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Comparative infectivity and biocontrol potential of *Acrobeloides* k29 and entomopathogenic nematodes on the leopard moth borer, *Zeuzera pyrina*

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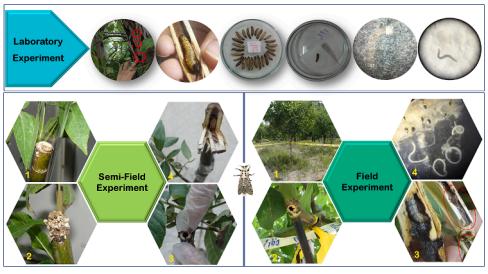
HIGHLIGHTS

- Infectivity of a native free-living nematode and two EPNs evaluated against *Zeuzera pyrina*.
- *Acrobeloides* K29 provided effective control of *Z. pyrina* in laboratory and natural conditions.
- *Acrobeloides* K29 indicated comparable reproduction to two tested EPNs on insect hosts.
- *Steinernema carpocapsae* showed highest virulence to *Z. pyrina* in lab, semi-field and field tests.

GRAPHICAL ABSTRACT



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ABSTRACT

The leopard moth, *Zeuzera pyrina* L. (Lepidoptera: Cossidae) is a serious pest of walnut trees in Iran. Due to special habitat of this cossid, the application of entomopathogenic nematodes (EPNs) may offer an efficient strategy in suppressing populations of this pest. The efficacy of two commercially available EPNs *Steinernema carpocapsae* and *Heterorhabditis bacteriophora*, as well as a local insect-killing nematode (*Acrobeloides* K29) were investigated against *Z. pyrina* larvae in laboratory, semi-field, and field conditions. Laboratory experiments included pathogenicity assessment of aforementioned nematodes in plates using a range of concentrations [5, 10, 20, 50 and 100 infective juveniles (IJs) per larva], as well as evaluation of their reproduction potential on *Z. pyrina* larvae with concentrations of 5 and 20 IJs per larva. Application of increasing concentrations of nematodes induced higher mortality on *Z. pyrina* larvae with mean mortality ranging from 28.3% to 100% after 72 h. All three nematodes successfully reproduced and recovered from pest's cadavers. The highest reproduction was recorded for *H. bacteriophora* at 5 IJs larva^{-1} in *Z. pyrina*. Semi–field experiments using potted walnut seedlings and field trials in a heavily infested walnut orchard during two consecutive years revealed the ability of the aforementioned nematodes to infect and kill *Z. pyrina* larvae inside the galleries of walnut branches. Overall, this research highlights the ability of all three examined nematodes to find, invade, recycle and effectively infect *Z. pyrina* larvae; however, *S. carpocapsae* is comparatively more effective than *H. bacteriophora* and *Acrobeloides* K29 under laboratory, semi–field and field conditions. Of significance, the free-living nematode causes high to moderate levels of mortality in *Z. pyrina* larvae in laboratory and natural conditions.

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1. Introduction

The leopard moth, *Zeuzera pyrina* L. (Lepidoptera: Cossidae) is a key insect pest of walnut trees in Iran and causes economic damage to the branches and trunks. In the past few decades, this pest has caused serious yield losses in walnut orchards in Kerman Province, Iran (Hosseini Gharalari and Kolyai, 2014). The polyphagous larvae of this cossid pest are cryptic wood-borers attacking a wide variety of deciduous trees, shrubs, nursery plants, and young plantations in over 30 plant families (Castellari, 1986; Kutinkova et al., 2006). The damage by this xylophagous pest is primarily caused by the emerging larvae which bore tunnels into the tree and feed within larval galleries inside the thin branches. The cryptic late-stage larvae bore deep into twigs, major branches, and trunk and eventually lead to withering or complete death of host trees (Långström et al., 2004; Alford, 2007).

Entomopathogenic nematodes (EPNs) with their mutualistic bacteria are considered one of the eco-friendly and sustainable biological control agents against a variety of cryptic wood-borer pests (Burnell and Stock, 2000; Canhilal and Carner, 2006; Lacey and Georgis, 2012). The special habitat of *Z. pyrina* provides an optimal environment required for these entomopathogenic agents. Therefore, EPNs may offer an efficient performance to suppress outbreaks of this insect pest within the framework of an integrated pest management approach.

EPNs of the genera *Steinernema* and *Heterorhabditis* have been reported to be pathogenic to *Z. pyrina* in apple trees in field trials causing 20% to 90% larval mortality, depending upon the nematode species and time and method of application (Abdel-Kawy et al., 1988). Several other laboratory and field investigations have similarly described the effectiveness of native and commercial EPNs against *Z. pyrina* larvae (Abdel-Kawy et al., 1992; Saleh et al., 1994; Shamseldean et al., 2009; Ashtari et al., 2011; El-Kholy et al., 2014; Salari et al., 2015; El-Ashry et al., 2018).

EPNs have shown variation in virulence, reproductive capacity and foraging behavior that influence their efficacy as biocontrol agents against different insect pests (Shapiro-Ilan et al., 2003, 2012; Lezama-Gutiérrez et al., 2006). Several studies on the infectivity of free-living nematodes in the family of Cephalobidae and Rhabditidae have indicated strong potentials for infecting some insect pests (Torres-Barragan et al., 2011; Park et al., 2012; Baquiran et al., 2013). For example, laboratory and greenhouse studies on biological control potentials of *Rhabditis blumi* Sudhaus against major cruciferous lepidopteran insect populations including *Artogeia rapae* L. (Lep.: Pieridae), *Mamestra brassicae* L. (Lep.: Noctuidae) and *Plutella xylostella* L. (Lep.: Plutellidae) indicated high potency of this free-living nematode comparable with heterorhabditid nematodes (Park et al., 2012). Also, *Oscheius carolinensis* was described as a potential EPN. It showed capacity to penetrate, kill and successfully reproduce inside different developmental stages of all five tested insect species under laboratory conditions (Torres-Barragan et al., 2011). However, infectivity potential of *Acrobeloides nanus* (De Man) was reported in natural populations of earthworm cocoons (Kraglund and Ekelund, 2002), but the detailed studies concerning the infectivity of *Acrobeloides* nematodes against insect pests are lacking. Thus, in order to successfully utilize these parasitic nematode groups for controlling indigenous insect pests, isolation, identification and virulence evaluation of the locally-collected insect-parasitic nematodes are required.

