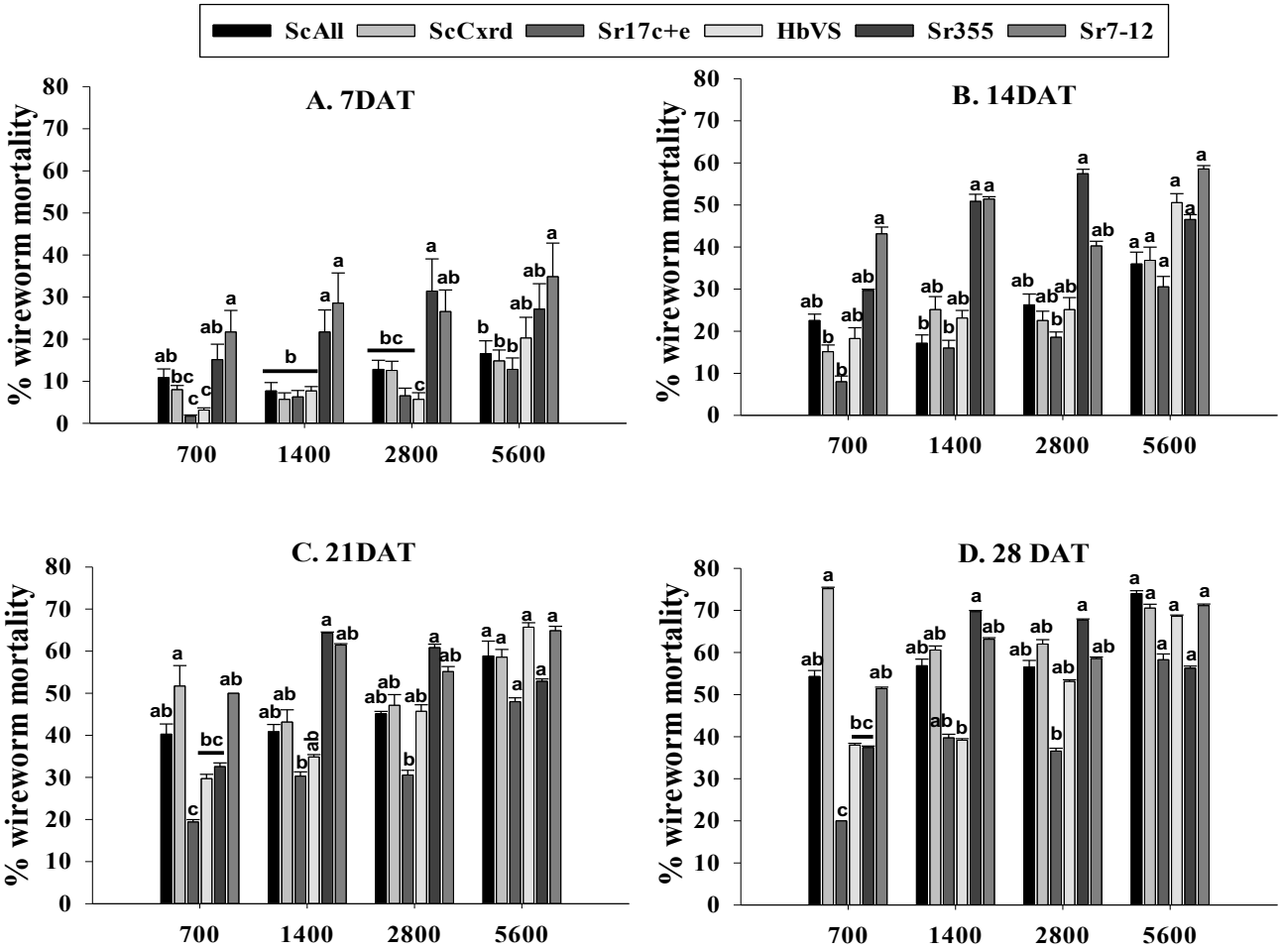


Figure 1. Average percentage mortality of larval *Limonius californicus* after exposure to entomopathogenic nematodes at the rate of 700, 1400, 2800, and 5600 Infective juveniles/larva concentrations at (A) 7DAT, (B) 14DAT, (C) 21DAT, and (D) 28DAT. ScAll = *Steinernema carpocapsae* All; ScCxrd = *Steinernema carpocapsae* Cxrd; Sr17c + e = *Steinernema rarum* 17c + e; HbVS = *Heterorhabditis bacteriophora* VS; Sr355 = *Steinernema riobrave* 355; Sr7-12 = *Steinernema riobrave* 7–12. Different letters above bars indicate statistical significance among strains at different concentrations ($P \leq 0.05$, Tukey-Kramer test); DAT = Days after Treatment; No larval mortality was observed in the control.

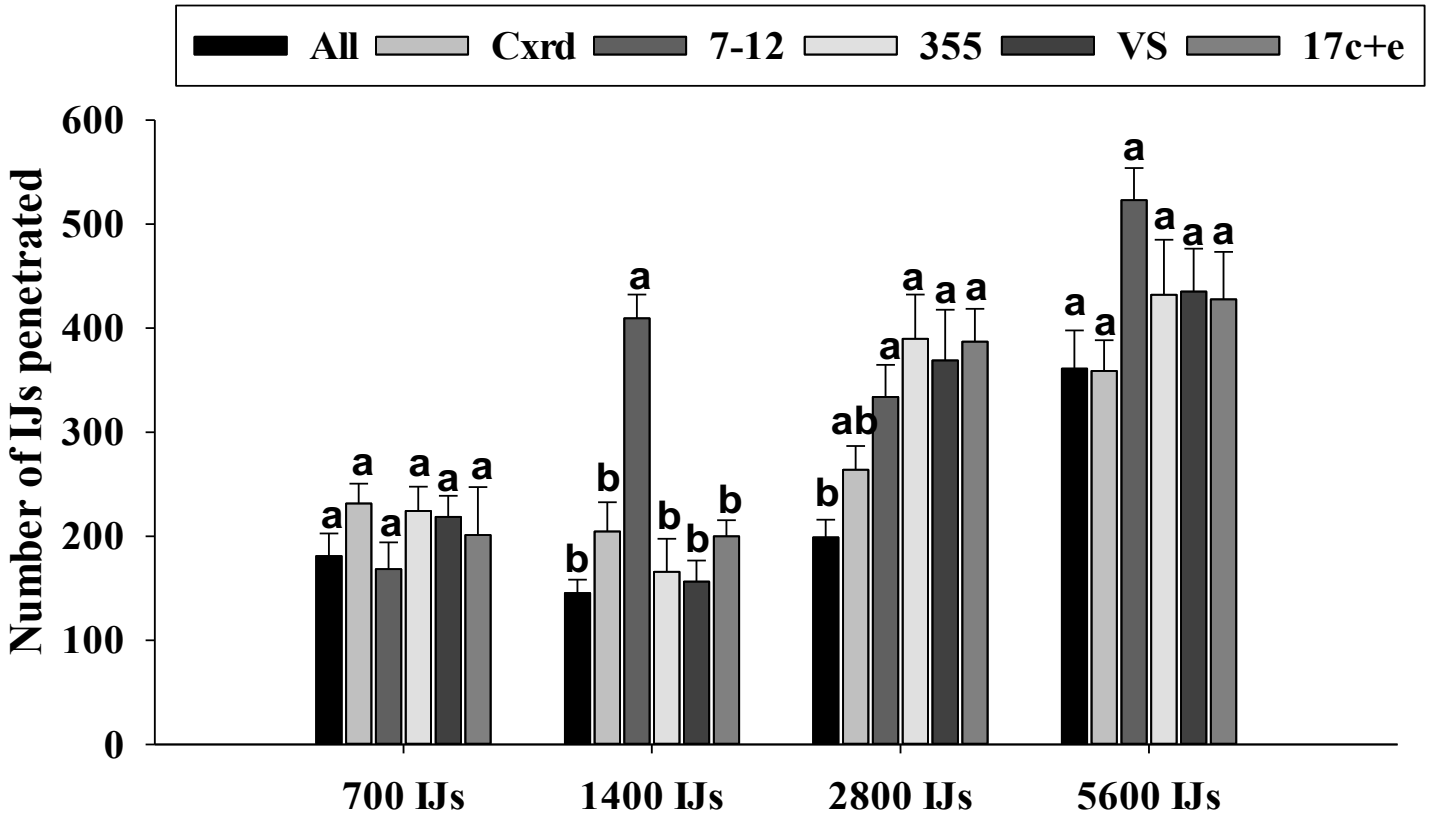


Figure 2. Average number of Infective Juveniles that penetrated *Limonius californicus* larvae in laboratory bioassay; All = *Steinernema carpocapsae* All; Cxrd = *Steinernema carpocapsae* Cxrd; 17c + e = *Steinernema rarum* 17c + e; VS = *Heterorhabditis bacteriophora* VS; 355 = *Steinernema riobrave* 355; 7–12 = *Steinernema riobrave* 7–12. Different letters above bars indicate statistical significance ($P \leq 0.05$, Tukey-Kramer test); DAT = Days after treatment.

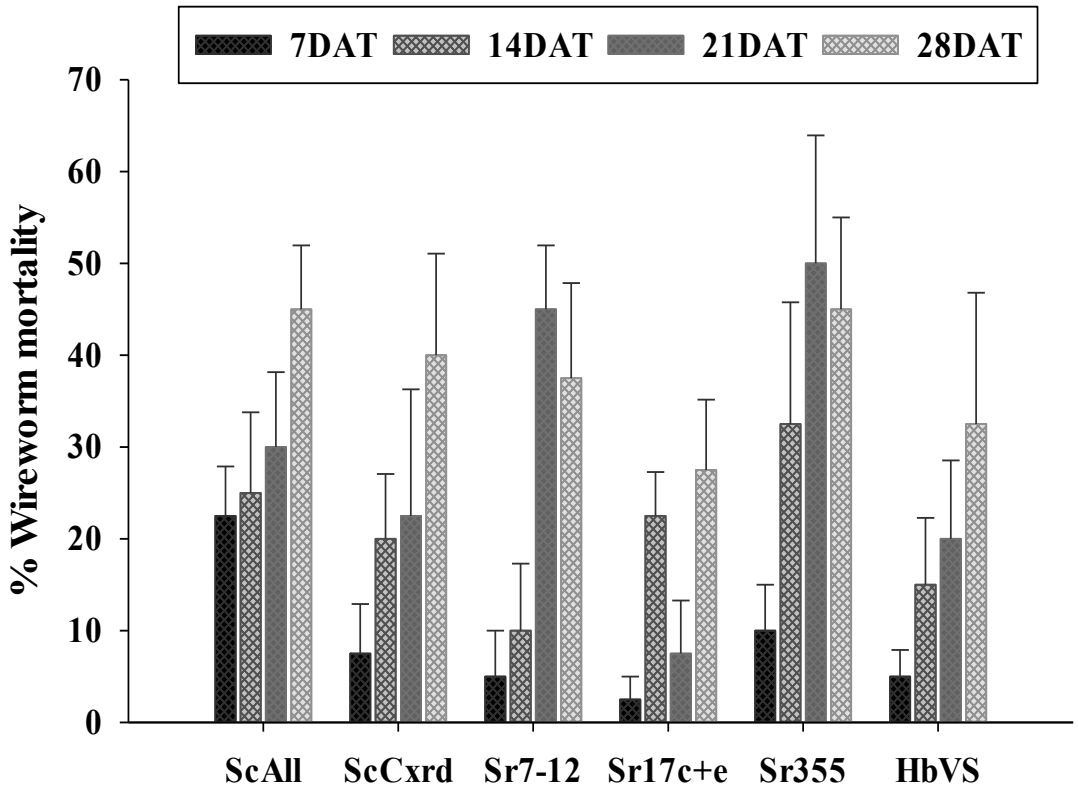


Figure 3. Average percentage mortality of larval *Limonius californicus* after exposure to entomopathogenic nematodes in shade house in 2018. ScAll = *Steinernema carpocapsae* All; ScCxrd = *Steinernema carpocapsae* Cxrd; Sr355 = *Steinernema riobrave* 355; Sr7-12 = *Steinernema riobrave* 7-12; Sr17c+e = *Steinernema rarum* 17c+e; HbVS = *Heterorhabditis bacteriophora* VS. Different letters above bars indicate statistical significance among EPN strains ($P \leq 0.05$, Tukey-Kramer test); DAT = Days after Treatment; no larval mortality was observed in control treatments.

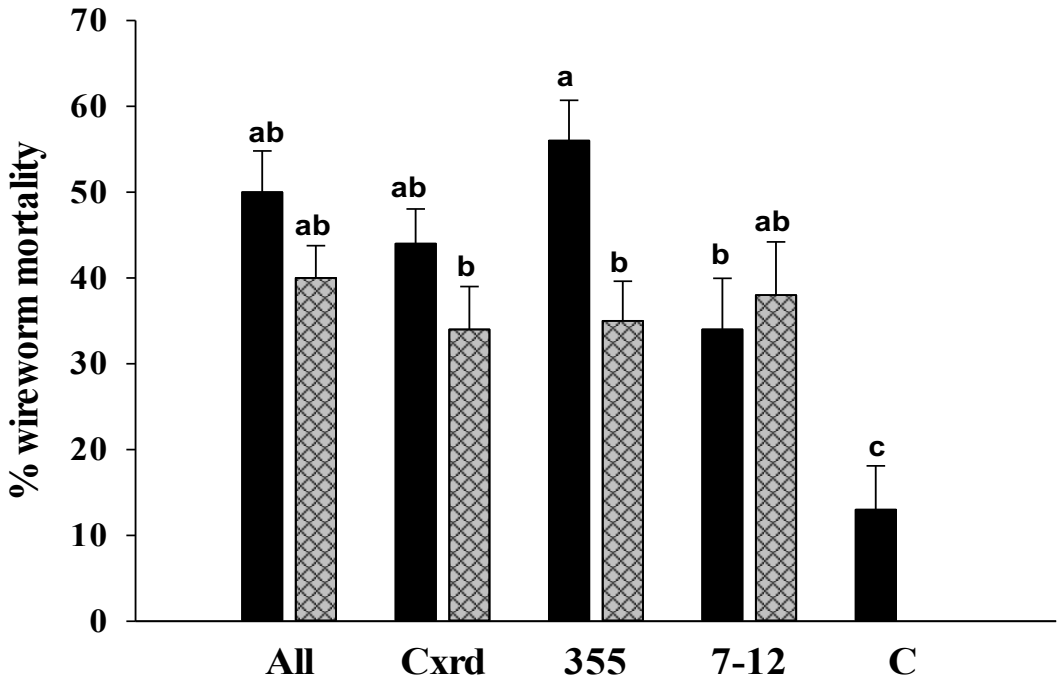


Figure 4. Average percentage mortality of larval *Limonius californicus* after exposure to entomopathogenic nematodes at 80,000 Infective Juveniles (IJs)/pot and 10,000 IJs/pot in shade house in 2019. All = *Steinernema carpocapsae* All; Cxrd = *Steinernema carpocapsae* Cxrd; Sr355 = *Steinernema riobrave* 355; Sr7-12 = *Steinernema riobrave* 7-12; C = Control. Different letters above bars indicate statistical significance ($P \leq 0.05$, Tukey-Kramer test).

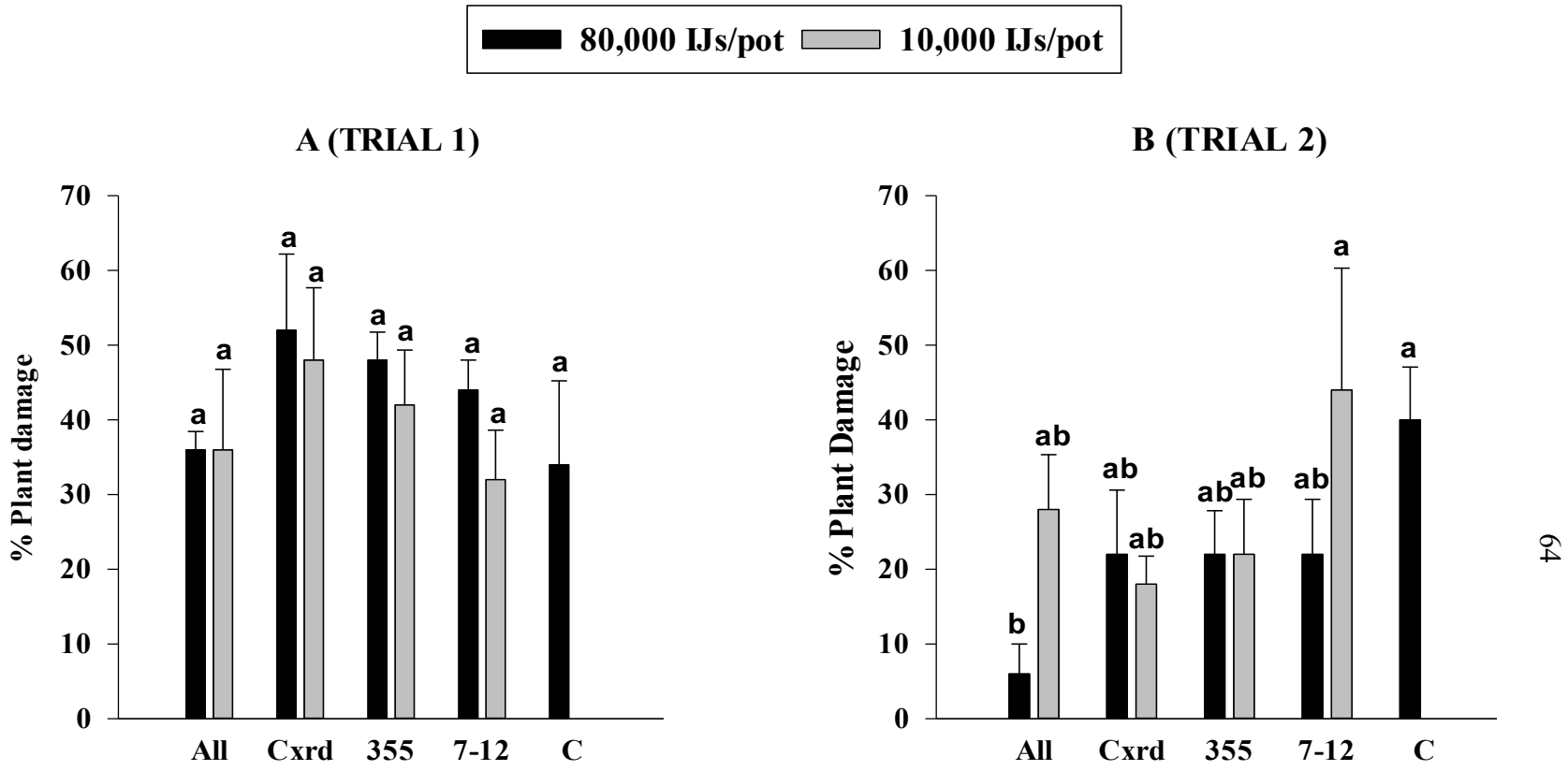


Figure 5. Average percentage plant damage by larval *Limonius californicus* after exposure to entomopathogenic nematodes at 80,000 Infective Juveniles (IJs)/pot and 10,000 IJs/pot in trial 1 (A) and trial 2 (B) in shade house in 2019. All = *Steinernema carpocapsae* All; Cxrd = *Steinernema carpocapsae* Cxrd; Sr355 = *Steinernema riobrave* 355; Sr7-12 = *Steinernema riobrave* 7-12; C= Control. Different letters indicate statistical significance ($P \leq 0.05$, Tukey-Kramer test).

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CHAPTER THREE

FIRST RECORD OF NATIVE ENTOMOPATHOGENIC NEMATODES FROM MONTANA AGROECOSYSTEMS

Contribution of Authors and Co-Authors

Manuscript in Chapter 3

Author: Ramandeep Sandhi

Contributions: Conceived and implemented the study design, data collection and wrote the manuscript.

Co-Author: Ratnasri Pothula

Contributions: Conducted molecular analysis including DNA extraction and PCR analysis for species identification. Provided feedback in manuscript improvement.

Co-Author: Satyendra K. Pothula

Contributions: Conducted molecular analysis including DNA extraction and PCR analysis for species identification. Provided feedback in manuscript improvement.

Co-Author: Byron J. Adams

Contributions: Provided guidance and funding for molecular analysis including DNA extraction and PCR analysis for species identification. Provided feedback in manuscript improvement.

Co-Author: Gadi V P Reddy

Contributions: Provided feedback in manuscript improvement and financial support for different experiments.

Manuscript Information Page

FIRST RECORD OF ENTOMOPATHOGENIC NEMATODES IN THE GOLDEN TRIANGLE REGION OF MONTANA

Ramandeep Kaur Sandhi, Ratnasri Pothula, Satyendra K. Pothula, Byron J. Adams, Gadi V.P. Reddy

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FIRST RECORD OF ENTOMOPATHOGENIC NEMATODES IN THE GOLDEN TRIANGLE REGION OF MONTANA

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Abstract

A total of 30 different agricultural fields in the Golden Triangle Region of Montana, USA were surveyed, and 150 soil samples were evaluated for the presence of entomopathogenic nematodes (EPNs). The authors isolated EPNs from 10% of the collected samples. The recovered isolates were identified as *Steinernema feltiae* and *Heterorhabditis bacteriophora* by using morphological and molecular analysis. *Steinernema feltiae* was found from two fields, Kalispell (*S. feltiae* 1) and Choteau (*S. feltiae* 2). *Steinernema feltiae* (1 and 2) differed significantly from each other in terms of morphological characters for infective juveniles (distance from anterior end to excretory pore and nerve ring) and 1st generation males (body length, spicule length, gubernaculum length, oesophagus, tail, and anal body diameter). *Steinernema feltiae* 2 and *H. bacteriophora* were recovered from the same field in Choteau. All these species were recovered from wheat fields with sandy clay loam and loam soils with 3.3 to 3.4% organic matter content and pH 8.

Key words: Entomopathogenic nematodes, Golden Triangle Region of Montana, *Heterorhabditis*, *Steinernema*.

Introduction

Entomopathogenic nematodes (EPNs), which occur naturally in soils, are obligate parasites of soil-inhabiting insects. EPNs were first described in 1923 with the identification of *Aplectana kraussei* Steiner (now known as *Steinernema kraussei*) (Nguyen and Hunt, 2007). Steinernematidae and Heterorhabditidae are two major families of EPNs with potential for managing insect populations (Kaya and Gaugler, 1993; Georgis et al., 2006). EPNs are associated with endosymbiotic bacteria belonging to the genera *Xenorhabdus* Thomas and Poinar and *Photorhabdus* Boemare, Akhurst, and Mourant, respectively (Boemare et al., 1993). EPNs, in association with their bacterial symbionts, are able to kill a wide range of insect hosts, usually within 24 to 48 hr. EPNs penetrate the insect host body and release symbiotic bacteria, causing septicemia that ultimately kills the host.

EPNs are widely distributed throughout the world and have been reported from different kinds of natural and managed habitats and a wide variety of soils (Hominick, 2002; Adams et al., 2006, 2007). The only continent where they have not been found is Antarctica (Griffin et al., 1990). About 95 species of *Steinernema* and 16 species of *Heterorhabditis* have been described so far (Hunt and Nguyen, 2016). However, there are reports of new EPN species being found and described from different parts of the world (Stock et al., 2019; Lephoto and Gray, 2019; Půža et al., 2020; Katumanyane et al., 2020). The distribution and abundance of EPNs varies depending upon the season, habitat, and geographic region. The presence and survival of EPNs is affected by various factors,

especially soil texture, moisture content, temperature, and host availability (Shapiro-Ilan et al., 2012; Stuart et al., 2015).

The rapid killing of the insect host by EPNs and their feasibility of mass production (Ehlers, 2001) has increased interest in searching for and using EPNs in integrated pest management systems (Georgis et al., 2006). Therefore, search for additional potential EPN species is being carried out in different parts of the world on a regular basis. (Malan et al., 2016; Cimen et al., 2016; Majić et al., 2018; Stock et al., 2019; Godjo et al., 2019). Indigenous EPNs have been used successfully as biological control agents to suppress various insect populations (Shapiro-Ilan et al., 2002). Indigenous EPNs are more suitable for inundative application against insect pests because of their better adaptation to local environmental conditions, allowing for more persistence and thus greater biological control efficacy. Such native nematodes can be developed as new biological control agents against important insect pests (Burnell and Stock, 2000; Grewal et al., 2002; Lewis et al., 2006). Some previous surveys have focused on finding new EPN species to control important agricultural and horticultural pests under specific conditions (Campos-Herrera and Gutiérrez, 2009). Native EPN species have been isolated from different areas that have showed better heat tolerance (Solomon et al., 2000), foraging ability, virulence (Yu et al., 2010), reproductive potential, or cold adaptation (Ivanova et al., 2001). In addition, the use of exotic EPNs can result in the suppression of native nematodes (Duncan et al., 2003).

Montana is the fourth largest state in the United States with a wide range of habitats, and it can potentially harbor an equally diverse group of EPNs. Until now, no organized survey has been conducted to locate EPNs in this area. Therefore, the objective of this

study was to survey EPN diversity in a variety of agricultural habitats in the Golden Triangle Region of Montana and to isolate and identify these EPNs.

Material and methods

Site description and soil sampling

Montana is a landlocked state in the northwestern United States and its economy is primarily based on agriculture, including ranching and production of grain (www.mt.gov). The Golden Triangle is situated in north central Montana (N46.965260, W109.533691). Average annual precipitation in this region is 380 mm. Mean daytime temperatures range from −2.2°C in January to 29.2°C in July (Montana Office of Tourism, FAQ 2013). Annual relative humidity varies from 75.5 to 81.7%. Annual snowfall varies from 300 inches (7620 mm) in some parts of the mountains in the western half of the state, to around 20 inches (508 mm) at some locations northeast of the Continental Divide. The Golden Triangle region includes the area north of Great Falls through Cut Bank to the south of the Canadian border and from Great Falls through Havre to the Canadian border. The three points of this triangle in north-central Montana are Havre, Conrad, and Great Falls. This region is named for its good dryland wheat-growing conditions.

In the summer of 2018 (May to September), a survey was conducted in some parts of the Golden Triangle region. The areas covered during the survey are shown in Table 1. The survey was mainly orientated toward 30 cultivated fields in the region including wheat, lentils, chickpea, peas, alfalfa, and fallow without any crops (Table 1) covering almost all the crops grown in the region. In all the fields, five random 10 to 15 cm deep soil samples were taken within each of the five random plots (8-10 m^2) with the help of a hand shovel.

Between samples, the shovel was thoroughly rinsed with 70% ethanol to prevent further contamination. Five random samples from each plot were combined to make one composite sample, providing five composite samples from each field. Overall, there were 150 composite samples from 30 fields. The collected samples were then placed in polyethylene bags to prevent water loss and kept in coolers (10°C) during transit to the laboratory. At each field site, data on sampling location, habitat (vegetation), longitude, latitude and elevation were recorded. For each sampling site, a subsample (*ca* 300 g) was analyzed for the physical and chemical characteristics: pH, organic matter, sand, silt, and clay content (Agvise laboratories, North Dakota) (Table 1).

Nematode isolation

EPNs were isolated from the soil samples using insect baiting techniques (Bedding and Akhurst, 1975). Within a week of soil sampling, a 300 g subsample was transferred to a 500 ml plastic container. Five larvae of *Galleria mellonella* L. (Lepidoptera: Pyralidae) were added as bait into each cup. The containers were kept in the dark at room temperature (22 ± 2°C). After five to six days of incubation, the dead larvae were removed and rinsed with tap water. The dead larvae that exhibited signs of possible infection with EPNs (e.g., flacid, soft, odorless larvae that were dark brown, orange, dark red, pale yellowish, brown, or black in color) were washed and placed in modified White traps (White, 1927). The larvae on the White traps were checked after one week and daily thereafter for emergence of infective juveniles (IJs). All IJs emerging from cadavers from a given soil sample were collected and considered as one isolate. In the case of negative results, the isolation process was repeated once to confirm results of the first isolation following the same procedure.

To confirm the virulence of the collected nematodes and to establish new cultures, collected IJs were used to infect fresh *G. mellonella* larvae (Kaya and Stock, 1997). Dead larvae were collected and placed on White traps. The emerged IJs were collected in distilled water for 10 days and stored in tissue culture flasks at 10°C. These nematodes were re-cultured monthly.

Morphological characterization

The extracted IJs from different fields were cultured on last instar larvae of *G. mellonella.* Ten *G. mellonella* larvae were inoculated with 1,000 to 2,000 IJs (100- 200 IJs/larva) for each isolate in 9 cm-diameter Petri dishes. These Petri dishes were observed for nematode infection daily. Infected cadavers were dissected in Ringer's solution according to the procedure of Kaya and Stock (1997). Overall, 20 males and IJs were observed for all the test isolates. The adults and IJs were killed in hot water at 60°C and fixed in an equal parts mix of hot TAF (60°C) and Ringer's solution. The nematodes were left in the fixative for two days, after which they were processed through glycerin in a vacuum desiccator as explained by Kaya and Stock (1997).

