ORIGINAL ARTICLE

Formulation of *Steinernema yirgalemense* in gel for long-term storage at room temperature

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Abstract

The use of entomopathogenic nematodes (EPNs) offers the potential of reducing the use of chemical insecticides in the agricultural industry. Hydrogels for the long-term storage of EPNs may have the potential to improve the storage ability of EPNs. In this study, infective juveniles (IJ) of a South African isolate *Steinernema yirgalemense* were formulated in different gels, which included a laboratory grade and consumer grade of agar, as well as STOCKOSORB® 660, and compared it with IJ in water. Their survival and pathogenicity of the IJ were investigated at 25 °C. The last instar larvae of *Tenebrio molitor* were used to test the pathogenicity of the IJs 6 weeks postformulation. IJs stored in 1% and 1.5% agar formulations were found to provide the best survival of 77% and 82% after 6 weeks in formulation. Results indicate consumer grade agar to be comparable with laboratory grade and can be used as a cheaper alternative for the formulation of *S. yirgalemense*. When comparing the STOCKOSORB® 660 formulations, 80% IJ survival was recorded in a formulation of 1 g in 50 ml of water, with 50% of the IJs surviving in a formulation of 1 g in 100 ml of water. The IJs formulated and stored in STOCKOSORB® 660 gel were found to be more virulent to *Tenebrio molitor* than were the IJs formulated in agar.

Keywords Agar · Gel · Shelf life · *Steinernema yirgalemense* · STOCKOSORB® 660

Introduction

The use of chemicals to control insect pest crop infestation often leads to insect chemical resistance, pollution of the environment and accumulation in animal bodies. Therefore, it is of importance to source and use such eco-friendly alternatives as biopesticides and biological control agents to prevent agricultural crop losses caused by insect pests (Ganguly et al. 2008). In recent years, entomopathogenic nematodes (EPNs) have been well studied and proven to work well as biological control agents of both above- and below-ground insects (Le Vieux and Malan 2015; Platt et al. 2020). When it comes to integrated pest management (IPM) programmes, EPNs are of great importance, as they can be incorporated when controlling insect pests of a wide range of agricultural crops, especially when the use of chemicals is not allowed, such as close to harvest (Heriberto et al. 2017).

EPNs are insect parasites of the genera *Steinernema* and *Heterorhabditis*, which carry a symbiotic bacterium inside their intestines. *Xenorhabdus* spp. bacteria are associated with *Steinernema* and *Photorhabdus* spp. being associated with *Heterorhabditis* (Poinar 1990). Infective juveniles (IJs) of EPNs are the only non-feeding free-living stages in the EPN life cycle. They penetrate the insect through its natural openings, and when inside the haemocoel, they release the bacteria with toxins, causing septicaemia, which leads to the death of the insect within 24–48 h. EPNs feed on the bacteria-converted insect gut tissues, developing and reproducing (1–3 generations), where after a new cohort of IJs leaves the cadaver to search for a new host (Stock 2015).

In South Africa, EPNs have been tested against such key pest as codling moth, false codling moth, weevils, mealybugs, *Bradysia* spp. and thrips (Malan and Hatting 2015; Hatting and Malan 2017; Malan and Ferreira 2017; Katumanyane et al. 2018; Dlamini et al. 2019a, b), showing great potential in all cases. Great progress has been made in terms of EPN formulation techniques, with EPNs being formulated in wettable powder, dispersible granules, sponge and water-soluble gel forms, which have become available both in Europe and on the American continent (Kaya et al.

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2006; Kagimu et al. 2017; Nxitywa and Malan 2021a). Market turnover of EPNs has come to assume second position in the biocontrol industry, after *Bacillus thuringiensis* products (Ehlers 1998). Nevertheless, the success that has been attained regarding formulation technology has mostly been challenged by the short shelf life of the product (Shapiro-Ilan et al. 2012), due to the necessity of maintaining the biological activity of the EPNs during storage (Georgis 1990). For the development of a successful EPN formulation, Ehlers (2007) suggests that the formulation should be done as soon as possible after the production cycle, or process, has been completed.

