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Effect of Storage Temperature and Duration on Survival and Infectivity of *Steinernema innovationi* (Rhabditida: Steinernematidae)

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Abstract: Entomopathogenic nematode species differ in their optimum storage temperature; therefore, we conducted a study on the survival and infectivity of the recently described *Steinernema innovationi* from South Africa at five storage temperatures (5°C, 10°C, 15°C, 20°C, and 25°C) over 84 d using 20,000 infective juveniles (IJ) in 25 ml aqueous suspension containing 0.1% formalin. Our results showed that survival was highest and most stable at 15°C, ranging from 84% to 88% after 84 d. Infectivity of IJ against *Galleria mellonella* larvae was >90% for all temperatures except for 5°C at which survival decreased to 10% after 84 d. In addition, we stored 2.5 million IJ on a sponge formulation in 15 ml of 0.1% formalin solution for 84 d at the optimum 15°C followed by 2 wk storage at 25°C. Storage of the IJ on a sponge formulation for 14 d at 25°C post 15°C storage for 84 d did not have a detrimental effect on IJ survival (87%) or infectivity to *G. mellonella* (95%).

Key words: entomopathogenic nematode, infective juvenile, infectivity, optimum temperature, sponge formulation, survival.

Entomopathogenic nematodes (EPN) belonging to the genera *Steinernema* and *Heterorhabditis* have been successfully used over decades as biocontrol agents for the management of key agricultural pests. This is because of their ease of culture, high virulence, and environmental compatibility (Grewal, 2002). The IJ is the nonfeeding stage and relies solely on energy reserves for survival and infectivity and is the stage used as a biopesticide. Therefore, minimizing IJ mortality during storage prolongs shelf life and enables easier transportation of the product prior to application. However, their short shelf life at room temperature is a major factor limiting EPN from reaching their full potential as biopesticides (Grewal, 2002). At elevated temperatures, nematode physiological activity is high, increasing the consumption of stored energy, and resulting in limited shelf life (Andalo et al., 2011).

Methods of energy conservation for IJ include dehydration or desiccation (Georgis et al., 1995; Strauch et al., 2000; Divya et al., 2011; Hussein and Abdel-Aty, 2012) and maintenance at low temperatures to improve EPN-based biopesticide shelf life (Westerman, 1992; Patel et al., 1997; Patel and Wright, 1997; Strauch et al., 2000; Goud et al., 2010; Andalo et al., 2011; Gulcu and Hazir, 2012). However, dehydration presents many challenges including poor nematode survival and difficulty in application because the carriers can block spray nozzles (San-Blas, 2013). Thus, low temperature storage is the most common and important factor affecting nematode survival in formulations, but even at low temperatures, EPN species differ distinctly in terms of the optimum storage temperature (Strauch et al., 2000; Goud et al., 2010; Andalo et al., 2011; Mejia-Torres et al., 2013). For these reasons research focus has recently moved toward improving liquid formulations by using polymer gels for postapplication survival (Navon et al., 2002; Andalo et al., 2010; Hussein and Abdel-Aty, 2012; De Waal et al., 2013). In the history of EPN research, product formulation has been the area with the least publications (about 20% of publications, including quality control, in the period from 1980 to 2010). Furthermore, information about product formulations is usually kept as a trade secret by commercial companies. A fairly simple method of storage or formulation is to concentrate nematodes to form a slurry, which is then absorbed into a polyurethane sponge (Bedding, 1984). Besides these main approaches, a variety of carriers such as charcoal, vermiculite, clay, dispersible granules, and capsules have been used to formulate nematode products (Deol et al., 2011; Hiltpold and Turlings, 2012).

Many new species of EPN are being described, but characterization of these new species for biocontrol purposes is lagging (Lacey et al., 2015). Thus, it is critical to characterize the various biological aspects of new species so that we can determine their potential for commercialization. The objectives of our study were to (i) determine the influence of storage temperatures on survival and infectivity of an indigenous species, *Steinernema innovationi* (Cimen et al., 2014) originally isolated from a soil sample collected near the town of Fouriesburg, Free State, South Africa (Hatting et al., 2009) and (ii) test a concentrated sponge formulation for IJ survival and infectivity following storage at the predetermined optimum temperature.

Materials and Methods

Rearing of experimental insects: Galleria mellonella, Linnaeus (Lepidoptera: Pyralidae) were produced using an artificial medium containing 22% ground wheat, 22% ground maize, 11% honey, 11% glycerol, 11% milk powder, 5.5% yeast extract, and 17.5% bee wax in a glass

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jar at 25°C ± 4°C in the laboratory (Han and Ehlers, 2000).