The processed males and IJs were mounted on glass slides and observed for different morphological characters: body length (L), maximum body width (D), tail length (TL), anal body width (ABD), distance from the anterior end to oesophagus (ES), distance from anterior end to excretory pore (EP), and distance from anterior end to nerve ring (NR). In addition, males were observed for spicule length (SP) and Gubernaculum length (GU). The different morphological measurements were recorded using ToupView 3.7 software (Zhejiang, China). According to their morphology, all isolates were placed into different

species groups using taxonomic criteria as suggested by Hominick et al. (1997). The morphological characteristics of IJs and males of the EPN isolates were compared statistically by using two-sided *t*-test ($\alpha = 0.05$) in R 3.5.2 (R Development Core Team, 2020).

Molecular characterization

For DNA extraction, pooled EPN IJs of each isolate were macerated with a plastic pestle in 1.5 ml centrifuge tube and genomic DNA was extracted using Qiagen DNeasy® Blood and Tissue kit (Waltham, MA) by following manufacturer's protocol (Qiagen DNeasy® Blood & Tissue Handbook, 2006). The extracted DNA was concentrated to 20 µl using Eppendorf Vacufuge Plus Vacuum Concentrator (Hamburg, Germany). A part of rDNA comprising the internal transcribed spacer regions (ITS), ITS1 and ITS2 including 5.8S were sequenced using two sets of primers. Primer set ITS-F (5'-TTGAACCGGGTAAAAGTCG-3 and ITS-R (5'-TTAGTTTCTTTTCCTCCGCT-3') was used to sequence the entire ITS1, 5.8S and ITS2 regions (Nadler et al., 2000) while primer set Fnema18S (5'-TTGATTACGTCCCTGCCCTTT-3') and rDNA1.58S (rev) (5'-ACGAGCCGAGTGATCCACCG-3') pair targeted the ITS1 region (Cherry et al., 1997). Each PCR reaction was carried out in a total volume of 30 µl consisting of 9 µl of DNA template, 15 µl of JumpStart™ REDTaq® ReadyMix (Sigma-Aldrich, St. Louis, MO), 2.4 µl of each primer and 1.2 µl of molecular grade water. The PCR conditions included initial denaturation at 94 ^{0}C for 5 minutes, 40 cycles of denaturation at 94 ^{0}C for 30 s, 40 cycles of annealing at 48 ^{0}C for 30 s, 40 cycles of extension at 0.5 ^{0}C/sec for 90 s and a final extension at 72 ^{0}C for 5 minutes. The PCR products were analyzed for expected DNA band

weights on 1% agarose gel run at 150V for 20 minutes. PCR products were treated with ExoSAP-IT™ PCR Product Cleanup Reagent (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol to digest excess primers and nucleotides. The products were sequenced bidirectionally with their PCR primers using Bigdye reaction chemistry on an ABI ABI3730xl. Primer sequences were removed from chromatograms and sequences were aligned and edited manually in Geneious Prime 2019.2.1 (http://www.geneious.com). Each species was identified via BLASTn (NCBI; http://www.ncbi.nlm.nih.gov) against the nucleotide collection (nr/nt) database using default search parameters.

Results

Overall, 150 composite samples (750 single point samples) were collected from the 30 fields in the survey area. Nematodes were recovered from only 15 of the 150 samples. The total percent nematode recovery from samples was 10%. These nematodes can be any kind of nematodes including bacteriophore nematodes. However, out of these 15 samples, we were able to re-culture only three nematode isolates from two fields (one isolate from Ron De Yong (Kalispell); two isolates from Mike Leys (Choteau)) (Table 1; supplementary figure 1). These three isolates were considered EPNs as they were able to reproduce in *G. mellonella*. The soils in most of the surveyed areas were alkaline, with soil pH ranging from 6.8 to 8.2. However, the EPN positive isolates were found in soils with a pH of 8.0 to 8.1. The successfully cultured isolates were from sandy clay loam and loam textural classes with organic matter of 3.3 to 3.4% as presented in Table 1. All the successfully cultured EPNs were extracted from wheat fields.

The different morphological characteristics of IJs and males for all the EPN isolates are provided in Tables 2 and 3, respectively. Two isolates collected from Mike Leys (Choteau) were found to be different on the basis of morphological data, resulting in two different isolates from the same field. On the basis of morphological characteristics, the isolate from Ron De Yong (Kalispell) was observed to be from the Steinernematidae. However, one isolate from Mike Leys (Choteau) belongs to Heterorhabditidae while the other is from the Steinernematidae.

BLASTn analysis showed that the steinernematid recovered from Ron De Yong (Kalispell), and Mike Leys (Choteau) were conspecific to *Steinernema feltiae* (Filipev, 1934). *Steinernema feltiae* recovered from Ron De Yong (Kalispell) and Mike Leys (Choteau) are referred to as isolates *S. feltiae* 1 (GenBank accession: MN647603) and *S. feltiae* 2 (GenBank accession: MN647604), respectively. The two isolates of *S. feltiae* (1 and 2) differed from each other for some morphological characters studied for IJs and males (Tables 2 and 3). In case of IJs, *S. feltiae* 1 had significantly greater distance from anterior end to excretory pore and nerve ring with significantly higher E% (EP/T × 100) as compared to *S. feltiae* 2 isolate (Table 2). Similarly, body length, spicule length and gubernaculum length were significantly higher for males of *S. feltiae* 1 as compared to *S. feltiae* 2 isolate (Table 3). However, oesophagus length, tail length and anal body diameter were significantly higher in case of *S. feltiae* 2 males as compared to *S. feltiae* 1 isolate (Table 3).

The second species from Mike Leys (Choteau) was observed to be identical to *Heterorhabditis bacteriophora* (Poinar, 1976) (GenBank accession: MN647605). Overall,

the IJs and males of this species were shorter in overall length and other morphological characteristics studied (Tables 2 and 3). In this study, *S. feltiae* 1 was observed to be 100% identical to *S. feltiae* isolates as found in NCBI (GenBank accession: MN044870.1, KT809344.1, KM016378.1, KM016374.1, KM016366.1, KM016361.1, KM016345.1, JN098451.1, AB243439.1). Similarly, *S. feltiae* 2 species was found 100% identical to *S. feltiae* (Accessions: MK294325.1, MK294320.1, KM016352.1, KM016339.1, AF121050). *Heterorhabditis bacteriphora* was 100% conspecific to a number of *H. bacteriophora* isolates in NCBI (MK072810.1, MK421482.1, MG551676.1, KT378450.1, KT378448). In addition, different morphological characters for females and 2nd generation males of three isolates were studied and presented in supplementary table 1 (*S. feltiae* 1), supplementary table 2 (*S. feltiae* 2), and supplementary table 3 (*H. bacteriophora*).

Discussion

The purpose of this survey was to see if EPNs were present in the Golden Triangle area of Montana, and if so, to explore the patterns of their diversity and distribution. Here, we established the occurrence of native EPNs for the first time in Montana, although the recovery percentage of EPNs was very low. The nematodes present in some soil samples caused *G. mellonella* mortality but were unable to reproduce further. These nematodes might be other rhabditids or opportunistic bacterivore nematodes that feed on saprobic bacteria in the cadaver but cannot reproduce in the cadaver. Different studies were focused toward the co-occurrence and effect of free living, opportunistic bacteriophagous nematodes such as *Oscheius* spp. (Campos-Herrera et al., 2015) and *Pellioditis* sp. (Rhabditida: Rhabditidae) (Duncan et al., 2003) on survival, infectivity and reproduction

of EPN species. Duncan et al. (2003) observed the reduced development of *Steinernema riobrave* Cabanillas, Poinar, and Raulston (Rhabditida: Steinernematidae) in the presence of *Oscheius* sp in a laboratory experiment. However, Blanco- Pérez et al. (2017) observed that EPNs were able to reproduce in freeze killed insect cadavers in the presence of scavenger bacteriophagous nematodes (*Oscheius*), though with lower progeny as compared to IJs produced in alive larvae. The reason behind the successful EPN reproduction in this study can be the already freeze-killed insect cadavers. The low recovery rate of nematodes might also be due to the failure of the EPN extraction method (Mráček et al., 2005), unsuitability of the laboratory environment for EPN culture and reproduction (Grewal et al., 1996), or the unsuitability of *G. mellonella* as a host because some EPNs are known to infect only certain hosts (Mráček et al., 2005; Klingen and Haukeland, 2006). In addition, there is a possibility that symbiotic bacterial cells may have been unable to effectively help EPNs reproduce in the hosts. Burnell and Stock (2000) emphasized that a critical number of bacterial cells are required to infect the host insects.

The prevalence of EPNs in agro-ecosystems are largely dependent on a number of biotic factors including host insects, predators such as mites, parasites, pathogens, free living non-EPNs, etc. and abiotic edaphic factors such as temperature, moisture, and UV light (Lewis et al., 2015; Stuart et al., 2015). Mostly, the fields surveyed in this study were cultivated with dryland farming, and low soil moisture levels might have been one of the most important factors for the absence of EPNs and the low recovery rate. When the level of soil moisture is unfavorable, EPNs can go into a resting phase known as “anhydrobiosis”

(Grewal, 2000). The negative effect of pesticides used in these agricultural fields may also be responsible for the absence of EPNs.

The measurements of the morphological characters of the three isolates were observed to be similar to those found by Hominick et al. (1997), with some differences in distance from anterior end to excretory pore, nerve ring and oesophagus for IJs and males. The two isolates of *S. feltiae* differed from each other in respect to spicule length, tail length, anal body diameter, oesophagus, and body length in males and oesophagus in case of IJs. This indicates that despite the genetic similarity between the isolates, there was high morphological variability among isolates. Campos-Herrera et al., (2006) cultured the same Rioja strain of *S. feltiae* (found in Spain) on three insect hosts, *G. mellonella*, *Spodoptera littoralis* Boisd. (Lepidoptera: Noctuidae) and *Bibio hortulanus* L. (Diptera: Bibionidae) and found significant differences in morphometric measurements of the IJs developed in different host insects. Similarly, Campos-Herrera and Gutiérrez (2014) also observed the intraspecific differences in 14 different populations of *S. feltiae* in terms of percentage *G. mellonella* mortality, time to kill the insect, penetration rate with sex-ratio being biased toward females. These morphological differences have been related to the different geographic origin, environmental conditions and host interactions (Stock et al., 2000) and might have been the reasons behind the differences in the morphology of two *S. feltiae* isolates found in the present study. *Steinernema feltiae* in the present study was recovered from two cultivated fields with sandy clay loam and loam textural soil class pH of 8 and 3.3% organic matter. Stock et al. (1999) reported similar results in a survey in California. Similarly, *H. bacteriophora* was found in a wheat field with sandy clay loam texture, pH

8, and 3.4% organic matter. The preference of sandy soils by *H. bacteriophora* has been reported in earlier studies as well (Stock et al., 1999; Campos-Herrera et al., 2012; Stuart et al., 2015). However, Jaffuel et al., (2018) reported different Swiss isolates of *H. bacteriophora* in areas with high clay content and low pH which is in contrast with the previous studies.

Steinernema feltiae and *H. bacteriophora* have a near-global distribution and a wide-range of habitats including pastures, roadsides, forests and gardens where human interference is minimal (Hominick et al., 1996; Hominick, 2002; Rosa et al., 2000). Recently, *S. feltiae* and *H. bacteriophora* have been found in Croatia (Majić et al., 2018, 2019). *Steinernema feltiae* can tolerate a wider range of environmental conditions than any other known EPN species. This species has been recovered in fields and grasslands in the UK, the Netherlands, and Germany (Sturhan and Liskova, 1999), and our findings reporting EPN presence from cultivated fields support these results.

Native EPNs already adapted to local environment are thought to be well suited as inundative biological control agents to suppress different insect pests (Shapiro-Ilan et al., 2002; Lewis et al., 2006). These native nematodes can persist longer in the soil, resulting in better biological control efficacy (Koppenhöfer and Fuzy, 2009). More surveys are needed because of the probability of the presence of additional and more virulent EPN species which can be added to the indigenous gene bank for further research. This will increase our understanding of the diversity and biogeography of EPNs. The new species and strains might be utilized in future ecological and biological control studies against

different economically important insect pests in Montana as well as other parts of the world with a similar climate.

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Table 1. Different agricultural field sites surveyed in Montana, USA, for native entomopathogenic nematodes during 2018.

Sampling site	**Farmer**	**Latitude**	**Longitude**	**Elevation (m)**	**Vegetation**	**Soil texture**	**Soil pH**	**Organic Matter %**	**Presence /Absence**	**Re-culture**
Pendroy	John Stoltz	N48.04130°	W112.16945°	4220	Wheat/Canola	Clay loam	8.2	3.9	**+(3)**	**No**
Pendroy	Kevin Johnson	N48.04206°	W112.20099°	4328	Wheat	Sandy clay loam	7.9	4.4	**+(1)**	**No**
Choteau	Joe Miller	N47.56785°	W112.13926°	3906	Fallow	Clay	7.8	3.2	**-**	**-**
Choteau	Mike Leys	N47.90238°	W112.3802°	-	Wheat	Sandy clay loam	8	3.3	**+(2)**	**Yes (1 Steinernematid and 1 Heterorhabditid)**
Valier	John Majerus	N48.18454°	W111.55524°	-	Wheat	Sandy clay loam	8.1	3.4	**--**	
Conrad	Mark Grub	N48.6334°	W111.53175°	-	Peas	Sandy clay loam	7	2.9	-	-
Conrad	Mark Orcutt	N48.16259°	W111.52896°	3498	Fallow	Clay loam	8.1	2.3	-	-
Conrad	Garett Grub	N47.56786°	W112.13930°	-	Wheat/Vegetables	Sandy clay loam	7.8	2.4	**+(2)**	**No**
Kalispell	Ron De Yong	N48.1945°	W114.2341°	-	Fallow/Winter wheat	Loam	8.1	3.4	**+(1)**	**Yes (1 Steinernematid)**
Valier	Jeremy Curry	N48.3032°	W112.4055°	3749	Fallow/Wheat	Sandy clay loam	8.2	2.1	**+(2)**	**No**
Conrad	Zane Drishinski	N48.77764°	W111.8948°	3508	Alfalfa	Clay loam	6.8	2.8	-	-
Conrad	WTARC	N48.3076°	W111.9255°	-	Fallow/Wheat	Clay loam	7.8	2.9	-	-
Conrad	Garman	N48.27808°	W111.92616°	1116.05	Alfalfa	Clay loam	8.1	3.7	-	-
Choteau (Knees)	A Killion	N48.00729°	W111.36596°	1081.26	Chickpea	Clay loam	8.2	2.5	-	-
Knees	A Killion	N48.00729°	W111.36596°	1081.26	Flax	Clay loam	7.5	3.5	**+(1)**	**No**
Brady	Winterhood	N47.98468°	W111.84313°	1112.80	Fallow/Winter wheat	Sandy clay loam	7.3	1.9	**+(1)**	**No**

Collins	Chickenhead	N47.95588°	W111.90971°	1122.68	Chickpea	Clay	7.8	2.3	**+(1)**	**No**
Choteau		N47.91965°	W112.04917°	1177.36	Hemp	Clay	8.2	2.8	-	-
Choteau	Nesbitt	N47.79007°	W111.16589°	1224.32	Durum Wheat	Clay loam	6.6	4.4	-	-
Dutton	Tims	N47.83529°	W111.53990°	1144.20	Lentils	Clay loam	7.9	3.4	-	-
Shelby west	John Wigand	N48.56643°	W111.98537°	1103.57	Peas	Sandy clay loam	6.9	3.9	-	-
Shelby west	John Wigand	N48.57870°	W111.99317°	1087.12	Triticale	Sandy loam	7.6	2.6	-	-
Shelby West	John Wigand	N48.54374°	W111.99096°	1071.82	Fallow	Clay loam	7.6	2.3	-	-
Potter Rd Shelby	John Wigand	N48.65339°	W111.96044°	1057.40	Fallow	Clay loam	7.9	2.7	-	-
Sunburst	Koeye Fauque	N48.82692°	W111.85641°	1093.77	Chickpea	Sandy clay loam	8	2.9	**+(1)**	**No**
Sunburst	Koeye Fauque	N48.82701°	W111.81002°	1102.46	Spring wheat	Clay loam	8.2	2.3	-	-
Tiber	Duncan	N48.19011°	W111.25206°	993.03	Fallow	Clay loam	8.2	2.6	-	-
Tiber	Paul Kolstad	N48.17184°	W111.17372°	960.91	Fallow	Clay loam	8.1	2.3	-	-
Tiber	Wicks	N48.23188°	W111.14511°	1029.37	Lentil/Fallow	Clay loam	7.7	2.5	-	-
Tiber	Broadhurst	N48.27280°	W111.14571°	930.70	Fallow	Clay loam	8.1	2.6	-	-

Notes: The number in the parenthesis represents the number of positive samples with nematodes. *Reculture means if we were able to reculture EPN isolates found from the samples in *G. mellonella.*

Table 2. Morphological characters of 3rd stage infective juveniles for three entomopathogenic nematode isolates from Montana, USA (n=20).

Character	*Steinernema feltiae* 1	*Steinernema feltiae* 2	ANOVA results	*Heterorhabditis bacteriophora*
L	820.25 ± 11.16 (711-900)[a]	807.85 ± 9.60 (743-906)[a]	t=0.82, df=37, P=0.11	602.25 ± 9.48 (513-654)
D	28.48 ± 0.73 (22-34.4)[a]	29.93 ± 0.57 (26-35)[a]	t=-1.63, df=36, P=0.11	24.74 ± 0.64 (18-31)
EP	64.45 ± 1.94 (48-80)[a]	55.25 ± 1.72 (43-67)[b]	t=3.45, df=37, P=0.001	77.35 ± 2.24 (55-95)
NR	72 ± 2.83 (42-91)[a]	59.65 ± 2.01 (48-81)[b]	t=3.47, df=34, P=0.001	78.2 ± 1.32 (68-90)
ES	90.2 ± 2.97 (61-114)[a]	87.7 ± 2.02 (48-81)[a]	t=0.68, df=33, P=0.50	106.5 ± 3.10 (93-140)
T	79.34 ± 1.49 (61-90)[a]	80.85 ± 1.09 (74-89)[a]	t=-0.82, df=35, P=0.42	91.26 ± 4.52 (56-140.3)
A	29.15 ± 0.81 (24.58-39.68)[a]	27.19 ± 0.6 (23.23-32.78)[a]	t=1.90, df=36, P=0.07	24.34 ± 0.60 (14.93-28.5)
B	9.31 ± 0.36 (7.45-13.15)[a]	9.31 ±0.25 (7.40-11.13)[a]	t=0.0, df=34, P=1.00	5.65 ± 0.18 (3.063-5.52)
C	10.40 ± 0.21 (8.67-13.14)[a]	10.02 ± 0.16 (8.78-11.19)[a]	t=1.63, df=37, P=0.11	6.60 ± 0.29 (2.10-9.16)
D%	72.42 ± 2.36 (55.24-87.91)[a]	63.62 ± 2.37 (39.81-83.33)[a]	t=-1.63, df=36, P=0.11	72.63 ± 2.61 (72.29-59.14)
E%	81.60 ± 2.63 (63.16-108.11)[a]	68.42 ± 2.11 (51.81-86.84)[b]	t=3.82, df=36, P=0.0005	84.75 ± 5.04 (49.52-98.21)

Figures showing means ± standard error (µm). L= body length; D= Maximum body diameter; EP=distance from anterior end to excretory pore; NR= distance from anterior end to nerve ring; ES= oesophagus length; T=Tail length; A=L/D; B=L/ES; C=L/T; D%= EP/ES × 100; E% = EP/T × 100. For *Steinernema felitae* isolates, means ± SE followed by the same letters are not significantly different at the ($\alpha = 0.05$).

Table 3. Morphological characteristics of males of three entomopathogenic nematode isolates from Montana, USA (n=20).

Character	*Steinernema feltiae* 1 (1st gen.)	*Steinernema feltiae* 2 (1st gen.)	t-test results	*Heterorhabditis bacteriophora* (2nd gen.)
L	1570.35 ± 36.84 (1259-1813) [a]	1304.8 ± 43.3 (913-1570) [b]	t=4.55, df=37, P<0.0001	802.1 ± 20.16 (610-920)
D	96.93 ± 3.01 (76-130) [a]	96.05 ± 3.14 (65-119) [a]	t=0.20, df=38, P=0.85	62.3 ± 1.99 (50-78)
EP	90.06 ± 2.25 (73-115) [a]	93 ± 1.55 (84-109) [a]	t=-1.05, df=34, P=0.30	77.9 ± 1.17 (70-90)
NR	98.35 ± 1.63 (88-113) [a]	100 ± 1.18 (88-110) [a]	t=-0.80, df=35, P=0.43	80.45 ± 1.32 (70-91)
ES	145.55 ± 2.80 (121-167) [b]	164.55 ± 1.72 (154-180) [a]	t=-5.63, df=32, P<0.0001	110.25 ± 1.97 (97-129)
T	41.65 ± 0.75 (36-48) [b]	52.75 ± 2.62 (34-72) [a]	t=-3.96, df=22, P=0.0006	61.58 ± 3.59 (41-110)
ABD	44.72 ± 1.07 (35-51) [b]	62.85 ± 3.00 (45-91) [a]	t=-5.56, df=24, P<0.0001	34.51 ± 0.67 (30-39.4)
SP	80.35 ± 1.41 (67-92) [a]	71.5 ± 2.21 (58-98) [b]	t=4.49, df=36, P<0.0001	41 ± 1.26 (32-51)
GU	49.95 ± 1.11 (41-59) [a]	45.85 ± 0.86 (40-53) [b]	t=2.85, df=36, P=0.007	22.95 ± 0.74 (14-28)
D%	62.09 ± 1.47 (49.08-72.34) [a]	56.56 ± 0.86 (51.14-65.19) [a]	t=0.20, df=38, P=0.85	70.90 ± 1.19 (63.2-83.50)
SW%	181.27 ±4.51 (145.65-235.90)[a]	117.34 ± 6.81 (63.74-168) [a]	t=7.63, df=33, P<0.0001	119.83 ± 4.69 (91.37-164.52)
GS%	62.74 ± 2.13 (49.40-85.51) [a]	66.35 ± 2.16 (51.19-91.38) [a]	t=-1.16, df=38, P=0.25	56.85 ± 2.61 (41.18-87.5)

Figures showing means ± standard error (µm). L= Body length; D= Maximum body diameter; EP=Distance from anterior end to excretory pore; NR= Distance from anterior end to nerve ring; ES= Oesophagus length; T=Tail length; ABD= Anal Body Diameter; SP=Spicule length; GU=Gubernaculum length; D%= EP/ES × 100; SW% = SP/ABD × 100; GS% = GU/SP ×100. For *Steinernema felitae* isolates, means ± SE followed by the same letters are not significantly different at the ($\alpha = 0.05$).

Appendices

Supplementary table 1. Morphological characteristics of females and 2nd gen. males of *Steinernema feltiae* 1 from Montana, USA (n=20).

Character	1st gen female	2nd gen female	2nd gen male
L	2697.95 ± 195.88 (1723-4942)	2205.65 ± 47.34 (1836-2546)	1189.4 ± 24.85 (980-1464)
D	140.85 ± 4.71 (119-207)	92.5 ± 2.88 (73-129)	86.32 ± 2.65 (65-119)
SL	8.1 ± 0.43 (6-15)	7.4 ± 0.40 (5-11)	-
SW	10.95 ± 0.60 (6-16)	10.15 ± 0.70 (6-20)	-
EP	84.75 ± 2.52 (72-106)	100.15 ± 3.4 (59-124)	69.65 ± 1.023 (60-78)
NR	110.3 ± 3.16 (91-141)	98.95 ± 2.51 (69-115)	93.6 ± 1.35 (82-103)
ES	165.85 ± 3.49 (134-193)	144.25 ± 4.02 (95-164)	135.2 ± 1.94 (110-149)
T	67.7 ± 3.15 (48-85)	50.2 ± 1.72 (34-67)	47.35 ± 0.61 (41-52)
ABD	44.35 ± 2.45 (28-73)	40.15 ± 1.88 (26-58)	45.5 ± 1.20 (38-56)
AV	1425.85 ± 61.1 (970-2000)	1188.15 ± 31.83 (1021-1690)	-
SP	-	-	69.85 ± 0.93 (62-77)
GU	-	-	49.4 ± 1.03 (40-56)
V	58.05 ± 4.42 (23.48-93.44)	54.52 ± 2.20 (40.55-89.42)	
D%	-	-	51.72 ± 1.05 (45.45-62.5)
SW%	-	-	156.033 ± 5.18 (114.29-202.63)
GS%	-	-	71.11 ± 1.97 (53.25-83.08)

Figures showing means ± standard error (µm). L= Body length; D= Maximum body diameter; SL= Stoma length; SW= Stoma width; EP=Distance from anterior end to excretory pore; NR= Distance from anterior end to nerve ring; ES= Oesophagus length; T=Tail length; ABD= Anal Body Diameter; AV= distance from anterior end to vulva; SP=Spicule length; GU=Gubernaculum length; V%= AV/L ×100; A=L/D; B=L/ES; C=L/T; D%= EP/ES × 100; % = SP/ABD × 100; GS% = GU/SP ×100.

Supplementary table 2. Morphological characters of females and 2nd gen. males of *Steinernema feltiae* 2 isolate from Montana, USA (n=20).

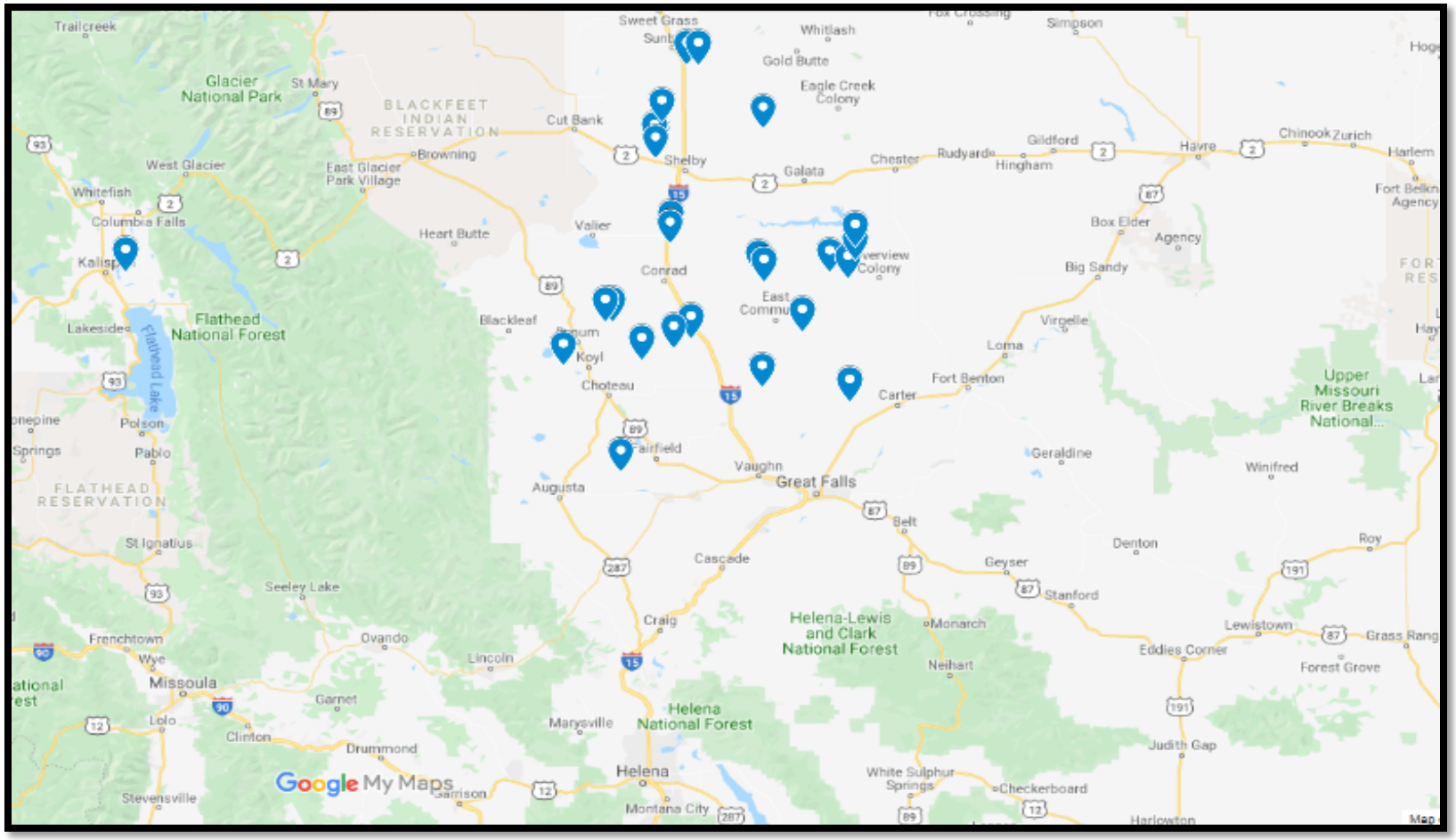
Character	1st gen female	2nd gen female	2nd gen male
L	2636.2 ± 87.81 (2013-3800)	1368.85 ± 32.62 (1089-1672)	1442.5 ± 43.20 (1117-1940)
D	157.9 ± 3.51 (112-178)	83.74 ± 1.83 (62-93)	94.1 ± 3.50 (62-137)
SL	18.6 ± 0.91 (12-27)	6.79 ± 0.31 (4-9)	
SW	17.9 ± 0.87 (10-25)	10.25 ± 0.53 (7-18)	
EP	115.7 ± 5.34 (79-160)	89.15 ± 2.46 (66-103)	93.1 ± 1.29 (83-107)
NR	140.45 ± 5.57 (89-201)	115.1 ± 1.87 (101-131)	109.05 ± 1.52 (98-120)
ES	179.25 ± 3.20 (160-212)	175.65 ± 1.69 (160-185)	155.95 ± 1.50 (143-168)
T	71.25 ± 3.00 (55-114)	58.4 ± 1.67 (44-78)	64.75 ± 1.57 (53-82)
ABD	93.75 ± 2.50 (77-117)	42.55 ± 1.00 (35-49)	59.75 ± 1.64 (48-73)
AV	1239.6 ± 30.58 (1044-1540)	670.75 ± 12.13 (557-760)	
SP			75.65 ± 1.96 (63-91)
GU			50.52 ± 1.45 (43-66)
V	47.022 ± 1.41 (36.29-59.26)	49.42 ± 1.25 (38.84-64.92)	
D%			59.83 ± 1.09 (52.17-72.29)
SW%			127.94 ± 3.88 (91.30-163.46)
GS%			67.36 ± 2.09 (48.86-90.47)

Figures showing means ± standard error (µm). L= Body length; D= Maximum body diameter; SL= Stoma length; SW= Stoma width; EP=Distance from anterior end to excretory pore; NR= Distance from anterior end to nerve ring; ES= Oesophagus length; T=Tail length; ABD= Anal Body Diameter; AV= distance from anterior end to vulva; SP=Spicule length; GU=Gubernaculum length; V%= AV/L × 100; D%= EP/ES × 100; SW% = SP/ABD × 100; GS% = GU/SP ×100.

