Cent. Eur. J. Biol. • 7(1) • 2012 • 77-82
DOI: 10.2478/s11535-011-0090-0

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Central European Journal of **Biology**

Effectiveness of the entomopathogenic nematode *Steinernema feltiae* in agar gel formulations against larvae of the Colarado potato beetle, *Leptinotarsa decemlineata* (Say.) (Coleoptera: Chrysomelidae)

Research Article

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Received 31 May 2011; Accepted 26 September 2011

Abstract: The entomopathogenic nematode *Steinernema feltiae* strain Ustinov Russia was used on potato foliage to control larvae of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say.) (Coleoptera: Chrysomelidae). Nematodes were applied in formulations of agar at 4%, 2%, 1% and 0.5% concentrations and compared to a control application of nematodes in water for nematode survival. Agar formulation significantly improved efficacy by increasing nematode survival through reduction in desiccation when compared to water-based formulation. More than 70% of infective juvenile nematodes (IJs) died after being incubated in the highest concentration of agar for 12 h, while only 8% mortality was recorded at the 1% concentration. Suspension of nematodes in 1% agar gel was shown to be efficacious in both laboratory and greenhouse tests for extension of the nematodes' survival. Agar formulation droplets dried slower than control droplets by 127.8 min. *Leptinotarsa decemlineata* mortality significantly increased when insects were exposed to infective juvenile nematodes for four hours after application. In conclusion, the agar formulation enhanced nematode survival by providing a suitable environment thereby delaying dryingand increasing the possibility for nematodes to invade their host on the foliage.

Keywords: *Anti-desiccant • Foliar application • Drought • Foliar treatments • Extended survival time*

1. Introduction

Colorado potato beetles, *Leptinotarsa decemlineata,* are a serious pest in most areas of the United States. Although the potato is the beetles preferred food, it will also feed on tomato, eggplant, tobacco, pepper, ground cherry, petunia, and even cabbage crops. It also attacks a number of common weeds including jimson weed, henbane, horse nettle, belladonna, thistle and mullein [1]. In Europe, *L. decemlineata* is prevalent in all potato-growing areas except the United Kingdom and Ireland. In warm and dry regions, it regularly occurs in high abundances [2] and can develop through two complete generations per year. Without control measures, the beetle causes severe reductions in tuber size affecting both tuber yield and quality.

For the use of the entomopathogenic nematode, *Steinernema carpocapsae,* on cabbage foliage to control larvae of the diamondback moth, *Plutella xylostella*, a formulation containing 0.3% of the surfactant Rimulgan and 0.3% of the polymer xanthan was tested. Leaf bioassays were used to compare the efficacy of the formulation to nematodes applied in water. Compared to water, the surfactant–polymer formulation reduced *P. xylostella* larval mobility and at the same time improved conditions for nematode invasion [3].

The impacts of adjuvant on sedimentation of dauer juveniles in the spraying suspension, runoff from the leaf surface and host invasion were demonstrated by Schroer *et al.* and Piggott *et al.* [4,5]. All these investigations were conducted in the artificial setup of leaf disc assays that do not reflect conditions in the field.

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Enhancing nematode survival through the use of cross-linked polyacrylamide and the benefits of anti-desiccants combined with surfactant–polymer formulation was studied by Piggott *et al*. [6]. Anti-desiccants are polymers with water swelling capacity and thus provide nematodes with moisture for an extended period. All adjuvants significantly increased the efficacy of nematodes against *P. xylostella* on leaves and the differences in efficacy between the adjuvants were not significant. These experiments were conducted with *P. xylostella*, a small lepidopteran but conditions differ when the control of larger insects with nematodes is attempted. Navon *et al.* developed an edible alginate-nematode-gel formulation for the control of *Spodoptera littoralis* (Boisduval) and *Helicoverpa armigera* (Hübner) [7]. The invasion process of different nematode species into various insect pests can differ [8,9] where it needs support to enable their mobility. When passive uptake is the main form of entrance into the host, then prolonging survival of the nematodes becomes a priority. Dauer juveniles can be entrapped in a formulation that prevents them from emigrating and preserves their energy resources [7,10]. Improved formulation technology can enable the integration of entomopathogenic nematodes into a biological control strategy for the control of foliage-feeding insects.

The aims of this study were to determine the activity, viability and the percentage mortality of the entomopathogeic nematode, *Steinernema feltiae,* in agar formulations at different time intervals. Followed by, the application of *S. feltiae* formulations on potato plants for control of *L. decemlineata* larvae in laboratory and greenhouse tests.