The main objectives of this study were to: 1) evaluate infectivity of an indigenous isolate of insect-killing *Acrobeloides* nematode (*Acrobeloides* K29) and two commercially available EPNs, *Steinernema carpocapsae* and *Heterorhabditis bacteriophora*, against *Z. pyrina* larvae in laboratory, semi-field and field conditions, 2) investigate reproductive potential of these three nematodes in *Z. pyrina* and *G. mellonella* larvae, and 3) establish an optimal seasonal time frame for efficacious application of these biocontrol agents in walnut orchards.

2. Materials and methods

2.1. Isolation of an indigenous nematode populations

During a survey of EPNs in Kerman region, located in southeastern Iran, an *Acrobeloides* nematode in the family Cephalobidae was recovered from soil samples using the insect-baiting technique (Bedding and Akhurst, 1975) and designated as *Acrobeloides* K29. After collecting soil samples and transferring to the laboratory, five last-instar larvae of the greater wax moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae) were exposed to fifty grams of moistened soil sample in plastic containers (10-cm diameter). The containers were maintained at room temperature, turned upside down and examined daily for larval mortality. The dead larvae suspected of being infected with nematodes, were rinsed several times with double-distilled water and transferred to a White trap (White, 1927). Nematode juveniles emerging from the cadavers were collected and kept in 0.1% formaldehyde prior to identification. Morphological studies with light microscopy and scanning electron microscopy, as well as molecular analyses using 18S, ITS and 28S regions of ribosomal DNA were used for identification of this isolate. It is important to note that no natural entomopathogenic or entomophilic nematode populations were recovered from the experimental field site in walnut orchards by baiting soil samples with wax moth larvae.

2.2. Nematodes and insect cultures

All experiments were conducted using the native nematode *Acrobeloides* K29, along with *Steinernema carpocapsae* (Capsanem) and *Heterorhabditis bacteriophora*, (Larvanem) commercial products (Koppert B.V., Berkel en Rodenrijs, The Netherlands). All three nematodes were cultured at 25 ± 1 °C in parallel on last-instar larvae of *G. mellonella* (Kaya and Stock, 1997). Following harvest on White traps (White, 1927), infective juveniles (IJs) were aerated and stored in sterile distilled water at 8 °C for about one week before using in experiments. Prior to semi-field and field application, the survival rate of IJs was determined under a stereomicroscope and when more than 98% of IJs were viable, the water suspensions of them were used for the experiments.

Larvae of *Z. pyrina* were collected from infested walnut orchards in Deh Zarchi region (29° 19′ 60″ N 56° 37′ 60″ E; 2,250 m above sea level), Kerman Province, Iran, during late May and early October 2014 and 2016 and maintained in laboratory under the diet and condition as described previously (Salari et al., 2015), and only healthy mid to late instar larvae were used for experiments.

2.3. Virulence of selected nematode against Z. pyrina larvae in laboratory experiments

In order to examine the susceptibility of larval stages of *Z. pyrina* to these biocontrol agents, 200 μl of aqueous suspension of each tested nematode at a range of 5, 10, 20, 50 and 100 IJs per larval concentrations were applied into a Petri dish (5 × 5 cm) lined with two sheets of sterile filter paper (Whatman No. 1). In control treatments, only 200 μl of distilled water was added to Petri dishes. Larvae of *Z. pyrina* were then individually placed in Petri dishes and pieces of fresh walnut wood were provided as food. After treatment, the Petri plates were sealed with Parafilm and kept in a controlled growth chamber at 25 ± 1 °C and 65 ± 10% RH. Twenty replicates, one larva/rep were used per nematode isolates and concentration and each experiment was repeated twice. Larval mortalities were recorded in all experiments after 24, 48 and 72 h of treatment followed by dissection of cadavers under the stereomicroscope and confirmation of nematode infection.

2.4. Nematode reproductive potential on Z. pyrina and G. mellonella larvae

To assess the reproduction capabilities of *Acrobeloides* K29, *S. carpocapsae* and *H. bacteriophora* on the larvae of *Z. pyrina*, the mean number of IJs produced per mg of host was measured and the results were compared to that of positive control with *G. mellonella* larvae. This trail was designed based on previous studies of nematode reproduction potential on the insect hosts (Campos-Herrera et al., 2008). Nematode IJs were placed in 200 μl of sterile distilled water and added to a 5-cm-diameter glass Petri dish lined with two pieces of filter paper (Whatman No. 1). A single healthy larva of each moth was weighed and placed in each dish test for inoculating with concentrations of 5 and 20 IJs per larva of each nematode population, separately, as described above for virulence testing. The experiment with twenty weighed larvae of each insect host per treatment was conducted. The mean ± SE weight of *Z. pyrina* larvae was recorded as 298.9 ± 6.3 mg, while the mean ± SE fresh weight of *G. mellonella* larvae was 279.1 ± 14.4 mg. The Petri dishes were then sealed with Parafilm and maintained in a controlled growth chamber at 25 ± 1 °C until hosts' death. The dead larvae, with signs of nematode infection, were rinsed in sterile distilled water to eliminate nematodes from the cadaver's surfaces and individually placed in separate White traps (Kaya and Stock, 1997) to assess nematode reproduction. The White traps were maintained in a controlled growth chamber to provide sufficient time for nematode reproduction. The numbers of emerging IJs were recorded until the last IJs emerged and total numbers of emerged IJs (no. IJs per mg larva) were enumerated for each nematode population. The entire experiment was repeated twice with the same number of replicates.