Supplementary table 3. Morphological characters of females of *Heterorhabditis bacteriophora* isolate from Montana, USA (n=20).

Character	Hermaphrodite	2nd gen female
L	3182.5 ± 121.15 (2260-4300)	1647.35 ± 85.89 (1100-2740)
D	179.15 ± 4.07 (147-213)	117 ± 3.49 (92-150)
SL	8.25 ± 0.38 (6-11)	6.27 ± 0.26 (4-8)
SW	9.85 ± 0.52 (6-13)	9.65 ± 0.38 (7.5-13)
EP	147.15 ± 1.85 (138-168)	92.25 ± 1.42 (79-104)
NR	159.5 ± 3.44 (148-216)	89.55 ± 2.16 (72-108)
ES	249.25 ± 8.90 (202-327)	126.85 ± 1.77 (109-141)
T	111.55 ± 2.20 (98-125)	64.2 ± 2.77 (46-90)
ABD	54.7 ± 1.38 (43-65)	64.35 ±3.36 (41-91)
AV	1404 ± 33.52 (1080-1650)	862.5 ± 38.06 (610-1290)
V	45.15 ± 1.81 (35.12-60.25)	55.35 ± 4.49 (32.34-117.27)

Figures showing means ± standard error (µm). L= Body length; D= Maximum body diameter; SL= Stoma length; SW= Stoma width; EP=Distance from anterior end to excretory pore; NR= Distance from anterior end to nerve ring; ES= Oesophagus length; T=Tail length; ABD= Anal Body Diameter; AV= distance from anterior end to vulva; V= AV/L × 100.



Supplementary figure 1. Different agricultural field sites surveyed in Montana, USA, for native entomopathogenic nematodes during 2018.

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CHAPTER FOUR

MONTANA NATIVE ENTOMOPATHOGENIC NEMATODE SPECIES AGAINST *LIMONIUS CALIFORNICUS* (COLEOPTERA: ELATERIDAE)

Contribution of Authors and Co-Authors

Manuscript in Chapter 4

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Contributions: Conceived and implemented the study design, data collection and wrote the manuscript.

Co-Author: David Shapiro-Ilan
Contributions: Provided input on experimental design, implementation, data analysis and manuscript improvement.

Co-Author: Gadi V P Reddy
Contributions: Provided feedback in manuscript improvement and financial support for different experiments.

Manuscript Information Page

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MONTANA NATIVE ENTOMOPATHOGENIC NEMATODE SPECIES AGAINST *LIMONIUS CALIFORNICUS* (COLEOPTERA: ELATERIDAE)

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Abstract

Wireworms are destructive soil inhabiting polyphagous pests in the Pacific Northwest and Intermountain region of the United States. Continuously increasing wireworm populations and damage in small grain crops such as spring wheat in Northern Great Plains has become a challenge for growers. Due to unavailability of effective control measures, alternative methods, including biological control agents such as entomopathogenic nematodes (EPNs) are needed. Native/indigenous EPN species are expected to have better potential than exotic species to control the local insect pests. Two Montana native EPN species (*Steinernema feltiae* and *Heterorhabditis bacteriophora*) were tested against, *Limonius californicus* (Coleoptera: Elateridae) in laboratory and shade house studies. In the laboratory bioassay, two isolates of *S. feltiae* at the rate of 28,000 IJs/five larvae killed 48–50% of the insects within 4 wk. *Heterorhabditis bacteriophora* was not able to cause >30% *L. californicus* larval mortality. None of the two isolates of *S. feltiae* performed well against *L. californicus* when tested in different soil types. Similarly, two isolates of *S. feltiae* that were tested killed only 20–25% wireworms in a shade house trial that did not differ significantly from the control treatment. Four weeks after EPN treatment in the shade house trial, the percentage of wheat plant damage from *L. californicus* ranged from 30 to 40% in the presence of *S. felitae*, not differing statistically from control. These results suggest that *S. felitae* have limited potential in managing wireworm populations.

Key words: *Limonius californicus*, biological control, entomopathogenic nematodes, spring wheat, Montana

Introduction

Wireworms are the larval stages of click beetles and their continuously growing populations have become a great threat to the small grain crops growers in the Northern Great Plains. *Limonius californicus* (Mannerheim) (Coleoptera: Elateridae) is reported as the dominant wireworm species in the Pacific Northwest (Milosavljević et al. 2015) and Intermountain region (Morales-Rodriguez et al.2014; Rashed et al. 2015, 2017) of the United States. According to Morales-Rodriguez et al. (2014), *L. californicus* and *Hypnoides bicolor* (Eschscholtz) were the most common wireworm species in the Golden Triangle Region of Montana in field surveys from 2011 to 2012.

Wireworms feed on different plant parts including seeds, roots, stems, and tubers inhibiting plant growth eventually leading to plant death (Traugott et al. 2015, Knodel and Shrestha 2018). Crop losses in the United States, Canada, and United Kingdom due to wireworms can reach up to 25% (Jansson and Seal 1994). With the ban of persistent synthetic insecticides such as lindane in 2009 due to negative effects on the environment (Vernon et al. 2009), no effective control tactics (chemical or biological) are available for wireworms. Neonicotinoids as seed treatment suppress the wireworm damage to a limited extent, but resurgence can occur, causing more crop damage (Vernon et al. 2009, Barsics et al. 2013). Additionally, these insecticides might not always be effective because even low wireworm populations can cause plant damage (Parker and Howard 2001, La Forgia and Verheggen 2019). Recently, Labrie et al. (2020) reported the effectiveness of neonicotinoid seed treatments in only 5% of the corn and soybean crop fields treated by neonicotinoids in Quebec and suggested to not to use these insecticides prophylactically.

These insecticides can also cause contamination of agricultural soils, freshwater resources, wetlands, and nontarget vegetation in addition to negative effects on pollinators (Paquet-Walsh et al. 2019, Labrie et al. 2020). Hence, there is a need for alternative methods that are environmentally friendly and can be included in Integrated Pest Management programs for suppressing wireworm populations or at least preventing crop damage. Different cultural crop protection techniques have potential for such use, including trap cropping, soil amendments, soil drying or flooding, and crop rotation (Andrews et al. 2008, Landl and Glauninger 2011, Esser et al. 2015, Sharma et al. 2018). Entomopathogenic fungi and nematodes are important biological control agents that can be effective and are being evaluated against wireworms (Ansari et al. 2009, Ensafi et al. 2018, Sharma et al. 2019, Sandhi et al. 2020a).

Steinernema (Rhabditida: Steinernematidae) and *Heterorhabditis* (Rhabditida: Heterorhabditidae) are two of entomopathogenic nematode genera that can be pathogenic to many soil-dwelling insect pests (Grewal et al. 2005). *Steinernema* spp. and *Heterorhabditis* spp. are associated mutualistically with symbiotic bacteria in the genera *Xenorhabdus* and *Photorhabdus* (Enterobacteriacae), respectively (Lewis and Clarke 2012). Infective juveniles (free living, third juvenile stage) locate the insect hosts in the soil and enter through the spiracles, mouth, anus, and via cuticle in some cases (Shapiro-Ilan et al. 2012b). Infective juveniles then release the symbiotic bacteria inside the insect host hemolymph which cause septicemia in the host insect due to release of toxins; ultimately the insect will die within 24–72 h. Entomopathogenic nematodes molt and develop within the insect host body, and IJs leave the insect cadaver to look for new hosts.

Non-native EPN species have the advantage of being easily available commercial products for insect pest control (Kaya et al. 2006, Lacey et al. 2015). However, potential nontarget effects of exotic EPNs has always been a matter of concern among certain researchers (Rojht et al. 2009, Abate et al. 2017). Alternatively, local and native EPNs were observed to suppress pests with no additional nontarget effects (Shapiro-Ilan et al. 2002, Duncan et al. 2003, Dillon et al. 2006, Lewis et al. 2006, Campos-Herrera 2015, Sandhi and Reddy 2019). These EPNs are suitable for inundative applications against different insect pests because of their ability to adapt to environmental conditions in the local areas. However, Harvey et al. (2016) reported that due to their relative persistence and other factors, native EPNs can pose higher risks to nontargets when compared with exotically introduced EPNs. Lacey et al. (2001) reported native EPN species to be as effective as exotic EPN species against *Popillia japonica* Newman (Coleoptera: Scarabaeidae). In addition, native EPNs have been purported to have higher persistence and greater biological control efficacy (Shields et al. 2018).

We investigated the virulence of two Montana native EPN species, *S. feltiae* and *H. bacteriophora* (Sandhi et al. 2020b) against wireworms in both laboratory and shade house in the present study. The main objective of the study was to determine whether Montana native EPN species are able to kill and suppress sugar beet wireworm, *L. californicus* in the Golden Triangle Region of Montana. In addition, we also studied if the efficacy of two Montana native EPN species against *L. californicus* differs depending on different soil types in Montana as well as varied moisture levels.

Material and methods

Wireworm Collection and Study site

Wireworm larvae were collected near Pendroy (N 48.04130°, W 112.16945°), Conrad (N 47.56786°, W 112.13930°), and Kalispell (N 48.1945°, W 114.2341°) in the Golden Triangle Region of Montana. Although *L. californicus*, *H. bicolor*, and *Aeolus mellilus* (Say) were found, we used only *L. californicus* in all experiments because it was the dominant species. Stocking bait traps (nylon stockings filled with wheat and barley grain mixture) were used for wireworm collection as described by Reddy et al. 2014. The bait traps were soaked in water for 24 h and buried 15–20 cm deep in soil of fields with high wireworm pressure. To maintain ambient temperature and moisture for seed germination and wireworm survival, the traps were covered with black plastic sheets. The traps were then collected from the field and brought back to the laboratory for extraction after 15 d. Wireworms were sorted from the traps using Berlese funnels and kept in deli plastic cups in sterilized sandy loam soil with soaked wheat seeds as food for larvae at 8–10°C. Medium-sized wireworm larvae (0.8–1.5 cm (measuring scale); >1 yr old) were used in all the experiments. All the experiments were conducted at the Western Triangle Agricultural Research Center (WTARC), Conrad, MT from 2018 to 2019.

EPN Culturing

Two isolates of *S. feltiae* (*S. feltiae* 1 and *S. feltiae* 2) and one isolate of *H. bacteriophora* (Sandhi et al. 2020b) were tested against *L. californicus*. These native EPN isolates were extracted from agricultural sites in Montana (Sandhi et al. 2020b). EPN isolates were reared in last-instar larvae of greater wax moth, *Galleria mellonella* L.

(Lepidoptera: Pyralidae) as explained by Sandhi et al. (2020a). All nematodes were cultured simultaneously but handled separately at different times in the workspace to avoid cross-contamination. For rearing, 1000–2000 IJs (100–200 IJs/larva) per 1 ml water were applied in 9 cm dia Petri dish lined with a single layer of Whatman No. 1 filter paper and 10–20 wax moth larvae. After 4–5 d, EPN infected wax moth cadavers were placed on White traps. These White traps were incubated at room temperature (~22°C) and observed daily for IJ emergence. The emerging IJs from the White traps were washed 2–3 times, collected in tissue culture flasks in fresh tap water and stored in an incubator at 8°C.

Laboratory Concentration Response Bioassay

Heterorhabditis bacteriophora, *S. feltiae* 1, and *S. feltiae* 2 were tested against medium sized *L. californicus* larvae in a laboratory bioassay. Approximately 150 g of autoclaved sandy loam soil (78% sand, 12% silt, and 10% clay, pH 7.7, and 1.4% organic matter) was filled in 500 ml plastic deli cups (soil surface area: 114 cm^2). In each cup, five wireworm larvae were introduced along with 8–10 soaked wheat seeds as food. The larvae that were unable to enter the soil within 12 h were removed and replaced with new larvae. Four concentrations—3,500 IJs/cup (30 IJs/cm^2), 7,000 IJs/cup (60 IJs/cm^2), 14,000 IJs/cup (120 IJs/cm^2), and 28,000 IJs/cup (240 IJs/cm^2)—were tested within 10–15 d of culturing the three EPN isolates. EPNs were kept at room temperature (~22°C) for 12 h for acclimatization and checked for viability before application in the cups. The IJs were applied in each cup in 1 ml of water via pipette and control cups received 1ml of water only. The final soil moisture was maintained at 18% (v/w). The bioassay was conducted twice on various dates from June to August 2018 with five replications for four

concentrations. Approximately 10 holes were poked in the cup lids for air and placed in an incubator at 22°C and 75% RH under dark conditions. Larval mortality was observed weekly for 4 wk.

Effect of Soil Texture (with Standardized Moisture) on Native EPNs Virulence Against *L. californicus*

Only *S. feltiae* 1 and *S. feltiae* 2 were tested in this laboratory bioassay. Four different types of soils, as described in Table 1, were used in this study. Subsamples of these different soil types were sent to Agvise laboratories, North Dakota to analyze pH, organic matter, sand, silt, clay content, and field capacity (1/3 bar pressure potential). Soils were sterilized in an autoclave at 121°C for 1 h to kill natural nematode populations and other microorganisms and kept at room temperature for at least 2 wk for acclimatization. Deli plastic cups (500 ml) were filled with 150 g of soil (surface area: 140 cm^2) for different soils. Five medium-sized wireworm larvae were introduced into each cup with 8–10 germinated wheat seeds. The larvae unable to enter the soil in cups within 12 h were replaced with healthy larvae. After 24 h, 7,000 IJs/cup (50 IJs/cm^2) were inoculated in 1 ml of water into each cup. Control cups received just 1 ml of water without EPN IJs. This concentration was prepared by following the same procedure as mentioned earlier. The soil moisture in the cups for all soil types was standardized at 18% (v/w) for all the soil types. The reason for standardizing the moisture was to compare the soil at same moisture level. After inoculation, the cups were kept in an incubator at 22°C and 75% RH in dark conditions. Water was provided every 2–3 d to maintain the moisture in the cups. The wireworm mortality was assessed weekly for 4 wk. There were five replications for each

treatment including control and the experiment was conducted in two trials with an interval of 2 wk. Different nematode cultures were used for both trials.

Effect of Soil Texture (Field Capacity Moisture Levels) on *Steinernema feltiae* 2 Virulence Against *L. californicus*

Steinernema feltiae 2 was tested in this laboratory bioassay. The soil types were the same as those used in the previous experiment (Table 1). These soils were sterilized in an autoclave as described in previous sections. The moisture content for all the soils was adjusted to field capacity levels as given in Table 1, which differed by soil type. Thus, in this experiment all soils had an equal level of available moisture. The rest of the procedure was same as described in previous section. Wireworm mortality was recorded at weekly intervals for 1 mo. The experiment was conducted twice with an interval of 2 wk between trials and different nematode cultures were used for both trials.

Efficacy of Selected EPN Species against *L. californicus* in a Shade House

The experiment was set up in a completely randomized block design with *S. feltiae* 1 and *S. feltiae* 2 as treatments. Approximately 1.7 kg of sterilized field-collected sandy loam soil (78% sand, 12% silt, and 10% clay, with pH 7.7 and 1.4% organic matter) was filled in plastic pots (14 cm diameter; 9 cm depth; surface area: 150 cm^2). Ten wheat seeds were planted in each pot. After 10 d, five wireworms were introduced into each pot and larvae that did not enter the soil were replaced with healthy wireworms after 24 h. Two concentrations - 60,000 IJs/pot (400 IJs/cm^2) and 7,500 IJs/pot (50 IJs/cm^2)—were tested for each of the two *S. feltiae* isolates. The same procedure as described earlier was followed for standardization of two concentrations of nematodes: 6,000 and 750 IJs/ml applied in 10

ml tap water. The pots intended for the control received 10 ml of water without IJs. There were five replicates (pots) for each concentration and the two *S. feltiae* isolates. The pots were placed in a shade house (a greenhouse structure made from net and plastic material to allow required sunlight, moisture, and air) and soil moisture was maintained in the pots by watering daily. After 4 wk, the pots were destructively sampled, and wireworm mortality was observed. Wireworms not found in the pots were considered to be dead as reported by Ensafi et al. (2018) and Sandhi et al. (2020a). A soil moisture meter (Spectrum Technologies Inc., IL) and a soil thermometer (Taylor, IL) were used to measure soil moisture and temperature three times each week for the whole month. Average air temperature in the shade house was recorded as 31°C (26–35°C) with soil temperature and soil moisture in the pots as 22°C (11–35°C) and 21 ± 5%, respectively. The experiment was repeated again from May to August 2019 with an interval of 10 d between the trials. At the end of experiment, plant damage (number of wheat seedlings injured by wireworms) was also observed and the average percentage of plant damage was recorded. To record plant damage, the presence of wilted or dead central leaf and/or seedling death was carefully observed.

Statistical Analysis

A generalized linear model (GLM) with quasibinomial distribution and logit link function was used for laboratory bioassays data analysis to deal with the over dispersion problem. The response variable was wireworm mortality, and EPN species, concentration, and time were the predictor variables in the model in the laboratory screening bioassay. EPN species, soil types, and time were the predictor variables with mortality proportion as

the response variable in the laboratory bioassays with different soil types. For wireworm mortality and plant damage data in shade house experiment, GLM with binomial distribution was used for the analysis. EPN species and concentration were the independent factors in these models. Tukey–Kramer test ($\alpha = 0.05$) was used for pairwise comparisons if the predictor in the model had significant effect. As the model was formulated in terms of transformed probabilities (logit transformation to the log odds scale), the results were back transformed to an original probability scale after analysis for graphical representation. All statistical analyses were performed in R 3.5.2 software (R Development Core Team 2020).

Results

Laboratory Concentration Response Bioassay

There were no significant differences among two trials in terms of wireworm mortality ($\chi^2 = 0.097$, df = 1, $P = 0.76$), which lead to pooling of data for further analysis. No three-way interaction was found between EPN species, concentration, and time to mortality ($\chi^2 = 14.81$, df = 27, $P = 0.97$), and, therefore, this factor was not included in the model. EPN species ($\chi^2 = 445.38$, df = 3, $P < 0.0001$), concentration ($\chi^2 = 59.13$, df = 3, $P < 0.0001$), and time periods ($\chi^2 = 171.22$, df = 3, $P < 0.0001$) showed significant effects on wireworm larval mortality. The interactions between EPN species and concentration ($\chi^2 = 24.26$, df = 9, $P = 0.004$) and between EPN species and time to mortality ($\chi^2 = 16.96$, df = 9, $P = 0.05$) had significant effects on the wireworm larval mortality proportions. However, concentration and time did not interact with each other ($\chi^2 = 2.13$, df = 9, $P = 0.98$) in terms of wireworm larval mortality.

On average, wireworm larval mortality was higher for higher nematode concentrations, and the significantly lowest mortality was caused by EPN species, when applied at the rate of 3,500 IJs/cup ($P < 0.001$). However, there was no significant differences among three concentrations (7,000 IJs, 14,000, and 28,000 IJs/cup) in killing the wireworm larvae ($P > 0.07$). Wireworm mortality at all the time intervals differed significantly ($P < 0.001$) except at 21 and 28 d after treatment (DAT) ($P = 0.08$) (Fig. 1). Mortality increased gradually with time and the EPN isolates killed 50% wireworms within 28 d of treatment (Fig. 1). At 7 DAT, there was no interaction between EPN and concentrations ($P = 0.23$). Overall, the larval mortality did not exceed 14% for any of the three tested EPNs at all the concentrations at 7 DAT (Fig. 1A). This mortality trend remained almost same at 14 DAT, with mortality ranging from 12 to 30%, with 30% mortality caused by *S. feltiae* 1. *Steinernema feltiae* 1 caused significantly higher mortality (30%) at 14,000 IJs/cup when compared with only 2% mortality caused by *H. bacteriophora* when applied at the rate of 3,500 IJs/cup ($P = 0.05$) (Fig. 1B). However, larval mortality increased at 21 DAT (Fig. 1C). *Steinernema feltiae* 1 at the rate of 14,000 IJs/cup and *S. feltiae* 2 at the rate of 28,000 IJs/cup killed significantly more *L. californicus* larvae (44%) compared with 12% by *H. bacteriophora* at 3,500 IJs/cup (Fig. 1C). Mortality ranged from 14 to 34% in the other EPN × dose combinations (Fig. 1C). Wireworm mortality increased by 28 DAT, and significantly higher mortality (48–52%) was caused by the two isolates of *S. feltiae* at the rate of 14,000 and 28,000 IJs/cup (Fig. 1D) compared with 24% mortality caused by *H. bacteriophora* at 28,000 IJs/cup. At 28 DAT, *H.*

bacteriophora and *S. feltiae* 2 caused only 14% mortality when applied at 3,500 IJs/cup. No wireworm died in the control cups.

Effect of Soil Texture (Standardized Moisture) on
Native EPNs Virulence Against *L. californicus*

No significant differences were detected between two trials for *L. californicus* larval mortality (trial: $\chi^2 = 1.15$, df = 1, $P = 0.28$; trial × EPN species: $\chi^2 = 0.07$, df = 2, $P = 0.96$). Additionally, the interaction between EPN species and time was not significant ($\chi^2 = 1.55$, df = 6, $P = 0.96$); therefore, data for cumulative wireworm mortality at 28 DAT were analyzed further. Two EPN species had a significant effect on *L. californicus* mortality ($\chi^2 = 74.29$, df = 2, $P < 0.0001$). However, wireworm mortality did not differ significantly for the four soil types tested ($\chi^2 = 0.36$, df = 3, $P = 0.95$). Similarly, no significant interaction was observed between EPN species and the four soil types ($\chi^2 = 2.54$, df = 6, $P = 0.86$). No wireworm larvae were observed dead in the control treatment cups. *Limonius californicus* mortality caused by two isolates of *S. feltiae* did not differ significantly from the control ($P = 0.99$) in different soil types. However, *S. feltiae* 2 caused significantly higher mortality (12–25%) when compared with only 6–12% *L. californicus* mortality caused by *S. feltiae* 1 in four soil types ($P = 0.003$) (Fig. 2).

Effect of Soil Texture (Field Capacity) on
Native EPNs Virulence against *L. californicus*

No significant differences were observed in two trials in terms of *L. californicus* larval mortality (trial: $\chi^2 = 1.70$, df = 1, $P = 0.19$; trial × soil type: $\chi^2 = 4.50$, df = 3, $P = 0.21$), therefore, the data were pooled for further analysis. The different soil types did not have significant interactions with time intervals in terms of *L. californicus* mortality ($\chi^2 =$

9.75, df = 9, $P = 0.37$). Soil type had a significant effect on *L. californicus* mortality ($\chi^2 = 51.29$, df = 3, $P < 0.0001$). Similarly, *L. californicus* mortality varied significantly at different time intervals ($\chi^2 = 52.68$, df = 3, $P = 0.0001$). Mortality caused by *S. feltiae* 2 did not differ from control treatments with no mortality recorded in control (Tukey–Kramer, $P = 0.98$). *Limonius californicus* mortality did not differ significantly between sandy loam and clay soils ($P = 0.99$) or between sandy clay loam and clay loam soils ($P = 0.72$). However, overall, there were significant differences among clay loam and clay, sandy clay loam and clay, sandy loam and clay loam, and sandy loam and sandy clay loam ($P < 0.0001$) in terms of wireworm mortality. The wireworm mortality caused by *S. feltiae* at 7 DAT was significantly lowest from mortality at 14, 21, and 28 DAT ($P < 0.01$). Wireworm mortality at 14 and 21 DAT and at 21 and 28 DAT did not differ significantly among each other ($P > 0.25$). Overall, the wireworm mortality caused by *S. feltiae* 2 did not exceed 32%, which was observed in sandy loam soil followed by 24% mortality in clay soil at 28 DAT (Fig. 3). Only 10–13% mortality was recorded in cups with sandy clay loam and clay loam soils (Fig. 3).

Efficacy of Selected EPN Species against *L. californicus* in Shade House

The two trials repeated over time did not differ significantly for *L. californicus* larval mortality ($\chi^2 = 0.39$, df = 1, $P = 0.53$), and the data were pooled for further analysis. EPN species and concentrationdid not have significant effects on mortality (EPN species: $\chi^2 = 4.21$, df = 2, $P = 0.12$; Concentration: $\chi^2 = 0.76$, df = 1, $P = 0.38$). In addition, EPN species and concentration did not interact significantly ($\chi^2 = 0.41$, df = 2, $P = 0.81$) for wireworm mortality. *Steinernema feltiae* 1 and *S. feltiae* 2 did not differ significantly from

the control for *L. californicus* mortality (Fig. 4). Overall, *L. californicus* mortality ranged from 15 to 25% when EPNs were applied at 60,000 and 7,500 IJs/pot.

Two trials repeated over time differed significantly in respect to plant damage caused by *L. californicus* (χ^2= 4.77, df = 1, P = 0.03). However, the interaction between EPN species and trials was not significant (χ^2 = 4.91, df = 2, P = 0.09); therefore, the data were pooled for further analysis. EPN species did not have any significant effect on plant damage (χ^2 = 0.19, df = 2, P = 0.91). Larval mortality for *L. californicus* did not differ between the two concentrations (χ^2 = 0.87, df = 1, P = 0.35). There was no significant interaction found between EPN species and the concentrations applied (χ^2= 1.77, df = 2, P = 0.41). None of the two isolates of *S. feltiae* tested differed significantly from the control for plant damage (Fig. 5). The plant damage caused by *L. californicus* in the presence of *S. feltiae* ranged from 30 to 40%. *L. californicus* larvae were able to cause 36% plant damage in control treatment as well.

Discussion

Most studies on efficacy of EPNs against wireworms have been against *Agriotes* spp. (Ester and Huiting 2007, Ansari et al. 2009, Campos-Herrera and Gutierrez 2009, Morton and Garcia-del-Pino 2016). We demonstrated susceptibility of the sugarbeet wireworm to Montana native EPNs. Although, significant differences were not detected in all instances, S. *feltiae* was more virulent than *H. bacteriophora*. In the laboratory bioassay, *S. feltiae* killed up to 50% of *L. californicus* larvae, while *H. bacteriophora* did not kill more than 30% of larvae tested. However, wireworm mortality did not exceed 25% when either of two isolates of *S. feltiae* were applied in a shade house experiment.

Greater virulence of *S. feltiae* against *L. californicus* in the laboratory is similar to the reports of Toba et al. (1983), who also observed 50% *L. californicus* mortality from *S. feltiae* in laboratory experiments. Similarly, our observation of lower virulence of *S. feltiae* against *L. californicus* in the greenhouse is similar to studies with different wireworms (Ester and Huiting 2007, Ansari et al. 2009, Campos-Herrera and Gutierrez 2009, Sandhi et al. 2020a). *Heterorhabditis bacteriophora* caused low *L. californicus* mortality in our study, which supports the earlier reports of Morton and Garcia-del-Pino (2016), who recorded 26–35% mortality of *Agriotes obscurus* under laboratory conditions. Similarly, Morris (1985) reported no infection of *H. bacteriophora* in last instar *Ctenicera destructor*. However, 67% mortality of *A. lineatus* larvae from *H. bacteriophora* in the laboratory has been reported by Ansari et al. (2009). The difference in all these results can be related to size of wireworm larvae, different wireworm species tested, and variation in the study environment. Late instar wireworm larvae are hard-bodied when compared with early instars which helps them in avoiding EPN penetration and infection.