2. Experimental Procedures

2.1 Insects rearing

Colorado potato beetles, *L. decemlineata,* were collected from potato fields in the vicinity of České Budějovice, Czech Republic. The colony was reared through many generations on the native potato cultivars Desireé or Superior in a greenhouse at a constant temperature 24±2°C and on a light:dark photoperiod of 16:8 h.

2.2 Potato plants

Potato cultivars Desirée and Superior were propagated *in vitro* (meristem clones) at the Potato Research Institute, Havlíčkův Brod (Czech Republic).

Young plants were transferred to soil in pots and grown in greenhouses at 24±2°C with a photoperiod of 16:8 h (light:dark). For laboratory tests, leaves were cut from 1-2 months old plants, or when the plants had 6-8 true leaves, and their stems were wrapped in moist absorbent cotton paper and aluminum foil to reduce desiccation.

2.3 Nematode culture

Experiments were performed using a strain of *S. feltiae* that was isolated from soil sample near Ustinov, Russia and then reared for many generations in the laboratory using last instar caterpillars of the wax moth, *Galleria mellonella,* as a host. The emerging infective juvenile nematodes (IJs) were harvested from White traps and stored in sterilized distilled water at 10°C [11] for 10–21 days before use in experiments. Their viability was verified under a stereoscopic microscope (Arsenal, SZP 1102 ZOOM) by counting all moving, all curved, all moving after a mechanical stimulation and all dead nematodes.

2.4 Testing viability of IJs in formulations of different agar concentration

Four concentrations of agar formulation (Agar powder; Natural Co., Jihlava, Czech Republic) prepared at 40, 20, 10 and 5 g/L (*i.e.* 4%, 2%, 1% and 0.5%) were used to test the effectiveness and viability of IJs. Three replicates of each concentration were made by placing 20 ml of agar formulation at 25°C into a glass Petri dish (9 cm, diameter). To each dish, 100 IJs were added. Nematode states (dead or living) were recorded after incubation for 3, 6, 9 and 12 h. Infective juvenile nematodes were divided into three classifications: active nematodes, which were moving; curved nematodes, whose shape indicated that it were still living but not moving which would then start moving when stimulated mechanically and dead nematodes, which would not move even when stimulated mechanically.

2.5 Testing anti-desiccants to prolong IJs survival

Five droplets of agar at each concentration (4%, 2%, 1% and 0.5%) and the control were put on each leaf. The mean weight of a droplet was 0.0104±0.002 g. Five leaves were used for each concentration and the control. The leaves were incubated for 0, 2, 4 or 6 hours until all dry. Tests were conducted at 60-80% RH and 25°C. The experiment was repeated three times.

2.6 Percentage of invading nematodes

2.6.1 Laboratory test

Twenty potato leaves were sprayed with: 5 mL formulation containing 250 IJs in Petri dishes (9 cm, diameter). Three concentrations of agar gel (2%, 1% and 0.5%) were tested additional to the control (nematodes in water). One last instar larva of *L. decemlineata* was added to each Petri dish. The number of dead larvae was counted daily until the surviving larvae pupated.

Dead larvae were rinsed with tap water, transferred to clean Petri dishes and kept in moist conditions for an additional 3–4 days, to obtain the 1st adult nematode generation, and then dissected individually in distilled water. The number of IJs that successfully invaded the larvae and developed to adult inside each cadaver was recorded by dissection [12], using a stereoscopic microscope (Arsenal model SZP 1102 ZOOM). The numbers of male and female nematodes in the larval cadavers were counted separately. The experiment was repeated three times

2.6.2 Greenhouse test

In this test, five pots, each with three potato plants, were used to test nematode efficacy at each agar concentration (2%, 1% and 0.5%) and for the control (nematodes in water) . Potato plants were at the same stage as those used in the laboratory tests. Whole plants in each pot were fully sprayed with the agar formulations containing IJs (2500 IJs/50 ml/pot). Potato leaves were sprayed from above at a distance of 55 cm using a Teejet (TP8003E) flat-fan nozzle sprayer at a flow rate of 0.96 l min^{-1} at 20^5 Pa and 15 µl cm^{-2}. Control plants were sprayed with water. Twenty last instar *L. decemlineata* larvae were added to each pot. Twenty four hours after exposure to the treated plants, the larvae were transferred to clean Petri dishes (15 cm, diameter) to feed on control potato leaves. Mortality in *L. decemlineata* larvae, number of IJs successfully invading each cadaver, and time to death were recorded for each treatment.

