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Pest Management

Biocontrol of Wireworms (Coleoptera: Elateridae) Using Entomopathogenic Nematodes: The Impact of Infected Host Cadaver Application and Soil Characteristics

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Abstract

Wireworms have become a significant menace to cereals in the Northern Great Plains. Therefore, research toward developing effective control methods such as biological control with entomopathogenic nematodes (EPNs) is warranted. Two strains, each of two EPN species, *Steinernema carpocapsae* (Weiser) and *Steinernema riobrave* Cabanillas, Poinar, and Raulston in the form of infected *Galleria mellonella* (L.) cadavers were evaluated against wireworms in field and greenhouse. In field experiments, none of the four EPN strains were found effective against wireworms. However, in the greenhouse test, three of the strains, *S. carpocapsae* (All and Cxrd) or *S. riobrave* (355) applied in cadavers killed 50–68% of the sugarbeet wireworm, *Limonius californicus* (Mannerheim) was associated with 8–24% plant damage at 35 d after treatment (DAT), when seeds were treated with imidacloprid. The mortality range was 40–56% with 57–75% plant damage observed at 35 DAT, when seeds were planted without imidacloprid treatment. Synergistic effect among imidacloprid and *S. carpocapsae* (Cxrd) or *S. riobrave* (355) was observed in regard to *L. californicus* mortality. Additionally, effects of soil texture, moisture, and temperature on the infection rate of EPNs against *L. californicus* were examined in the laboratory. *Limonius californicus* mortality was not significantly affected by either soil moisture or soil types maintained at field capacity moisture levels. However, soil temperature showed a significant effect on *L. californicus* mortality. Overall, imidacloprid enhanced the infection and killing ability of EPNs against *L. californicus* and *S. carpocapsae* (All and Cxrd) strains were the virulent strains in different soil experiments.

Key words: Entomopathogenic nematode, imidacloprid, *Limonius californicus*, soil moisture, texture, temperature

Wireworms (Coleoptera: Elateridae) are generalists causing serious damage to wide variety of field, cereal, tuber, vegetable, and fruit crops worldwide (Rashed et al. 2017, Sandhi et al. 2020a). Entomopathogenic nematodes (EPNs) have become successful inundative biological control agents for different insect pests (Lacey and Georgis 2012). 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 (Sandhi et al. 2020a, b; and studies there within). 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). This study is in continuation in a series of studies (Sandhi et al. 2020a, b, c), aimed at evaluating EPNs for wireworm control, soil dwelling larval Elateridae, in the Golden Triangle of Montana (GTR).

Generally, EPNs are applied as 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 (Del Valle et al. 2008, Shapiro-Ilan et al. 2010, Raja et al. 2015, 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. Entomopathogenic nematodes applied as insect cadavers are reported to be superior to application in aqueous suspension in

laboratory and field with respect to better IJs survival, dispersal, and infection (Shapiro-Ilan et al. 2010).

Along with behavioral and morphological barriers of wireworms, poor susceptibility of wireworms to EPNs in field has also been related to unfavorable soil characteristics (Kaya 1990, Koppenhöfer and Fuzy 2006). 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, Shapiro-Ilan et al. 2006, 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 migration, survival, pathogenicity, and host-finding ability through soil (Koppenhöfer and Fuzy 2006). Entomopathogenic nematodes species such as *Steinernema glaseri* (Steiner), different *Heterorhabditis* spp. and *S. carpocapsae* all show better dispersal and efficacy in sandy soils with large pore size (Koppenhöfer and Fuzy 2006, Ensafi et al. 2018). However, some other studies reported high efficacy of EPNs in clay soil, when compared with sandier soils (Toledo et al. 2009, Toepfer et al. 2010). Outcomes are not always obvious. In substrates with large pore sizes (peat), cruiser *Heterorhabditis megidis* Poinar, Jackson, and Klein was observed dispersing better than the ambusher species, *S. carpocapsae* and yet, *S. carpocapsae* outcompeted *H. megidis* (Kruitbos et al. 2010).

Entomopathogenic nematodes need a thin film of free moisture in order 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). Shapiro-Ilan et al. (2014a) observed the highest level of desiccation tolerance in *S. carpocapsae* followed by *Steinernema feltiae* (Filipjev) and with heterorhabditids being least tolerant. *Steinernema riobrave* was observed to be intermediate in desiccation tolerance. Temperature is also one of the key factors affecting the infectivity of nematodes. Entomopathogenic nematodes 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. 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).