In general, a minimum field rate of 25 IJs/cm^2 is recommended for most applications though certain target pests will require higher rates (Shapiro-Ilan et al. 2012b). We intentionally used high EPN concentrations in laboratory bioassay when compared with the recommended concentrations for other pests to see if wireworm mortality would increase. Similarly, relatively high nematode concentrations were tested in the shade house because of the low infectivity against test wireworms in laboratory bioassays. Despite being tested at very high concentrations (>400 IJs/cm2), *S. feltiae* was not able to cause more than 25% *L. californicus* mortality in the shade house experiment. Other studies have

also reported unsatisfactory results even when EPNs were used at high concentrations against wireworms (Toba et al. 1983, Ensafi et al. 2018, Sandhi et al. 2020a).

EPNs were able to cause a significant mortality only 3–4 wk after treatment regardless of EPN concentration in laboratory trials (concentration response bioassay and soil texture experiment). Other studies have reported similar long times taken by different EPN species to kill different wireworm species (Ansari et al. 2009, Morton and Garcia-del-Pino 2016). Also, EPNs take some time to move through the soil to find the host before penetration. Time needed by IJs to penetrate after locating the wireworms are unknown and need to be investigated.

Soil texture can play an important role in affecting EPN virulence. EPNs are reported to have higher infectivity against insect pests in sandier soils (El-Borai et al. 2012). Ensafi et al. (2018) reported that by *S. carpocapsae* provided higher protection of wheat seedlings from *L. californicus* in sandy media compared with peat media. However, the opposite has also been reported where higher efficacy of EPNs was observed in clay soils when compared with sandy soils (Shapiro-Ilan et al. 2000, Toepfer et al. 2010). In our study, four soil types did not affect wireworm mortality caused by *S. feltiae* when soils were maintained at a standardized moisture level (18%) or when maintained at field capacity levels for the different soil types. This might be due to the inefficiency of the Montana native EPN species to penetrate inside wireworm larvae rather than an effect of the soil types themselves.

EPN efficacy can also be impacted by soil moisture and temperature. The shade house experiment was conducted outdoors, without any temperature or moisture control.

Variation in air temperature, soil temperature and especially, soil moisture would have affected EPN efficacy. During the night, temperatures were lower in the shade house compared with day time temperatures, affecting soil moisture in the pots. Similarly, other factors such as developmental stage, nematode species or isolate, food availability, and exposure time can all affect the EPN efficacy (Lewis et al. 2006; Shapiro-Ilan et al. 2012a, 2017).

In the shade house trial, *L. californicus* caused 36–40% plant damage in both the control and the various EPN treatments after 4 wk. Wireworms may have damaged the plants before EPNs could infect them, equalizing plant damage between control and treated pots. In addition, insects have been observed avoiding EPN contaminated areas (Mankowski et al. 2005, Wilson-Rich et al. 2007). Whether wireworms were able to avoid the repellent pressure of EPNs during the study is unknown. Indigenous/native EPNs have been used successfully as biocontrol agents in some instances (Lewis et al. 2006, Campos-Herrera and Gutiérrez 2009). These EPNs may be more suitable for inundative application because of their adaptation to local environment, resulting into greater persistence and biological control efficiency (Shields et al. 2018). Native EPN species have been extracted from different areas with better heat tolerance, foraging ability, virulence, reproductive potential, or cold adaptation (Solomon et al. 2000, Ivanova et al. 2001, Hussaini et al. 2004, Yu et al. 2010, Shapiro-Ilan et al. 2014). Montana native EPNs, such as *S. feltiae* and *H. bacteriophora*, are presumably well adapted to the local climate and soil conditions given the often extreme weather of Montana and were expected to be, therefore, more effective as biological control agents for *L. californicus.* However, this expectation was not met

since neither EPN species was found to be very effective against *L. californicus*. This may have been due to presence of physical barriers in wireworms, such as their thick sclerotized cuticle, the group of dense hairs in oral cavity, and hard musculature in the anus (Lewis et al. 2015). Some researchers have reported lower efficacy of native EPNs compared with exotic EPN species, on the basis of the foraging strategy and hosts targeted (Berry et al. 1997, Heve et al. 2017). Moreover, a recent study indicated that, due to their relative persistence and other factors, native EPNs can pose higher risks to nontargets relative to exotically introduced EPNs (Harvey et al. 2016). Future studies are required to compare the effects and impact of native Montana EPNs versus commercialized strains within the same species.

We conclude that the native EPNs that we tested have only limited potential for wireworm control. However, there are many wireworm species, in several genera such as *Hypnoides*, *Aeolus*, and *Agriotes* sp., that are pests in various crops in Montana, and some of these species may be more susceptible to the native EPNs. Perhaps, different commercially available EPN species as studied by Sandhi et al. (2020a) and additional species need to be evaluated and explored against different wireworm species present in the area instead of the native ones.

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Table 1. Characteristics of different soil types used in the laboratory bioassays

Soil type	**Sand%**	**Silt%**	**Clay%**	**Soil pH**	**Organic Matter (%)**	**Field capacity (% moisture)**
Sandy loam	78	12	10	7.7	1.4	8.0
Sandy clay loam	36	30	34	7.0	2.9	21.4
Clay loam	40	28	32	7.8	2.3	22.35
Clay	30	28	42	7.8	2.9	25.4

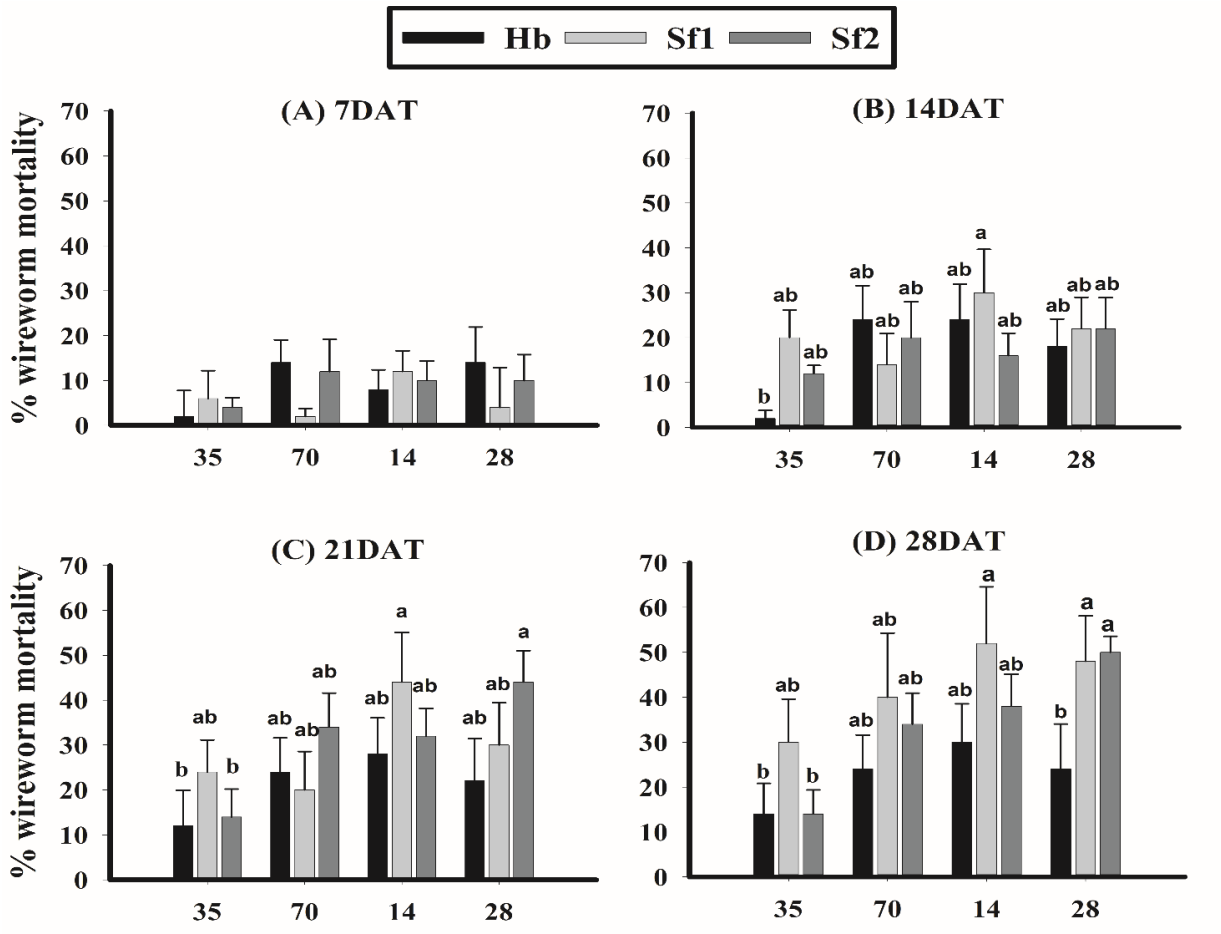


Figure 1. Average percentage mortality of *Limonius californicus* larvae after exposure to entomopathogenic nematodes at (A) 7 DAT, (B) 14 DAT, (C) 21 DAT, and (D) 28 DAT, where IJs = infective juveniles/cup; Sf1 = *Steinernema feltiae* 1; Sf2 = *Steinernema feltiae* 2; Hb = *Heterorhabditis bacteriophora*. Different letters above bars indicate statistical significance ($P \leq 0.05$, Tukey–Kramer test); DAT = days after treatment; No larval mortality was observed in the control.

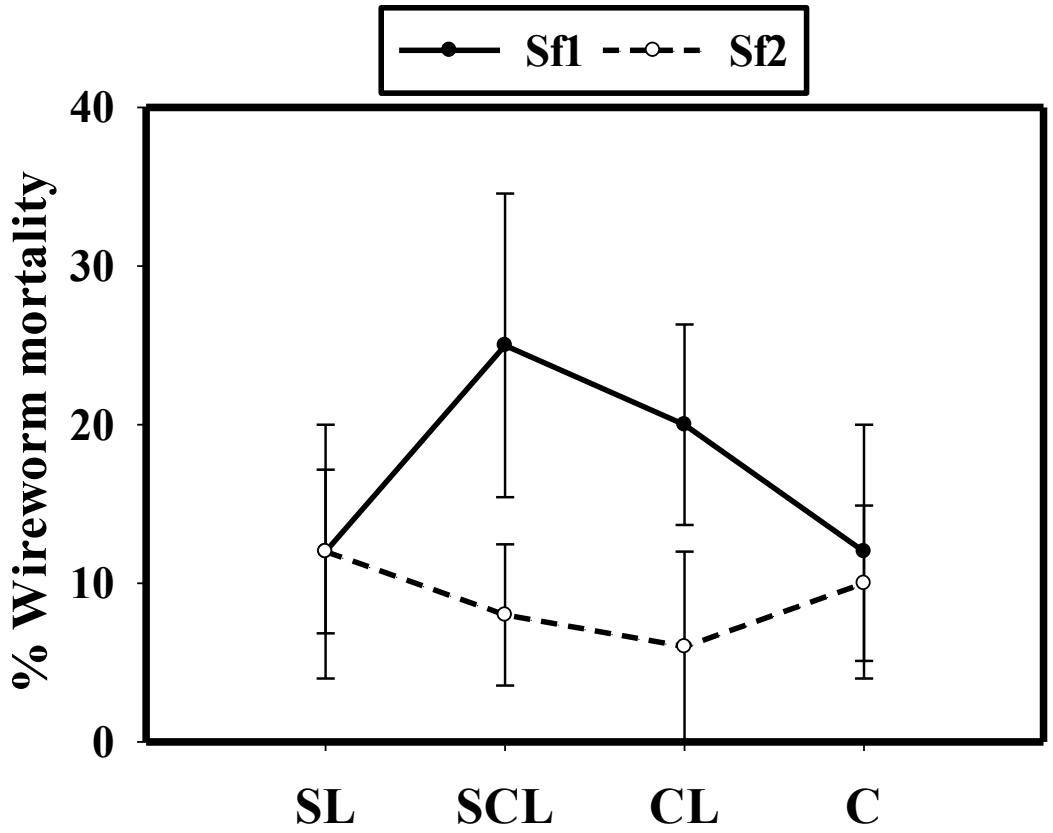


Figure 2. Average percentage mortality of *Limonius californicus* larvae after exposure to entomopathogenic nematodes at 7,000 infective juveniles/cup with soils maintained at standardized moisture, where Sf1 = *Steinernema feltiae* 1 and Sf2 = *Steinernema feltiae* 2, and SL = Sandy loam; SCL = Sandy clay loam; CL = Clay loam; C = Clay. No significant differences were observed among treatments ($P > 0.05$, Tukey–Kramer test). No larval mortality was observed in control treatments.

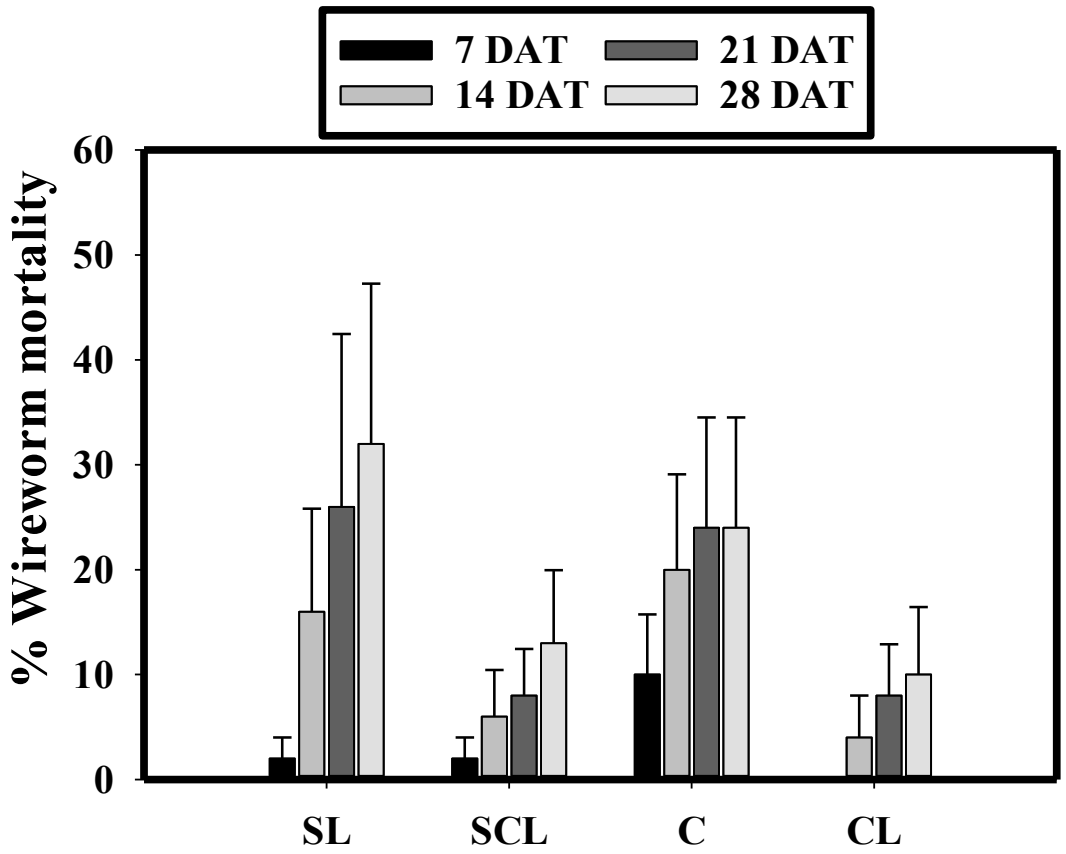


Figure 3. Average percentage mortality of *Limonius californicus* larvae after exposure to *Steinernema feltiae* 2 at 7,000 infective juveniles/cup with soils maintained at field capacity moisture levels, where SL = Sandy loam; SCL = Sandy clay loam; CL = Clay loam and C = Clay. No statistical differences were observed among EPN species ($P > 0.05$, Tukey–Kramer test). No larval mortality was observed in control treatments.

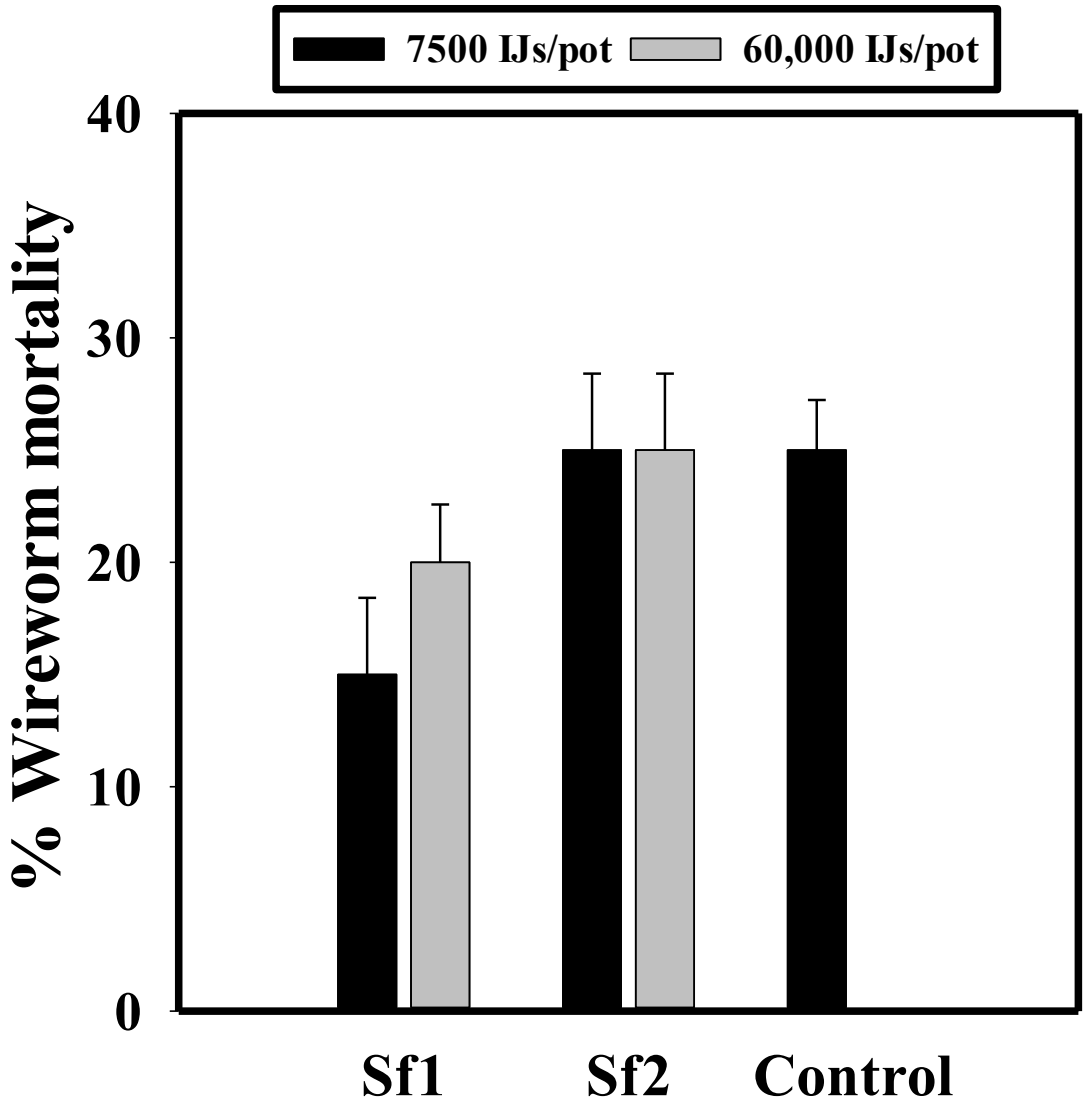


Figure 4. Average percentage mortality of *Limonius californicus* larvae after exposure to entomopathogenic nematodes at 7,500 infective juveniles (IJs) and 60,000 IJs/pot in shade house in 2019, where Sf1 = *Steinernema feltiae* 1, Sf2 = *Steinernema feltiae* 2, and Control = control treatment. No significant differences were observed among treatments ($P > 0.05$, Tukey–Kramer test).

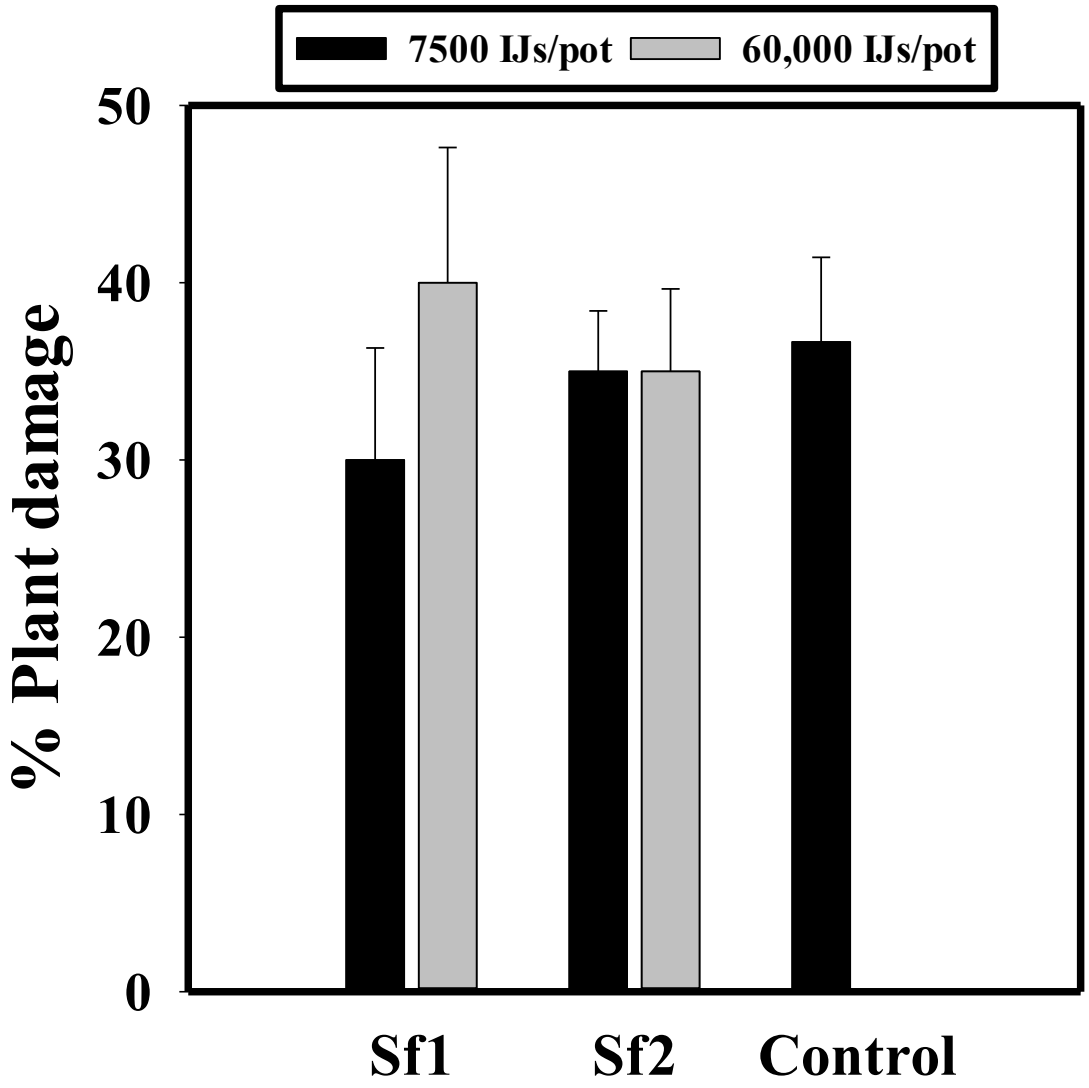


Figure 5. Average percentage plant damage by *Limonius californicus* larvae after exposure to entomopathogenic nematodes at 7,500 infective juveniles (IJs) and 60,000 IJs/pot in shade house in 2019, where Sf1 = *Steinernema feltiae* 1, Sf2 = *Steinernema feltiae* 2, and Control = control treatment. No significant differences were observed among treatments ($P > 0.05$, Tukey–Kramer test).

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CHAPTER FIVE

EFFICACY OF ENTOMOPATHOGENIC NEMATODE INFECTED INSECT CADAVERS AGAINST WIREWORMS IN GREENHOUSE AND FIELD

Contribution of Authors and Co-Authors

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Contributions: Developed experimental objectives, Provided input on experimental design, implementation, data analysis and manuscript preparation.

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EFFICACY OF ENTOMOPATHOGENIC NEMATODE INFECTED INSECT CADAVERS AGAINST WIREWORMS IN GREENHOUSE AND FIELD

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Abstract

Wireworms are economically important soil-inhabiting insect pests that attack variety of field crops worldwide. These pests have become a significant menace to spring wheat and barley in the Northern Great Plains because of absence of effective control measures. Therefore, research toward developing alternative control methods such as biological control with entomopathogenic nematodes (EPNs) is warranted. Four EPN strains, *Steinernema carpocapsae* (All and Cxrd) and *Steinernema riobrave* (355 and 7-12) in the form of infected *Galleria mellonella* cadavers were evaluated against wireworms (*Limonius californicus* (Mannerheim) and *Hypnoides bicolor* Eschscholtz) in two field sites and *L. californicus* in the greenhouse. In the field, none of the four EPN strains tested significantly affected the number of wireworms collected, plant count, plant height, moisture, protein, seed test weight, and yield for both the Pendroy and Choteau sites. This trend was similar for both doses (3 and 6 cadavers/plot) tested in the field. However, in the greenhouse test, one *G. mellonella* cadaver of *S. carpocapsae* All, *S. carpocapsae* Cxrd and *S. riobrave* 355 strains per plot killed 50-68% of *L. californicus*, when the seeds were treated with imidacloprid. The mortality range was 40-56% for *S. carpocapsae* (All and Cxrd) and *S. riobrave* 355 strains, when seeds were planted without imidacloprid treatment. The wireworm mortality range did not differ significantly different from imidacloprid treatments but was different from control treatment. However, the percentage of plant damage caused by different EPN strains was significantly lower in the imidacloprid treated plants (6-8% at 14 DAT (days after treatment) and 8-24% at 35 DAT) as compared to non-imidacloprid plants (40-57% at 14 DAT and 57-75% at 35 DAT). The *L. californicus* larvae were able to reduce 57% and 92% of non-imidacloprid plants in greenhouse at 14 and 35 DAT, respectively. This indicates that imidacloprid enhances the infection and killing ability of EPNs against *L. californicus*. Synergism was observed among imidacloprid and *S. carpocapsae* Cxrd and imidacloprid and *S. riobrave* 355 strains in regard to *L. californicus* mortality. However, *S. riobrave* 7-12 and *S. carpocapsae* All strains showed antagonism with imidacloprid. Overall, EPN alone treatment was not found to be as effective as the treatment combining imidacloprid and EPN strains.

Key words: Biological control, Entomopathogenic nematodes, imidaclorpid, *Limonius californicus*, Spring wheat, wireworm,

Introduction

This is the fourth in a series of studies aimed at evaluating entomopathogenic nematodes (EPNs) for the control of wireworms, soil dwelling larval Elateridae (Coleoptera), in the Golden Triangle of Montana. For background on the pests, rationale for this approach and general EPN biology, see Sandhi and Reddy (2019), Sandhi et al. (2020a), Sandhi et al. (2020b), Sandhi et al. (2020c) and references therein.

In previous laboratory experiments that screened 10 EPN strains for virulence against wireworms (Sandhi et al. 2020a), two EPN species *Steinernema carpocapsae* (All and Cxrd strains) and *Steinernema riobrave* (355 and 7-12 strains) were selected as being most effective against *L. californicus* (Sandhi et al. 2020a). The objective of the current study was to test the field efficacy of these selected EPN strains against different wireworm species (*L. californicus* and *Hypnoides bicolor* Eschscholtz), especially *L. californicus.* Generally, EPNs are applied in form of aqueous suspensions of infective juveniles (IJs) using different irrigation systems, sprayers, or injection techniques (Shapiro-Ilan et al., 2006). However, these nematodes have also been tested against different insect pests by using infected insect cadavers under laboratory, greenhouse, and field conditions (Shapiro-Ilan et al., 2003; Bruck et al., 2005; Del Valle et al., 2008; Shapiro-Ilan et al., 2010; Deol et al., 2011; Raja et al., 2015; Bal et al., 2017; Monteiro et al., 2020). In this method, EPN infected cadavers are placed in the field, IJs emerge from the cadavers and target the insect pests. EPNs applied as insect cadavers are reported to be superior to application in aqueous suspension in laboratory and field conditions with respect to better IJs survival, dispersal and infection (Shapiro and Glazer, 1996; Shapiro and Lewis, 1999; Perez et al., 2003;

Shapiro-Ilan et al., 2003; Shapiro-Ilan et al., 2010; Deol et al., 2011; Bai et al., 2016). In the present study, the cadaver method was used to mimic natural conditions. This approach will enable us to better understand the actual potential of EPNs against wireworms in Montana wheat-barley irrigated and dryland agroecosystems.

