Formulation of *Steinernema yirgalemense* by entrapment in alginate beads

Abongile Nxitywa and Antoinette P. Malan

Department of Conservation Ecology and Entomology, Stellenbosch University, Private Bag X1, Matieland, 7602, Stellenbosch, South Africa
e-mail: apm@sun.ac.za

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Summary. The large-scale use and commercialisation of entomopathogenic nematodes as biological control agents is impaired by their short shelf life. Developing a preservation method that can improve their storage, without negatively affecting their infectivity against the target pest insects, is, therefore, of key importance. *Steinernema yirgalemense* infective juveniles (IJ) were formulated in sodium alginate beads to improve their entrapment and storage capability. To improve the entrapment levels inside the beads, 2% and 4% sodium alginate, 0.5% and 2% calcium chloride ($CaCl_2$), with a hardening time of 20 and 60 min, were investigated. The beads were stored at 25°C and monitored weekly, for the number of IJ escaping for a period of 6 weeks. The disintegration of the beads in soil and the pathogenicity of IJ was investigated after 6 weeks using *Tenebrio molitor* as the trapping host. The highest number of IJ that escaped from the beads occurred with the treatment of 2% sodium alginate and 0.5% $CaCl_2$, with a hardening time of 60 min, and with a mean of 127 ± 11 escaped nematodes. The treatment with the least number of nematodes escaped after 6 weeks was 4% sodium alginate, 2% $CaCl_2$, 20 min hardening time, with a mean of 33 ± 16 escaped nematodes. None of the treatments negatively affected the infectivity of *T. molitor*. The beads applied to orchard soil successfully infected the mealworms, and disintegration took place after 2 weeks. Alginate concentration was found to be the most important factor in preventing the IJ from escaping from the beads.

Key words: endemic species, entomopathogenic nematodes, long-term storage.

The entomopathogenic nematodes (EPN) of the genera *Steinernema* and *Heterorhabditis* (Grewal, 2002) have a symbiotic relationship with bacteria of the genera *Xenorhabdus* and *Photorhabdus*, respectively (Poinar, 1990). The EPN are biological control agents that have been successfully used to control insect pests worldwide (Grewal, 2002). EPN are safe for the environment, as well as for humans (Shapiro-Ilan & Gaugler, 2002), and can be mass-cultured both *in vitro* and *in vivo* (Ehlers *et al.*, 1998). After penetrating the insect through its natural openings, the infective juveniles (IJ) of EPN release their associated bacteria into the haemocoel of the insect (Ehlers, 2001). The presence of the bacterial toxins, combined with those excreted by the nematodes themselves, lead to rapid insect death within 24 h to 48 h (Lewis & Clarke, 2012; Lu *et al.*, 2017). The IJ inside the insect feed on the bacterial bioconverted insect, growing into adults and reproduce, keeping the cuticle of the insect intact. When the food in the insect is depleted after one to three generations inside the insect (depending on its size), they leave the cadaver as a new cohort of IJ, in search of a host insect to infect (Stock, 2015).

Although formulation is the last, but very important, step in the successful commercialisation of EPN as biocontrol agents, their short shelf life is a major challenge hampering their use. The degree of success obtained in the commercialisation of formulated EPN depends on the extent of storage stability and infectivity attained. Therefore, recent studies have focused on advancing and improving the formulations involved, so as to enable the prolonged storage of the IJ, without negatively affecting their longevity and virulence (Shapiro-Ilan *et al.*, 2012; Grzywacz *et al.*, 2014; Ruiz-Vega *et al.*, 2018). The important components to consider when deciding on the formulation technique to be used are handling, transportation, application, persistence and storage. Regardless of the limitations on, and the challenges to, EPN survival in formulation, the improvement of formulation techniques is bound to enhance the EPN survival

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rate, to ease transportation problems and to improve application (Perry *et al.*, 2012).