The most significant reason for EPN formulation is the ability of the medium both to enable and condition the EPNs to survive in storage for lengthy periods and to improve their infectivity (Lacey et al. 2010). The shelf life of EPNs differs according to the species and strains, with the expected shelf life of *Heterorhabditis* spp. being 3–6 months and of *Steinernema* spp. being 6–12 months (Hazir et al. 2003). Therefore, it is important for researchers to choose the appropriate formulation media, which should possess properties that prolong the shelf life, and retain the pathogenicity, of EPNs to exceed expectations (Georgis 1990).

In South African plantation forestry, the survival and growth of seedlings during transplanting is enhanced by using the water-retaining polymer STOCKOSORB® 660 (Viero and Little 2006). The gel is commonly applied around the root zones of seedlings during planting, to retain moisture. It provides a potential medium for the application of EPNs in plantation forestry and other environments (Abate et al. 2019) where moisture depends on rainfall.

As the slight desiccation of EPNs is reported to reduce their metabolism and energy reserves, with it having been proved to extend the shelf life of EPNs (Lacey et al. 2010), it should be one of the properties to be considered when choosing the formulation media. The formulation of EPNs using gels serves to minimise the movement of the IJs, which leads to their reduced metabolism, thereby helping to maintain the energy reserves required for insect infection.

In the current study, a South African endemic EPN species was formulated in different types of gels and stored at 25 °C. The viability of formulated IJs was determined during storage as well as pathogenicity after 6 weeks of storage.

Materials and methods

Production of nematodes

Steinernema yirgalemense Nguyen, Tesfamariam, Gozel, Gaugler & Adams (Nguyen et al. 2004; Malan et al. 2011) was cultured using in vitro liquid culture (Ferreira et al. 2016; Dunn et al. 2020, 2022). The culture process used in short was as follows: *Xenorhabdus indica* Somvanshi, Lang, Ganguly, Swiderski, Saxena & Stackebrandt (Ferreira et al. 2016) associated with *S. yirgalemense* was cultured by means of inoculating autoclaved 30 ml of tryptic soy broth (TSB) in 250-ml Erlenmeyer flasks with 200 µl bacteria. The flasks were shaken in a 28 °C growth incubator, in a junior orbital shaker at 140 rpm, for 48 h. The bacterial cultures (4%) were inoculated into 30 ml of dried egg yolk-complex media, contained in 250-ml Erlenmeyer flasks and left to grow at 25 °C on an open OrbiShaker for 48 h. The Erlenmeyer flasks containing both the media and the bacteria were inoculated with 3×10^4 IJs (1000 IJs ml^{-1}) of *S. yirgalemense* and left to grow and reproduce for 14 days at 140 rpm. The flasks containing the IJs were then moved to cold storage (14 °C), where they were left on an orbital shaker for formulation from day 17 onwards, to ensure IJs are fully developed.

Downstreaming of nematodes

After harvesting the IJs from one Erlenmeyer flask using a 32-µm sieve [Clear Edge Filtration SA (Pty) Ltd, South Africa], they were washed thoroughly with filtered tap water. After being scooped into a weighing boat, the paste of IJs was used for formulation together with the different gel types. Each in vitro cultured Erlenmeyer flask delivered approximately 7.5×10^6 IJs.

Insect host

The larvae of mealworms, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae), used for the pathogenicity tests were purchased from a local pet shop and maintained in the dark at the Department of Conservation Ecology and Entomology, Stellenbosch University. The mealworms were fed bran, together with pieces of carrots as a source of moisture and kept in a container with an aerated lid (Van Zyl and Malan 2015).

Gels for formulation

Nematodes were formulated using two different gel media: agar and STOCKOSORB® 660 (Agro-Serve (Pty) Ltd, Bryanston, South Africa). Two different agar powders were used: agar powder (SIGMA-ALDRICH) and agar–agar powder (Health Connection Wholefoods). STOCKOSORB® 660, which is a super-absorbent homopolymer (hydrogel)-based acid potassium that is acrylamide-free, is used for both water and soil management in agriculture and horticulture (Table 1).