Musca domestica Linnaeus (Diptera: Muscidae) were cultured as follows: pupae of *M. domestica* in moist bran were supplied by Bio-Insectaries South Africa (BISA) (Pty) Ltd (www.bioinsectsa.com) and reared at ARC-SGI. A container with pupae in wet bran was placed without a lid in a cage with a mesh small enough to retain adults while allowing adequate ventilation. On adult emergence, flies were provided with Nespray milk powder (Nestlé, South Africa), *ad libitum*. In a separate container, adults were provided with water absorbed in cotton wool. From Day 6, about 100 g of wet bran was placed in the cage for adult females to lay eggs. Eggs were collected by removing the bran container and mixing bran-containing eggs with larval diet (2,000 g bran, 300 g Nespray milk powder, 6 g sodium benzoate, 20 g brewer's yeast, 3,000 ml lukewarm water) (Klunker and Kiesow, 1980). Eggs hatched in less than 24 hr and final instar were collected and frozen until used or left to pupate and progress to the next production cycle. This colony was reared at temperatures between 20°C and 28°C, under natural light conditions.

Optimum storage temperature: Steinernema innovationi IJ were produced in vivo according to the method described by Kaya and Stock (1997). The nematodes were cultured in the final instar of the greater wax moth. Nematodes were used immediately after harvesting.

To determine optimal storage temperature, 25 ml of IJ in 0.1% formalin solution were stored in 550-ml flat-angled tissue culture flasks (Lasec SA, www.lasecsa.co.za) at a concentration of 20,000 IJ per flask (Strauch et al., 2000). Three flasks were prepared for each test temperature (5°C, 10°C, 15°C, 20°C, and 25°C) and stored for a period of 84 d in total darkness. For evaluation, three samples of 120 µl each were pipetted into a 55-mm glass petri dish with 5-mm^2 grids drawn on the base with a diamond pen (Lasec SA, www.lasecsa.co.za). Sterile water was then added to the dish to cover the base to a depth of 2 mm. Survival was determined by counting, at random, live and dead IJ to a total of 100 IJ using a dissection microscope at 1.5 ×10 magnification (Nikon SMZ800). Immobile IJ were probed with a blunt needle to confirm their state. Survival and infectivity was assessed every 28 d for the duration of the experiment.

Infectivity tests: To determine the infectivity (as a function of live IJ) of the IJ, 600 µl (10 × 60 µl) of IJ suspension was sampled per flask every 28 d for a period of 84 d. The infectivity of IJ was assessed by covering the bottom of wells of a 24 well CELLSTAR culture plate (Lasec SA, www.lasecsa.co.za) with 0.5 g sterile loam sand. The wells were each inoculated with 50 live IJ suspended in 60 µl of 0.1% formalin solution (Hazir et al., 2001). Controls were inoculated with 60 µl of 0.1% formalin solution only. One final instar *G. mellonella* was added to each well. A total of 30 larvae (split into three repeats of 10 larvae as single replicates) were used per storage temperature and control. Each culture plate was placed inside a 330 × 215 mm ziplock bag (GLAD, www.glad.co.za) to conserve moisture and incubated at 25°C for 72 hr in complete darkness.

Survival and infectivity of formulated IJ at 15°C for 84 d: For this study, IJ were produced in vitro by using pureed larvae of the house fly, *M. domestica*.

For nematode culturing, 5 g of larval puree and 0.15 g canola oil were mixed with 0.5 g of sponge cubes that were cut in 0.5 cm^3, placed in a 100-ml Erlenmeyer flask, and autoclaved at 121°C for 15 min. After cooling to room temperature, flasks with production medium were inoculated with 0.5 ml (each) of 48 hr bacteria culture of *S. innovationi* and incubated at 27°C for 72 hr. Symbiotic bacteria were isolated from infected *G. mellonella* larva according to Ulug et al. (2015). The flasks were then inoculated with 0.5 ml of a monoxenic nematode culture (inoculum prepared according to Lunau et al. [1993]) and incubated at 22°C for 28 d. Nematodes were harvested (yield = 781,678 ± 221 IJ/5 g medium) by washing the sponges with 50 ml distilled water six times. Sponges were squeezed five times during each wash. Three subsamples were counted per flask to calculate the percentage survival of IJ using the dilution method described by Kaya and Stock (1997). Formulated IJ were prepared by sieving batches of harvested IJ through two milk filters (www.denvet.co.za) in a container with water to remove dead IJ. Live IJ were diluted to 2.5 million IJ in 15 ml of a 0.1% formalin solution. This "nematode paste" was then placed onto 7.5- × 5.0- × 2.5-cm sponges, and three sponges were individually placed in a plastic bag and sealed using a hot-element bag sealer (Verimark, Model: VP5201, www.verimark.co.za). Individual bags were stored at 15°C for 84 d in total darkness. Percentage survival was assessed by cutting out five pieces of 0.5 cm^3 sponges, rinsing out the IJ from each piece in a separate beaker and counting three subsamples as described above. This experiment was repeated two times.