In previous laboratory experiments that screened 10 EPN strains for virulence against wireworms, two EPN species *Steinernema carpocapsae* (Weiser) and *Steinernema riobrave* (Cabanillas et al.) were selected as most effective against *Limonius californicus* (Mannerheim) (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*, *Hypnoides bicolor* Eschscholtz, and *Aelous mellilus* Say), especially *L. californicus*. The infected *G. mellonella* cadaver approach instead of aqueous suspension was used to mimic the natural conditions. Montana has a wide range of soil types, dryland and irrigated farming systems and extreme weather that can affect the EPN efficacy against wireworms. Therefore, another objective 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 *L. californicus*. Three experiments were conducted to study the effects of soil texture, moisture, and temperature on efficacy of selected EPNs against wireworms under laboratory conditions. The knowledge gained from these studies will be important in assessing the potential of EPN-based biological control against wireworms in wheat-barley irrigated and dryland agroecosystems as well as in diverse environmental conditions of Montana.

Materials and Methods

EPN Source, Cadaver Production, and Wireworm Collection

Cultures of EPN strains of *S. carpocapsae* (All and Cxrd) and *S. riobrave* (355 and 7–12) were obtained from the USDA-ARS Entomopathogenic Nematode culture collection (Byron, GA). Entomopathogenic nematodes used in experiments were produced on greater wax moth larvae, *Galleria mellonella* L. (Lepidoptera: Pyralidae) obtained from the Bassett's Cricket Ranch (CA, USA). Ten *G. mellonella* larvae were exposed to approximately 200 freshly produced IJs of each of four selected EPN 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 d. The nematode-infected cadavers were then transferred to individual White traps (Kaya and Stock 1997) for another 4–5 d at room temperature to observe the initiation of IJs emergence. The cadavers were checked daily for the initiation of IJs. After 4–5 d, the cadavers that were about to release IJs were used in field experiments to reduce the chances of variation in emergence among replications. For the EPN rearing and dose preparation in laboratory experiments, the same procedure was followed as described in Sandhi et al. (2020a, c). The stored IJs were used within 10 d of collection. Wireworm larvae used in these studies were collected from May to August 2019 and 2020, in the GTR near Pendroy John Stultz Ranch Inc., Pondera Co. (N48.04130°, W112.16945°). The larvae were collected using stocking traps (Reddy et al. 2014). The collected wireworm larvae were stored in an incubator at 9°C in sandy loam.

Field Experiment

Experimental design

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 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 Pendroy site had Rothiemay-Niart clay loams, with 0–4% slopes and Choteau site had Niart-Crago gravelly loams soil with 0–4% slopes. 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 including 56 l of 'thirty-two' nitrogen, 20-10-5-10 (1,300 L/ha) and manure at the rate of 135 kg/ha were applied to the spring wheat before seeding. Beyond and Wildcard were applied at label rates for weed control in this field. Roundup at the rate of 1.17–1.46 L/ha and liquid nitrogen at the rate of 47 L/ha 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.

A completely randomized block design was used with a 17.6 m × 33.7 m section measured in each field. Within each section, there

were five 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. Five replications were maintained for all treatments and control plots. For treatments, two application rates (3 and 6 cadavers) were tested. Overall, there were 45 plots (four strains × two doses × five replications + five Controls) in each field. Five days after seeding (15 May 2019), cadavers were placed 5–8 cm beneath the soil surface (10 cm away). 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 a.m. 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%. The soil temperature and soil moisture were observed at 8–10 cm soil depth.

Pre- and post-harvest field efficacy

To estimate 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 until emergence stopped (3 wk) and counted with the serial dilution method (Glazer and Lewis 2000).

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 d after seeding for barley and 20 d after seeding for spring wheat. The traps were replaced five times at 2-wk intervals from June to August. Soil temperature and soil moisture were recorded at the time of wireworm trap collection by using soil thermometer (Taylor, Illinois) and soil moisture meter (Spectrum Technologies Inc., Illinois), respectively. Wireworm larvae were extracted using Berlese funnels (Bioquip Products, California). The collected wireworms were counted and identified using taxonomic keys by Etzler (2013).

To assess the wireworm damage to wheat plants, number of seedlings in each plot were randomly counted using a one m line-intercept method (Sharma et al. 2018). The first and second counts were taken 3 wk after plant germination and just before harvesting, respectively. At harvest, the height of the marked plants was also recorded. The wheat field was harvested on 29 August 2019 and the barley field on 12 September 2019. After harvesting, grain from each plot was cleaned (Almaco, Allan Machine Company, Iowa) and 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 levels were used to calculate yield.

IJs persistence using insect baiting technique

Infective juvenile survival was observed in all plots in August 2019 (3 mo after treatment) and May–June 2020 (1 yr after treatment). In 2019, five soil core samples (approximately 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. Entomopathogenic nematodes 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 10 *G. mellonella* larvae in each cup. The containers were kept in the dark at room temperature (22 ± 2°C) for 7 d. Dead larvae with possible EPN infection were removed, rinsed with tap water, and 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.