Tab 1 Different types of gels used for the formulation of nematodes

Gel type	Trade name	Supplier	Form
Agar 1	Agar powder (laboratory grade)	Sigma-Aldrich	Powder
Agar 2	Agar–agar powder (consumer grade)	Health connection wholefoods	Powder
Waterwise crystals	STOCKOSORB® 660	Agro-Serve (Pty) Ltd, Bryanston, South Africa	Granules

Preparation of gels

Agar types

For each of the two agar powders (agar, expensive laboratory grade and agar–agar, cheap consumer grade), six different concentrations (1%, 1.5%, 3%, 4% and 6%) were used per 50 ml of distilled water. The agar formulation was prepared by means of dissolving the agar powder in water in a microwave. When the temperature of the solution reached ± 28 °C, the nematodes were added and stirred, and the mixture was poured into Petri dishes to solidify. The Petri dishes were stored in a 25 °C incubator. The number of dead and live IJs was recorded every week for 6 weeks.

STOCKOSORB® 660

The water-retaining STOCKOSORB® 660 (polyacrylamide gel, Agro-Serve (Pty) Ltd, Bryanston, South Africa) is commercially available in the form of powder or granules, whereas the gel was prepared following the manufacturer's instructions, which involved mixing 5 g of powder with 500 ml sterilised water. Two compositions were prepared by means of adding 100 ml (C1) and 50 ml (C0.5) of nematodes in the nematode suspension in distilled water to 1 g of STOCKOSORB® 660 powder, upon which it was mixed. Each solution contained approximately 5×10^6 IJs. After the polymer had fully retained the water, the formulations were packaged in small plastic containers with lids and stored in a 25 °C incubator. For each combination, three replicates were used (involving three packaged containers per formulation). The experiment was repeated twice on a different test date, using a different nematode batch.

Survival of IJs in gels

Weekly, 0.5 g of the agar or gel formulation was stirred in distilled water on a magnetic stirrer, to facilitate the release of the IJs. From the solution, 1 ml was pipetted into a graded Petri dish, and both the live and the dead nematodes were observed under the stereomicroscope. The number of live and dead nematodes was counted of the first 100 nematodes observed, with the number being used to indicate the percentage survival. The experiment was repeated twice on a different test date, using a different nematode batch.

Pathogenicity tests

The formulated IJs were evaluated for infectivity after a 6 week of the storage period. The formulation was stirred on a magnetic stirrer in water, to facilitate release of the IJs. The concentration of the nematodes in the suspension was adjusted to the concentration required for the inoculation (100 IJs/50 μl). Twelve filter papers were fitted in alternate wells of a 24-bioassay plates, with mealworms being added on top of each filter-papered well. The mealworms were inoculated with 100 IJs/50 μl of the gel solution. To prevent the escape of the mealworms and to retain moisture, glass was fitted onto the bioassay lids. Five bioassays were used for each treatment concentration (with each consisting of 12 alternating wells) ($n = 60$). The bioassay plates were then placed in plastic tubs lined with moistened paper towels and maintained in a 25 °C incubator for 48 h. Mortality by infection was verified through the visual colour observation of the mealworms and dissection using a stereo microscope. The experiment was repeated with each batch of gel formulation, with the nematode species and storage conditions remaining the same as in the above description.