Material and methods

EPN source and production of cadavers

Cutures of EPN strains of *S. carpocapsae* (All and Cxrd strains) and *S. riobrave* (355 and 7-12 strains) were obtained from the USDA-ARS Entomopathogenic Nematode culture collection (Byron, GA). EPNs used in experiments were produced on greater wax moth larvae, *Galleria mellonella* L. (Lepidoptera: Pyralidae) obtained from the Bassett's Cricket Ranch (CA, USA). The larvae were stored in the containers provided by the supplier at 10°C until used for culturing. Ten *G. mellonella* larvae were exposed in a Petri dish to approximately 200 freshly produced IJs of each of four selected EPN strains: *S. carpocapsae* (All and Cxrd strains) and *S. riobrave* (355 and 7-12 strains) in a 90 mm diameter Petri dish, yielding 80 infected cadavers of each strain. The Petri dishes were held at room temperature (22°C) for 3-4 days. The nematode-infected cadavers were then transferred to individual White traps (Kaya and Stock 1997) for another 4-5 days at room temperature to observe the initiation of IJs emergence. The cadavers were checked daily for the initiation of IJs. After 4-5 days, the cadavers that were about to release IJs were used in field experiments to reduce the chances of variation in emergence among replications.

Study sites

The field trials were conducted in 2019 in a barley field (Pendroy: N48.04130°, W112.16945°) and a spring wheat field (Choteau: N47.9023°, W112.2330°) in the GTR. Both fields were selected on the basis of a history of moderate-to-high wireworm pressure. Prior to the experiment, the fields were evaluated and found negative for native EPNs. According to NRCS (1999), the soils at the Pendroy site had Rothiemay-Niart are clay loams, with 0–4% slopes and Choteau site had Niart-Crago gravelly loams soil with 0–4% slopes (NRCS 1999).

Farmers seeded Clearfield spring wheat on 09 May, 2019 and Hockett barley on 10 May, 2019. The Choteau site (spring wheat) was irrigated while the Pendroy site (barley) was unirrigated with row spacing of 19 cm and 25 cm, respectively. Seeding rate was 215 seeds/m^2 in barley field and was ~ 230 seeds/m^2 in spring wheat field. Fertilizers 56 litres of of 'thirty two' nitrogen, 20-10-5-10 (1300 liter/hectare) and manure at the rate of 135 kg/hectare were applied to the spring wheat before seeding. Beyond and Wildcard were applied at label rates for weed control in this field. Before seeding. Roundup at the rate of 1.17-1.46 litres/hectare and liquid nitrogen at the rate of 47 liter/hectare were applied before seeding in barley field. Imidacloprid (Gaucho® 600, Bayer Crop Science) was applied as seed treatment in both fields. The wheat received 5 cm of water via overhead irrigation weekly.

Experimental design

A completely randomized block design was used. (Fig. 1). A 17.6 m × 33.7 m section was measured in each field. Within each section, there were 5 replicate blocks, each with eight treatment plots plus a control plot (for a total of nine plots). Each plot was 1.5 m × 1.5 m and the plots and blocks were separated by 2.5 m to avoid interspecific competition between EPN strains. Plot order was randomized at each location.

Five replications were maintained for all treatment and control plots. For treatments, two doses (3 and 6 cadavers) were tested and control plots lacked cadavers. Overall, there were 45 plots (4 strains × 2 doses × 5 replications = 40 + 5 Controls). Five days after seeding the cadavers were placed 5-8 cm beneath the soil surface and at least 10 cm away from each other. Holes were dug and covered with a hand shovel after placing the cadaver carefully. In the wheat field, cadavers were placed starting at 9:00 AM under cloudy conditions, with an average soil temperature of 5.5±4°C, average air temperature of 16°C, and average soil moisture percentage of 24%. However, in the barley field, EPN cadavers were placed starting at 7:00 PM under cloudy conditions, with an average soil temperature of -6±2°C, average air temperature of 23°C, and average soil moisture percentage of 59%. Irrigation in wheat field began within 30 days of planting.

Plant Count

To assess the wireworm damage to wheat plants, the number of seedlings in each plot were randomly counted using a 1 m line-intercept method (Sharma et al. 2018). Two rows were selected from each plot (n=2) and the ends of each row (1 m length) were marked with iron nails and the individual plants marked. The first counts were taken three weeks

after plant germination. The second counts were made just before harvesting. At harvest, the height of the marked plants was also recorded.

Number of Wireworms/Wireworm sampling

Soil bait traps (Reddy et al. 2014) were used to determine wireworm density in the experimental plots. Traps were run twice a month starting 10 days after seeding for barley and 20 days after seeding for spring wheat. Trapping in the wheat was delayed because of rain. The traps were replaced five times at two-week intervals from June to August. Soil temperature and soil moisture were recorded at the time of wireworm trap collection by using soil thermometer (Taylor, IL, USA) and soil moisture meter (Spectrum Technologies Inc., IL, USA), respectively. Air temperature was also checked each time the traps were installed. Wireworm larvae were extracted using Berlese funnels (Bioquip Products, California, USA), the collected wireworms were counted and identified using taxonomic keys by Etzler (2013).

Emergence of IJs from cadavers used in field study

To determine IJ emergence rates from cadavers placed in the field, 15 randomly selected infected cadavers were removed from the treatment batches for each of the four EPN strains and placed individually on separate White traps at room temperature. Emerging IJs were collected, washed 2-3 times with tap water, and counted with the serial dilution method (Glazer and Lewis 2000). IJs were collected and counted until emergence stopped (3 weeks).

IJs persistence

The IJ's survival was observed in all plots in August 2019 and May-June 2020. In 2019, five soil core samples (approx. 100 g each) were taken from each plot with a hand shovel and mixed to make a composite sample. The hand shovel was washed with water and rinsed with 75% ethanol between plots to avoid contamination. Overall, there were 45 composite samples from each field. These composite samples were kept separately in plastic bags in a thermal cooler and taken to the laboratory. EPN IJs were recovered from the soil samples using the insect baiting technique (Bedding and Akhurst 1975). Approximately 300 g soil sample from each composite sample was transferred to a 500 ml plastic container with ten *G. mellonella* larvae in each cup. The containers were kept in the dark at room temperature (22 ± 2°C) for seven days, after which the dead larvae were removed and rinsed with tap water. Larvae that showed signs of EPN infection were dissected to confirm IJ presence. The number of EPN infected *G. mellonella* larvae were averaged over each replication to obtain the mean larval mortality. The same procedure was followed for nematode extraction and baiting in May 2020 except that only 25 random samples were collected from the whole plot area.

Post-harvest data collection

In 2019, the wheat field was harvested on 29 August and the barley field on 12 September. After harvesting, grain from each plot was cleaned (Almaco, Allan Machine Company, IA, USA). The plot and test weights were measured using a laboratory balance (Ohaus, Adventure™ Pro model AV8101). A sample of ca. 300 g from each plot was processed through a grain analyzer (Perten Instruments IM9500; Hägersten, Sweden) to

determine grain moisture and protein. Plot weight and moisture were used to calculate yield.

Preparation of *Galleria mellonella* cadavers for greenhouse experiment

Fifteen *G. mellonella* larvae in 60 mm diameter Petri dishes were exposed separately to approximately 200 freshly produced IJs of each of the four selected EPN strains; *S. carpocapsae* (All and Cxrd strains) and *S. riobrave* (355 and 7-12 strains) and held at room temperature (22 ± 2°C), RH: 75% in the dark for 3-4 days. Nematode infected cadavers were then transferred to individual White traps (Kaya and Stock 1997) and held for 4-5 days at room temperature, to observe initiation of IJ emergence. The traps were checked daily for the initiation of IJ emergence from the cadavers. Cadavers that were releasing IJs after 3-4 days were used in greenhouse experiments, to reduce the chance of variation due to stage of emergence between replications.

Greenhouse experiment

To explore the efficacy of EPN infected *G. mellonella* cadavers, a greenhouse experiment was conducted in conjuction with the imidacloprid seed treatment. The imidacloprid effect was tested by treating the wheat seeds with imidacloprid at the rate of 0.26 fluid ounces per 100 pounds. The experiment was conducted at the Plant Growth Center, Montana State University (MSU), Bozeman, in a completely randomized block design. Greenhouse pots (20 cm diameter) were filled with approximately 2.0 kg of sterilized sandy loam soil (MSU mix; sand 77% Silt 9%, clay 14%; pH=7.9, EC=0.38 mmhos/cm, 15.5% field capacity). Ten wheat seeds (variety Duclair) were seeded in each pot, and five medium-sized larvae (average length of 0.8 to 1.5 cm) were added after 24

hrs. Any larvae unable to enter the soil after five hours were replaced with other larvae. Next, one infected cadaver per EPN strain was placed in a 2 cm deep hole in the center of each pot and covered with soil. 60 pots made up the experiment with 20 pots of 4 EPNs × 5 replications, each with imidacloprid-treated plants plus wireworms and 20 pots of 4 strains of EPNs × 5 replications, but non-treated plants and wireworms. The last 20 were two types of controls: 10 pots, 5 with imidacloprid treatment and wireworms but no EPNs and 5 pots with imidacloprid treatment but no wireworms or EPNs; and 10 without imidacloprid, 5 of which had wireworms but no EPNs and 5 pots with no wireworms or EPNs).

The pots were watered daily. Soil temperature and moisture were recorded three times per week throughout the experiment using a soil moisture meter (Spectrum Technologies Inc., Illinois, USA) and soil thermometer (Taylor, Illinois, USA). Emergence success, probability of plant damage, and wireworm mortality were recorded for each pot. Plant damage was measured weekly for five weeks, determined by the presence of a wilted or dead central leaf, the presence of a point of feeding (just below the soil surface) at harvest, and/or seedling death. After five weeks, the pots were destructively sampled and the soil within each pot was checked for dead wireworms. If no wireworm was found, it was recorded as dead. Wireworm bodies decompose within 3 week and cannot be recovered after this point as explained in Chapters 2 and 4. The whole experiment was repeated after an interval of a week from the first trial.

Emergence of IJs from cadavers used in greenhouse

To determine IJ emergence rates from cadavers used in the greenhouse experiment, five randomly selected infected cadavers for each of the four EPN strains were removed from the treatment batches and placed individually on separate white traps at room temperature. Throughout the emergence period (three weeks) emerging IJs were collected, washed with tap water 2-3 times, counted by the serial dilution method (Glazer and Lewis 2000).

Statistical analysis

Data regarding IJs emergence in the laboratory, number of wireworms collected, yield, plant count, plant height, test weight, moisture and protein content were subjected to analysis of variance. Wireworm number data from barley was normalized using log transformation. The number of *G. mellonella* larvae in the field soil samples that were infected with EPNs was analyzed using the Generalized Linear Model (GLM) with quasibinomial distribution to avoid the overdispersion problem (Ramsey and Schafer 2012). A χ^2 test was used to test the interaction (synergistic, additive, or antagonistic) of imidacloprid and EPN strains on wireworm mortality in greenhouse. The wireworm mortality data was corrected for control mortality before analysis (Abbott 1925). The method of determining the type of interaction (synergistic, additive, or antagonistic) was first described by Finney (1964), and then modified by McVay et al. (1977). The expected additive proportional mortality (ME) for the EPN / imidacloprid combinations was calculated by ME = MN + MI (1-MN/100), where MN and MI are the observed proportional mortalities relatively caused by EPNs and imidacloprid alone. A χ^2 test was

then carried out using the formula $\chi^2 = (MNI - ME)^2 / ME$, where MNI represents the observed mortality for the EPN/imidacloprid combination. The calculated value from the χ^2 test was then compared with the χ^2 table value for 1 degree of freedom ($\chi^2_{1, 0.05} = 3.84$). The interactions were additive if $\chi^2 < 3:84$, antagonistic if $\chi^2 > 3:84$ and MNI < ME, and synergistic if $\chi^2 > 3:84$ and MNI > ME, where MNI is the observed mortality from the combination and ME is the expected mortality from the combination. For greenhouse trials, GLM with quasibinomial distribution was used for wireworm mortality and plant damage data to avoid the overdispersion problem (Ramsey and Schafer 2012). Trial, EPN strains, imidacloprid treatement, and time were the predictor variables in different models. The model was formulated in terms of transformed probabilities (logit transformation to the log-odds scale), the results were back transformed to an original probability scale after analysis for graphical representation. The Tukey-Kramer test ($\alpha = 0.05$) was used to find the significant differences between the treatments. Data were analyzed using the software statistical package R 2.15.1 (R Development Core Team, 2017)

Results

Field study

In June, the Pendroy (barley) and Choteau (spring wheat) sites had soil temperature varied from 6-14±5°C with 15-20°C air temperature. In July, both sites were observed with 15-22±5°C soil temperature and 22-27°C air temperature. In beginning of August, the soil temperature was higher in Pendroy site (20±5°C) with 16±2°C soil temperature at Choteau site. However, the soil moisture varied between two sites. At Pendroy site, the soil moisture content was almost twice as compared to Choteau site. The soil moisture content at Pendroy

site was 55.8-56.2±3%, 43-48±5%, and 55.38±5% in June, July, and first week of August, respectively. However, the soil moisture content at Choteau site was 22-29±5%, 25.88-32.24±7%, and 31.02±8% in June, July, and August, respectively. The IJs emerged from one cadaver in the laboratory varied significantly among four EPN strains ($F=8.55$, $df=3$, $p<0.0001$). EPN strains *S. carpocapsae* Cxrd, *S. riobrave* 7-12 and *S. riobrave* 355 produced significantly higher number of IJS (248,500 to 270,270) as compared to 181,860 IJs produced by *S. carpocapsae* All strain (Figure 2).

In barley, *L. californicus* was the numerically dominant species (~75%) followed by *A. mellilus* (~15%) and *H. bicolor* (~10%). However, in spring wheat, only *H. bicolor* was found (100%). Wireworm found at both the field sites were of multiple instars. The data for number of wireworms collected at different time intervals at both sites are provided in Table 1. Wireworm pressure was high in barley compared to spring wheat (Figure 3 and Table 1). In barley, there was no significant interaction between strain, dose and time ($F=0.53$, $df=16$, $p=0.93$) for number of wireworms collected. A significantly higher number of wireworms were collected after 60 days ($p<0.05$) and 75 days ($p<0.05$) as compared to 15, 30, and 45 days regardless of the EPN strains and dose. Overall, in barley, wireworm numbers trends remained almost the same from June to July, but more wireworms were collected in the beginning of August (Table 1). However, the number of wireworms collected remained the same throughout the collection time in spring wheat as there were no significant effects of time ($F=0.48$, $df=4$, $p=0.76$) as well as no significant interaction was detected between EPN strain, dose and time ($F=1.11$, $df=16$, $p=0.34$) (Table 1). Therefore, data regarding total number of wireworms collected throughout the

season were pooled and statistically analyzed further. In barley, the mean number of total wireworms (log transformed) collected over the season (Figure 3) did not differ significantly among EPN strains (F=0.62, df=4, p=0.65) and dose (F=0.11, df=1, p=0.74). The interaction between EPN strains and dose was also non-significant (F=0.12, df=4, p=0.97). Wireworm number did not vary significantly due to EPN strains (F=1.20, df=4, p=0.33) and dose (F=1.78, df=1, p=0.19). The interaction between EPN strain and dose was also not significant for wireworm numbers (F=0.46, df=4, p=0.76).

Weeds and volunteer plants at both the sites impacted plant counts at three weeks after planting and therefore, could not be considered accurate. and plant count data taken before harvesting is being analyzed further. In spring wheat, no parameters (yield, test weight, plant count, plant height, moisture, and protein) varied significantly with different treatments and dosages (Table 2). Similarly, none of the post-harvest and other parameters (yield, test weight, plant count, plant height, moisture and protein) differed significantly among EPN strains and doses in barley (Table 3).

In 2019, data regarding number of *G. mellonella* larvae infected in soil samples were analyzed separately for both fields. In barley, EPN dose had a non-significant effect on the *G. mellonella* mortality (χ^2=3.26, df=1, p=0.07). Similarly, the interaction of EPN strain and dose had no significant effect on *G. mellonella* mortality (χ^2=3.43, df=4, p=0.49). Although, EPN strains showed a significant effect on the *G. mellonella* mortality (χ^2=10.92, df=4, p=0.03) in combined model but did not show significant effect on *G. mellonella* mortality when applied at the rate of 3 cadavers/plot (χ^2=8.23, df=4, p=0.08) and 6 cadavers/plot (χ^2=6.94, df=4, p=0.17). In spring wheat, the interaction between EPN

strain and dose was found significant (χ^2=16.44, df=3, p<0.0001) in regard to *G. mellonella* mortality. However, dose did not have significant effect on the *G. mellonella* mortality (χ^2=1.50, df=1, p=0.22). When compared at two different doses separately, four EPN strains tested did not differ significantly among each other and from control treatment plots at 3 cadavers/plot dose (χ^2=5.97, df=4, p=0.20), but differed significantly among each other (χ^2=29.43, df=4, p<0.0001), when applied at the rate of 6 cadavers/plot. However, Tukey's test did not show any significant difference among these four EPN strains at 6 cadavers/plot dose. Overall, the percentage mortality of *G. mellonella* was very low in spring wheat as only 25% average mortality was observed in samples collected from plots with *S. carpocapsae* infected cadavers (Figure 4). However, in barley, the *G. mellonella* mortality was observed to be higher than in spring wheat. There were no significant differences among EPN strains in terms of percentage larval mortality in barley. The percentage *G. mellonella* mortality ranged from 30-45% at Pendroy. However, we observed no mortality of *G. mellonella* in soil samples collected from the field in June 2020.

Greenhouse experiment

For greenhouse experiments in 2020, the number of IJs that emerged from a cadaver in the laboratory varied significantly among the four EPN strains (F=11.61, df=3, p=0.0003). EPN strain *S. carpocapsae* Cxrd produced a significantly higher number of IJs (248,031 IJs; P <0.01) compared to *S. riobrave* 7-12 (161,077 IJs) and *S. riobrave* 355 (166,096 IJs). However, it did not differ significantly from *S. carpocapsae* All (206,525 IJs) (P>0.01) (Figure 5).

Antagonistic interactions of imidacloprid seed treatment were found with *S. carpocapsae* All and *S. riobrave* 7-12 (Table 4). However, synergism was observed among imidacloprid and *S. carpocapsae* Cxrd and imidacloprid and *S. riobrave* 355 strains in regard to *L. californicus* mortality (Table 4). Wireworm mortality did not differ significantly between the two trials (trial: $\chi^2 = 1.68$, df = 1, P=0.20; trial*EPN strain: $\chi^2 = 6.32$, df = 4, P=0.18), therefore the data were pooled for further analysis. The EPN strains significantly impacted the wireworm mortality ($\chi^2 = 65.42$, df = 4, P <0.0001) with moderate effect of imidacloprid treatment on wireworm mortality ($\chi^2 = 3.52$, df = 1, P = 0.06). Overall, all four EPN strains caused significantly higher mortality than the control (P<0.01) (Figure 5). However, the mortality caused by *S. carpocapsae* All, *S. carpocapsae* Cxrd, and *S. riobrave* 355 strains did not differ from each other, with mortality ranging from 40 to 68% with *S. carpocapsae* Cxrd strains causing 68% mortality when the plants were treated with imidacloprid. *Steinernema riobrave* 7-12 caused significantly higher mortality (26-36%) than the control (10-14%), but it was significantly lower than the other three EPN strains (P <0.01). Although, the mortality caused in imidacloprid treated plants and non-imidacloprid plants was not significantly different, percentages were higher for *S. carpocapsae* Cxrd, *S. riobrave* 355, and *S. riobrave* 7-12 in imidacloprid treatments except *S. carpocapsae* All strain which caused 56% mortality in non-imidacloprid plants as compared to only 50% mortality caused in imidacloprid treated plants (Figure 6).

In greenhouse trials, plant damage caused by *L. californicus* did not vary significantly betweeen 7DAT (Days after treatment) and 14DAT, but varied significantly at 21, 28, and 35DAT (P < 0.001). However, the plant damage occurred at 21, 28, and 35

DAT did not vary significantly among each other (P > 0.20). Therefore, the data regarding plant damage observed at 14DAT and 35DAT were chosen for further analysis (Figure 7A and B). The two trials differed significantly in regards to plant damage rate (trial: $\chi^2 = 4.98$, df = 1, P = 0.03), however there was no significant interaction detected between trial and EPN strain ($\chi^2 = 5.98$, df = 5, P = 0.31), therefore the data were pooled among trials for further analysis. The damage rate was significantly affected by EPN strain ($\chi^2 = 285.20$, df = 5, P < 0.001), time ($\chi^2 = 51.45$, df = 1, P < 0.0001), and imidacloprid treatment ($\chi^2 = 464.79$, df = 1, P < 0.001). Significant interaction was also found between EPN strains and imidacloprid treatment ($\chi^2 = 17.85$, df = 5, P = 0.003). However, the interaction between EPN strain and time ($\chi^2 = 10.61$, df = 5, P = 0.06), time and imidacloprid treatment ($\chi^2 = 0.53$, df = 1, P = 0.47) were not significant. Similarly, no three-way interaction was detected between EPN strain, time and imidacloprid treatment ($\chi^2 = 3.01$, df = 5, P = 0.69). At 14DAT, none of the EPN strains differed significantly for plant damage as compared to control pots (P>0.05) in both imidacloprid and non-imidacloprid treated plants (Fig. 7A). However, the percentage of plant damage observed was significantly higher (40-57%; P<0.01) in pots with non-imidacloprid plants as compared to only 6-9% plant damage observed in pots with imidacloprid treated plants (Fig. 7A). However, at 35DAT, the percentage of plant damage recorded was significantly lower in pots with *S. riobrave* 355 (59%), *S. carpocapsae* All (65%), and *S. carpocapsae* Cxrd (57%) as compared to 92% plant damage observed in control treatment pots (P >0.01), when the plants were not treated with imidacloprid (Fig. 7B). The EPN strain, *S. riobrave* 7-12 also caused 75% plant damage in the non-imidacloprid plants and did not differ significantly from other EPN

strains and control ($P < 0.05$) (Fig. 7B). The four EPN strains did not vary significantly from the control in regard to plant damage when the seeds were planted after imidacloprid treatment (Fig. 7B). The percentage of plant damaged ranged from 8-24% in these imidacloprid treated plants and was significantly lower than the damage in non-imidacloprid pots ($P<0.01$) (Fig. 7B). Additionally, the plants germinated well and had less damage (1-2%) occurred in pots without *L. californicus* larvae in both imidacloprid and non-imidacloprid controls (Fig. 7A and B). The percentage of plant damage observed at 7, 21, and 28DAT is also being presented in Table 5. The average soil temperature and moisture in the greenhouse were recorded as 22°C (17-27°C) and 22 ± 5%, respectively.

Discussion

EPNs have been evaluated against European wireworm species in laboratory, greenhouse and field conditions (Ester and Huiting, 2007; Ansari et al., 2009; Campos-Herrera and Gutierrez, 2009; Morton and Garcia-del-Pino, 2017). However, this approach did not turn out to be very successful in the field. The only report available on evaluating different EPN species against *L. californicus* is the work done by Toba et al. (1983) in which *Steinernema feltiae* (Filipjev) was able to kill 28% of this species under caged field conditions.

In addition, field studies against wireworms were so far only conducted with aqueous EPN suspensions (Toba et al., 1983; Schalk et al., 1993; Ester and Huiting, 2007; Arrington et al., 2015; Morton and Garcia-del-Pino, 2017). In the present study, we tested EPN infected cadavers as a delivery method to see if this approach can be better at managing wireworm populations. We hypothesized that IJs applied via EPN infected

cadavers will be more protected from unfavorable environmental conditions as compared to aqueous suspensions but unfortunately in the present study, EPNs applied in the form of infected cadavers did not prevent wireworm damage in crops or protect yields at either site. In addition, other parameters such as test weight, plant count, plant height, moisture, and protein percentages did not differ significantly among treatments at either field sites. Number of wireworms collected at both sites cannot be correlated with the overall yield and other post-harvest parameters as the distribution of wireworms on the basis of stocking trap collection cannot directly be related to the efficiency of the EPN treatments (Reddy et al., 2014; Sharma et al. 2018). All these results need to be studied further as factors such as poor germination due to mechanical damage and flooding due to excess early season rains (Pendroy site) and weed problems, could also be responsible for these results.

However, at least three EPN strains: *S. carpocapsae* All, *S. carpocapsae* Cxrd, and *S. riobrave* killed more than 50% of *L. californicus* larvae in greenhouse experiments. The mortality rates of *L. californicus* (50-68%) due to both strains of *S. carpocapsae* (All and Cxrd) observed in the present study differed from those of Ensafi et al. (2018), where only 20% *L. californicus* mortality was observed due to *S. carpocapsae* in a greenhouse experiment. However, Morton and Garcia-del-Pino (2017) and our previous study (Sandhi et al. 2020a) reported 50% wireworm mortality caused by *S. carpocapsae* in a greenhouse experiment. Despite no significant differences observed between the mortality caused in imidacloprid and non-imidacloprid treatments, the wireworm mortality was higher in the pots with imidacloprid treated plants. This indicates that the seed treatment might have been able to enhance the infection and killing ability of EPNs against *L. californicus* in the

present study. Similarly, in non-imidacloprid plants, *S. carpocapsae* All and Cxrd, and *S. riobrave* 355 strains killed 40-58% of the treated larvae which shows the virulence of these EPN species against the wireworms. Almost 14% wireworm mortality was observed in control pots as well, limiting the significance of the mortality caused by EPNs either in presence of imidacloprid or absence of this seed treatment.

In the present study, imidacloprid seed treatment was observed protecting wheat plants from *L. californicus* damage as compared to the non-imidacloprid treatments throughout the experiment. This trend was seen after the plants germinated and remained the same to the end of the experiment, i.e. 35 days after the treatment for the plants treated with imidacloprid. However, the EPN-only treatments did not protect the wheat plants to the same extent, and damage recorded after 14 days of the treatment was not even lower than the control treatment. But by the end of the experiment (35 DAT), the plant damage was lower in pots treated with *S. riobrave* 355, *S. carpocapsae* All and Cxrd strains than the in the control pots. These findings are in agreement with study of Ensafi et al. (2018) who reported that neonicotinoids only reduce plant damage by repelling wireworms and do not cause direct wireworm mortality. The lower efficacy of *S. riobrave* 7-12 strain as compared to other strains might be due to the comparatively lower number of IJs reproduced from cadavers, which needs to be studied further.

Although in some cases, EPNs applied as insect cadavers are reported to be superior to application in aqueous suspension (Shapiro and Lewis, 1999; Perez et al., 2003; Shapiro-Ilan et al., 2003; Shapiro-Ilan et al., 2010), other studies did not report any positive outcome from this approach (Bruck et al., 2005; Raja et al., 2015). The main reason may be cadavers

rupturing or sticking together during handling, transportation and application (Shapiro-Ilan et al., 2001). However, in the present study, the chances of this problem are considered to be very low because proper care was taken while transferring the cadavers from laboratory to the field and the greenhouse. Additonally, the cadaver approach has a limitation of lower dispersal of IJs as compared to aqueous suspension application of EPNs which could have been the reason behind the poor efficacy. The IJs dispersal from infected host cadavers happens in an aggregated pattern rather than a random or uniform distribution (Shapiro et al., 2014).

Cadavers might have ruptured or disintegrated after being placed in the soil because of the unfavorable environmental conditions especially in the field. The survival and infectivity of EPNs post-application has always remained one of the major challenges for utilization of EPNs in general. Reasons for the low post-application survival may be various abiotic factors such as ultraviolet radiation, soil texture, pH, temperature, relative humidity and moisture content (Shapiro-Ilan et al., 2006; Koppenhöfer and Fuzy, 2006; Shapiro-Ilan et al., 2012). When these abiotic factors are unfavorable, IJs tendency to desiccate will eventually deter their movement and survival and might have been the reason behind the unexpected results in the field. However, in the greenhouse experiment, the mortality rates observed and the presence of IJs in the dead wireworm larvae showed that the IJs were able to develop and reproduce well in the applied cadavers. The reason behind the higher efficacy of EPN cadavers against *L. californicus* in greenhouse experiment can be the well-maintained sorroundings in term of temperature and soil moisture in the pots. Additinally, dose of one EPN infected *G. mellonella* cadaver used in greenhouse

(equivalent to the rate of 161,000 to 248,000 IJs/pot) may have been very high as compared to the doses applied in the field and could be the cause of higher mortality observed in greenhouse.