Nematode infectivity was assessed by filling the wells of a 24-well tissue culture plate with 0.5 g sterile loam sand to which 50 live IJ were pipetted in 60 µl of distilled water per well. One final instar *G. mellonella* was added per well. Controls were inoculated with 60 µl distilled water only. A total of 30 larvae, split into three replicates of 10 each, were used per treatment and a control. Plates were then incubated at 25°C for 72 hr to determine infectivity.

Survival and infectivity of formulated IJ after 14 d storage at 25°C following storage for 84 d at 15°C: Nematode formulation sponges previously stored at 15°C for 84 d were cut into six pieces. Sponges were placed in a plastic bag, sealed as above, and maintained at 25°C for 14 d to mimic a commercial scenario from purchase to application, where cooling facilities may not be available. Nematodes were harvested by washing sponges with 50 ml distilled water six times. Sponges were squeezed five times during each wash. Three subsamples per flask

were diluted and counted (100 IJ per subsample) to determine percentage survival as described above. Infectivity tests were performed as described above. The experiment was repeated twice.

Statistical analysis: A split-plot analysis of variance (ANOVA), with temperature as main plot and days as subplot, was used to analyse optimum temperature data (SAS Institute, 1999). Thereafter, the means were separated using Fisher's unprotected *t* test (least significant difference) at the 5% level of significance (Fisher, 1970). Infectivity data were arcsine transformed before analysing with one-way ANOVA and mean separation with Tukey's test (SPSS, 2004) at the 5% level of significance.

Results

Optimum storage temperature: IJ percentage survival was the highest ($F = 42.13$; df = 4,116; $P < 0.05$) at 15°C reaching 88%, 85%, and 84% on sampling Day 28, 52, and 84, respectively (Fig. 1). There was no significant difference in percentage survival between 10°C and 15°C on the three sampling days. However, survival at 20°C was 86% on Day 28 but dropped to 59% after 52 d. At 25°C survival dropped to 57% in the first 28 d but remained stable up to Day 84. There was no significant difference between 20°C and 25°C on Day 52 and 84. The lowest percentage survival was recorded at 5°C (35%, 17%, and 13% on Day 28, 52, and 84, respectively) and it was significantly different from all other temperatures. Survival at different temperatures was lowest at 5°C, followed by 10°C, then a peak at 15°C, followed by a decline at 20°C and 25°C after 84 d (Fig. 1).

Pathogenicity tests with IJ stored at 5°C to 25°C showed more than 80% infectivity and there was only a statistical difference between treatments and control on Day 28 ($F = 56.74$; df = 5,12; $P < 0.0001$) and Day 56 ($F = 270.13$; df = 5,12; $P < 0.0001$) (Fig. 2). However, on Day 84, the infectivity of nematodes stored at 5°C dropped significantly to 10% ($F = 77.94$; df = 5, 12; $P < 0.0001$), whereas IJ stored between 10°C and 25°C exhibited more than 90% mortality (Fig. 2).

Survival and infectivity of formulated IJ at 15°C for 84 d: Mean survival of IJ produced in vitro and stored on a sponge formulation was 91 ± 4.06% at 15°C. Pathogenicity against *G. mellonella* was 100% (data not shown) with a significant difference between control and treatment ($t = 7.497$; $P < 0.0001$).

Survival and infectivity of formulated IJ after 14 d storage at 25°C following storage for 84 d at 15°C: Average percentage of IJ survival among the three replicates was 87% ± 4%. Virulence of the nematodes against *G. mellonella* was above 93% for the three replicates which differed significantly from control mortality ($t = 8,013$; $P < 0.0001$).

Discussion

Temperature had an important effect on IJ storage, survival, and infectivity. Survival of *S. innovationi* was better at 10°C and 15°C compared to all other temperatures and it was lowest at 5°C. In general, survival decreased with increased temperature (>15°C) as well as the duration of storage. Boff (2001) also found that survival and performance was better when *Heterorhabditis megidis*, Poinar, Jackson, and Klein (strain NLH-E87.3) was stored at 10°C or 15°C than at 5°C and 20°C. Low temperatures also reduced the growth of contaminants that compete for available oxygen in storage, thereby causing harmful environmental conditions for the IJ (Strauch et al., 2000). Similarly, when studying *Steinernema rarum* (de Doucet), Cagnolo and Campos (2008) noticed a reduction in IJ activity at 23°C ± 1°C after 7 d storage, yet after 12 wk storage 95% of the IJ survived at this temperature compared to 15% survival at 8°C. Clearly, EPN species differ distinctly in their optimum storage temperature (Strauch et al., 2000; Goud et al., 2010; Andalo et al., 2011; Koppenhofer et al., 2013; Mejia-Torres and Saenz, 2013).