Statistical analysis

Statistical analyses were conducted using STATISTICA 13.2 software (StatSoft. Inc). Where the results were not normally distributed, bootstraps were performed on the data to obtain least significance difference (LSD) multiple comparisons. In other instances, the means were accordingly separated by means of the administration of Fisher's least significant difference or the Games-Howell post hoc test.
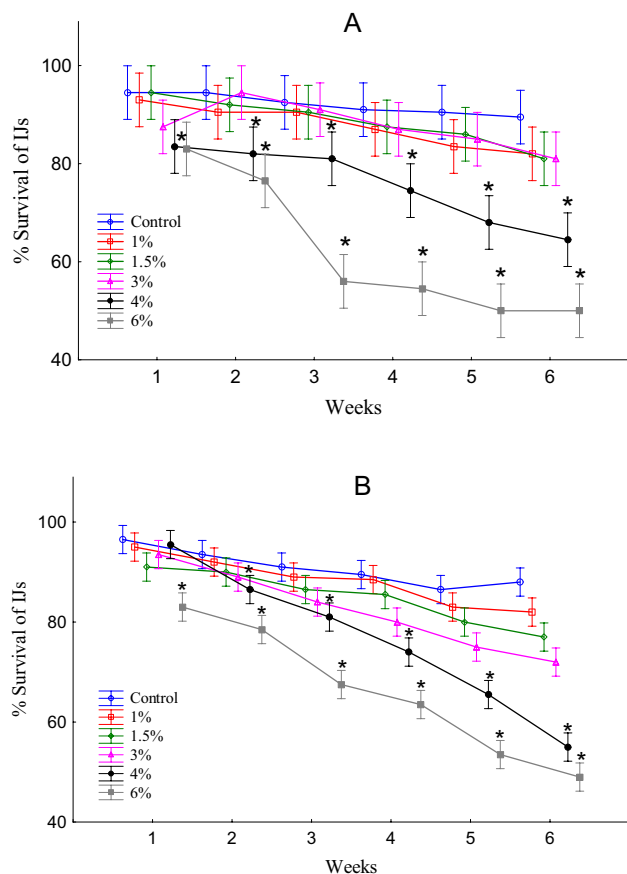
Results

Agar types

The analysis of the data of laboratory grade agar showed no significant difference ($p > 0.05$) between the two batches in terms of time and treatments, allowing for the data to be pooled and analysed. In week 1, the control, 1%, 1.5% and 3% agar formulation did not differ significantly ($p > 0.05$) from each other, but they did differ significantly ($p < 0.05$) from the 6% formulation. Within the tested treatments

(excluding the control), the formulation with 1.5% agar had the highest mean percentage survival of IJs, with a mean percentage of $93.00 \pm 1.41\%$, and the formulation with 6% agar had the lowest mean percentage survival of $83.00 \pm 2.83\%$. From week 2 to week 6, the control, 1%, 1.5% and 3% agar formulation were not significantly different from each other ($p > 0.05$), but significantly ($p < 0.05$) differed from the 4% and 6% formulation. The formulations made of 4% and 6% did not differ significantly ($p > 0.05$) from each other. During the last week of the experiment, the treatment with the highest mean percentage survival was that which was made of 1% agar ($81.00 \pm 2.23\%$), while that with the lowest mean percentage survival was that which was made of 6% agar ($50.00 \pm 1.41\%$) (Fig. 1).

Analysis of the data of agar–agar used showed no significant difference ($p > 0.05$) between the two batches in terms of time and treatments, allowing for the data to be pooled and analysed. The data analysis of IJs survival formulated in the agar–agar 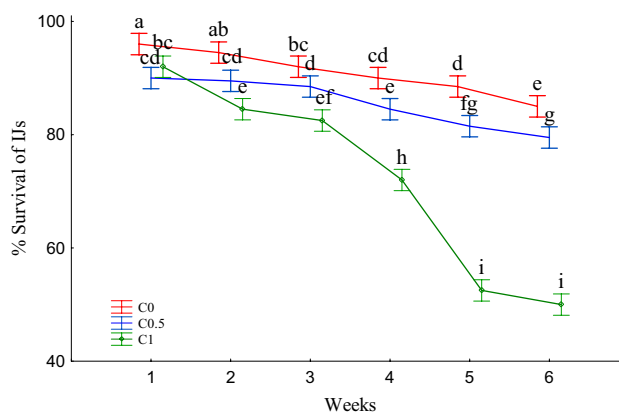media showed a relatively slow decrease over 6 weeks for all agar percentages that were investigated, except for in the case of the IJs that were stored in 4% and 6% agar–agar gel formulation where a fast decline was observed. Significant differences were observed between some of the percentages in the different agar tested, 1% agar–agar differed significantly from 3%, 4% and 6% from week 2 to week 6. Six per cent was always significantly lower than the other percentages, while 4% was significantly lower than 3% from week 4 to week 6. The IJs stored in 6% agar–agar formulation gave the lowest mean percentages throughout the 6-week period of the experiment. In week 6, the IJs stored in 6% agar–agar had the lowest mean survival rate of $49.00 \pm 1.47\%$ (Fig. 1B).