The short shelf life of EPN has always been a factor limiting their use. As the IJ depend on energy reserves, reducing their metabolic rate influences their activity levels, thus prolonging their shelf life and improving their viability (Qiu & Bedding, 2002; Chen & Glazer, 2005). Chen & Glazer (2005), mention two main steps that can be taken to improve and enhance the shelf life of IJ, namely physical trapping and their partial anhydrobiosis. Previously, investigation has been undertaken into using different types of trapping mechanisms (Georgis, 1990; Grewal, 1998). Chen & Glazer (2005) mentioned the use of glycerol and calcium alginate as promising. Glycerol improves the shelf life of IJ by inducing dormancy, resulting from the degree of osmotic pressure exerted, and leading to their improved adaptability to environmental stress (Chen *et al.*, 2000; Glazer & Salame, 2000). In fact, the shelf life of IJ is extended, due to the desiccation effect that is achieved when the formulation uses beads and/or capsules (Navon *et al.*, 2002). To commercialise and expand the use of EPN as biological control agents for the successful control of pest insects, certain research has come to focus on developing formulations that are cost-effective, that have an increased room temperature stability, that are relatively simple in terms of transportation requirements and easy application in fields or glasshouses (Grewal, 2000; Grzywacz *et al.*, 2014), as well as having high infectivity against the targeted pest insect and improved field persistence (Grewal, 2000; Shapiro-llan *et al.*, 2012). Navon *et al.* (2002) mention that formulations that contain such gelling materials as sodium and calcium alginate correlate with the above-mentioned requirements, as they provide EPN that are trapped in a hydrophilic environment that positively contributes to the prevention, or, at least, to the delayed desiccation, of the EPN, thereby improving their survival rate. The use of alginate in formulating the EPN contributes to improving their shelf life stability (Vemmer & Patel, 2013), as the biomaterials from the alginate are used for tissue regeneration (Szekalska *et al.*, 2016).

The method of entrapping IJ in sodium and calcium alginate beads was developed with the aim of protecting the IJ from both ultraviolet light (UV) and desiccation (Navon *et al.*, 2002), thereby improving the shelf life of EPN. Using such a method should also have a slow-release effect, as the nematodes tend to escape over time from the beads. However, the EPN formulation is still undergoing improvement, as, among others, the challenge of escaping IJ from the soft, unrefrigerated beads within a few days, has been reported by Hiltpold *et al.* (2012) and Kim *et al.* (2015), thus limiting the long-term storage using this type of formulation. Ideally, IJ should be retained inside the beads until they are required for use and they need to maintain high viability at room temperature storage condition, at least for a few months (Kim *et al.*, 2015). Recently, Kagimu & Malan (2019) reported only 10% to 20% of nematodes escaping from the alginate beads at 25°C during a 4-week storage period, which is regarded as a relatively low percentage, indicating an improvement in room temperature long-term stability.

In this study, the endemic EPN species was formulated in sodium alginate beads, so as to improve the entrapment and shelf life of the IJ for long-term storage at 25°C. The method was accomplished by determining the effect of different sodium alginate concentrations and of the hardening time on preventing the IJ escaping from the beads. Furthermore, the study also investigated the rate of escape and the infection of the nematodes from the beads, and the integration duration of the beads when broadcast on natural soil.

MATERIAL AND METHODS

Origin of the insect host. The mealworm *Tenebrio molitor* L. (Coleoptera: Tenebrionidae), which was used for the pathogenicity tests and for the soil experiments, was purchased from a local shop and maintained in the dark, at the Department of Conservation Ecology and Entomology, Stellenbosch University. Bran and carrots were added as a food source for moisture and the mealworms were kept in a container with an aerated lid (Van Zyl & Malan, 2015).

Mass-culture and concentration of infective juveniles. The South African isolate species, *Steinernema yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler & Adams, 2004 (Nguyen *et al.*, 2004; Malan *et al.*, 2011), was used in this study. The nematodes were mass-cultured in Erlenmeyer flasks, using a combined technique of Ferreira *et al.* (2015) and Dunn *et al.* (2019), which should be referred to for more detail. In short, bacteria associated with the species were cultured by means of adding 200 µl of stored (–80°C in 15% v/v glycerol) *Xenorhabdus indica* Somvanshi, Lang, Ganguly, Swiderski, Saxena & Stackebrandt, 2006 to 30 ml of tryptic soy broth (TSB) in 250-ml Erlenmeyer flasks. The flasks were shaken in a junior orbital shaker at 0.547 *g* for 48 h, in a 28°C growth incubator. Bacterial cultures (4% v/v) were

then inoculated into 250-ml Erlenmeyer flasks containing a complex medium, consisting of 30 ml of dried egg yolk as the protein source, and left to grow at 25°C on an open OrbiShaker for 48 h. Erlenmeyer flasks containing the media and bacteria were inoculated with IJ (1,000 IJ ml^{-1}) of *S. yirgalemense*, which were left to grow and reproduce for 14 days at 0.547 *g*. The inoculum used, which was from previous cultures, was stored on orbital shakers at 14°C. The culture flasks containing the IJ were moved to cold storage (14°C) on day 17 for formulation.