Greenhouse Experiment

To explore the efficacy of EPN-infected *G. mellonella* cadavers, a greenhouse experiment was conducted in conjunction 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. For EPN-infected cadavers, 15 *G. mellonella* larvae in 60 mm diameter Petri dishes were exposed separately to approximately 200 freshly produced IJs of each of the four EPN strains. Rest of the procedure was same as described for the field experiment.

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 *L. californicus* larvae (average length of 0.8–1.5 cm) were added after 24 h. Any larvae unable to enter the soil after 5 h 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. Sixty pots made up the experiment with 20 pots of four EPNs × five replications, each with imidacloprid-treated plants plus wireworms and 20 pots of four strains of EPNs × five replications, but nontreated 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, and soil temperature and moisture were recorded three times per week using a soil moisture meter (Spectrum Technologies Inc.) and soil thermometer (Taylor). Probability of plant damage and wireworm mortality were recorded for each pot. Plant damage was measured weekly for 5 wk, 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 5 wk, the pots were destructively sampled and the soil within each pot was checked for dead wireworms. The whole experiment was repeated after 1 wk. To estimate IJ emergence 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 (3 wk) emerging IJs were collected and counted by the serial dilution method (Glazer and Lewis 2000).

Effect of Soil Characteristics on EPN Efficacy

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

Four different types of soils (Table 1) were used. The soil from each textural class was sieved (2 mm mesh) and sterilized in an

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

autoclave at 121°C to kill microorganisms and any natural nematode populations. Deli plastic cups (500 ml) were filled with 150 g 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 h were replaced. After 24 h, 7,000 IJs/cup (50 IJs/cm^2) were inoculated in 1 ml of water. Control cups received only 1 ml of water without IJs. Finally, the moisture contents for all the soils were adjusted to field capacity levels (Table 1). Later, 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 d to maintain the moisture. The wireworm mortality was assessed at weekly intervals for 4 wk. There were five replications for each treatment. The experiment was conducted twice with an interval of 2 wk between trials and different nematode cultures were used for both trials.

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

This experiment was also conducted at the Western Triangle Agricultural Research Center (WTARC), Conrad, MT in 2019. Same procedure was used for preparation of treatment cups as described in soil texture experiment with only sandy clay loam soil type. Sandy clay loam (Table 1) is the common soil type in the GTR and the common habitat soil type for wireworms. Five wireworm larvae were introduced into each cup and allowed to go down for 12 h. 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 (100 g) at 60°C and were calculated on a wet weight (wt:wt) basis. Soil moisture levels were adjusted to get the approximate target percentage for each moisture level, and the appropriate amount of water was added and mixed into the soil. The control cups received plain water without IJs. The experiment was repeated once again after 10 d with five replications and the mortality was observed at weekly intervals.

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

The experiment studying the effect of soil temperature on EPN efficacy was conducted at Marsh laboratory, Montana State University, Bozeman in 2020. The experiment was conducted by following the same procedure as described 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 evaluated. The experiment was conducted twice, with an interval of 2 wk between trials and different nematode cultures were used for both trials.

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 infected *G. mellonella* larvae from field soil samples were analyzed using the Generalized Linear Model (GLM) with quasibinomial distribution. A χ^2 test was also used to test the interaction (synergistic, additive, or antagonistic) of imidacloprid and EPN strains on wireworm mortality in greenhouse. The wireworm mortality data were corrected for control mortality before analysis (Abbott 1925). 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. Trial, EPN strains, imidacloprid treatment, and time were the predictor variables in different models. For the laboratory experiments, data for *L. californicus* mortality were analyzed just for 28 days after treatment (DAT) in all the experiments because the mortality rates were not very high at 7, 14, and 21 DAT. For soil texture at field capacity level, soil moisture and soil temperature, data were analyzed by using Generalized Linear Model (GLM) with quasibinomial distribution and logit link with EPN strain, soil type, moisture level, and temperature as predictor variables in different models and dead wireworm as response variable. The quasibinomial distribution was used to deal with the overdispersion problem (Ramsey and Schafer 2012). The Tukey–Kramer test ($\alpha = 0.05$) was used to find the significant differences between the treatments. Data were analyzed using R 2.15.1 (R Development Core Team 2017).