STOCKOSORB® 660

No difference was found in the main effects of survival over time in the two test dates, and the data were pooled and analysed. The mean percentage survival of IJs in the control treatment differed significantly ($p < 0.05$) from both the treatments (C0.5 and C1) investigated throughout the 6-week period. In week 1, the formulation with half the concentration of water did not differ significantly from that with the recommended concentration of water. However, a change was noticed from week 2 to week 6, wherein the mean percentage survival of IJs in C0.5 and C1 differed significantly ($p > 0.05$) from each other, and C0.5 maintained the highest mean percentage survival throughout the period. The mean percentage survival of IJs in C1 dropped significantly at week 5, from $72.00 \pm 1.41\%$ in week 4 to as low as $52.50 \pm 0.71\%$ in week 5 (Fig. 2).
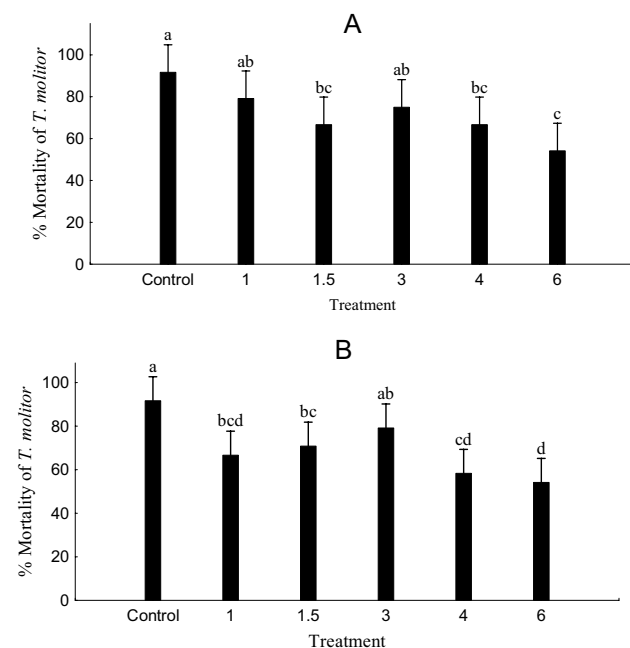

Fig. 1 Mean percentage survival (95% confidence level) of *S. yirgalemense* infective juveniles (IJs) in **A** agar formulation ($F_{25, 36} = 3.468$; $p < 0.01$) and **B** agar–agar formulation ($F_{25, 36} = 11.723$; $p < 0.01$). Stars indicate significant differences ($p < 0.05$) between treatment concentrations and the number of IJs to survive over time

Fig. 2 Mean percentage survival (95% confidence level) of *Steinernema yirgalemense* infective juveniles (IJs) in STOCKOSORB® 660 formulation, prepared by adding 1 g to 100 ml (C1) and 50 ml (C0.5) of nematodes to the nematode suspension in distilled water, as well as in the control (C0), using nematodes in water only ($F_{10, 18} = 75.079$; $p < 0.01$). Different letters above the bars indicate significant differences ($p < 0.05$) between time in weeks and the percentage of IJs survival

Pathogenicity of agar gels

No significant ($p > 0.05$) difference was obtained between the two batches of all the gels in terms of the main effects of treatments and time, allowing for the data to be pooled and analysed using a one-way ANOVA. The control mortality did not differ significantly in the case of either the 1% ($p = 0.15$) or the 3% ($p = 0.07$) agar mortality, but it did differ significantly from that caused by the 1.5% ($p = 0.017$), 4% ($p = 0.017$) and 6% ($p = 0.0026$) agar. The percentage mortality caused by the IJ formulations with 1%, 1.5%, 3% and 4% agar did not significantly differ ($p > 0.05$). The percentage mortality caused by IJ formulations with 6% agar was significantly different from that of those with 1% ($p = 0.0026$) and 3% ($p = 0.034$) agar but did not differ significantly from those with 1.5% ($p = 0.15$) and 4% ($p = 0.15$) agar. Within the treatments (excluding the control), the formulation that caused the highest mean mortality was that with 1% ($79.17 \pm 5.38\%$) agar, whereas the one that caused the lowest mean mortality was that with 6% ($54.17 \pm 5.38\%$) agar (Fig. 3A).