2.5. Efficacy of selected nematodes against Z. pyrina larvae in semi-field trials

In order to corroborate results of laboratory trials and determine if these native and commercial nematode populations could reach, penetrate and infect *Z. pyrina* larvae inside larval galleries, the infectivity of all three aforementioned nematodes were examined under semi-field conditions. Experimental units included a one-year-old healthy seedlings of Superior walnut variety planted in plastic pots (12 cm height and 14 cm diameter) containing approximately 2 kg of autoclaved sandy-clay loam soil collected from walnut orchards of Deh Zarchi region, Kerman Province, Iran (each pot = one replicate). The soil was 51% sand, 28% silt, and 21% clay, with salinity of 0.37 dS/m and pH 7.9.

Prior to the experiment, one mid-size healthy *Z. pyrina* larva was introduced into each cylindrical clip cage (18 mm inside diameter × 100 mm length) on the tip of each seedling. After almost two hours, all larvae started boring into the pith of the healthy seedlings, entering the tips, and as a result, producing sawdust-like feces in the clip cages. This was followed by injecting *Acrobeloides* K29, *S. carpocapsae* and *H. bacteriophora* directly into the gallery entrances at concentrations of 160 IJs cm^{-2} (5000 IJs in total per galleries), and 300 IJs cm^{-2} (10,000 IJs in total per galleries), in 5 ml distilled water using a 10-ml syringe. Control pots were treated with only 5 ml water without nematodes.

There were fifteen pots per treatment and the whole test was repeated twice. After introduction of tested nematodes, all pots were maintained inside a greenhouse with direct sunlight at 27 ± 1 °C, 65% RH and a photoperiod of 16:8 (L:D) h. The pots were monitored daily and after 5 days, the tip of each seedling was dissected and the surviving and dead larvae were enumerated and inspected for nematode infection.

2.6. Efficacy of selected nematodes against Z. pyrina larvae in field trials

Field pathogenicity assessments were carried out in walnut orchards, naturally infested with *Z. pyrina* in Deh Zarchi region, Kerman Province, Iran, in two consecutive years, 2015 and 2016. The first field experiment was conducted at the beginning of July 2015 and was repeated in the third week of September 2016 to determine the efficiency of *Acrobeloides* K29, *S. carpocapsae* and *H. bacteriophora* against *Z. pyrina* larvae on heavily infested walnut trees. The average minimum and maximum daily temperature were 20.1 °C and 35.5 °C for 2015 (July trial), and 9.4 °C and 26.9 °C for 2016 (September trial), from the date the application was made to seven days post-application. Prior to the experiments, the same height infested walnut trees (average height of 3.25 m) were selected and tagged with neon yellow tapes. Experimental units were naturally infested branches of 25-year-old walnut trees planted in sandy-clay loam soil, with active holes of *Z. pyrina* larvae distinguishable by accumulated frass at the entrance of the larval galleries.

The infectivity of the suspension of nematodes was then evaluated against *Z. pyrina* larvae at the final concentration of 10,000 IJs per galleries in 25 ml distilled water directly injected into the larval tunnels using a 60-mL sterile Luer Lock syringe. Each trial comprised of twenty trees with symptoms of *Z. pyrina* infestation and for each nematode treatment, two annual branches with active larval galleries were treated in each tree, individually. In control treatments, active holes of walnut branches received only water without nematodes. After seven days, the marked branches were removed from the trees and transferred to the laboratory to examine larvae for nematode infection. For this purpose, after dissecting branches and enumerating the survived and dead larvae, some cadavers were randomly dissected under a stereomicroscope to confirm nematode infection. The others were rinsed with distilled water and transferred to individual White traps and maintained in a controlled growth chamber for inspection of the reproduction ability of the nematodes under natural condition.

2.7. Statistical analyses

Efficacy of tested nematodes in causing insect mortality was corrected by control mortality using Abbott's correction (Abbott, 1925). The data were tested for normality (Shapiro-Wilcoxon test) and homogeneity of variance by Leven's test (SAS version 9.1; SAS Institute) and they were transformed to arcsine square root when necessary. In the nematode pathogenicity assay, the larval mortality data was analyzed by running a two-way factorial ANOVA (nematode population × nematode concentrations) followed by Tukey's HSD with significance level at $P \leq 0.05$. To assess the LC_{50} and LC_{90} for each nematode at $P \leq 0.05$ probability, larval mortality resulted from examined nematodes and their concentrations was subjected to probit analysis using Polo-Plus software. Parallelism test was applied to regression lines, and when 95% fiducial limits did not overlap, the LC_{50} and LC_{90} differences between nematode populations were considered significant. To calculate the mean numbers of reproductive capacity of selected nematodes, a two-way factorial ANOVA (nematode concentrations × host species) was used. Reproduction rate of nematodes compared by Tukey's HSD test and differences among means were considered significant at $P \leq 0.05$.

Non-parametric Kruskal-Wallis (K-W) test was used to compare the effects of the three nematodes on *Z. pyrina* larvae susceptibility in semi-field experiments. To determine the efficacy of the three nematodes against *Z. pyrina* larvae inside larval galleries in walnut orchards, the data were subjected to one-way analysis of covariance in which examined nematodes were considered main factors and the total number of *Z. pyrina* larvae was regarded as a covariate. When covariate was significant ($P \leq 0.05$), LSD test was used to determine the differences among mean values (SAS version 9.1; SAS Institute).