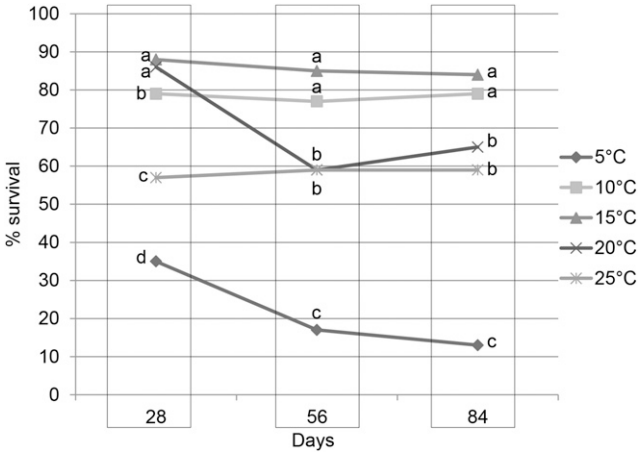

Fig. 1. Percentage survival of *Steinernema innovationi* infective juveniles stored in 0.1% formalin solution for a period of 84 d. Lines with different letters within a given column differed significantly at the 5% test level.

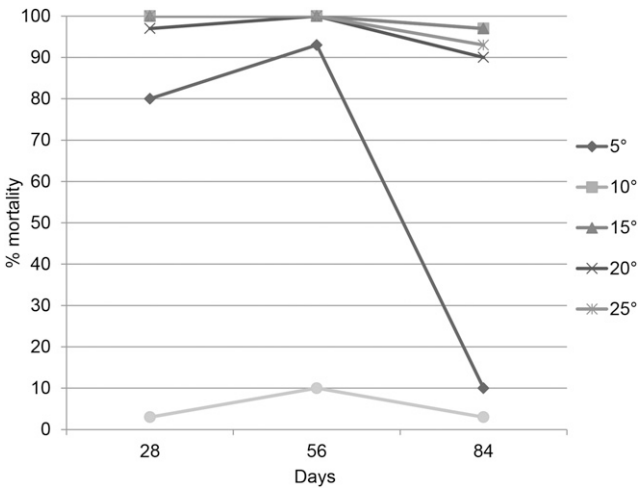

Fig. 2. Infectivity of *Steinernema innovationi* infective juveniles expressed as mortality of *Galleria mellonella* post storage at different temperatures over a period of 84 d.

The use of artificial sponges as carriers for IJ storage has been successful in many studies (Bedding, 1984; Strauch et al., 2000; Ley and Mundo-Campos, 2004; San-Blas, 2013). Using a physical carrier as compared to water suspension can reduce stress by simulating a natural environment because it provides a large surface area for oxygenation through perforation (San-Blas, 2013). Demonstrating the utility of sponge formulations, Andalo et al. (2011) achieved 89.3% and 57.5% survival of *Steinernema carpocapsae* (Weiser) in sponge formulation (3,000 IJ/ml) after 90 and 180 d storage, respectively, at 16°C ± 1°C.

Previous studies have shown that formulation of active nematodes require refrigeration during transportation and storage, which increases the cost of EPN products (Westerman, 1992; Strauch et al., 2000; Goud et al., 2010). If IJ could be stored even for a short time at room temperature (e.g., between the time of purchase and application) this would be seen as a great advantage in commercial utility. Our results indicate that *S. innovationi* can be stored for 84 d at 15°C and then for 2 wk at room temperature. Infectivity against *G. mellonella* was above 90% for the duration of the experiment except for IJ stored at 5°C, which declined to 10% after 8 wk storage. Although the literature suggests that survival and infectivity are conserved at lower temperatures due to the tendency of IJ to be less active (Grewal, 2002), 5°C did not support *S. innovationi* survival and infectivity. Some EPN species are naturally more cold tolerant, whereas others are more heat tolerant. Fan and Hominick (1991) recorded a higher percentage survival of *Steinernema feltiae* (Filipjev) at 5°C compared to 15°C, but infectivity of IJ stored at 5°C declined to 30% in the first 2 wk followed by an unexpected increase to 70% in Week 3 and 4. Similarly, Koppenhofer et al. (2013) showed that increases in infectivity occurred when *Steinernema scarabaei* (Stock and Koppenhofer, 2003) was stored at 8°C over 12 wk. Low infectivity followed by an increase in infectivity instead of a gradual decrease indicates that the cold temperatures may induce a state of dormancy, followed by switching back to an active "risk-taking behaviour" when IJ face starvation (Fushing et al., 2008).

In conclusion, our study demonstrated that the optimum storage temperature for *S. innovationi* was 15°C. Other studies have suggested that factors other than temperature and storage time (such as IJ concentration and type of carrier) affect virulence and infectivity of IJ. Therefore, further investigation will be needed to determine how these factors affect *S. innovationi*.

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