No significant ($p > 0.05$) difference was obtained between the two batches of all the gels in terms of the main effects of treatments and time, allowing for the data to be pooled and analysed through one-way ANOVA. The mortality percentage of the IJ control did not differ significantly ($p = 0.10$)
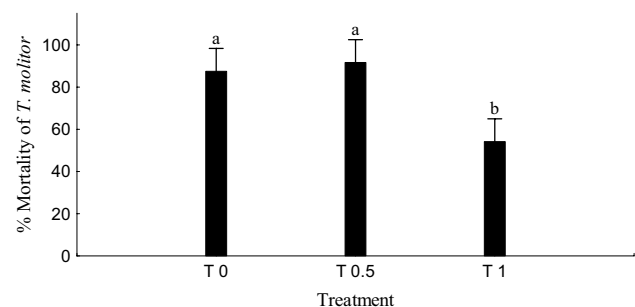
from that of the IJs formulated in 3% agar, but it did differ significantly from that of formulations made with 1%, 1.5%, 4% and 6%, with p-values of 0.01, 0.02 < 0.001 and < 0.00, respectively. Within the treatments (excluding the control), the formulation that caused the highest mean mortality was that with 3% agar ($79.17 \pm 4.50\%$), whereas the one that caused the lowest mean mortality was that with 6% agar ($54.17 \pm 4.50\%$) (Fig. 3B).

Pathogenicity of STOCKOSORB® 660

The mortality percentage of the IJs in the treatment in which half of the recommended water was used for the formulation did not differ significantly ($p = 0.45$) from that of the control, but it did differ significantly ($p < 0.001$) from the recommended water addition. The highest mean mortality of *T. molitor* L. occurred with the T.0.5 ($91.67 \pm 0.00\%$), whereas the lowest mean mortality of *T. molitor* L. occurred with the T1 ($54.17 \pm 5.89\%$) (Fig. 4).

Discussion

The short shelf life of EPNs has been seen as being among the limiting factors in the commercialisation and expanded use of EPNs as the biological control agents of pest insects. Maintaining the balance in the water retention, both inside and in the surrounding area of the IJs is important for prolonged IJ viability since they are aquatic multicellular organisms (Chen and Glazer 2005; Nxitywa and Malan 2021a, b). In the current study, to maintain the water balance in and around the IJ bodies, different gel media were used. Although the IJ survival in the gels tended to be lower than was that in the water control, some IJ survival rates did not differ significantly from that in the control. The performance

Fig. 3 Mean percentage (95% confidence level) of infected *Tenebrio molitor* inoculated with infective juveniles of *Steinernema yirgalemense* formulated in **A** agar ($F_{5, 6} = 5.6800$; $p = 0.0282$) and **B** agar–agar ($F_{5, 6} = 9.400$; $p < 0.01$), stored at 25 ° for 6 weeks. The letters above the bars indicate significant differences ($p < 0.005$) between the treatments and the percentage mortality of *T. molitor*

Fig. 4 Mean percentage (95% confidence level) of the infected *Tenebrio molitor* L. inoculated with the infective juveniles of *Steinernema yirgalemense* formulated in agar and stored at 25 °C for 6 weeks ($F_{2, 3} = 36.5$; $p < 0.01$). The letters above the bars indicate significant differences ($p < 0.05$) between the treatments and the percentage nematode mortality of *T. molitor*

of *S. yirgalemense* IJs in the gels varied in the different gel types used.