3. Results

3.1. Virulence of native and commercially tested nematodes for Z. pyrina larvae

Pathogenicity assessment indicated that all three examined nematodes were capable of infecting and killing *Z. pyrina* larvae. Analysis of variance revealed that larval mortality was significantly influenced by

nematode population ($F_{2, 60} = 7.45$, $P = 0.0013$). Also, total mortality of *Z. pyrina* larvae significantly increased as nematode concentrations increased ($F_{4, 60} = 68.34$, $P < 0.0001$), with mean mortality ranging from 26.7% to 100%, when they were treated with 5 to 100 IJs larva^{-1} of all three tested nematodes. However, there were no statistical differences between the interaction of nematode population and concentration on the susceptibility of host larvae ($F_{8, 60} = 0.90$, $P = 0.5191$).

Our results showed that *S. carpocapsae* was able to kill the same number of *Z. pyrina* larvae with fewer IJs concentrations compared to *Acrobeloides* K29 ($P < 0.05$). All three nematodes caused maximum larval mortality (>95%) after 72 h at 100 IJs larva^{-1} concentration. However, the lowest mortality was recorded for *Acrobeloides* K29 (20%) at 5 IJs larva^{-1} concentration (Fig. 1).

The calculated values of LC_{50} and LC_{90} together with their upper and lower fiducial limits as well as Chi-square (χ^2) for aforementioned nematodes are given in the Table 1. The LC_{50} for *Acrobeloides* K29 was 12.9 IJs per larva, followed by those of *S. carpocapsae* and *H. bacteriophora* with LC_{50} of 6.2 and 8.7 IJs per larva after 72 h of incubation, respectively. In addition, the LC_{90} of *Acrobeloides* K29, *S. carpocapsae*, and *H. bacteriophora* for *Z. pyrina* larvae after 72 h was 54.5, 30.1, and 22.7 IJs larva^{-1}, respectively. Analysis of χ^2 confirmed that the regression lines were considered parallel after 72 h ($\chi^2 = 2.93$, df = 2, $P = 0.231$) (Table 1).

3.2. Nematode reproductive potential on Z. pyrina and G. mellonella larvae

Our results indicate that all three nematodes successfully reproduced in the *Z. pyrina* larvae and their offsprings were emerged from the cadavers. No statistical difference was detected between the different hosts (*Z. pyrina* and *G. mellonella* larvae) on the reproduction of *Acrobeloides* K29 ($F_{1, 76} = 0.10$, $P = 0.7559$). The reproduction of *S. carpocapsae* in *Z. pyrina* larvae was significantly lower than that of *G. mellonella* ($F_{1, 76} = 7787.56$, $P < 0.0001$), while the average number of *H. bacteriophora* IJs per mg of *Z. pyrina* larvae was significantly higher than that of infected *G. mellonella* ($F_{1, 76} = 698.08$, $P < 0.0001$). Although the reproductive potential of *Acrobeloides* K29 and *S. carpocapsae* in the larvae of *Z. pyrina* and *G. mellonella* significantly increased as rate of IJs increased (*Acrobeloides* K29: $F_{1, 76} = 1401.09$, $P < 0.0001$; *S. carpocapsae*: $F_{1, 76} = 109.56$, $P < 0.0001$), the number of *H. bacteriophora* IJs per mg of host larvae at the lower concentration (5 IJs larva^{-1}) were significantly higher than that at 20 IJs larva^{-1} ($F_{1, 76} = 44.24$, $P < 0.0001$) (Fig. 2).

No significant interaction occurred between the IJs concentrations and the host species for *Acrobeloides* K29 and *S. carpocapsae* (*Acrobeloides* K29: $F_{1, 76} = 0.13$, $P = 0.7158$; *S. carpocapsae*: $F_{1, 76} = 2.51$, $P = 0.1169$), while the reproductive potential of *H. bacteriophora* was significantly induced by interactive effects of the IJs concentrations and the host species ($F_{1, 76} = 147.95$, $P < 0.0001$). Slicing of the interaction effects between host species and nematode concentration by host for *H. bacteriophora* showed that the reproductive potential of this EPN per mg of the *Z. pyrina* and *G. mellonella* larvae was significantly influenced by both host species (*Z. pyrina*: $F_{1, 76} = 177.00$, $P < 0.0001$; *G. mellonella*: $F_{1, 76} = 15.19$, $P = 0.0002$).

The highest reproductive potential was observed with *H. bacteriophora* at 5 IJs larva^{-1} in the infected larvae of *Z. pyrina* and was 1164.6 ± 52.92 IJs per mg of larva (n = 20). The reproductive potential of *Acrobeloides* K29 at 20 IJs larva^{-1} in the infected larvae of *Z. pyrina* was 102.2 ± 12.76 IJs/mg larva (n = 20), relatively higher than *S. carpocapsae* at this rate (84.7 ± 15.74 IJs per mg *Z. pyrina* larvae). Overall, the results revealed that irrespective of hosts, the mean reproduction rate varied from 27.1 to 1164.6, and 84.7–853.8 per mg host for concentrations of 5, and 20 IJs larva^{-1}, respectively (Fig. 2).