Coating of EPN infected cadavers (mostly *G. mellonella*) with formulations such as clay, calcareum powder, talc powder, gelatin capsules, gluten, lignin, and starch coverings (Shapiro-Ilan et al., 2001; Del Valle et al., 2008) have been tested with some promising results. In addition, mechanized equipment has been developed to place the infected cadavers in field (Zhu et al., 2011). All these formulations and techniques should be tested to improve the efficacy of EPN infected cadaver applications for wireworm control.

Soil texture affects wireworm population, their movement in the soil and ability to cause damage in various crops (Milosavljević et al., 2016; Ensafi et al., 2018). Sandier soils are reported to be associated with higher wireworm damage (Hermann et al. 2013, Rashed et al. 2017). Similarly, EPNs such as *S. carpocapsae* reduced wireworm damage to wheat seedling damage in sand dominated soil media (Ensafi et al. 2018) which is in agreement with our greenhouse results. Both our field sites had clay loam/gravelly loam soils which may have been one of the explanations for the low efficacy of EPNs in the field. Space between soil particles is needed for adequate circulation of air and EPN movement through soil (Kung et al., 1990; Koppenhöfer and Fuzy, 2006). EPN migration, survival, pathogenicity and host-finding ability decrease in clay soil due to small pore sizes (Kung et al., 1990). It is possible that after emergence from cadavers IJs could not disperse well because of the clayey soils in the fields.

Similarly, adequate soil moisture is necessary for EPN movement, survival, and efficacy, and therefore, pre- and post-irrigation application in fields might help in improving EPN efficacy and persistence (Georgis et al., 2006). Dry soils have no consistent film of water and are associated with inhibition of EPN movement and dispersal (Koppenhöfer and Fuzy, 2006; Lewis et al. 2006). In the present study, the barley field followed dryland culture while the wheat field was irrigated. Counterintuitively, in the barley, the soil moisture content was recorded in the range of 43-55% which is high enough to inhibit EPN movement and dispersal, and may have contributed to poor efficacy. However, at the spring wheat site, soil moisture content varied from 22-31%. At both sites, the soil temperature was low i.e. 6-14°C in the beginning of the season but increased and reached 16-22°C later in the crop season. In the present study, soil temperature does not seem to be a huge problem as temperatures below 0°C and above 40°C are lethal to most EPNs and reduce nematode survival and infectivity (Ulu and Susurluk, 2013). However, the lower temperatures observed in early season in field might have limited the EPN movement and survival leading to poor efficacy. Additionally, varied soil temperature and soil moisture levels throughout the season might have contributed to the lack of efficacy of EPNs in the present studies. Therefore, some cold-tolerant EPN species such as *S. feltiae* or the timing of EPN application to avoid cold weather need to be studied in future for effective wireworm control.

We strongly believe that in barley field, low germination because of mechanical damage at seeding might have been the major factor for the unfortunate outcomes of these experiments. It is evident from 30-45% *G. mellonella* mortality observed in the barley field

soil samples that EPNs were able to survive and persist in this field. The presence of high number of wireworms in spring wheat field could be the reason behind the higher persistence as the EPNs might have been able to infect the wireworms and reproduce further. At the spring wheat site, wireworm numbers were very low anyway and we cannot actually relate the effect of EPN treatments on the yield. In addition, we are not sure if EPNs had a favorable soil environment for survival as only 25% *G. mellonella* larvae died after baiting the soil samples collected from this site. It is important to mention here that the seeds planted were imidacloprid treated in both the fields but this cannot be correlated with the plant damage results in the present study because of the possibility of all above mentioned factors.

In our study, we found no significant differences among EPN strains in terms of protecting the crops from wireworm damage in field and greenhouse. In addition, dosage (3 vs. 6 cadavers) also did not differ in terms of efficacy against wireworms. On the basis of IJs emergence in laboratory, three EPN strains *S. carpocapsae* Cxrd and *S. riobrave* (7-12 and 355 strains) infected cadavers released an average of 250,000 IJs/cadaver which makes the total IJs released 0.75 million IJs/plot (3 cadavers; 330,000 IJs/m^2) and 1.5 million IJs/plot (6 cadavers; 670,000 IJs/m^2). However, the *S. carpocapsae* All infected cadavers released 182,000 IJs/cadaver on average making the final dose as 0.55 million IJs/plot (3 cadavers); 240,000 IJs/m^2) and 1.1 million IJs/plot (6 cadavers; 490,000 million IJs/m^2). Despite the difference in the IJs emergence for these EPN strains, we are unable to differentiate the efficacy of EPN strains because of poor results. Wireworm size may have been another factor in the study because later instar larvae have very hard cuticles

that are difficult for EPNs to penetrate. Sandhi et al. (2020a) reported 50-60% of medium sized *L. californicus* larval mortality with the same EPN strains under laboratory and green house when a very high dose (>200 IJs/cm^2) was used. As already mentioned, all the larval instars were found in barley field throughout the season, and it is possible that EPNs were unable to penetrate the later instar larvae due to their hard cuticle, narrow mouth and strong anal musculature. It is possible that wireworms avoided the EPN infection by moving around the plots. Movement and distribution of the EPNs from the cadavers needs further study.

Although, greenhouse studies have shown moderate to high infection of *L. californicus* larvae and reduced wheat damage due to *S. carpocapsae* and *S. riobrave* released via infected cadavers, especially when combined with the imidacloprid seed treatment, further field studies should concentrate on testing different formulations for coating EPN infected cadavers in addition to seed treatment. The aqueous suspension approach should also be tested and compared with the EPN infected cadaver approach. The efficacy of these EPN species against different wireworm species should be examined in different soil types. In addition, the ability of EPNs to persist longer in the applied area should be explored in order to hopefully achieve a cost-efficiency control method for wireworms. Lastly, different cold tolerant EPN strains and investigation of timings when temperatures are more conducive for EPN application should be explored.

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Table 1. Number of wireworms collected from treatment plots in 2019.

Pendroy (Barley)*						
Treatment	12 June 2019	27 June 2019	10 July 2019	25 July 2019	9 August 2019	**Total**
Sr7-12(3)	12	9	10	16	30	77
Sr7-12(6)	9	15	9	21	22	76
Sr355(3)	3	26	16	30	36	111
Sr355(6)	13	14	5	12	35	79
ScAll(3)	13	17	13	24	24	91
ScAll(6)	7	23	9	21	38	98
ScCxrd(3)	2	12	9	35	57	115
ScCxrd(6)	9	21	22	25	26	103
Control	18	33	14	26	56	147

Choteau (Spring wheat)						
Treatment	3 June 2019	18 June 2019	3 July 2019	17 July 2019	31 July 2019	**Total**
Sr7-12(3)	4	0	2	0	4	10
Sr7-12(6)	0	0	1	2	1	4
Sr355(3)	7	0	1	2	2	12
Sr355(6)	0	2	3	1	2	8
ScAll(3)	2	4	2	3	1	12
ScAll(6)	3	4	0	2	4	13
ScCxrd(3)	1	6	3	0	3	13
ScCxrd(6)	2	4	1	1	1	9
Control	2	3	3	3	2	13

*At the Pendroy site, wireworm sampling was delayed for nine days compared to the Choteau site because of heavy rain at Pendroy during that nine days period.

Table 2. Plant count, plant height, moisture, protein, seed test weight, and yield in the EPN treated plots (Mean ± SE) in Choteau (Spring wheat) in 2019.

Treatment	Plant Count	Plant height (cm)	Moisture (%)	Protein (%)	Test weight (kg/ha)	Yield (kg/ha)
Sr7-12(3)	33.8±2.3[a]	81.7±1.52[a]	11.89±0.30[a]	12.32±1.92[a]	77.89±1.17[a]	2050.15±330.91[a]
Sr7-12(6)	37±2.07[a]	69.6±3.31[a]	11.79±0.06[a]	11.06±0.44[a]	81.19±1.83[a]	1525.90±160.81[a]
Sr355(3)	34.5±2.80[a]	69.6±5.01[a]	11.42±0.16[a]	13.89±0.85[a]	76.49±1.69[a]	1495.58±265.91[a]
Sr355(6)	36±3.5[a]	76.1±5.28[a]	11.82±0.23[a]	11.69±1.30[a]	79.05±0.98[a]	1598.19±371.44[a]
ScAll(3)	34.6±2.58[a]	74.1±3.59[a]	11.78±0.20[a]	11.90±1.27[a]	80.42±1.14[a]	1818.65±353.65[a]
ScAll(6)	30.9±1.6[a]	70.3±3.52[a]	11.69±0.32[a]	13.28±1.61[a]	78.61±0.64[a]	1845.98±249.22[a]
ScCxrd(3)	31.8±1.54[a]	68.0±3.84[a]	12.16±0.16[a]	10.70±0.67[a]	78.68±2.65[a]	1540.48±247.68[a]
ScCxrd(6)	39.3±2.12[a]	79.7±2.66[a]	11.54±0.30[a]	14.73±1.61[a]	76.24±0.99[a]	1969.97±237.57[a]
Control	38.7±1.68[a]	74.4±3.65[a]	11.80±0.30[a]	12.05±1.44[a]	77.07±1.37[a]	1689.78±106.56[a]
Treatment	F_4=1.74, p=0.16	F_4=0.26, p=0.90	F_4=0.18, p=0.95	F_4=0.26, p=0.90	F_4=1.22, p=0.32	F_4=0.37, p=0.83
Dose	F_1=1.41, p=0.24	F_1=0.04, p=0.85	F_1=0.26, p=0.61	F_1=0.18, p=0.68	F_1=0.13, p=0.72	F_1=0.002, p=0.97
Treatment:Dose	F_4=1.65, p=0.18	F_4=3.03, p=0.03*	F_4=0.95, p=0.45	F_4=1.49, p=0.22	F_4=1.51, p=0.22	F_4=0.88, p=0.49

Where ScAll = *Steinernema carpocapsae* All; ScCxrd = *Steinernema carpocapsae* Cxrd; Sr355 = *Steinernema riobrave* 355; Sr7-12 = *Steinernema riobrave* 7-12. "3" and "6" in the parentheses represents number of cadavers. None of the treatment had significant effect on different parameters (α=0.05, Tukey-Kramer test).

Table 3. Plant count, plant height, moisture, protein, seed test weight, and yield in the EPN treated plots (Mean ± SE) in Pendroy (Barley) in 2019.

Treatment	Plant Count	Plant height (cm)	Moisture (%)	Protein (%)	Test weight (kg/ha)	Yield (kg/ha)
Sr7-12(3)	8.6±1.70 [a]	79.3±6.82 [a]	9.70±0.19 [a]	12.3±0.72 [a]	60.28±1.55 [a]	1623.96±261.86 [a]
Sr7-12(6)	8.0±1.80 [a]	79.4 ±4.92 [a]	9.67±0.19 [a]	12.59±0.89 [a]	61.27±1.19 [a]	2169.25±315.46 [a]
Sr355(3)	8.2±1.50 [a]	72.6±5.81 [a]	9.99±0.34 [a]	12.71±0.59 [a]	59.22±1.17 [a]	1737.86±304.71 [a]
Sr355(6)	8.3±1.51 [a]	71.8±2.91 [a]	10.18±0.34 [a]	12.54±0.80 [a]	59.26±1.23 [a]	1617.07±343.27 [a]
ScAll(3)	11.2±1.33 [a]	84.3±5.01 [a]	9.81±0.27 [a]	12.64±0.53 [a]	59.41±2.34 [a]	2135.89±387.31 [a]
ScAll(6)	8.5±0.97 [a]	80.0±5.77 [a]	9.79±0.27 [a]	11.94±0.48 [a]	57.40±2.39 [a]	1812.46±351.64 [a]
ScCxrd(3)	11.6±1.34 [a]	91.5±4.23 [a]	9.61±0.26 [a]	11.84±0.54 [a]	61.49±2.08 [a]	1879.59±383.32 [a]
ScCxrd(6)	9±2.21 [a]	76.8±8.48 [a]	10.30±0.44 [a]	13.39±1.12 [a]	58.58±2.09 [a]	1531.80±495.99 [a]
Control	9±1.88 [a]	71.9±6.60 [a]	10.13±0.12 [a]	12.88±0.56 [a]	58.83±1.72 [a]	1860.02±261.80 [a]
Treatment	F_4=0.62, p=0.65	F_4=1.83, p=0.14	F_4=1.04, p=0.40	F_4=0.05, p=0.99	F_4=0.56, p=0.70	F_4=0.28, p=0.89
Dose	F_1=1.24, p=0.27	F_1=1.11, p=0.29	F_1=0.87, p=0.36	F_1=0, p=0.99	F_1=0.47, p=0.50	F_1=0.15, p=0.70
Treatment:Dose	F_4=0.35, p=0.84	F_4=0.57, p=0.69	F_4=0.63, p=0.65	F_4=0.67, p=0.61	F_4=0.40, p=0.81	F_4=0.59, p=0.67

Where ScAll = *Steinernema carpocapsae* All; ScCxrd = *Steinernema carpocapsae* Cxrd; Sr355 = *Steinernema riobrave* 355; Sr7-12 = *Steinernema riobrave* 7-12. "3" and "6" represents number of cadavers. None of the treatment had significant effect on different parameters (α=0.05, Tukey-Kramer test).

Table 4. Interaction of entomopathogenic nematodes and imidacloprid seed treatment against *Limonius californicus* larvae at 35 DAT (days after treatment) in greenhouse in 2020.

EPN strain	Observed mortality (%)[a]	Expected mortality (%)[b]	χ^2	Type of Interaction
Steinernema carpocapsae All	40.00±7.89	52.80±5.74	20.36±4.25	Antagonistic
Steinernema carpocapsae Cxrd	58.00±8.14	47.60±6.65	70.94±32.65	Synergistic
Steinernema riobrave 355	40.00±7.30	39.60±7.17	40.93±31.66	Synergistic
Steinernema riobrave 7-12	26.00±8.46	28.00±7.52	----------	Antagonistic

a. Observed mortality was corrected for control mortality with Abbott's formula (Abbott 1925)
b. Expected mortality ME = MN + MI (1-MN/100), where MN and MI are the observed proportional mortalities caused by EPNs and imidacloprid, respectively.

Table 5. Average percentage plant damage by larval *Limonius californicus* after exposure to entomopathogenic nematodes at 7, 21, and 28 DAT (days after treatment) in greenhouse in 2020.

Strain	**7DAT**		**21DAT**		**28DAT**	
	IMIDA	**NON-IMIDA**	**IMIDA**	**NON-IMIDA**	**IMIDA**	**NON-IMIDA**
All	6.00±2.58	58.00±8.32	5.00±2.45	63.00±9.45	6.0±2.45	65.0±9.64
Cxrd	10.00±4.79	41.00±6.22	10.00±5.55	55.0±5.74	17.0±8.55	57.0±6.81
355	8.00±3.74	44.00±6.84	8.00±3.74	55.0±7.48	14.0±6.24	58.0±8.21
7-12	7.00±2.00	56.00±8.67	7.00±2.81	75.0±6.23	9.0±3.45	75.0±6.23
CW	12.00±3.58	57.00±6.45	16.00±4.03	80.0±6.78	24.0±5.61	89.0±3.22
CNW	1.00+1.00	2.00±1.22	1.00±1.00	2.0±1.22	1.0±1.00	2.0±1.22

Where, All = *Steinernema carpocapsae* All; Cxrd = *Steinernema carpocapsae* Cxrd; 355 = *Steinernema riobrave* 355; 7-12 = *Steinernema riobrave* 7-12; CW = Control with wireworms; and CNW = Control without wireworms and DAT = Days after treatment.

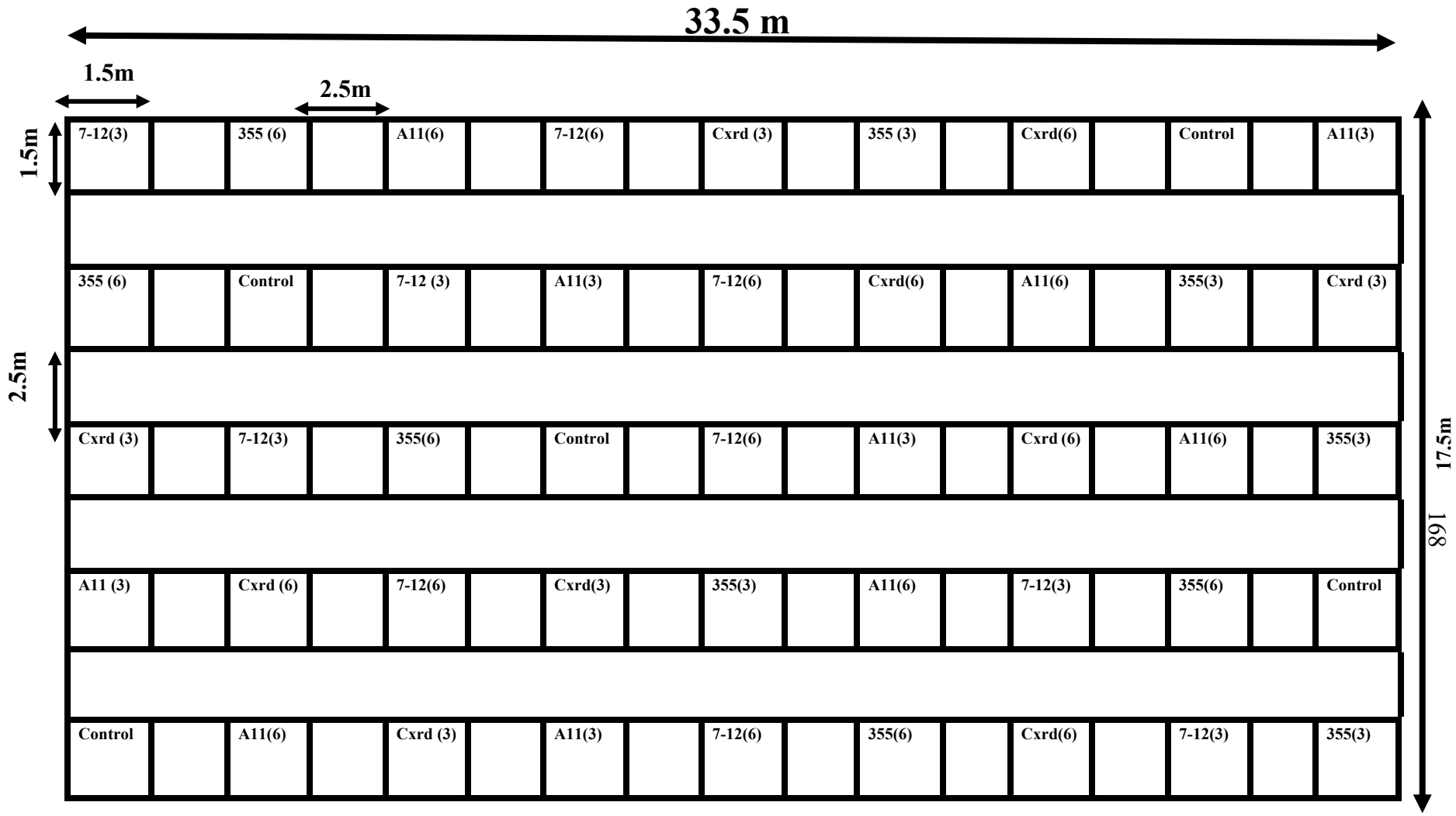


Figure 1. Schematic diagram of field layout at Choteau and Pendroy sites in 2019. All = *Steinernema carpocapsae* All; Cxrd = *Steinernema carpocapsae* Cxrd; 355 = *Steinernema riobrave* 355; 7-12 = *Steinernema riobrave* 7-12; C = Control. The numbers "3" and "6" represent number of EPN infected cadavers.

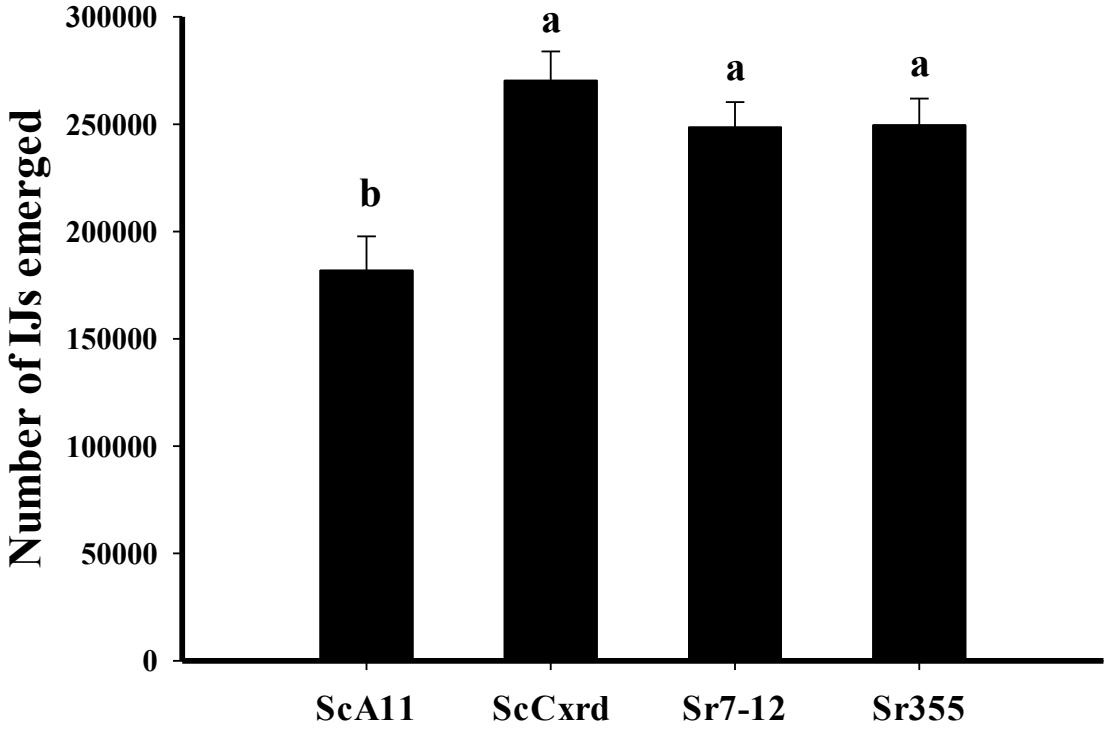


Figure 2. Mean number of IJs emerged from a cadaver infected with different EPN strains for field experiment in 2019, where ScAll = *Steinernema carpocapsae* All; ScCxrd = *Steinernema carpocapsae* Cxrd; Sr355 = *Steinernema riobrave* 355; Sr7-12 = *Steinernema riobrave* 7-12.

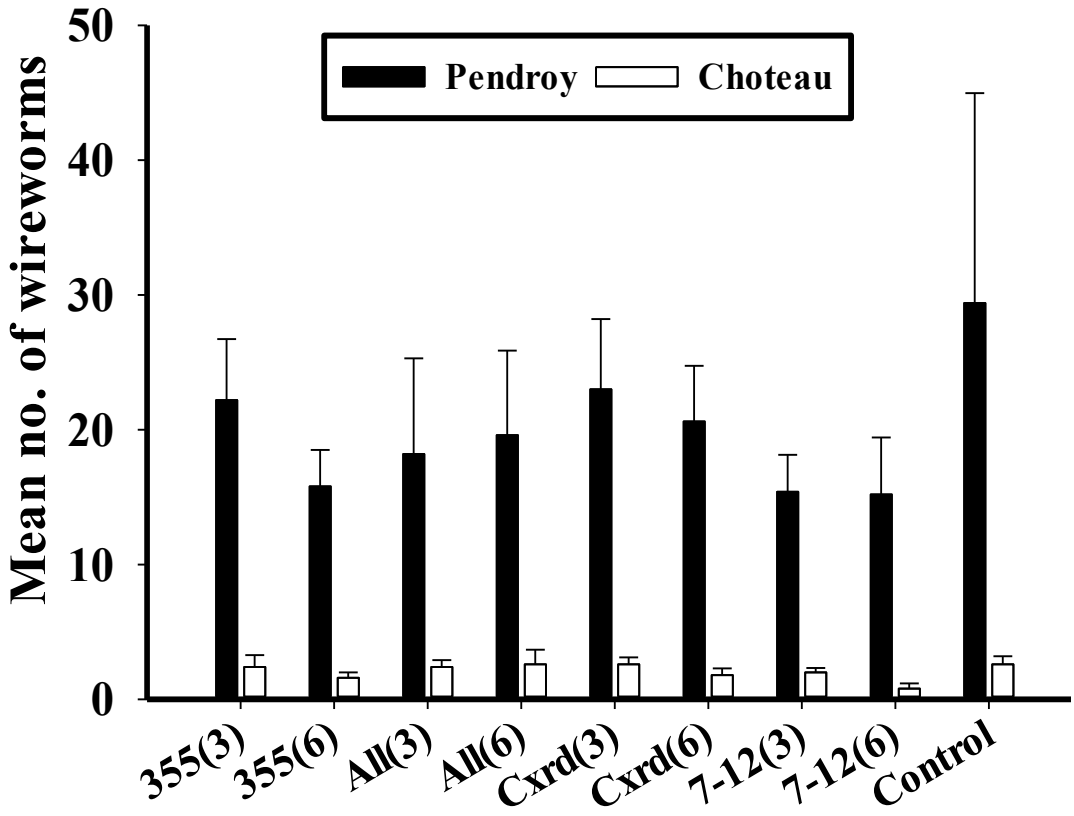


Figure 3. Total mean numbers of wireworms collected in bait traps after 75 days in 2019 (n=5). No significant difference was found between treatments (α = 0.05, Tukey-Kramer test), where ScAll = *Steinernema carpocapsae* All; ScCxrd = *Steinernema carpocapsae* Cxrd; Sr355 = *Steinernema riobrave* 355; Sr7-12 = *Steinernema riobrave* 7-12.

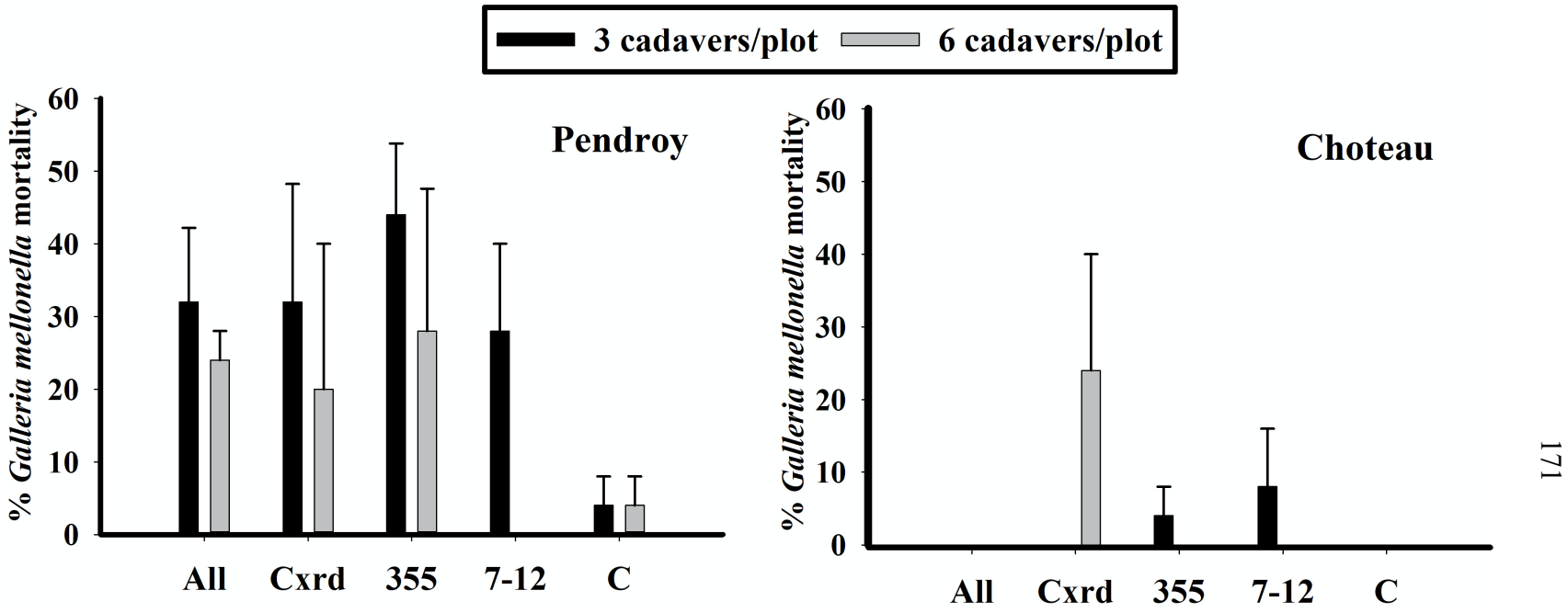


Figure 4. Average percentage of *Galleria mellonella* infected with EPNs in collected soil samples. 355 = *Steinernema riobrave* 355; 7-12 = *Steinernema riobrave* 7-12; All = *Steinernema carpocapsae* All; Cxrd = *Steinernema carpocapsae* Cxrd; C = Control. No significant differences were observed among the treatments (Tukey-Kramer test; α=0.05)

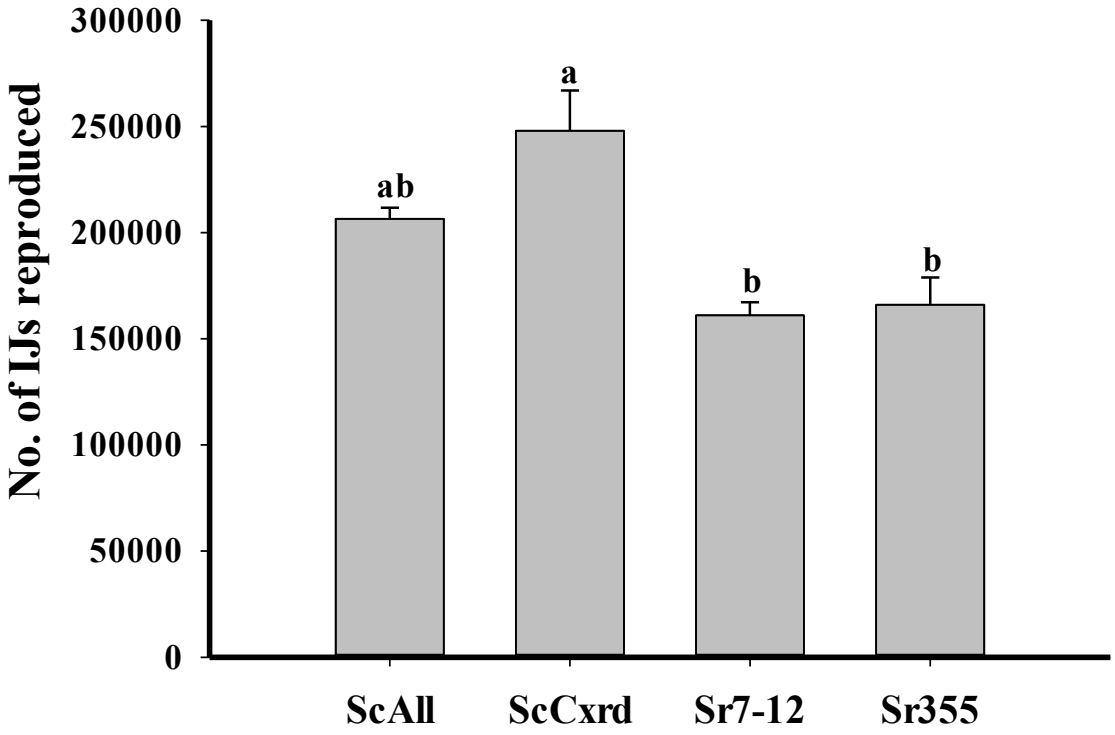


Figure 5. Mean number of IJs emerged from a cadaver infected with different EPN strains for greenhouse experiment in 2020, where where ScAll = *Steinernema carpocapsae* All; ScCxrd = *Steinernema carpocapsae* Cxrd; Sr355 = *Steinernema riobrave* 355; Sr7-12 = *Steinernema riobrave* 7-12.