The IJ were harvested from one flask at a time, using a 32-µm sieve (Clear Edge Filtration SA (Pty) Ltd, South Africa), and washed with clean running tap water. The remaining water below the sieve was dried off with paper towels (SCOTT® KIMDRI*, Bedfordview, South Africa) and the IJ paste was scooped up with a plastic spoon into a container for further use.

Encapsulation of infective juveniles. IJ were encapsulated by means of modifying the ionic gelation method of Kagimu & Malan (2019) and Kim *et al.* (2015), at room temperature. Different concentrations of sodium alginate, calcium chloride ($CaCl_2$) and hardening periods were used to measure the escape of nematodes from the beads (Table 1). Two solutions were utilised for this experiment, with the first being a 20 ml alginate solution containing 2% to 4% sodium alginate (FMC Biopolymer, Cape Town, South Africa), 10% glycerol, 0.075% formaldehyde, Moir's red or green food colouring and 50,000 IJs ml^{-2} in distilled water. The second solution, consisting of 20 ml Ca^{2+} solution, contained 0.5% or 2% calcium chloride ($CaCl_2$) ((Merck SA (Pty) Ltd, 10% glycerol and 0.075% formaldehyde in distilled water. Droplets of alginate solution (with the nematodes) were dripped using a glass-graduated pipette dropper into Ca^{2+}, which immediately resulted in beads forming. The Ca^{2+} was shaken with an orbital shaker (Benchmark's ORBI-SHAKERTM JR) at 0.547 *g* for 20 or 60 min to prevent the beads from sticking together. The beads were then removed from the solution, rinsed thoroughly with distilled water and dried on paper towels. After packaging the beads in Petri dishes sealed with Parafilm, they were stored at 25°C in an incubator. The survival and escape of the IJ was monitored weekly for 6 weeks.

Sodium alginate concentration. Two concentrations of sodium alginate were tested at two different hardening times for bead forming, on the escape and survival of nematodes from the beads. The following concentrations were used: 2% sodium alginate with 0.5% $CaCl_2$ for 20 min for treatment 1 (T1), and for 60 min for treatment 2 (T2), as well as with 2% $CaCl_2$ for 20 min for treatment 3 (T3), and for 60 min for treatment 4 (T4). A 4% sodium alginate concentration was used with 0.5% $CaCl_2$ for treatment 5 (T5) for 20 min, and for 60 min for treatment 6 (T6), as well as with 2% $CaCl_2$ for 20 min for treatment 3 (T7), and for 60 min for treatment 4 (T8) (Table. 1).

Survival in, and the escape of IJ from, the alginate beads. The survival and escape of *S. yirgalemense* formulated in alginate beads was monitored weekly for 6 weeks. Three alginate beads, previously described, were either crushed with a disposable tissue grinder pestle (Axygen®, Axygen Biosciences, Union City, USA) in a 1.5-ml micro-centrifuge tube, or dissolved in 0.5% citric acid (Chen & Glazer, 2005). To determine the survival rate, the number of both the dead and the live IJ was counted and compared to that which was obtained on the first day of formulation. The escape of the IJ from the beads was also determined by means of comparing the number of IJ in the beads with the number of IJ that were present on the day of formulation.