3.3. Efficacy of selected nematodes against Z. pyrina larvae in semi-field trials

Semi-field experiment demonstrated that all three nematodes infected *Z. Pyrina* larvae inside branches of walnut seedlings. Comparison of the susceptibility of *Z. Pyrina* larvae to *Acrobeloides* K29, *S. carpocapsae* and *H. bacteriophora* showed that all three injected nematodes could reach, infect and cause > 50% mortality following exposure to concentration of 300 IJs cm^{-2} (Fig. 3). The results indicated that *S. carpocapsae* was the most virulent EPN to *Z. pyrina* larvae and caused maximum larval mortality of 62.5% and 73.3% when applied at the rate of 160 and 300 IJs cm^{-2}, respectively. However, at the concentration of 160 IJs cm^{-2}, the virulence of *H. bacteriophora* (43.7%) was higher than *Acrobeloides* K29 (35.5%) for *Z. pyrina* larvae, but after injection of 300 IJs cm^{-2}, *Acrobeloides* K29 was more virulent (66.7%). There were no significant differences in virulence of the nematodes for *Z. Pyrina* larvae infesting walnut seedlings after application of 160 IJs cm^{-2} (K-W: $\chi^2 = 2.125$; df = 2; $P = 0.34$) and 300 IJs cm^{-2} (K-W: $\chi^2 = 1.328$; df = 2; $P = 0.51$).

3.4. Efficacy of selected nematodes against Z. pyrina larvae in field trials

Field pathogenicity trials revealed similar results as the pot experiment and after 7 days of treatment with *Acrobeloides* K29, *S. carpocapsae* and *H. bacteriophora*, and the infected larvae of *Z. pyrina* were found inside the galleries of walnut trees in both consecutive years of

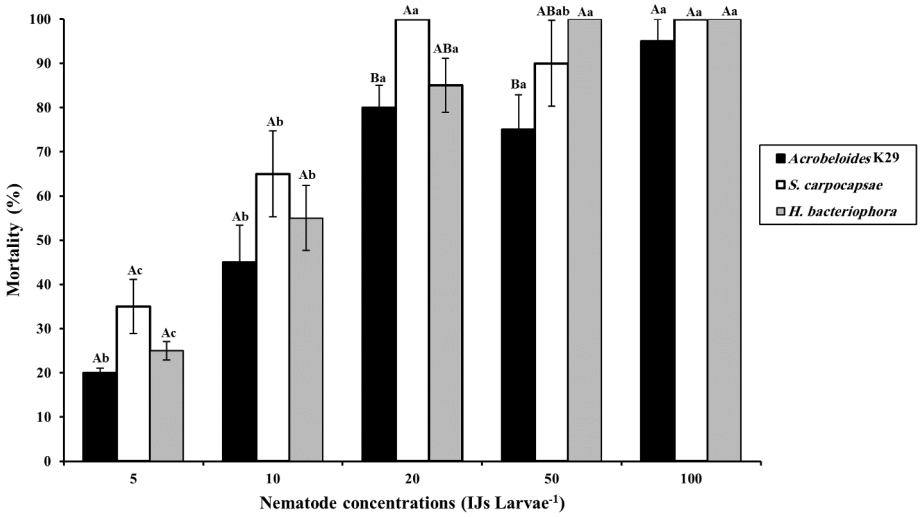

Fig. 1. Average percentage mortality of *Zeuzera pyrina* larvae after exposure to *Acrobeloides* K29, *Steinernema carpocapsae* and *Heterorhabditis bacteriophora* at the rate of 5, 10, 20, 50 and 100 infective juveniles (IJs) per larval concentrations. Different upper case letters above bars indicate statistical significance among different nematodes at same concentrations. Different lower case letters indicate statistical significance among different concentrations of each nematode populations ($P \leq 0.05$, Tukey's HSD test).

Table 1
The calculated LC_{50} and LC_{90} values (IJs $larva^{-1}$) for *Acrobeloide* K29, *Steinernema carpocapsae*, and *Heterorhabditis bacteriophora* on *Zeuzera pyrina* larvae in the laboratory bioassays.

EPN species	LC_{50} (95% CL)[a]	LC_{90} (95% CL)[a]	Intercept ± SE[a]	Slope ± SE[b]	χ^{2c} (df = 3)	P-value[d]
Acrobeloides K29	12.9 (7.9–18.7)	54.5 (34.7–129.7)	−2.28 ± 0.54	2.05 ± 0.41	0.74	0.86
S. carpocapsae	6.2 (2.9–9.3)	30.1 (19.4–75.8)	−1.48 ± 0.50	1.87 ± 0.44	0.40	0.94
H. bacteriophora	8.7 (6.3–11.3)	22.7 (16.5–43.3)	−2.89 ± 0.67	3.08 ± 0.64	0.35	0.95

[a] Concentration are expressed in IJs $larva^{-1}$; [b] SE, standard error; [c] Pearson χ^2 of the slope; [d] P-values represent the probability of the slope.