2.7 Statistical analysis

Results are expressed as mean ± SE. GraphPad Prism 5 (GraphPad Software Inc.) was used to present the descriptive statistics. Statistical analysis was done using two-way ANOVA and Tukey's honest significant difference test (HSD) of ANOVA (CSS StatSoft, Tulsa, OK, USA).

3. Results

3.1 Effect on viability

No significant differences were recorded in the viability of nematodes among the different treatment concentrations three hours post-application (Figure 1; P=0.1320, F=1.560, df=8). Six hours after application, significant differences between concentrations (4%, 2%, 1%, 0.5% and water as control) were observed (Figure 2). The viability of nematodes in a 4% agar formulation was significantly less than the control (P<0.0001, F=9.952, df=8).

By increasing the amount of time that the nematodes remained in the different agar formulations, the variation in nematode activity increased between the control and other concentrations. Nine hours after application (Figure 3), the viability of nematodes in a 4 and 2% was significantly less than the control (P<0.0001, F=6.930,

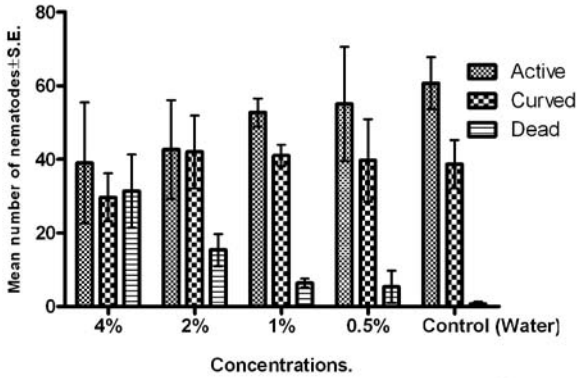

Figure 1. Viability of 100 IJs nematodes divided into three states (active, curved and dead) in different concentrations of agar gel, after 3 hours of treatment. Not significant (P=0.1320, F=1.560, Df 8).

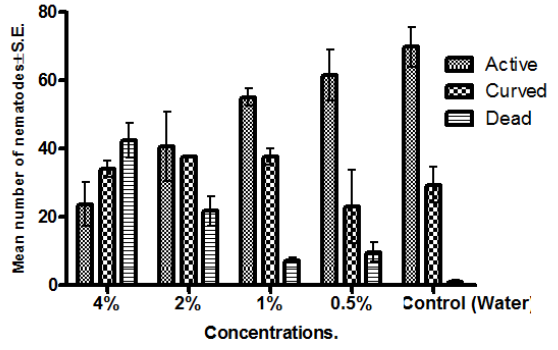

Figure 2. Viability of 100 IJs nematodes divided into three states (active, curved and dead) in different concentrations of agar gel, after 6 hours of treatment. Highly significant (P<0.0001, F=9.592, Df 8).

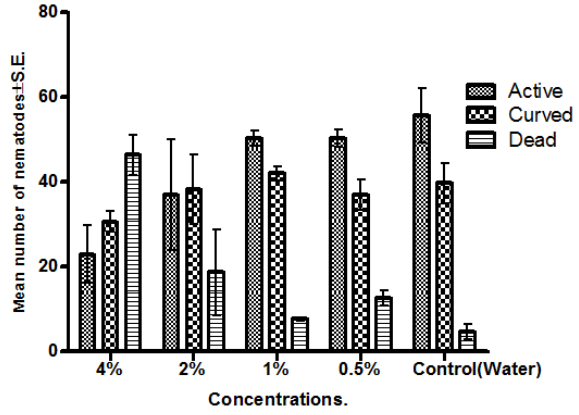

Figure 3. Viability of 100 IJs nematodes divided to three state (active, curved and dead) in different concentrations of agar gel , after 9 hours of treatment. Highly significant (P<0.0001, F=6,930, Df 8).

df=8). Twelve hours after application, the nematode mortality inside the agar formulation increased in the 4% concentration to more than 70%, and this high percentage may be related to the highly gelatinous nature of the 4% suspension in comparison with other concentrations and the control (Figure 4).

Figure 5 shows that no significant differences were recorded between the control and the 1% concentration at 12 h after application in terms of the activity, viability and percentage of nematode mortality (P=0.0708, F=3.284, df=2).