The concentration that had better survival stability for the *S. yirgalemense* IJs in both the agar and the agar–agar formulations was 1%. The results of the current study are consistent with those of Hussein et al. (2012), who reported that IJ of *Steinernema feltiae* (Filipjev) Wouts, Mráček, Gerdin & Bedding in 1% agar formulation had better survival and viability than did those in the other concentrations (2 and 4%). In addition to the above, they, further, discussed that the higher concentrations condensed during spraying, which was more like the similar experience in the current study, where facilitating the release of the IJs from the higher concentrations, being the 3%, 4% and 6%, was found to be difficult, as the solutions were inclined to clump. Therefore, using relatively high agar concentrations is not suitable when the delivery method to fields or greenhouses is conducted through spraying, as the presence of the agar particles can lead to the blockage of the spray nozzles. However, to deliver slow release in the blocks in the case of such potted plants as blueberries and cucumbers produced under cover would be ideal. After 6 weeks of storage, in both agar formulations survival of IJ was 50% and 49%, respectively, which indicated that the cheaper consumer grade of agar will be suitable for formulation of *S. yirgalemense*.

The survival of the IJs in the STOCKOSORB® 660, where half of the recommended amount of water (C0.5) was added, was higher than that where the recommended amount of water (C1) was added. At week 6, C0.5 had 80% IJ survival, whereas C1 had 50% IJ survival. The results clearly indicate that the free water in the formulation had a negative effect on the survival of *S. yirgalemense* IJs. The results of the current study concur with those that were obtained by Abate et al. (2019), who reported 66% survival at 6 weeks when they formulated the same species, *S. yirgalemense*, in STOCKOSORB® 660. However, their mortality results differed from those that were obtained in the present study, as they reported 71% mortality of *G. mellonella*, whereas 54% mortality of *T. molitor* was obtained in the current study. The difference obtained in the percentage might have been due to the host insect susceptibility. The use of STOCKO-SORB® 660 in the transplanting of tree seedlings in forestry in South Africa creates a suitably moist environment in which to increase the postapplication survival rate of the EPNs (Abate et al. 2019). The authors also suggest that the EPNs could be directly applied to the soil to form a soil-gel mix to enhance their survival rate.

The use of a slurry to stiffen the gels as the storage medium for the IJs could be the way forward in striving to attain long-term survival of EPNs, as, in the study conducted by Hokkanen and Menzler-Hokkanen (2002), the polyacrylate *S. feltiae* gel formulation survived for a period up to 1 year at 4 °C. Applying *S. carpocapsae* in a gel formulation has been found to protect IJs from UV damage, with the IJs in such a formulation being found to be more effective, in terms of infecting and killing the larvae of *G. mellonella*, than are the IJs in aqueous solution (Dito et al. 2016).

In this study storage was at a constant 25 °C, with good results for both high- and low-quality agar, as well as using STOCKOSORB® 660 with survival of > 80% after 6 weeks. Kagimu and Malan (2019) used alginate beads for storage of three South African EPN isolates. Their results showed poor survival and loss of virulence at low temperatures for both formulations. However, with the alginate beads the nematodes were successfully retained with a longer storage time. Kagimu et al. (2022) also indicated that all the South African isolates used cannot be stored in a fridge at 8 °C. The disadvantage of storing nematodes at room temperature is the tread of getting contaminated with the inability of using temperature to restrict movement and contamination with micro-organism (Nxitywa and Malan 2021b).

The application of nematodes in a gel mixture not only protects the IJs from such abiotic factors as UV radiation, desiccation and high temperatures, but it can also help with soil moisture retention, by reducing the amount of water required for irrigation (Brixey et al. 2006). Although the agar gel formulation investigated in the current study was found to have a relatively high IJ survival and infectivity rate against mealworms, further greenhouse experiments or field evaluations are recommended for where the gels with a relatively high percentage of concentration require placing near the root zone, or just below the soil surface, so as to investigate whether the nematodes will be able to escape the agar and infect the target pests (above- and below-ground pests).

Declarations

Conflict of interest All authors declare no conflict of interest.

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