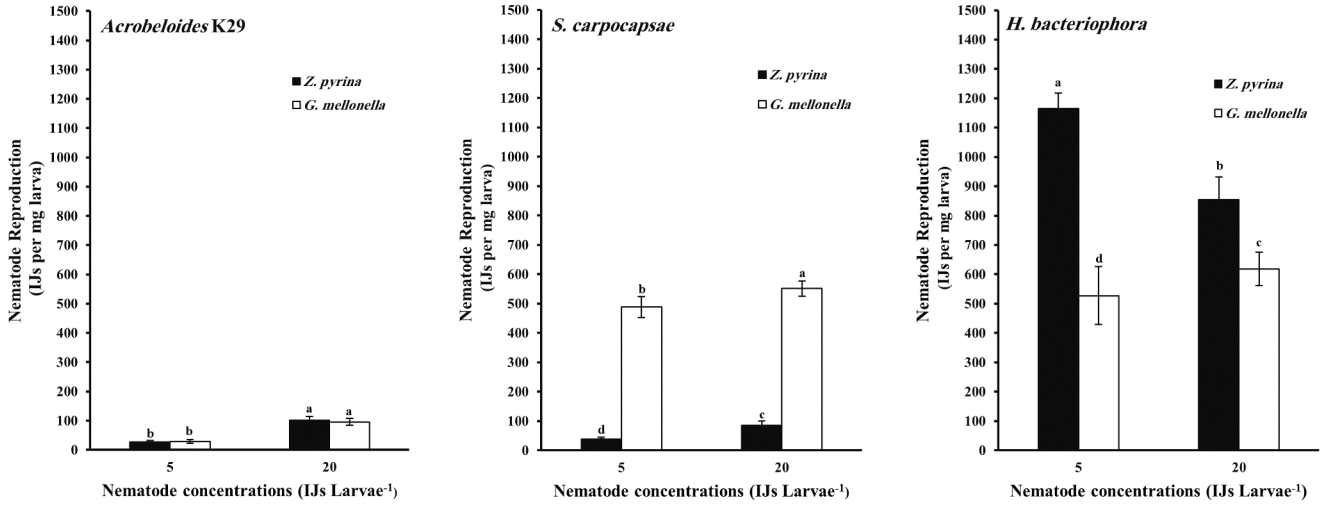

Fig. 2. Average reproduction (IJs per mg larva) of *Acrobeloides* K29, *Steinernema carpocapsae* and *Heterorhabditis bacteriophora* on *Zeuzera pyrina* and *Galleria mellonella* larvae in the laboratory. Different letters indicate statistical significance between different hosts and concentrations for the same nematode populations at $P \leq 0.05$ (two-way factorial ANOVA).

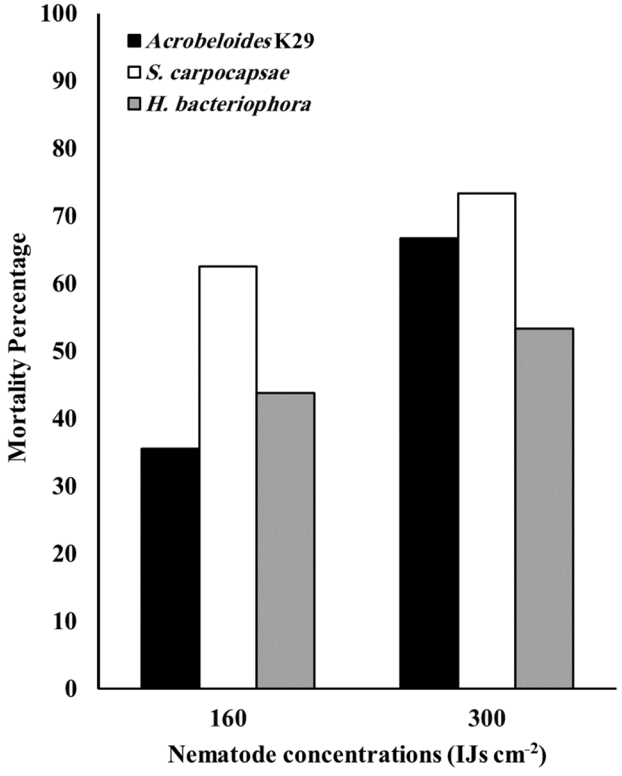

Fig. 3. Average percentage mortality of *Zeuzera pyrina* larvae after injection of *Acrobeloides* K29, *Steinernema carpocapsae* and *Heterorhabditis bacteriophora* at the rate of 160 and 300 IJs cm^{-2} in potted walnut seedlings (Non-parametric Kruskal-Wallis (K-W) test at $P \leq 0.05$).

experiment. In both 2015 and 2016 experiments, the analysis of covariance indicated that *Z. pyrina* larval mortality was significantly influenced by the total numbers of larvae inside the tunnels (In 2015: $F_{1, 116} = 44.81$, $P < 0.0001$; In 2016: $F_{1, 116} = 65.94$, $P < 0.0001$). All three nematode treatments had significant effects on larval mortality in 2016 (September trial) ($F_{2, 116} = 7.09$, $P = 0.0012$); however, there was no significant differences among nematode populations on larval mortality in 2015 (July trial) ($F_{2, 116} = 1.54$, $P = 0.2188$).

Comparison of the efficacy of the examined nematodes revealed that the highest mortality of 71.1% and 58.4% in larvae inside tree was achieved by injecting *S. carpocapsae* suspension in September and July trials, respectively; however, *S. carpocapsae* was more virulent in September than in July trial. September trial indicated that the virulence of *Acrobeloides* K29 was significantly higher than that of *H. bacteriophora* in *Z. pyrina* larvae inside the larval galleries in walnut trees ($p < 0.05$). At the concentration of 10,000 IJs/galleries, *Acrobeloides* K29 caused 56.8% larval mortality, while *H. bacteriophora* induced 37.1% mortality at 7 days post-application. However, when nematodes were applied in July, *H. bacteriophora* was more virulent than *Acrobeloides* K29 to *Z. pyrina* larvae with percent mortality of 50.2% and 41.5%, respectively, but their differences were not statistically significant ($p = 0.3759$) (Fig. 4).

4. Discussion

This investigation has shown that all three tested nematodes were virulent to *Z. pyrina* larvae and caused larval mortality under laboratory, semi-field and field conditions. Although, the susceptibility of *Z. pyrina* larvae to these biocontrol agents varied depending on nematode population and concentration as well as experimental condition.