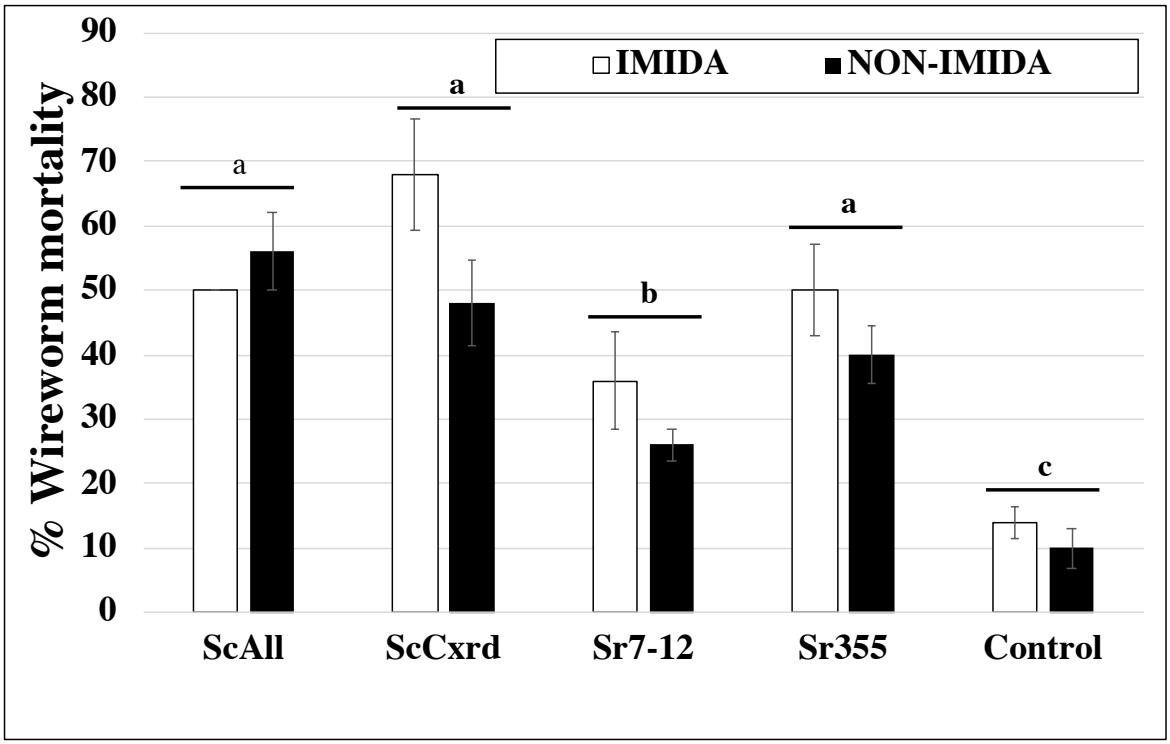


Figure 6. Average percentage mortality of larval *Limonius californicus* after exposure to entomopathogenic nematodes at one EPN infected *Galleria* cadaver/pot in greenhouse in 2020. ScAll = *Steinernema carpocapsae* All; ScCxrd = *Steinernema carpocapsae* Cxrd; Sr7-12 = *Steinernema riobrave* 7-12; Sr355 = *Steinernema riobrave* 355; Different letters above the line points indicate statistical significance ($P \leq 0.05$, Tukey-Kramer test).

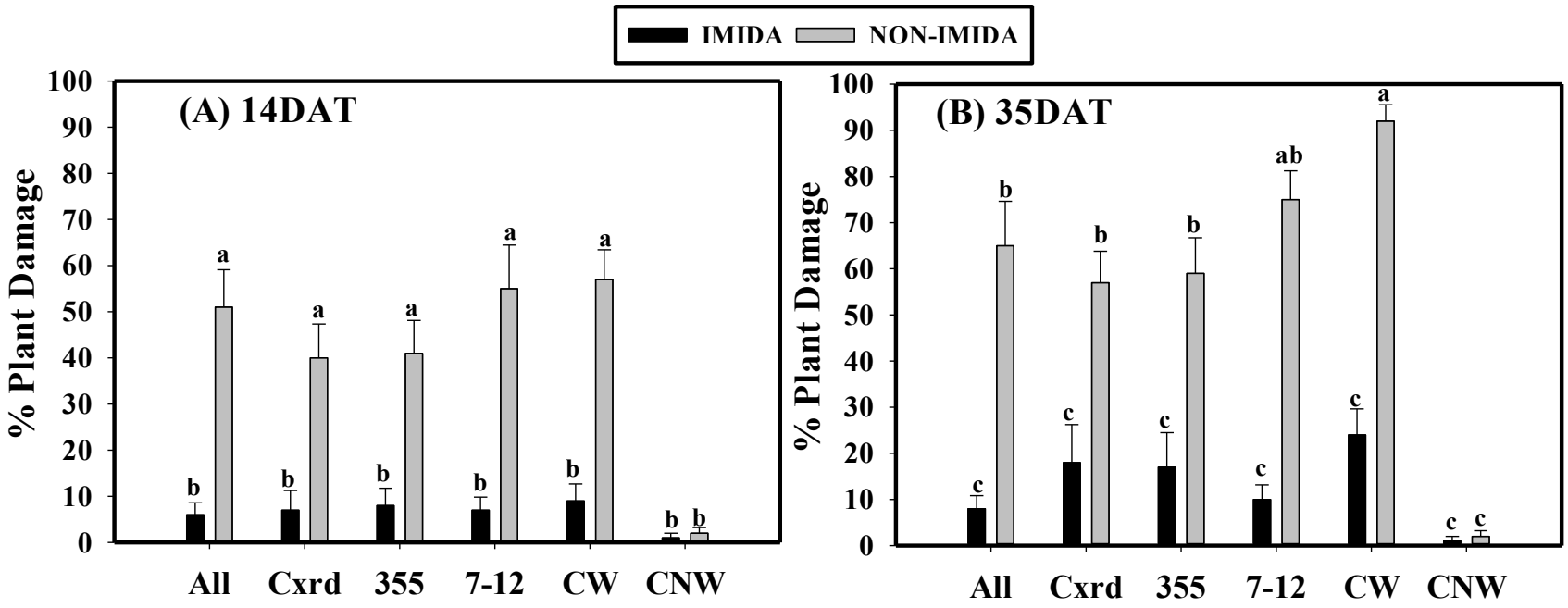


Figure 7. Average percentage of plant damage by larval *Limonius californicus* after exposure to entomopathogenic nematodes at one EPN infected *Galleria* cadaver/pot at (A) 14DAT and (B) 35DAT in greenhouse in 2020. All = *Steinernema carpocapsae* All; Cxrd = *Steinernema carpocapsae* Cxrd; 355 = *Steinernema riobrave* 355; 7-12 = *Steinernema riobrave* 7-12; CW = Control with wireworms; and CNW = Control without wireworms. DAT = Days after treatment. Different letters above the line points indicate statistical significance ($P \leq 0.05$, Tukey-Kramer test).

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CHAPTER SIX

EFFECT OF SOIL TEXTURE, SOIL MOISTURE, AND SOIL TEMPERATURE ON EFFICACY OF ENTOMOPATHOGENIC NEMATODE STRAINS AGAINST WIREWORMS UNDER LABORATORY CONDITIONS

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EFFECT OF SOIL TEXTURE, SOIL MOISTURE, AND SOIL TEMPERATURE ON EFFICACY OF ENTOMOPATHOGENIC NEMATODE STRAINS AGAINST WIREWORMS UNDER LABORATORY CONDITIONS

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Abstract

Entomopathogenic nematodes (EPN) are effective biological control agents against different insect pests especially soil dwelling insects. However, abiotic factors such as soil moisture, texture and temperature greatly influence the EPN dispersal, movement and ability to find host insects. In the present study, effects of soil texture, moisture, and temperature on the infection rate of *Steinernema carpocapsae* and *Steinernema riobrave* against *Limonius californicus* were examined under laboratory conditions. Soil type did not have significant effect on the *L. californicus* mortality when maintained at either standardized moisture content (18%) or field capacity moisture levels. *Steinernema carpocapsae* All and Cxrd strains caused 40 and 52% *L. californicus* mortality in sandy loam and clay loam soil type, respectively, when soils were maintained at 18%. Similarly, *S. carpocapsae* Cxrd killed 46% *L. californicus* larvae in sandy loam soil which was the highest among all the soil type and EPN strain combinations, when soils were maintained at field capacity. Soil moisture too did not significantly affect the *L. californicus* mortality. Although not significantly different from All strain, *S. carpocapsae* Cxrd strain caused the highest mortality i.e. 58%, when soil was maintained at 16% moisture level. However, soil temperature showed a significant effect on *L. californicus* mortality. At 15°C, *S. carpocapsae* Cxrd caused 60% *L. californicus* as compared to only 4-20% mortality caused by *S. riobrave* (355 and 7-12 strains) and control treatment. However, *S. carpocapsae* (All and Cxrd strains) and *S. riobrave* 355 strains caused 38 to 66% mortality as compared to only 34% *L. californicus* mortality caused by *S. riobrave* 7-12 and 12% being caused in control treatment at 25°C. However, at 30°C temperature, none of the four EPN strains, even causing significantly higher mortality than control, differed significantly from each other in regard to *L. californicus* mortality and it ranged from 38 to 58%.

Key words: Soil moisture, soil texture, temperature, entomopathogenic nematodes, *Limonius californicus*

Introduction

Entomopathogenic nematodes (EPNs) have become successful inundative biological control agents for different insect pests (Lacey and Georgis 2012; Shapiro et al. 2012). In nature, EPNs from families Steinernematidae and Heterorhabditidae are obligate parasites of insects. Infective Juvenile (IJ) is the only free-living life stage of EPNs that is able to penetrate and kill the insect pest species. The IJs find the insect hosts in the soil and penetrate into the hemocoel through mouth, anus, spiracles, and sometimes through cuticle as well (Campbell and Gaugler 1991; Shapiro-Ilan et al. 2012). IJs then release the symbiotic bacteria inside the insect host hemolymph, ultimately killing the hosts within 24-72 hrs.

Wireworms (Coleoptera: Elateridae) are generalists causing serious damage to wide variety of field, cereal, tuber, vegetable, and fruit crops worldwide (Seal et al. 1992; Kohno and Miyai 1993; Parker and Howard 2001; Traugott et al. 2015; Milosavljević et al. 2017; Rashed et al. 2017). Wireworms feed on plant roots, bore into stems and cause plant wilting which causes thin plant stand and bare spots in field (Simmons et al. 1998; Vernon et al. 2009). High species diversity, long larval lifespan, poorly known taxonomy and undocumented life history makes it difficult to control wireworm populations (Traugott et al. 2015). Even with the use of neonicotinoids (imidacloprid, clothianidin and thiamethoxam) and the pyrethroid tefluthrin as seed treatments, insufficient control is common causing a worldwide wireworm resurgence in cereal crops such as spring wheat and barley (Morales-Rodriguez et al. 2014; Rashed et al, 2017). Pesticide resistance is so common that Labrie et al. (2020) suggested abandoning neonicotinoids prophylactically

against wireworms. Various crop protection and cultural control methods such as soil amendments, soil drying, flooding, manipulation of planting or harvesting dates, crop rotation, trap crops. Biological control of wireworms with entomopathogenic fungi and nematodes is also being explored under laboratory and field conditions (Andrews et al. 2008; Ansari et al. 2009; Landl and Glauninger 2013; Esser et al. 2015; Sharma et al. 2018; Ensafi et al. 2018; Sharma et al. 2019; Sandhi et al. 2020).

Considering their broad host range and their cohabitation with wireworms within the soil, EPNs represent a significant option for wireworm control. Some earlier studies have shown high wireworm mortality caused by several EPN species in the laboratory (Ansari et al. 2009; Morton and Garcia-del-Pino 2016; Sandhi et al. 2020a; Sandhi et al., 2020b). However, field studies on the susceptibility of wireworms to EPNs have been less promising (Ester and Huiting 2007; Campos-Herrera and Gutierrez 2009; Půža and Mráček 2010; Arrington et al. 2015). Lewis et al. (2015) associated the inability of EPNs to infect wireworms with wireworms' narrow mouth, obstructing oral filters, septa covered spiracles, and strong anal musculature. Poor susceptibility of wireworms to EPNs in field has also been related to unfavorable soil characteristics (Kaya 1990; Koppenhöfer and Fuzy 2006), lack of alternative hosts in the soil (Brust 1991; Susurluk 2005), application failures (Cabanillas et al. 2005), use of EPN species that were not adapted to the local conditions (Georgis et al. 2006), and competition with native EPN species (Georgis et al. 2006, Campos-Herrera et al. 2008). To use EPNs against wireworms, it is necessary to consider the ecology of the nematode species, physiology of the host species, and biotic and abiotic factors of the soil environment (Campos-Herrera and Gutiérrez 2009).

As Yeo et al. (2003) reported for microbial control agents such as fungi, selection of EPN species and strains should not be based solely only on their virulence to the target host, but should also consider their ability to perform over the range of abiotic conditions (including soil conditions) that they find in the agroecosystem. Soil characteristics such as soil texture, moisture, temperature, pH, organic matter, and bulk density can affect the survival and activity of EPNs (Kaya 1990; Grewal et al. 1994; Shapiro-Ilan et al. 1999; Shapiro-Ilan et al. 2006; Koppenhöfer and Fuzy 2006; Gruner et al. 2007; Lacey and Georgis 2012). Soil texture plays a crucial role in EPN movement and survival in the soil (Kaya 1990). The space between soil particles is required for adequate circulation of air and EPN movement through soil (Kung et al. 1990, Koppenhöfer et al. 1995; Koppenhöfer and Fuzy 2006). The survival and movement of different EPN species varies significantly across soil textures. Generally, EPN migration, survival, pathogenicity and host-finding ability decrease in clay soil due to small pore sizes (Kung et al. 1990). EPN species such as *Steinernema glaseri* (Steiner), different *Heterorhabditis* spp. and *Steinernema carpocapsae* (Weiser) all show better dispersal and efficacy in sandy soils with large pore size (Georgis and Poinar 1983; Kung et al. 1990; Koppenhöfer and Fuzy 2006; Ensafi et al. 2018). However, some other studies reported high efficacy of EPNs in clay soil when compared to sandier soils (Shapiro-Ilan et al. 2000; Toledo et al. 2009; Toepfer et al. 2010). Outcomes are not always obvious. In substrates with large pore sizes, like peat, ambusher *Heterorhabditis megidis* Poinar, Jackson, and Klein was observed dispersing better than the cruiser species, *S. carpocapsae* and yet, *S. carpocapsae* outcompeted *H. megidis* for host finding in peat (Kruitbos et al. 2010).

Soil moisture has a direct relationship with soil texture in determining EPN efficacy. EPNs need a thin film of free moisture in order to to move through the soil and find a host. Continuity of these films is inconsistent in dry soils preventing nematode movement and dispersal (Koppenhöfer and Fuzy 2006, Lewis et al. 2006). EPN efficacy is reported to be highest at the moderate soil moisture in a range of soil water potentials between −10 to −200 kPa (Koppenhöfer and Fuzy 2007). Pilz et al. (2014) emphasized that light sandy soils will only favor persistence as long as moisture is not a limiting factor, but in drier regions, sandy soils will have desiccation issues that affect EPN survival. Shapiro-Ilan et al. (2014) observed the highest level of desiccation tolerance in *S. carpocapsae* followed by *Steinernema feltiae* (Filipjev) and with heterorhabditids being least tolerant. *Steinernema riobrave* (Cabanillas, Poinar, and Raulston) was observed to be intermediate in desiccation tolerance.

Temperature is also one of the key factors affecting the infectivity of nematodes (Hazir et al. 2001). Optimum temperatures for infection and reproduction vary among EPN species and strains (Grewal et al. 1994). EPN species such as *Heterorhabditis indica* (Poinar, Karunakar and David), *S. glaseri* and *S. riobrave* are relatively heat tolerant and can maintain efficacy at temperatures of 29°C and above. Lacey and Unruh (1998) reported that *S. riobrave* can remain effective at soil temperatures above 35°C. In contrast, *S. feltiae*, *H. megidis* and *Heterorhabditis marelatus* (Liu and Berry) are more cold tolerant, maintaining efficacy at 15°C and below (Grewal et al. 1994; Radová and Trnková 2010). Likewise, *Steinernema kraussei* (Steiner) is favored by low temperatures and can infect insects at temperatures below 10°C (Torr et al. 2007). Generally, temperatures below 0°C

and above 40°C are lethal to most EPNs and reduce the nematode survival and infectivity (Ulu and Susurluk 2013), although the lethal effect of temperature depends on exposure time (Koppenhöfer 2000). Overall, both high and low temperatures can be harmful for EPNs by inhibiting or diminishing their infectivity depending on species.

The main purpose of this study was to examine the impact of different soil characteristics (found in Montana) on the efficacy of EPN species that were found virulent to the wireworm *Limonius californicus* (Mannerheim) in previous laboratory and shade house experiments (Sandhi et al. 2020a). Montana has wide range of soil types, dryland and irrigated farming systems and extreme weather that can affect the EPN efficacy against wireworms. Three experiments were conducted to study the effects of soil texture, moisture, and temperature on the efficacy of selected EPNs against wireworms under laboratory conditions. The knowledge gained from this study will be important to assessing the potential of EPN based biological control strategies against wireworms in the diverse environmental conditions of Montana.

Material and methods

Study site and wireworm collection

The experiments studying the effect of soil texture and soil moisture were conducted at the Western Triangle Agricultural Research Center (WTARC), Conrad, MT in 2019. The experiment studying the effect of soil temperature on EPN efficacy was conducted at Marsh laboratory, Montana State University, Bozeman in 2020. Wireworm larvae used in these studies were collected from May to August 2019 and 2020, in the

Golden Triangle Region of Montana near Pendroy (John Stultz Ranch Inc., Pondera Co. (N48.04130°, W112.16945°).

The larvae were collected using stocking traps (Reddy et al., 2014) in which nylon stockings were filled with a half cup of wheat grain or a mixture of wheat and barley grain. These baits were soaked in water for 24 hrs and buried 15–20 cm below the soil surface in wireworm-infested fields. The traps were then covered with black plastic sheets to maintain the ambient temperature and moisture for wireworm survival. After 2 weeks, the traps were collected from the field in plastic buckets and brought back to the laboratory for extraction. The larvae were sorted from the traps manually using Berlese funnels. The collected wireworm larvae were stored in an incubator at 9°C in the plastic cups with sterilized sandy loam soil and soaked wheat seeds as food. The cups were sprayed with water once a week to provide enough moisture for wireworm survival. Medium-sized wireworm larvae (0.8–1.5 cm) were used in all experiments.

Source of nematodes and culturing

Four EPN strains (*S. carpocapsae* All and Cxrd strains and *S. riobrave* 7-12 and 355 strains) were used in different experiments. The EPN strains were obtained from the USDA-ARS Entomopathogenic Nematode Culture Collection (Byron, GA). EPNs were reared in last-instar greater wax moth larvae, *Galleria mellonella* L. (Lepidoptera: Pyralidae), according to the procedure explained by Woodring and Kaya (1988). Wax moth larvae were obtained from Bassett's Cricket Ranch (Visalia, CA, USA). All nematodes were cultured in parallel to avoid differential age effects but handled separately in the workspace to avoid cross-contamination. For EPN rearing, the same procedure was

followed as described in Chapter 4. EPN infected cadavers were then transferred to White traps according to the protocol described by Kaya and Stock (1997). The infective juveniles (IJs) emerging from white traps were collected and stored at 8°C in tissue culture flasks. The stored IJs were used within 10 days of collection. The concentrations used in different experiments were prepared by counting out the desired number of IJs into 100 µl to 1 ml of tap water (depending on the concentration) in a nematode counting slide (Chalex, LLC, Park City, UT, USA) under a compound microscope by following the formula of Glazer and Lewis (2000).

Effect of soil texture on efficacy of EPNs against *L. californicus* at standardized moisture content (18%)

Four different types of soils (Table 1) were used in this study. The soil from each textural class was sieved (2 mm mesh) and sterilized in an autoclave at 121° C to kill microorganisms and any natural nematode populations. For acclimatization, soil was left at room temperature for at least two weeks. Deli plastic cups (500 ml) were filled with 150 gm of soil (soil surface area: 140 cm^2) for different soils. Five medium-sized wireworm larvae were placed with 10 germinated wheat seeds as food in each cup. The larvae that did not enter soil within 12 hrs were replaced. After 24 hrs, 7000 IJs/cup (50 IJs/cm^2) were inoculated in one ml of water. The reason behind using high concentrations was to see if they would cause higher mortality than in previous experiments (Chapter 2 and 4). Control cups received only one ml water without IJs. The final moisture content was standardized at 18% (wt: wt) for all the soil types. Finally, the cups were placed in an incubator at 23°C and 75% RH in dark conditions. More water in form of two to three sprays was provided

in cups every 2-3 days to maintain the moisture. The wireworm mortality was assessed at weekly intervals for four weeks. There were five replications for each treatment. The experiment was conducted twice with an interval of two weeks between trials and different nematode cultures were used for both trials.

Effect of soil texture on efficacy of EPNs against *L. californicus* at field capacity

Following the above procedure, the moisture content for all the soils was adjusted to field capacity levels (Table 1). The wireworm mortality was assessed at weekly intervals for four weeks. The experiment was conducted twice with an interval of two weeks between trials and different nematode cultures were used for both trials.

Effect of soil moisture on efficacy of EPNs against *L. californicus*

Deli plastic cups (500 ml) were filled with 150 gm sieved and autoclaved sandy clay loam soil (surface area -140 cm^2) for the bioassay. Sandy clay loam (table 1) is the common soil type in the Golden Triangle Region of Montana and the common habitat soil type for wireworms. The soil was left at room temperature for 10 days for acclimatization. Five wireworm larvae were introduced into each cup and allowed to go down for 12 hrs. Four different soil moisture levels; 21.4% (w/w) (100% of field capacity), 16% (w/w) (75% of field capacity), 10.7% (w/w) (50% of field capacity), and 5.35% (w/w) (25% of field capacity) were tested. Approximately 7,000 IJs (50 IJs/cm^2) were inoculated into the cups in one ml of water with a pipette. Soil moisture contents were obtained by weighing and oven drying soil samples (100g) at 60 °C and were calculated on a wet weight (wt:wt) basis. Soil moisture levels were adjusted to get the approximate target perecentage for each

moisture level, and the appropriate amount of water was added and mixed into the soil. These soil moisture percentage levels were also confirmed by using a soil moisture meter (Spectrum Technologies Inc., Illinois, USA). The control cups received plain water without IJs. There were five replicates per treatment. The mortality was observed at weekly intervals for a month. The experiment was performed twice under the same conditions with different batches of nematodes with an interval of 10 days.

Effect of temperature on efficacy of EPNs against *L. californicus*

The experiment was conducted by following by the same procedure as above except the final moisture content was standardized at 21.4% (w/w), the field capacity for sandy clay loam soil. Three temperature levels (15, 25, and 30° C) were tested for studying the effect of different EPN species against *L. californicus*. The experiment was conducted twice, with an interval of two weeks between trials and different nematode cultures were used for both trials.

Statistical analysis

All statistical analyses were performed in the R statistical language (version 3.5.2, R Core Team 2020). The data for *L. californicus* mortality was analyzed just for 28 DAT (Days after treatment) in all the experiments because the mortality rates were not very high at 7, 14, and 21 DAT. For soil texture at standardized moisture content and field capacity levels, data were analyzed by using Generalized Linear Model (GLM) with quasibinomial distribution and logit link with EPN strain, soil types, and time as predictor variable and dead wireworms as response variable. For soil moisture and soil temperature data, GLM

with quasibinomial distribution with logit link was used with EPN strain and time as predictor variables with moisture levels and temperature as the third predictor variable in soil moisture and temperature data, respectively. The quasibinomial distribution was used to deal with the overdispersion problem in the data (Ramsey and Schafer 2012). The models were simplified using stepwise removal of predictors with no significant effect ($P>0.05$) and the lack of fitness test was performed to compare the models. As the model was formulated in terms of transformed probabilities (logit transformation to the log-odds scale), the result was back transformed to an original probability scale after analysis for graphical representation. If significant differences were observed among treatments, Tukey-Kramer test ($\alpha = 0.05$) was used for pairwise comparisons.

Results

Effect of soil texture on efficacy of EPNs against *L. californicus* at standardized moisture content (18%)

No mortality was observed in the un-inoculated control treatment, therefore its data was excluded from the model. The two wo trials did not differ significantly in terms of wireworm mortality ($\chi^2=0.14$, df= 1, $P=0.71$) and did not have an interaction with EPN strain ($\chi^2=2.79$, df= 3, $P=0.43$), therefore their data was pooled for two trials. *Limonius californicus* mortality was significantly affected by EPN strains ($\chi^2=36.30$, df=3, $P<0.0001$), but not by soil type ($\chi^2=2.46$, df=3, $P=0.49$). The interaction between EPN strain and soil type was also not found significant ($\chi^2=14.15$, df=9, $P=0.12$). At 28DAT, *S. carpocapsae* All and Cxrd strains caused significantly higher mortality (28-40% for All and 30-52% for Cxrd) than *S. riobrave* 355 and 7-12 strains ($P<0.001$) and did not differ

significantly from each other (P=0.92) (Figure 1). *Steinernema carpocapsae* All and Cxrd strains caused 40 and 52% *L. californicus* mortality in sandy loam and clay loam soil type, respectively (Figure 1). *Steinernema riobrave* 7-12 strains was the least mortality producing strain with only 12-18% *L. californicus* mortality caused after 28 days of treatment for all the soil types (Figure 1).