3.2 Time needed to dry droplets

Droplets of formulation with high concentrations of agar, *i.e.* 4% , 2% and 1%, dried significantly slower when compared to the control (water; P<0.001). Droplets of the highest concentration (4%) of agar formulation took 2.36 h to dry compared to 2.21, 2.13 and 1.44 h for concentrations 2%, 1% and 0.5%, respectively (control, 1.24 h). The lowest concentration (0.5%) showed a borderline significant difference from the control (P<0.05). Clear significant differences between the agar concentrations of 4% and 2% and the control were recorded in terms of the time needed for the drops to dry. The 1% concentration was more suitable for application as compared to the higher ones 4% and 2% concentrations because it was not highly gelatinous during spraying and the time needed to dry droplets was (127.8 min.) but (53.4 min.) for control (Figure 6).

3.3 Number of invading nematodes

3.3.1 Laboratory tests

In the laboratory test, the count of adult nematodes inside cadavers of *L. decemlineata* for each concentration showed that the suspension of 1% agar concentration recorded the highest invasion at 52.35±28.38 (mean ±SE) where the mean number of females (42.6) was generally higher than that of males (29.4). The number of adult nematodes that invaded with agar formulation concentrations 2%, 0.5% and control, were 35.35±3.72 (28.8 female and 18.2 male), 32.80±3.79 (23.4 female and 14.6 male) and 35.25±4.01 (24.6 females and 14.2 males), respectively (Figure 7). Highly significant differences in invasion were recorded between the 1% concentration and the concentrations of 2%, 0.5% and control (P<0.01, F=3.885).

3.3.2 Greenhouse tests

The same trend of results were obtained in greenhouse experiments as with the laboratory tests, the number of adult nematodes inside cadavers was higher for the 1% concentration (77.28±31.18) as compared to the other concentrations 2%, 0.5% and the control (43.67±3.72, 38.24±6.67 and 42.44±8.48, respectively). Tukey's multiple comparison test showed highly significant differences in invasion were recorded between the 1% concentration and the 2%, 0.5% and control P<0.0001, F=118.4. (Figure 8)

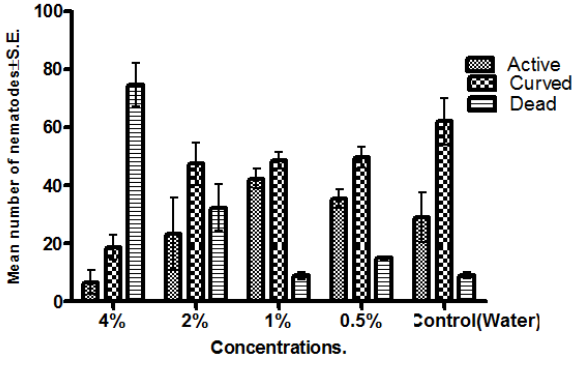

Figure 4. Viability of 100 IJs nematodes divided into three states (active, curved and dead) in different concentrations of agar gel , after 12 hours of treatment. Highly significant (P<0.0001, F=16.59, Df 8).

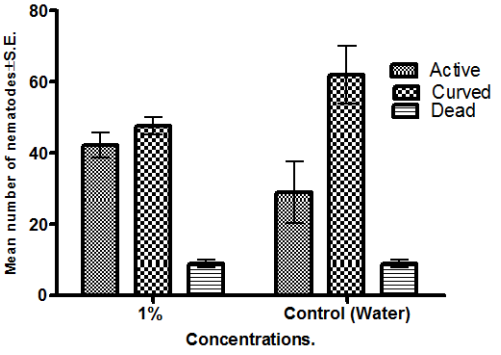

Figure 5. Viability of 100 IJs nematodes divided to three state (active, curved and dead) in 1% concentration of agar gel comparing to control, after 12 hours of treatment. Not significant (P=0,0708, F=3.284, Df 2).

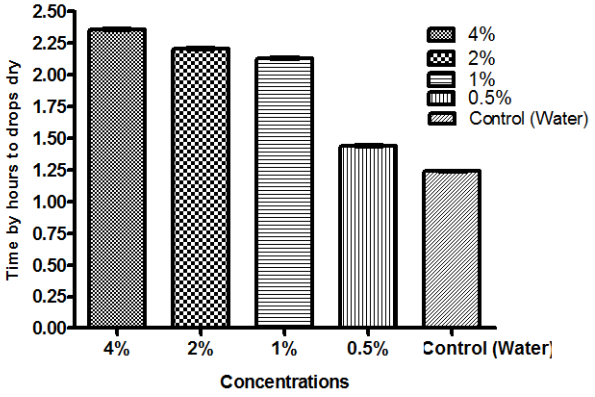

Figure 6. Time required to desiccate the droplets gel suspension sprayed on the leaves of each concentration. Tukey's multiple comparison test showed highly significant differences between concentrations 4%, 2% and 1% with control (water) P<0.001, while it was marginally significant with concentration 0.5%,P < 0.05.