The results of laboratory, semi-field and field trials confirmed that *S. carpocapsae* is the most efficacious entomopathogen in comparison to

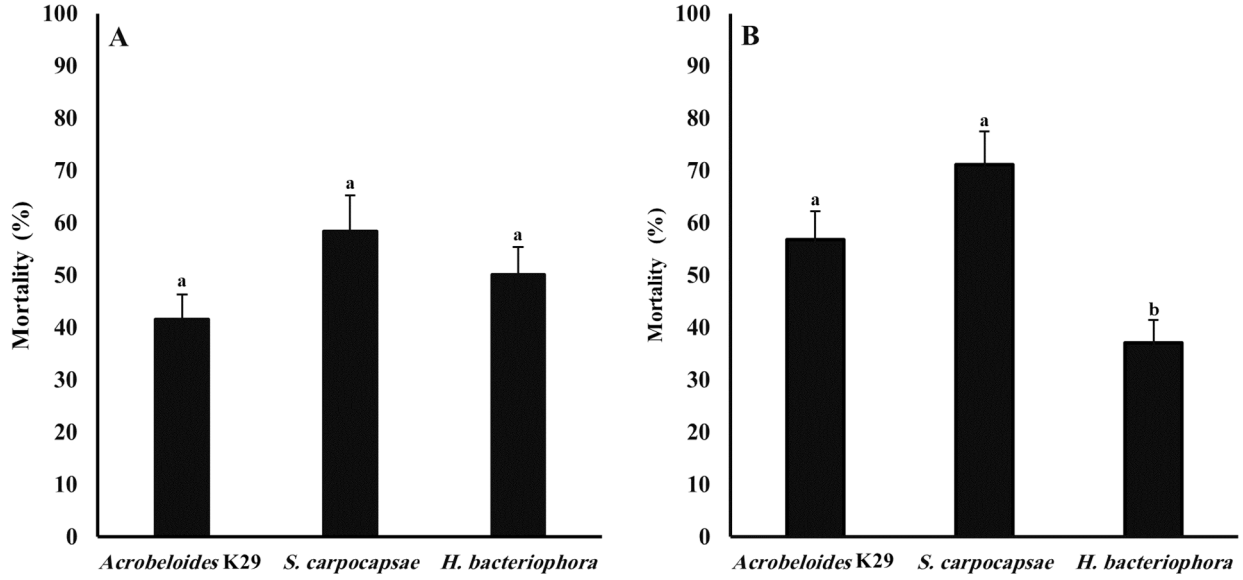

Fig. 4. Average percentage mortality of *Zeuzera pyrina* larvae after injection of *Acrobeloides* K29, *Steinernema carpocapsae* and *Heterorhabditis bacteriophora* at the final concentration of 10,000 IJs per galleries in a heavily infested walnut orchard in two consecutive years (A: July trial and B: September trial). Letters indicate statistical significance between different nematode populations at $P \leq 0.05$ (one-way ANCOVA).

H. bacteriophora and *Acrobeloides* K29. However, a wide variation was observed in its infectivity against *Z. pyrina* larvae at various concentrations, causing up to 100% larval mortality in the laboratory bioassay and about 58–73% mortality under semi-field and field conditions. The comparatively high virulence of the *S. carpocapsae* tested against *Z. pyrina* larvae in this investigation is in agreement with the results of some studies reporting 100% (El-Kholy et al., 2014), 63–100% (Ashtari et al., 2011) and 31–88% (Abdel-Kawy et al., 1992) mortality in *Z. pyrina* caused by *S. carpocapsae*, under laboratory or field experimental conditions. However, other studies have reported that orchard application of Steinernematids caused lower levels of virulence than the Heterorhabditids against *Z. pyrina* larvae (Shamseldean, 2000; El-Ashry et al., 2018). Similar results of better performance of *Steinernema* species compared with *Heterorhabditis* species have been reported in the case of other cryptic and wood borer pests including *Cydia pomonella* (Odendaal et al., 2016), *Rhagium bifasciatum* (Harvey et al., 2012), and *Synanthedon pictipes* (Cottrell et al., 2011) in laboratory and field trials.

Our study provides the first empirical evidence of the infectivity of *Acrobeloides* K29 as an insect-killing *Acrobeloides* nematode in Iran. Of significance, this indigenous free-living nematode has the ability to find, invade, rapidly kill and successfully develop and reproduce inside *Z. pyrina* larvae. It causes mortality from high to moderate level in *Z. pyrina* larvae in laboratory, semi-field and field experiments. This well adapted nematode to walnut orchard conditions shows promising potential for the management of this cossid pest inside larval galleries. Although the indigenous *Acrobeloides* K29 displayed great infectivity against healthy live hosts and were able to reinfect new hosts with high potency, it may be too early to consider this native free-living nematode as an entompathogen. Regarding the suggested criteria to differentiate the border line among pathogenic and non-pathogenic nematodes (Dillman et al., 2012; Zhang et al., 2019), *Acrobeloides* K29 should be characterized as an insect-parasitic nematode. However, its remarkable infectivity under different conditions, acceptable reproduction, low LC_{50} value, and also its foraging ability (unpublished data) make the nematode a potential biological control agent against insect pests.

Reproductive potential of EPNs is one of the most influential life characteristics in assessing their performance and justification of their development and commercialization as biocontrol agents (Glazer et al., 2001; Campos-Herrera et al., 2008; Susurluk and Ehlers, 2008; Salame et al., 2010). In our earlier study, it has been reported that *S. carpocapsae* and *H. bacteriophora* were able to invade and penetrate into the larvae of *Z. pyrina* and the greater penetration rate was recorded for *S. carpocapsae* (Salari et al., 2015). There has been no report of the ability of these tested nematodes to reproduce in *Z. pyrina* larvae. In the present study, it has been reported that *Z. pyrina* larvae were highly suitable host for reproduction of these biocontrol agents and that all three tested nematodes successfully recovered from pest's cadavers.