Results

Field Experiment

In June, the Pendroy (barley) and Choteau (spring wheat) sites had soil temperature varied from 6 ± 5°C to 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 the beginning of August, the soil temperature was higher at barley site (20 ± 5°C) with 16 ± 2°C soil temperature at spring wheat site. However, the soil moisture varied between two sites. At barley site, the soil moisture content was almost twice as compared to the spring wheat site. The soil moisture content at barley site was 56. 2 ± 3%, 45. 5 ± 5%, and 55. 38 ± 5% in June, July, and first week of August, respectively. However, the soil moisture content at Spring wheat site was 22. 29 ± 5%, 25. 88 ± 7%, and 31. 02 ± 8% in June, July, and August, respectively.

Pre- and Post-harvest Field Efficacy

The IJs emerged from one cadaver in the laboratory varied significantly among four EPN strains ($F = 8.55$, df = 3, $P < 0.0001$). The EPN strains *S. carpocapsae* (Cxrd), *S. riobrave* (7–12) and *S. riobrave* (355) produced significantly higher number of IJs, i.e., 270, 270 ± 13,637, 248, 513 ± 11,821, 249, 592 ± 12,384, respectively as compared to only 181, 860 ± 15,957 IJs produced by *S. carpocapsae* (All). In barley, *L. californicus* was the numerically dominant species (~75%) followed by *Aelous mellilus* (~15%) and *H. bicolor* (~10%). However, in spring wheat, only *H. bicolor* was found (100%). Wireworms found at both the field sites were of multiple instars and the wireworm pressure was high in barley compared to spring wheat (see Supp table 1 [online only]). In both spring wheat and barley, there was no significant EPN effect ($P > 0.33$) or significant interaction among EPN strain, dose, and time (barley: $F = 0.53$, df = 16, $P = 0.93$; spring wheat: $F = 1.11$, df = 16, $P = 0.34$). However, in barley, number of wireworms collected varied with time ($F = 1.11$, df = 16, $P = 0.34$) with significantly higher numbers of wireworms collected after 60 and 75 d ($P < 0.05$) as compared to 15, 30, and 45 d regardless of the EPN strains and dose. Time effect was not detected in spring wheat ($P = 0.76$). Overall, total number of wireworms collected ranged from 76 to 147 in barley and 4–13 in spring wheat field irrespective of the EPN strain treatment (see Supp table 1 [online only]).

Weeds and volunteer plants at both the sites impacted plant counts at 3 wk after planting and therefore could not be considered accurate and plant count data taken before harvesting is being analyzed further. In spring wheat and barley field, no parameters (plant count, plant height, moisture, protein, and test weight) varied significantly with different treatments and dosages. In spring wheat, the ranges for different parameters observed were the following: plant count (33–39 plants), plant height (70–81 cm), moisture (11–12%), protein content (11–14%), and test weight (76–81 kg/ha) (see Supp table 3 [online only]). In barley, the ranges for different parameters observed were: plant count (8–12 plants), plant height (72–91 cm), moisture (9–10%), protein content (12–13%), and test weight (57–61 kg/ha) (see Supp table 2 [online only]). Similarly, no significant differences were observed among EPN treatments in terms of yield (kg/ha) at both the field sites with barley yield ranging from 1,532 to 2,169 kg/ha for barley and 1,496 to 2,050 kg/ha for spring wheat (see Supp table 2 & 3 [online only]).

IJs Persistence Using Insect Baiting Technique

In barley, mortality of *G. mellonella* did not vary significantly with EPN strains at either rate of three or six cadavers/plot ($P > 0.08$) and was not affected by application rate ($P = 0.07$), and there was no significant interaction ($P = 0.49$). In spring wheat, *G. mellonella* mortality was not affected by rate of application ($P = 0.22$); there was significant interaction between EPN strain and rate ($\chi^2 = 16.44$, df = 3, $P < 0.0001$). EPN strains had no effect on *G. mellonella* mortality at three cadavers/plot ($P = 0.20$) and had a significant effect at six cadavers/plot ($\chi^2 = 29.43$, df = 4, $P < 0.0001$). In general, higher *G. mellonella* mortality was observed in barley (30–45%) than spring wheat (25% on average; Fig. 1). No mortality of *G. mellonella* was seen in soil samples collected from the fields in June 2020 (1 yr after application).