Effect of soil texture on efficacy of EPNs against *L. californicus* at field capacity

No *L. californicus* mortality was observed in the control treatment and therefore, not included in analysis further. Two trials did not differ significantly in respect to wireworm mortality (Trial: χ^2=2.72, df= 1, P=0.10; Strain*Trial: χ^2=0.53, df= 2, P=0.77), and the data for wireworm mortality were pooled for two trials for further analysis. Three EPN strains significantly affected the *Limonius californicus* mortality (χ^2=9.90, df=2, P=0.007). However, the four soil types tested were not found significantly different in terms of *L. californicus* mortality at 28 DAT (χ^2=5.71, df=3, P=0.13). Similarly, the interaction between EPN strain and soil type was also not significant (χ^2=8.17, df=6, P=0.23). *Steinernema carpocapsae* Cxrd strain caused significantly higher wireworm mortality (30-46%) than *S. riobrave* 355 strain (12-40% mortality), irrespective of the soil type (P=0.008) (Figure 2). However, *S. carpocapsae* All strain did not differ significantly from *S. riobrave* 355 and *S. carpocapsae* Cxrd strain (P >0.06; Tukey-Kramer test) and caused 28-36% *L. californicus* mortality. Overall, after 28 days, *L. californicus* mortality could not exceed 50% with 46% mortality caused by *S. carpocapsae* Cxrd strain for sandy loam soil which was the highest among all the soil type and EPN strain combinations (Figure 2).

Effect of soil moisture on efficacy of EPNs against *L. californicus*

No wireworm mortality was observed in the control treatment and therefore, not included in further analysis. Two trials differed significantly in terms of wireworm mortality (χ^2=6.33. df= 1, P=0.001). However, the interaction between EPN strains and trials (χ^2=1.51, df= 2, P=0.47) and moisture levels and trials (χ^2=2.74, df=3, P=0.43) were not significant, therefore data for two trials were pooled for further analysis. *Limonius californicus* mortality was significantly affected by EPN strains (χ^2=24.35, df=2, P<0.0001). However, moisture levels did not differ significantly in terms of *L. californicus* mortality (χ^2=5.99, df=3, P=0.11). Similarly, EPN strain and moisture did not interact significantly (χ^2=8.81, df=6, P=0.18) with regards to *L. californicus* mortality. Overall, *Steinernema carpocapsae* (All and Cxrd strains) did not differ significantly at different moisture levels (P=0.34) and were able to cause 32 to 58% *L. californicus* mortality at 28DAT (Figure 3). However, these two strains differed significantly from *S. riobrave* 355 in terms of *L. californicus* mortality (P<0.01). Although not significantly different, *S. carpocapsae* Cxrd strain caused the highest mortality i.e. 58%, when soil was maintained at 16% moisture level (Figure 3).

Effect of soil temperature on efficacy of EPNs against *L. californicus*

Two trials did not differ significantly in terms of *L. californicus* mortality (Trial: χ^2=0.28, df= 1, P=0.60; EPN strain*Trial: χ^2=1.40, df= 4, P=0.84), therefore data for two trials were pooled together for further analysis for wireworm mortality at 28DAT. *Limonius californicus* mortality was significantly affected by EPN strains (χ^2=92.15, df=4, P<0.0001) and temperature (χ^2=19.09, df=2, P<0.0001). Additionally, interaction between

EPN strain and temperature was also found significant (χ^2=47.10, df=8, P<0.0001) with regards to wireworm mortality. At 15°C, *S. carpocapsae* Cxrd strain caused significantly higher mortality (60%) as compared to only 4-20% mortality caused by *S. riobrave* (355 and 7-12 strains) and control treatment (Figure 4). However, *S. carpocapsae* All strain did not differ from *S. carpocapsae* Cxrd with 34% *L. californicus* mortality. *Steinernema carpocapsae* All strain caused significantly higher mortality (66%) as compared to only 34% mortality caused by *S. riobrave* 7-12 and 12% beong caused in control treatment at 25°C (Figure 4). However, it did not differ from *S. carpocapsae* Cxrd and *S. riobrave* 355 strains which were also able to cause 58 and 38% *L. californicus* mortality, respectively, when tested at 25°C. However, at 30°C temperature, none of the four EPN strains, even causing significantly higher mortality than control, differed significantly from each other in regard to *L. californicus* mortality (Figure 4). The *L. californicus* mortality ranged from 38 to 58% with only 12% mortality observed in control (Figure 4). Overall, *S. carpocapsae* All and Cxrd strains were the virulent strains at all temperatures as compared to *S. riobrave* 7-12 strain.

Discussion

Different abiotic factors impact the survival, infectivity, and persistence of EPNs. Soil being the natural habitat of these nematodes as well as wireworms, the effect of the soil environment on nematode activity need to be considered for successful utilization of EPNs is a pre-requisite for the successful use of EPNs against wireworms. Although not statistically different, different EPN species showed variation in terms of virulence to *L. californicus* larvae at different soil texture and soil moisture levels under laboratory

conditions. When at standardized at 18% moisture, *S. carpocapsae* All and Cxrd strains caused higher *L. californicus* mortality as compared to *S. riobrave* 355 and 7-12 strains with the highest mortality (52%) caused by *S. carpocapsae* Cxrd strain in clay loam soil type. Similarly, when the soil moistures were maintained at field capacity, wireworm mortality caused by *S. carpocapsae* All and Cxrd strains ranged from 30 to 46% in all the four soil types after 28 days of treatment with 40% mortality observed by *S. riobrave* 355 strain as well in sandy loam soil.

Overall, wireworm mortality was found to be higher in sandy loam than the other three soil types at the field capacity moisture levels, although not significantly different from other soil types. This higher virulence capacity of EPNs in the sandier soils has been reported earlier by Koppenhöfer and Fuzy (2006) and Ensafi et al. (2018). Ensafi et al. (2018) observed less wheat plant damage due to wireworms in a sand-dominated medium in greenhouse experiments. This better efficacy of EPNs in these studies might be due to better movement and survival of EPNs in these porous sandier soils (Kung et al., 1990; Kaya and Gaugler, 1993). The poor dispersal of EPNs in soils with high clay contents is mainly due to the restricted pore space and poor aeration.

This trend was not seen in the experiment with soil types maintained at standardized moisture levels where no significant differences were observed among four soil types. *Steinernema carpocapsae* was observed to cause higher mortality in clayey soils at standardized moisture. Other studies were also observed with higher efficacy of EPNs in clay soil as compared to sandier soils against different insect pests (Shapiro-Ilan et al., 2000; Toledo et al., 2009; Toepfer et al., 2010). In this experiment, *S. carpocapsae*'s higher

efficacy in clay loam soils might be due to changes in wireworm behavior. The other reason behind this may be the high moisture retaining capacity of clay soils (Grant and Villani 2003). EPNs might still be able to locacter their hosts easier in clay soils because of ease in the movement in the cracks formed by insect hosts' movement as compared to sandy soils. The difference of *S. carpocapsae* infectivity in different soils in both soil texture experiments can be related to the minor changes in the environmental conditions such as moisture level variations. The clayey soils maintained at 18% moisture levels were probably at field capacity levels whereas, for sandy soil 18% might have been very high moisture, making them wet for EPNs to move, disperse and show poor efficacy. Koppenhöfer and Fuzy (2007) suggested that EPN activity can vary based on differences in soil water potential among soil types for the same EPN species. Various physical and chemical characteristics such as organic matter, pH, and electrical conductivity of different soils can also alter the nematode virulence (Shapiro-Ilan et al., 2006; Kaspi et al., 2010).

The high efficacy of *S. carpocapsae* against *L. californicus* found in the present study supported the findings of Ansari et al. (2009), Morton and Garcia-del-Pino (2016), and Sandhi et al. (2020a) and were not in conformity with other studies (Campos-Herrera and Gutierrez 2009; San-Blas et al. 2012; La Forgia et al. 2020) where very low efficacy of this species was observed against different wireworm species in laboratory bioassays. However, the actual field efficacy of this species needs to be determined as field soils will have larger aggregated chunks even under good farming practices which may also affect the nematode movement and survival.

In the experiment testing the effect of soil moisture, the reason for choosing sandy clay loam soil was that it is the most common soil in Montana under spring wheat and barley cultivation and with wireworm infestation. The present findings regarding the effects of soil moisture strongly indicate that moisture levels did not really affect the survival and efficacy of EPNs against the wireworms. *Limonius californicus* mortality was lowest at the 5% moisture levels and increased with increased soil moisture. Wireworm mortality ranged from 30 to 40% for all the four EPN strains at 5 and 10% moisture percentage levels. *Steinernema carpocapsae* All and Cxrd strains caused highest wireworm mortality at four moisture levels especially at 16 and 24% as compared to *S. riobrave* 355 strain. These finding are in agreement with the studies of Koppenhöfer et al. (1995) and Grewal (2000), where low virulence of *S. carpocapsae* has been observed due to lack of moisture between the soil pores in soils with low moisture contents (4-5%). EPN species such as *S. carpocapsae* are known to enter "anhydrobiosis" due to low available moisture in the soil, which ultimately will reduce the metabolism and activity of EPNs. *Steinernema carpocapsae* is able to reach a lower metabolic rate than *S. riobrave* or *S. feltiae* (Grewal 2000). High water content in the soil was found to be favorable to nematode virulence and survival (Shapiro et al. 2000). However, *S. carpocapsae* infectivity and survival was observed to be low at high moisture levels as well (Kung et al. 1991; Glazer 2002). Overall, considering the dryland farming practices of Montana farmers, it is really important to establish if *S. carpocapsae* can persist for extended time periods under dryland conditions.

Similar results indicating better efficacy of *S. carpocapsae* against *L. californicus* were observed in the temperature experiments. Temperature is known to affect EPNs'

infectivity, virulence and reproductive capacity and most species are most active at 20 to 30 °C (Kaya 1990; Grewal et al. 1994; Shapiro-Ilan et al. 2006). Although, the ability of *S. carpocapsae* to kill *L. californicus* larvae increased as the temperature increased and 25 °C had the highest wireworm mortality, it was affected less by temperature. The higher efficiency of *S. carpocapsae* at higher temperatures has been reported in other studies (Grewal et al. 1994, Brown et al. 2002; Shapiro-Ilan et al. 2011). Despite not being able to cause higher mortality than *S. carpocapsae*, *S. riobrave* was also able to cause at least 40% *L. californicus* mortality at 25 °C. *Steinernema riobrave* killed highest number of *L. californicus* (not significantly different from other strains) with increase in the temperature as compared to *S. carpocapsae*. which was expected. Different strains of *S. carpocapsae* and *S. riobrave* were reported to be highly pathogenic to plum curculio, *Conotrachelus nenuphar* (Herbst) (Coleoptera: Curculionidae), at 25 °C under laboratory conditions (Shapiro-Ilan et al. 2011). Hussaini et al. (2005) have also observed the high virulence of *S. carpocapsae* towards *G. mellonella* and *Agrotis ipsilon* (Hufnagel) caterpillars at temperatures of 25 to 32 °C, and with lethal time being directly proportional to temperature increase.

Above all, a major reason of wide variations in wireworm mortality due to different EPN strains in present studies even under the same experimental conditions could be the morphological barriers and behavioral defenses of hard-bodied wireworm larval instars (Lewis et al. 2015, Sandhi et al. 2020a). Moreover, the wireworm mortality rates observed in laboratory in this study cannot be directly extended to EPN field efficacy and all these

environmental factors should be explored further before making a final decision on field application.

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Table 1. Soil types used in the laboratory bioassays

Soil type	Sand%	Silt%	Clay%	Soil pH	Organic Matter (%)	Field capacity (% moisture)
Sandy loam	78	12	10	7.7	1.4	8.0
Sandy clay loam	51	25	24	8.0	2.5	19.4
Clay loam	40	28	32	7.8	2.3	22.35
Clay	30	28	42	7.8	2.9	25.4

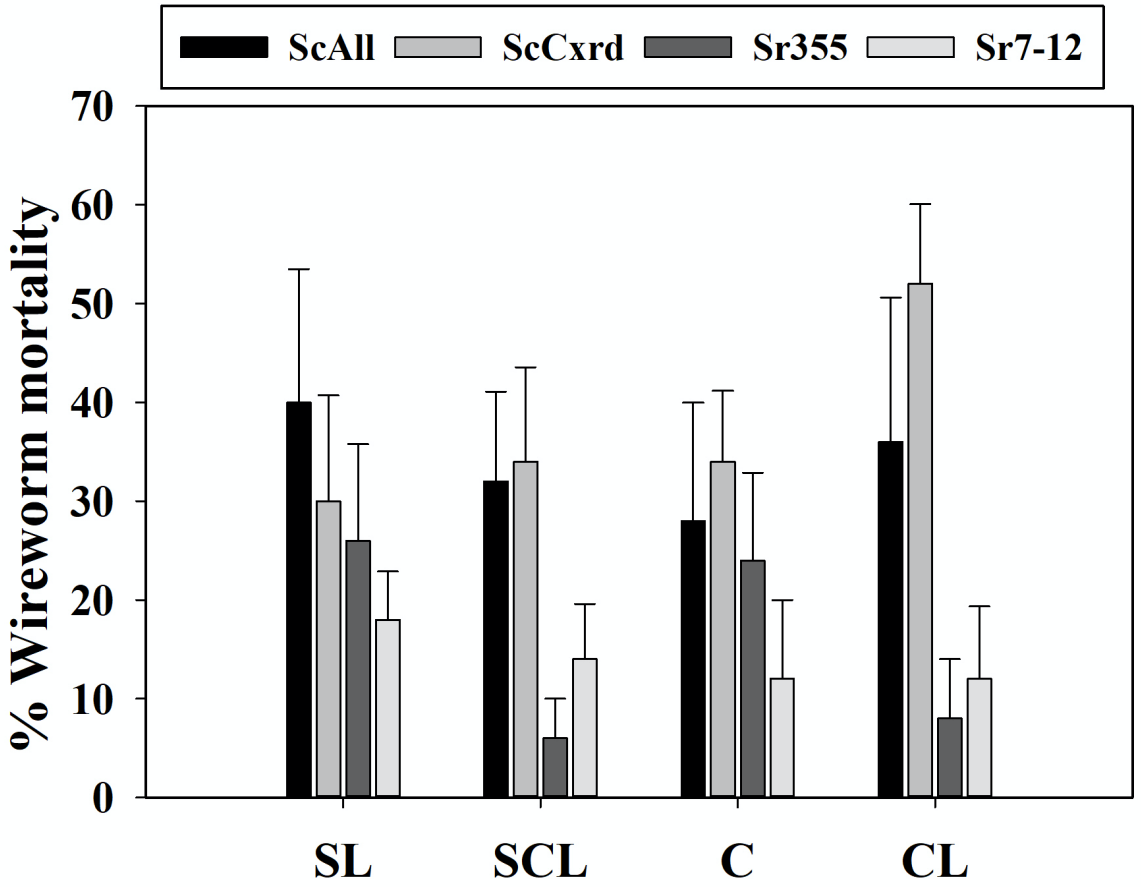


Figure 1. Average percentage mortality of larval *Limonius californicus* after exposure to entomopathogenic nematodes at the rate of 1400IJs/larva (7000 IJs/cup) concentration at (A) 7 DAT, (B) 14 DAT, (C) 21 DAT, and (D) 28 DAT with 18% standardized moisture content in different soil types. DAT=Days after Treatment; ScAll = *Steinernema carpocapsae* All; ScCxrd = *Steinernema carpocapsae* Cxrd; Sr355 = *Steinernema riobrave* 355; Sr7-12 = *Steinernema riobrave* 7-12. SL = Sandy loam; SCL = Sandy clay loam; C = Clay; CL = Clay loam soil types; No larval mortality was observed in the control.

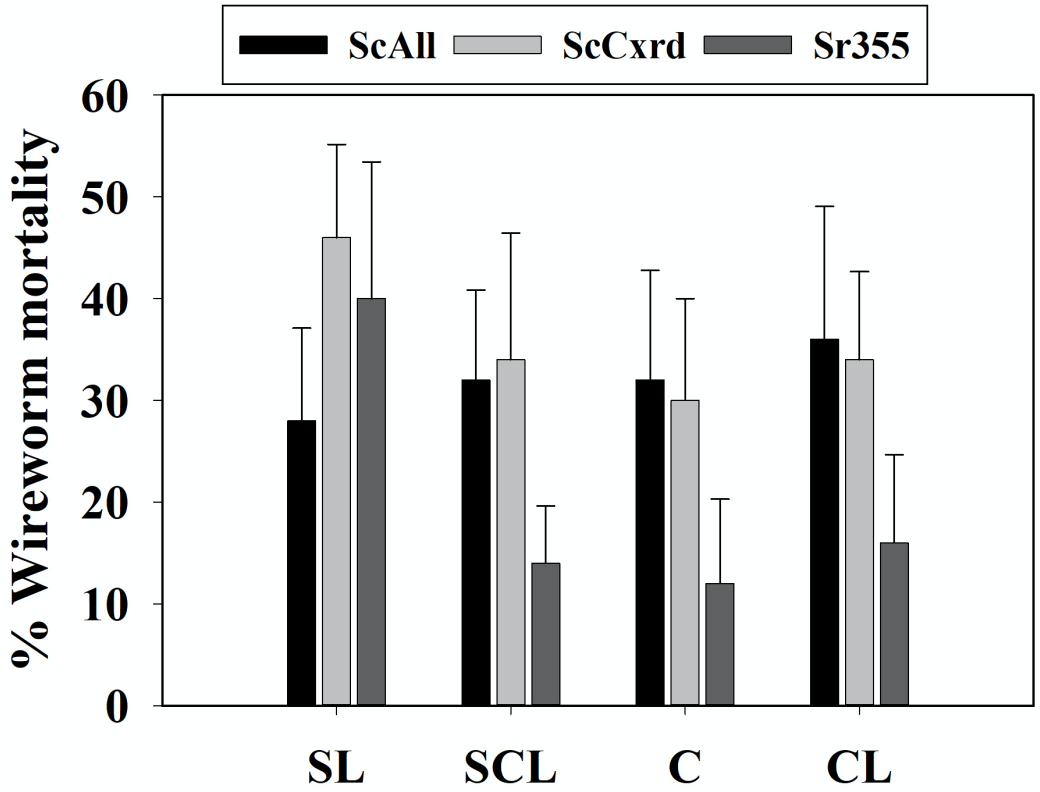


Figure 2. Average percentage mortality of larval *Limonius californicus* after exposure to entomopathogenic nematodes at the rate of 1400IJs/larva (7000 IJs/cup) concentration at 28 days after treatment with soil moisture content at field capacities in different soil types. DAT=Days after Treatment; ScAll = *Steinernema carpocapsae* All; ScCxrd = *Steinernema carpocapsae* Cxrd; Sr355 = *Steinernema riobrave* 355. SL = Sandy loam; SCL = Sandy clay loam; C = Clay; CL = Clay loam soil types; No larval mortality was observed in the control.

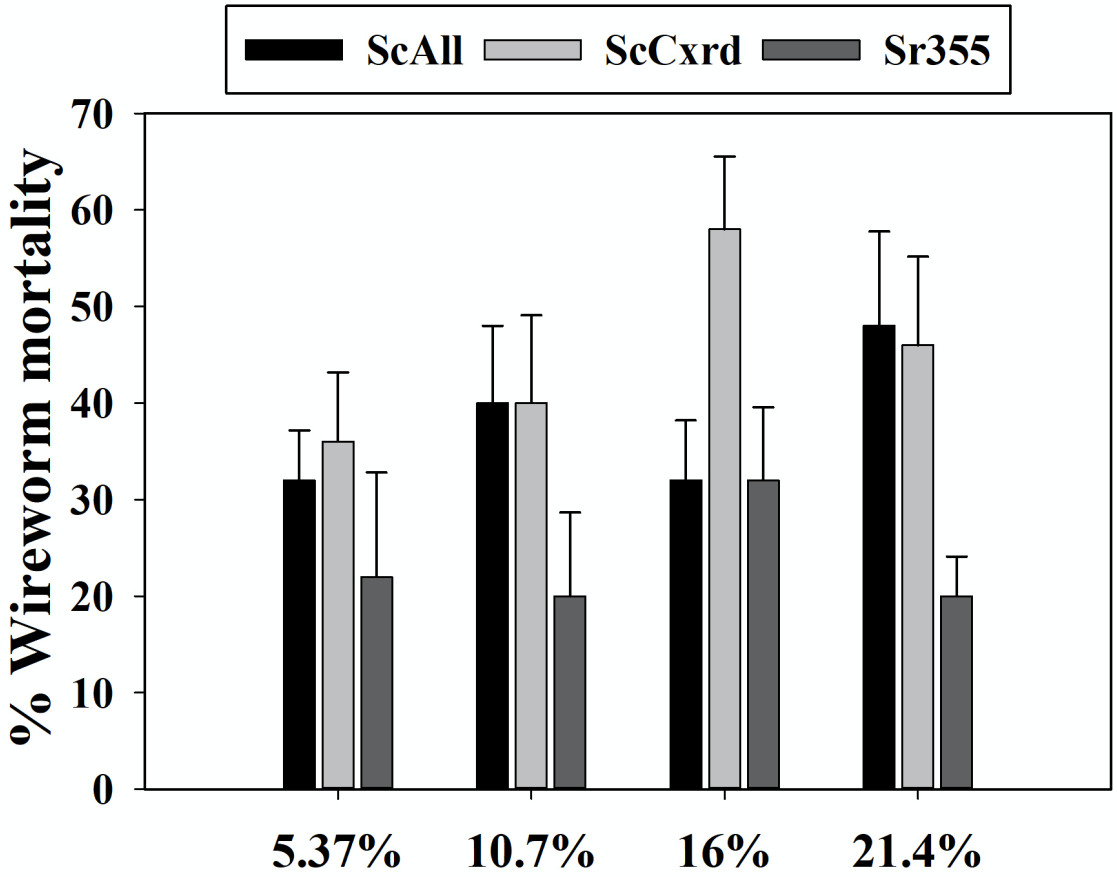


Figure 3. Average percentage mortality of larval *Limonius californicus* after exposure to entomopathogenic nematodes at the rate of 1400IJs/larva (7000 IJs/cup) concentration at 28 days after treatment and different soil moisture levels in sandy clay loam soil. DAT=Days after Treatment; ScAll = *Steinernema carpocapsae* All; ScCxrd = *Steinernema carpocapsae* Cxrd; Sr355 = *Steinernema riobrave* 355. No larval mortality was observed in the control.

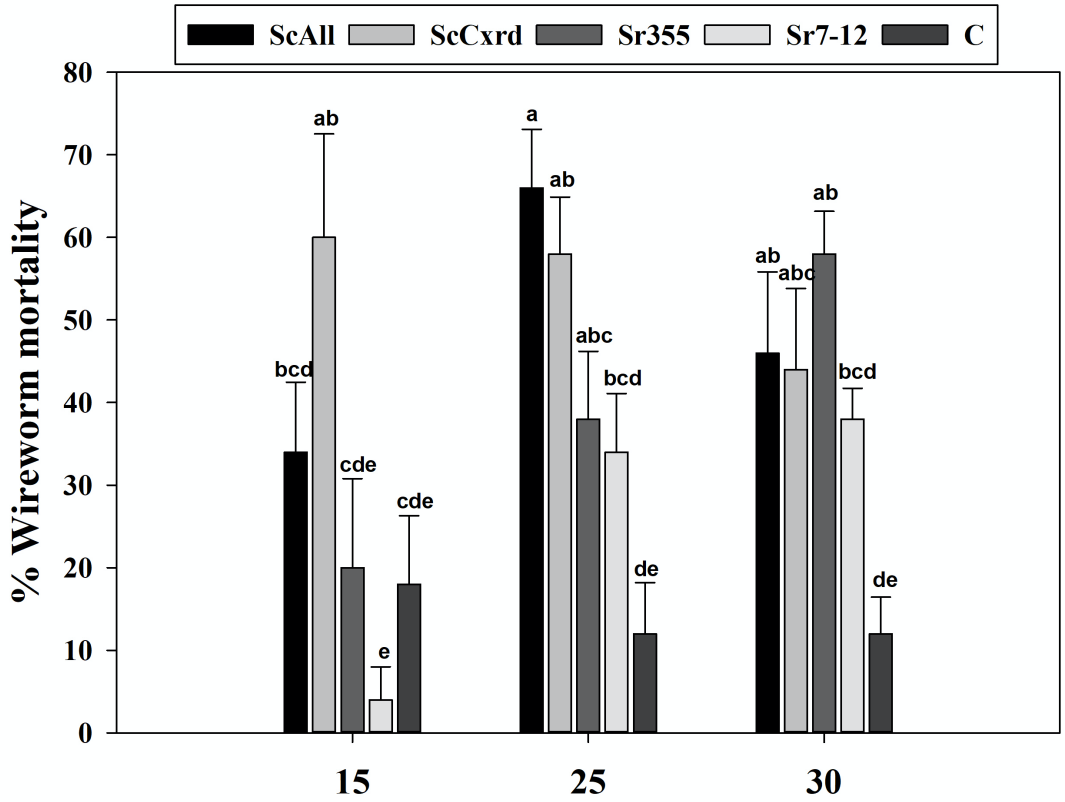


Figure 4. Average percentage mortality of larval *Limonius californicus* after exposure to entomopathogenic nematodes at the rate of 1400IJs/larva (7000 IJs/cup) concentration at 28 days after treatment and different temperature ranges (15, 25, and 30°C) in sandy clay loam soil. DAT=Days after Treatment; ScAll = *Steinernema carpocapsae* All; ScCxrd = *Steinernema carpocapsae* Cxrd; Sr355 = *Steinernema riobrave* 355; Sr7-12 = *Steinernema riobrave* 7-12; C = Control treatment.

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CHAPTER SEVEN

CONCLUSIONS

Wireworms are significant pests of small grain crops and have become a serious threat to spring wheat and barley growers in the Northern Great Plains because of a lack of effective control measures. This research work was focused on finding an effective biological strategy for wireworm management in Montana. Soil being the habitat for both wireworms and entomopathogenic nematodes (EPNs), the potential of these nematodes to suppress the wireworms was explored in spring wheat and barley. The goal of the present study was to test efficacy of different EPN species against wireworms. Firstly, ten different entomopathogenic nematodes (EPNs) were evaluated against *Limonius californicus* (Mannerheim) in laboratory and greenhouse and *Steinernema carpocapsae* (Weiser) (All and Cxrd strains) and *Steinernema riobrave* Cabanillas, Poinar, and Raulston (355 and 7-12 strains) were found to have potential against the wireworm in spring wheat when a high concentration (>200 IJs/cm^2) is used (Chapter 2).

To explore further the efficacy of EPNs against wireworms, Golden Triangle area of Montana was surveyed for native EPN species, and occurrence of Montana native EPNs (*Steinernema feltiae* and *Heterorhabditis bacteriophora*) was reported for the first time (Chapter 3). These Montana native EPNs were thought to be well adapted to the local climate and soil conditions, considering the extreme weather of Montana and to be more effective as against *L. californicus.* However, neither native EPN species was found very

virulent towards the wireworms in laboratory and greenhouse and showed limited potential for wireworm control (Chapter 4).

Next, the field efficacy of non-native EPN species (*S. carpocapsae* and *S. riobrave*) was evaluated with EPN species applied in the form of infected *Galleria mellonella* cadavers to confirm the virulence of these EPNs towards *L. californicus* previously observed in laboratory and greenhouse studies. Overall, none of the EPN strains were found very effective in suppressing wireworms or even protecting from plant damage (Chapter 5). However, *S. carpocapsae* and *S. riobrave* applied as *G. mellonella* cadavers were found as effective as treatment of combination of EPN infected host cadavers and imidacloprid seed treatment in causing *L. californicus* mortality and reducing wheat plant damage in a greenhouse pot experiment (Chapter 5).

The ineffectiveness of EPNs against wireworms in the field might be due to the effect of various edaphic factors such as soil texture, moisture, and temperature. To explore further, the effect of these three abiotic factors on the infection rate of *S. carpocapsae* and *S. riobrave* against *L. californicus* were examined under laboratory conditions. No significant differences were observed among different soil types tested, with highest mortality observed in sandy loam and clay loam. The moisture level of 16% and 25% temperature were found to be the most effective levels for EPNs to kill the highest number of *L. californicus*. Overall, *S. carpocapsae* was observed to be more effective than *S. riobrave* in all the experiments (Chapter 6).

Future studies are required to evaluate *S. carpocapsae* and *S. riobrave* against other wireworm genera such as *Hypnoides*, *Aeolus*, and *Agriotes* spp. that are pests in various

crops in Montana. These species might be more susceptible to native Montana EPNs and should be studied further. These new native Montana species might be utilized in future ecological and biological control studies against different economically important insect pests in Montana as well as other parts of the world with a similar climate.

The direct aqueous suspension application of EPNs should be directly compared with the cadaver approach under field conditions to have a better idea about the EPNs potential against wireworms. Using different formulations for coating EPN infected cadavers for enhancement of efficacy against different wireworm species should be explored as well. In addition, the ability of the applied EPNs to persist longer in the field should also be taken into consideration in order to attain cost-efficient control method for wireworms.

At last, sublethal effects of EPNs on wireworm physiology, behavior, and feeding in addition to direct killing should be examined. This will enhance our understanding of entomopathogenic nematode ecology and their overall efficacy against wireworms. The information gained from this research would contribute in to the development of Integrated Pest Management strategies for wireworms in small grain crop systems in Montana.

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