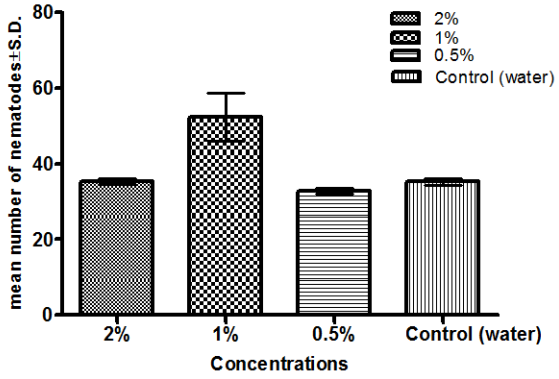

Figure 7. Mean number of adult nematodes inside each cadaver ±S.D. under laboratory conditions. Tukey's multiple comparison test showed significant differences between concentration 1% and 2%, 0.5% and control (water) ($P<0.01$, $F=3.885$.

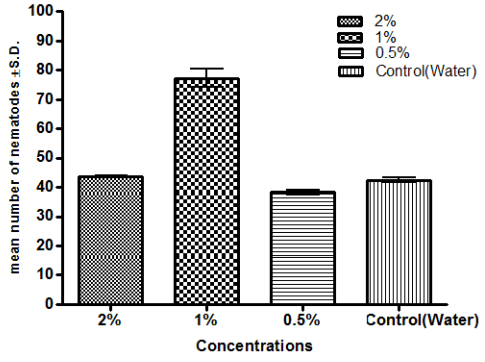

Figure 8. Mean number of adult nematodes inside each cadaver ±S.D. under greenhouse conditions Tukey's multiple comparison test showed highly significant differences between concentration 1% and 2%, 0.5% and control (water) ($P<0.0001$, $F=118.4$).

4. Discussion

Due to desiccation caused by the warm weather conditions in summer, the strategy for controlling foliage-feeding insects by entomopathogenic nematodes needs to be improved. For example Shapiro-Ilan *et al.* [13] stated that „the efficacy of aboveground applications using entomopathogenic nematodes can be limited due to harmful effects of ultraviolet radiation and desiccation". Shapiro-Ilan *et al.* [13] and Lacey and Shapiro-Ilan [14] concluded that improvements in formulations or application techniques may enhance the efficacy of above ground applications with entomopathogenic nematodes. The use of anti-desiccants or other adjuvants has been reported to provide improved above ground control of various foliar pests including the diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), the sweet potato whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae), and egyptian cotton leafworm, *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) [3,15-17]. In the present study, adding agar to a water suspension of *S. feltiae* prolonged the persistence of nematodes and increased the chance of IJs nematode attack on hosts and invasion of the target insect on foliage crops. Our findings are consistent with those of Piggot *et al.* [6] who studied the benefit of anti-desiccants combined with a surfactant-polymer formulation. Anti-desiccants are polymers with water swelling capacity and thus provide moisture for a longer period. Shapiro-Ilan *et al.* [18] concluded that nematode treatments followed by application of a sprayable gel such as Barricade can enhance control of the lesser peachtree borer, *Synanthedon pictipes* and possibly other above ground pests as well.

Our results indicate that adding agar to the water suspension of *S. feltiae* gave a better result at a 1% concentration compared to the other agar concentrations. This 1% concentration was more suitable for maintaining nematode viability and promoting invasion of the target insect on plant foliage. The highest concentrations, *i.e.* 4% and 2%, were more condense during spraying.

Schroer and Ehlers [3] stated that host penetration on the leaf occurs within the first hour after application. Our results indicate that prolonging the time that the droplets of the agar formulation remain moist relative to the water control, *i.e.* by 127.8 min in the 1% concentration, can increase the possibility for successful control of the Colorado Potato beetle by foliage application of entomopathogenic nematodes. Especially since the insect regularly occurs in high abundances in warm and dry regions [2] where it can develop two complete generations per year.

Acknowledgements

We are grateful to Dr. Zdeněk Mráček and to Mrs. Lenka Kropáčková at the entomopathogenic Nematodes Laboratory, as well as to the Laboratory of Experimental Ecology for providing us the entomopathogenic nematode strain. All the facilities for the completion of this work have been at the Biology Centre, Institute of Entomology, under Project Grant No. Z50070508 of the Academy of Sciences of the Czech Republic.

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