Greenhouse Experiment

For greenhouse experiments in 2020, the number of IJs that emerged from a cadaver in the laboratory varied significantly among EPN strains ($F = 11.61$, df = 3, $P = 0.0003$). EPN strain *S. carpocapsae* (Cxrd) produced a significantly higher number of IJs (248, 031 ± 18,909 IJs; $P < 0.01$) compared to *S. riobrave* (7–12) (161, 077 ± 6,195 IJs) and *S. riobrave* (355) (166, 096 ± 12,847 IJs). However, it did not differ significantly from *S. carpocapsae* (All) (206, 525 ± 5,190 IJs) ($P > 0.01$). Antagonistic interactions of imidacloprid seed treatment were found with *S. carpocapsae* All and *S. riobrave* (7–12) (Table 2). However, synergism was observed among imidacloprid and *S. carpocapsae* (Cxrd) and imidacloprid and *S. riobrave* (355) strains in regard to *L. californicus* mortality (Table 2). 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 ($P < 0.01$) (Fig. 2). 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) 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

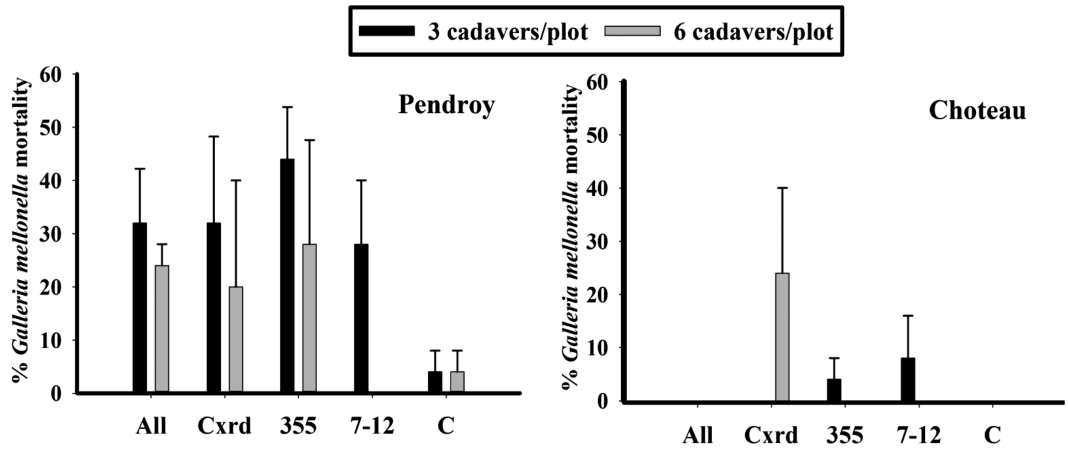

Fig. 1. Average percentage of *Galleria mellonella* infected with entomopathogenic nematodes in collected soil samples three months after treatments. ScAll = *Steinernema carpocapsae* (All), ScCxrd = *Steinernema carpocapsae* (Cxrd), Sr355 = *Steinernema riobrave* (355), Sr7-12 = *Steinernema riobrave* (7-12), C = Control. No significant differences were observed among the treatments (Tukey-Kramer test, $\alpha = 0.05$).

Table 2. Interaction of entomopathogenic nematodes and imidacloprid seed treatment against *Limonius californicus* larvae at 35 d 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	Antagonistic
Steinernema carpocapsae (Cxrd)	58. 00 ± 8.14	47. 60 ± 6.65	70.94	Synergistic
Steinernema riobrave (355)	40. 00 ± 7.30	39. 60 ± 7.17	40.93	Synergistic
Steinernema riobrave (7–12)	26. 00 ± 8.46	31. 2 ± 6.63	64.84	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.

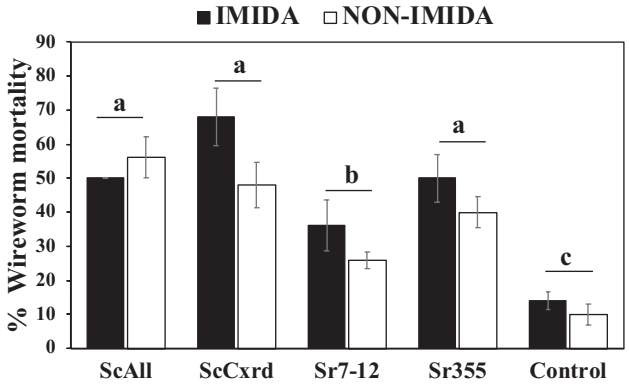

Fig. 2. Average percentage mortality of larval *Limonius californicus* after exposure to entomopathogenic nematodes (EPN) at one EPN-infected cadaver of Galleria mellonella/pot in greenhouse in 2020. ScAll = *Steinernema carpocapsae* (All), ScCxrd = *Steinernema carpocapsae* (Cxrd), Sr7-12 = *Steinernema riobrave* (7-12), Sr355 = *Steinernema riobrave* (355). IMIDA = plants with imidacloprid treatment, NON-IMIDA = plants without any treatment. Different letters above the line points indicate statistical significance ($P \leq 0.05$, Tukey-Kramer test).

were higher for *S. carpocapsae* (Cxrd), *S. riobrave* (355), and *S. riobrave* (7–12) in imidacloprid treatments except *S. carpocapsae* (All) which caused 56% mortality in non-imidacloprid plants as compared to only 50% mortality caused in imidacloprid treated plants (Fig. 2).