Quality control of beaded infected juveniles. The infectivity of the encapsulated IJ after six weeks was tested using the larvae of mealworms. The alginate beads, as was previously described, were dissolved in 0.5% citric acid, whereupon the number of live IJ was counted and their concentrations adjusted to 100 IJ $(50\ \mu l)^{-1}$. The infectivity bioassays were performed using mealworms in 24-well bioassay plates. Filter papers were fitted in 12 alternate wells of the 24-well bioassay plates, with a mealworm being added to each well, which was then inoculated with 100 IJ in 50 µl of citric acid distilled water. Distilled water only was used as a control. To prevent the escape of the mealworms from the bioassay plates and to maintain the existing moisture levels, a glass of the same size of the bioassay plate was fitted into the lids. Five bioassay plates were used per treatment. The plates were placed in plastic containers lined with moistened paper towels and stored in a 25°C incubator for 48 h, with the mortality of the mealworms being assessed and confirmed through the visual colour observation of mealworms, and by means of dissection, using a stereo microscope (LEICA MZ75). The experiment was repeated twice with a different nematode batch on a different test date.

Soil experiments. The soil used for the current study was obtained from the experimental farm at

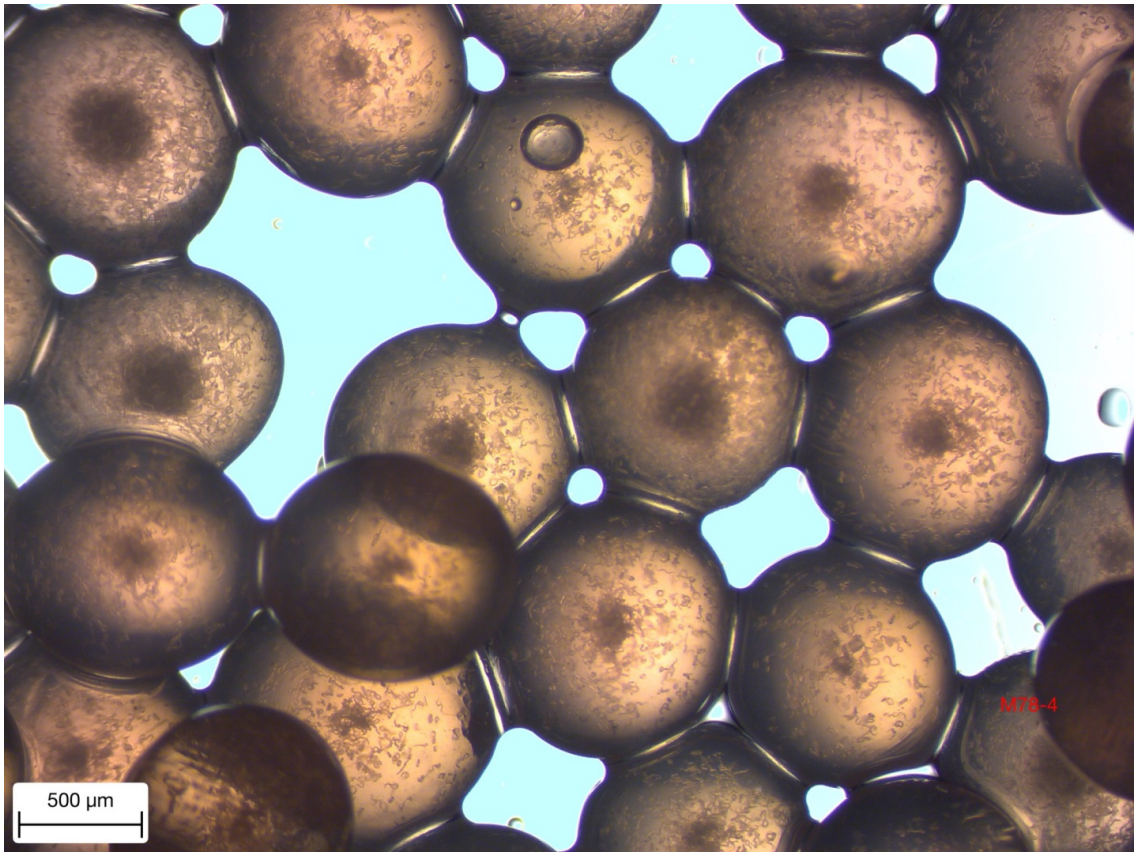

Fig. 1. *Steinernema yirgalemense* infective juveniles aggregated in the centre of alginate beads.

Table 1. Concentrations of sodium alginate, calcium chloride and hardening time (min) used to measure the escape of infective juveniles of *Steinernema yirgalemense* from the beads.