In comparison of the reproduction potential of the nematodes in *Z. pyrina* and *G. mellonella* larvae, it has been observed that substantial variations in the numbers of produced offspring. The reproduction rate of *H. bacteriophora* were greater than that of *S. carpocapsae* and *Acrobeloides* K29 at both low and high concentrations. These differences may be attributed to the fact that Heterorhabditids are hermaphroditic and produce more offspring than amphimictic Steinernematids (Mannion and Jansson, 1992; Yadav and Lalramliana, 2012). Our results are in accordance with the findings of other studies that reported *H. bacteriophora* produces more IJs than other *Steinernema* species per cadaver of infected larvae of *Dorcadion pseudopreissi* (Susurluk et al., 2009), *Helicoverpa armigera* (Jothi and Mehta, 2006), and *Maladera matrida* (Bhatnagar et al., 2004). However, this is not a general rule, as it was found that the number of IJs emerged from *R. bifasciatum* larvae infected with *H. downesi* was lower than that of *S. carpocapsae* (Harvey et al., 2012). It was also observed that although *H. bacteriophora* reproduced more offspring than *S. carpocapsae* in sixth-instar larvae of *Agrotis ipsilon*, the number of *S. carpocapsae* IJs emerged from fifth-instar larvae was higher than that of *H. bacteriophora* and *H. megidis* (Ebssa and Koppenhöfer, 2012). These discrepancies could be due to differences in nematode species and their concentrations, as well as differences in insect hosts and their life stages (Selvan et al., 1993; Ehlers, 2001). In this study, it was observed that *Acrobeloides* K29 indicated comparable reproduction to *S. carpocapsae* on *Z. pyrina* larvae. Therefore, the relatively high reproductive potential of *H. bacteriophora*, *S. carpocapsae* and *Acrobeloides* K29 in *Z. pyrina* larvae observed in our study reaffirms their potentials as biocontrol agents.

Semi–field experiments using potted walnut seedlings and field experiments in both consecutive years in heavily infested walnut orchards revealed the ability of tested nematodes to locate *Z. pyrina* larvae in their feeding galleries inside walnut branches and cause adequate mortality. Though, in the September trial it was observed that *S. carpocapsae* and *Acrobeloides* K29 were more effective against *Z. pyrina* larvae than in

July trial, but *H. bacteriophora* provided weaker control effects. Differences in several factors, including temperature and moisture inside feeding galleries of infested branches, host size, and host-seeking ability of these biocontrol agents may be responsible for these differences. During the dissection of marked branches, it was observed that sufficient moisture in almost all active galleries in both July and September trials. Thus, it is conceivable that lower maximum daily temperature (26.9 °C), and greater size of *Z. pyrina* larvae inside the galleries provide optimal conditions for *S. carpocapsae* and *Acrobeloides* K29 to be more effective in the September trial.

Our finding is in agreement with the results of several studies indicating that unlike *Steinernema* species, *H. bacteriophora* was more virulent at higher temperatures (Batalla-Carrera et al., 2013; Odendaal et al., 2016; El-Ashry et al., 2018). For example, it was stated that spring application of *S. carpocapsae* to control *Z. pyrina* in apple trees was more effective than autumn application due to higher temperature (El-Ashry et al., 2018). Studies have also shown that host size influences susceptibility of the majority of Lepidoptera and Coleoptera species to EPNs (Nielsen and Philipsen, 2004; Cottrell et al., 2011; Hodson et al., 2011; Khatri-Chhetri et al., 2011). In our previous investigation, it had been noticed that larger *Z. pyrina* larvae were more susceptible to *S. carpocapsae* (Salari et al., 2015). These findings support our hypothesis that host body size is an influential factor for better performance of *S. carpocapsae* and *Acrobeloides* K29 in September trial, among other factors.

It is also noteworthy that in our previous study it had been noticed that host-seeking ability of *H. bacteriophora* for different host cues of *Z. pyrina* was more directional and strongly higher than that of *S. carpocapsae* in Petri dishes containing agar (Salari et al., 2015). Based on these findings, it was expected to observe similar pattern in field experiments but surprisingly, it was noticed that *S. carpocapsae* showed higher efficacy against *Z. pyrina* inside the larval tunnels in comparison to other nematodes in both September and July trials.

This discrepancy may be explained by the habitat specialist hypothesis which states that nematodes such as ambusher *S. carpocapsae* with a sit and wait foraging strategy are able to adapt to habitats and use a cruise foraging strategy under certain circumstances (e.g., inside cryptic woody environments in nature) (Wilson et al., 2012). Similar studies have also indicated that field application of *S. carpocapsae* provided good control of the peachtree borer, *Synanthedon exitiosa*, a major cryptic living insect pest of peach orchards in the southeastern United States (Shapiro-Ilan et al., 2015, 2016). Thus, effective performance of *S. carpocapsae* against the cryptic living insect pests could be due to its ability as an ambusher to adjust foraging behavior as either “ambush” or “cruise” forager depending upon the environmental conditions and host habitats (Griffin, 2015; Wilson et al., 2012).

5. Conclusions

Our study highlights the ability of all three examined nematodes to search, find, recycle and effectively infect *Z. pyrina* larvae in laboratory, semi-field and field conditions, with *S. carpocapsae* showing greater efficacy. It is worth noting that application of *S. carpocapsae* during moderate or cool temperatures provides better control of host inside larval tunnels. Results of the semi-field and field trials indicate that total mortality of pests inside larval galleries declines in comparison to laboratory mortality rates. Nevertheless, these results hold promise and justify extended research on combined applications of these bioagents individually or in combination with chemical insecticides within the framework of integrated pest management systems. This is the first report of the biocontrol potential of the indigenous *Acrobeloides* K29. In addition to causing substantial infection of host inside larval tunnels, *Acrobeloides* K29 showed adaptation potential to *Z. pyrina* larval habitat in walnut orchards. Additional studies on the presence and features of a potential mutualistic bacterium associated with *Acrobeloides* K29 may warrant further insight to support the potential usefulness of this indigenous free-living nematode in biological control and pest management programs.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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