In greenhouse trials, plant damage caused by *L. californicus* did not vary significantly between 7 DAT and 14 DAT, but varied significantly at 21, 28, and 35 DAT ($P < 0.001$). 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 14 DAT and 35 DAT were chosen for further analysis (Fig. 3A and B). Two trials differed significantly in regards to plant damage (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. No three-way interaction was detected between EPN strain, time and imidacloprid treatment ($\chi^2 = 3.01$, df = 5, $P = 0.69$). At 14 DAT, none of the EPN strains differed significantly for plant damage as compared to control pots ($P > 0.05$) in both imidacloprid and non-imidacloprid treatment (Fig. 3A). However, the percentage of plant damage observed was significantly higher (40–57%; $P < 0.01$) in pots with plants without imidacloprid treatment as compared to only 6–9% plant damage observed in pots with imidacloprid treated plants (Fig. 3A).

At 35 DAT, 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. 3B). The percentage of plant damage ranged from 8 to 24% in imidacloprid treated plants and was significantly lower than the damage in pots without imidacloprid treatment ($P < 0.01$) (Fig. 3B). 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. 3A and B). The average soil temperature and moisture in the greenhouse were recorded as 22°C (17–27°C) and 22 ± 5%, respectively.

Effect of Soil Characteristics on EPN Efficacy

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

No *L. californicus* mortality was observed in the control treatment. Two trials did not differ significantly in respect to wireworm mortality (Trial: $\chi^2 = 2.72$, df = 1, $P = 0.10$; Strain × Trial: $\chi^2 = 0.53$, 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 *L. californicus* mortality ($\chi^2 = 9.90$, 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 ($\chi^2 = 5.71$, df = 3, $P = 0.13$). Similarly, the interaction between EPN strain and soil type was also not significant ($\chi^2 = 8.17$, df = 6, $P = 0.23$), therefore the data are not being presented graphically. Overall, *Steinernema carpocapsae* (Cxrd) caused significantly higher wireworm mortality (30–46%) than only 12–40% mortality caused by *S. riobrave* (355) irrespective of the soil type ($P = 0.008$).

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

The interaction between EPN strains and trials ($\chi^2 = 1.51$, df = 2, $P = 0.47$) and moisture levels and trials ($\chi^2 = 2.74$, 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 ($\chi^2 = 24.35$, df = 2, $P < 0.0001$). However, moisture level independently as well as interaction of EPN strains and moisture levels did not have significant effect on *L. californicus* mortality ($P > 0.11$). Overall, *S. carpocapsae* (All and Cxrd) did not differ significantly at different moisture levels ($P = 0.34$) and were able to cause significantly higher i.e., 32 to 58% *L. californicus* mortality at 28 DAT as compared to other strains irrespective of soil type ($P < 0.05$). *Steinernema riobrave* 355 strain was not able to cause > 32% wireworm mortality irrespective of the moisture levels. No wireworm mortality was observed in the control treatment.

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

Two trials did not differ significantly in terms of *L. californicus* mortality (Trial: $\chi^2 = 0.28$, df = 1, $P = 0.60$), therefore data for two trials were pooled together for further analysis. Wireworm mortality was significantly affected by EPN strains ($\chi^2 = 92.15$, df = 4, $P < 0.0001$) and temperature ($\chi^2 = 19.09$, df = 2, $P < 0.0001$). Additionally, interaction between EPN strain and temperature was also found significant ($\chi^2 = 47.10$, df = 8, $P < 0.0001$) with regards to wireworm mortality. At 15°C, *S. carpocapsae* (Cxrd) caused significantly higher mortality (60%) as compared to only 4–20% mortality caused by *S. riobrave* (355 and 7–12) and control treatment (Fig. 4). However, *S. carpocapsae* (All) did not differ from *S. carpocapsae* (Cxrd) with 34% *L. californicus* mortality. *Steinernema carpocapsae* (All) caused significantly higher mortality (66%) as compared to only 34% mortality caused by *S. riobrave* (7–12) and 12% being caused in control treatment at 25°C (Fig. 4). *Steinernema carpocapsae* (Cxrd) and *S. riobrave* (355) were also able to cause 58 and 38% *L. californicus* mortality, respectively, at 25°C. However, at 30°C temperature, none of the four EPN strains that caused significantly higher mortality than control, differed significantly from each other in regard to *L. californicus* mortality (Fig. 4).

Discussion

Entomopathogenic nematodes have been evaluated against European wireworm species in laboratory, greenhouse and field conditions (Ester and Huiting 2007, Campos-Herrera and Gutierrez 2009, Morton and Garcia-del-Pino 2016). 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), where *S. feltiae* 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, Ester and Huiting

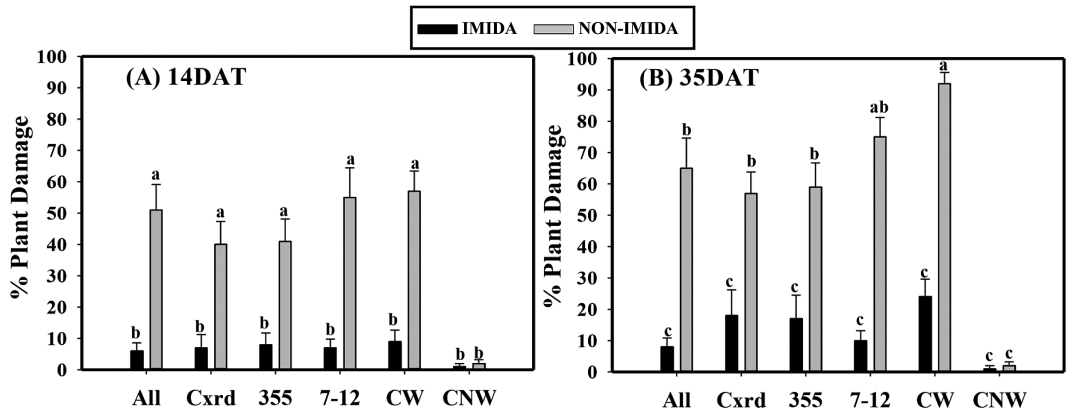

Fig. 3. Average percentage of plant damage by larval *Limonius californicus* after exposure to entomopathogenic nematodes at one EPN infected Galleria mellonella cadaver/pot at (A) 14DAT and (B) 35DAT in greenhouse in 2020. All = *Steinernema carpocapsae* (All), Cxrd = *Steinernema carpocapsae* (Cxrd), 355 = *Steinernema riobrave* (355), 7-12 = *Steinernema riobrave* (7-12), CW = Control with wireworms, and CNW = Control without wireworms. IMIDA = plants with imidacloprid treatment, NON-IMIDA = plants without any treatment. DAT = Days after treatment. Different letters above the line points indicate statistical significance ($P \leq 0.05$, Tukey-Kramer test).

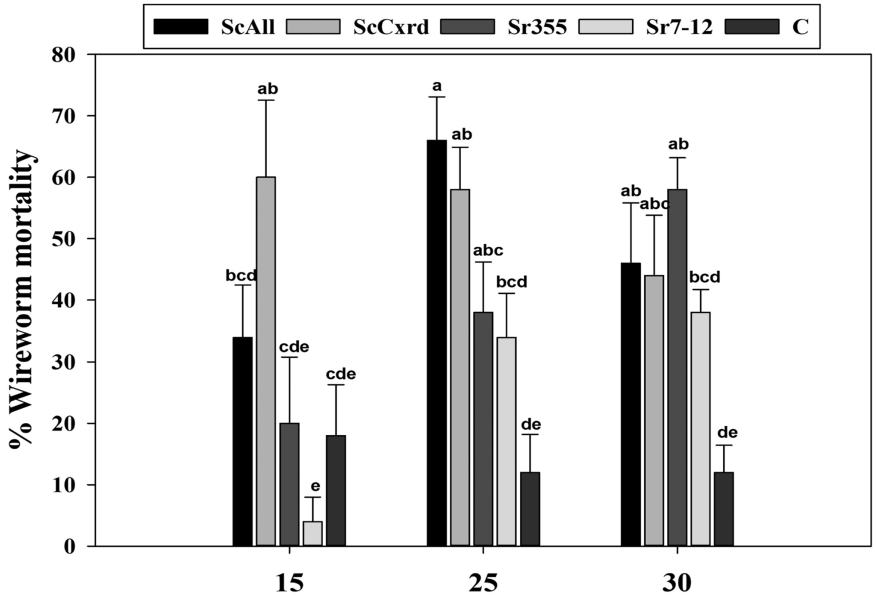

Fig. 4. Average percentage mortality of larval *Limonius californicus* after exposure to entomopathogenic nematodes (EPNs) at the rate of 1400IJs/larva (7000 IJs/cup) concentration at 28 days after treatment and different temperature ranges (15, 25, and 30°C) in sandy clay loam soil. ScAll = *Steinernema carpocapsae* (All), ScCxrd = *Steinernema carpocapsae* (Cxrd), Sr355 = *Steinernema riobrave* (355), Sr7-12 = *Steinernema riobrave* (7-12), C = Control without EPNs.

2007, Arrington et al. 2015, Morton and Garcia-del-Pino 2016). 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, this approach did not prevent wireworm damage in crops or protect yields at either site. Number of wireworms collected at both sites cannot be correlated with the overall yield 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).

However, at least three EPN strains *S. carpocapsae* (All), *S. carpocapsae* (Cxrd), and *S. riobrave* (355) killed more than 50% of *L. californicus* larvae in greenhouse experiments. These results 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 2016) 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 imidacloprid and non-imidacloprid treatments, the seed treatment was able to enhance the infection and killing ability of EPNs against *L. californicus* as well as protecting the plants in the present study. Koppenhöfer and Fuzy (2008) and many studies there within have reported synergism between imidacloprid and EPNs, when applied as aqueous suspensions. The present study is the first report of possibility of synergistic interactions between imidacloprid and EPNs, when applied in the form of infected cadavers. 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.

Although in some cases, EPNs applied as insect cadavers are reported to be superior to application in aqueous suspension (Shapiro-Ilan et al. 2010), other studies did not report any positive outcome from this approach as compared to aqueous suspension application (Raja et al. 2015). The main reason may be cadavers rupturing or sticking together during handling, transportation and application (Shapiro-Ilan et al. 2001). Additionally, the cadaver approach shows greater dispersal of IJs as compared to aqueous suspension application of EPNs (Shapiro-Ilan and Glazer 1996) 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-Ilan et al. 2014b) due to which IJs might have been unable to infect the moving wireworms.

Cadavers might have ruptured or disintegrated after being placed in the soil because of the unfavorable environmental conditions especially in the field. Reasons for the low postapplication EPN survival and efficacy in the field might have been due to IJ desiccation because of abiotic factors such as ultraviolet radiation, soil texture, pH, temperature, relative humidity, and moisture content as reported by Shapiro-Ilan et al. 2006, Koppenhöfer and Fuzy 2006, Shapiro-Ilan and Dolinski 2015. However, in the greenhouse experiment, comparatively positive results showed that the IJs were able to develop and reproduce well due to well-maintained surroundings in terms of temperature and soil moisture in the pots.

Soil being the natural habitat of these nematodes as well as wireworms, the effect of the soil environment on EPNs need to be considered for their successful utilization against wireworms. *Steinernema 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. Adequate soil moisture is also necessary for EPN movement, survival, and efficacy, and therefore, pre- and post-irrigation application in fields might help in improving EPN efficacy and persistence. 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 to 31%. *Steinernema carpocapsae* caused highest wireworm mortality at four moisture levels especially at 16 and 24% under laboratory conditions (not statistically different though).

At both sites, the soil temperature was low i.e., 6–14°C in the beginning of the season that might be the other potential reason for limited EPN movement and survival leading to poor outcomes. These observations were the main reasons for testing the effect of temperature on EPN efficacy against wireworms in laboratory conditions. Although, the ability of *S. carpocapsae* to kill *L. californicus* larvae increased as the temperature increased and 25°C had the highest wireworm mortality in the laboratory, it was affected less by temperature. The higher efficacy of *S. carpocapsae* at higher temperatures has been reported in other studies (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. Nonsignificant results in regards to wireworm mortality at different moisture levels, in different soil types, as well as some temperature combinations observed in the present study might just be the low susceptibility of wireworms to the tested EPN species. 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 in addition to managing the populations.

We strongly believe that in barley field, low germination because of mechanical damage at seeding and heavy rains might have been the major factor for the unfortunate outcomes. It is evident from 30 to 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 barley 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 spring wheat. 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 greenhouse study because of the possibility of all above mentioned factors. The dosage (three vs six cadavers) also did not differ in terms of efficacy against wireworms. 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 in the field. Additionally, 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.

In future research, aqueous suspension approach should also be tested and compared with the EPN-infected cadaver approach. 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) should be tested to improve

the efficacy of EPN-infected cadaver applications for wireworm control. Shapiro-Ilan et al. (2010) indicated some potential in using the tape-formulation approach for applying EPNs against *Diaprepes abbreviatus* L. (Coleoptera: Curculionidae), where infected cadavers were formulated using a mechanized tape-packaging machine. This approach can be explored further in future for field application of EPN-infected cadavers. Moreover, the possibilities of developing mechanized operators to scatter the cadavers in the field are intriguing and might be better in handling the delicate EPN-infected cadavers. In addition, the ability of EPNs to persist longer in the applied area should be explored in order to hopefully achieve a cost-effective 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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