Formulation	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**	**T8**
Alginate (%)	2	2	2	2	4	4	4	4
$CaCl_2$ (%)	0.5	0.5	2	2	0.5	0.5	2	2
Hardening time (min)	20	60	20	60	20	60	20	60

T1 (2% sodium alginate, 0.5% $CaCl_2$, 20 min); **T2** (2% sodium alginate, 0.5% CaCl2, 60 min); **T3** (2% sodium alginate, 2% CaCl2, 20 min); **T4** (2% sodium alginate, 2% CaCl2, 60 min); **T5** (4% sodium alginate, 0.5% CaCl2 , 20 min); **T6** (4% sodium alginate, 0.5% CaCl2, 60 min); **T7** (4% sodium alginate, 2% CaCl2, 20 min); **T8** (4% sodium alginate, 2% CaCl2, 60 min).

Stellenbosch University, South Africa. The soil was sieved with a 250-µm sieve to remove stones and root residues. The soil experiments were carried out in plastic containers containing 100 g sieved soil (n = 5). After spraying distilled water into the soil to obtain optimum soil moisture content, 10 mealworms and 50 alginate beads were added per plastic container. The containers were then closed with lids and stored in a 25°C incubator. The control container consisted of only soil and mealworms. The disintegration of beads into the soil and the mortality of the mealworms were monitored daily. To confirm whether the mortality of mealworms was due to the IJ, the mealworms were dissected, using a stereo microscope so as to be able to observe the nematodes inside the cadaver visually. The experiment was repeated twice on different test dates.

Statistical analysis. Statistical analyses were conducted using STATISTICA 13.2 software (StatSoft. Inc). If an ANOVA conducted on the two different test dates showed no significant difference between the two experiments, the resultant data were pooled and analysed. Where the results were not normally distributed, bootstraps were performed on the data to obtain least significant difference (LSD) multiple comparisons. In other instances, the

means were, accordingly, separated by means of the conducting of Fisher's least significant difference, or the Games-Howell post hoc test.

RESULTS

Behaviour of *S. yirgalemense* IJ in alginate beads. Most of the IJ, which were in the centre of the beads, formed aggregations, while some remained dispersed through the beads (Fig. 1).

Escape and survival of *S. yirgalemense* using 2% sodium alginate. Analysis of the data with all the beads containing 2% sodium alginate, with different $CaCl_2$ concentrations and two hardening times, showed no significant difference ($F_{15, 120} = 8.715$, $P < 0.01$) between the two batches and the test dates, thus allowing for pooling of the data and for the response variable to be tested against the treatment, using a one-way ANOVA. Since the escapements required analysis over a number of weeks, a two-way ANOVA was conducted, with the treatments and the number of weeks involved as the main effects, with a significant difference ($F_{5, 240} = 113.395$, $P < 0.01$) being obtained. A drastic increase occurred in terms of the mean number of nematodes moving out of the beads from week 5 in both T1 and T2, with, in week 4, the mean ± standard error of IJ escaping being 39.8 ± 10.5 and 25.3 ± 8.2, whereas, in week 5, 98.3 ± 8.1 and 104.3 ± 19.3, respectively, escaped. Treatment 1 did not differ significantly from T2 at week 5 ($P = 0.30$). On the overall treatments analysis, neither T1 nor T2 differed significantly ($P = 0.87$) from each other, whereas T3 and T4 did ($P = 0.03$) (Fig. 2).

Escape and survival of *S. yirgalemense* beads using 4% sodium alginate. Analysis of the data with all the beads containing 4% sodium alginate, with different $CaCl_2$ concentrations and two hardening times, showed no significant difference ($F_{15, 120} = 8.715$, $P < 0.01$) recorded between the two batches and the test dates, thus allowing for pooling of the data and for the response variable to be tested against the treatment, using a one-way ANOVA. Since the escapements required analysis over a number of weeks, a two-way ANOVA was conducted, with the treatments and the number of weeks involved as the main effects, with a significant difference ($F_{5, 240} = 113.395$, $P < 0.01$) being obtained (Fig. 3).

In week 1, no treatment differed significantly from another ($P > 0.05$). The highest mean number of 15.0 IJ was recorded for T8, followed by the mean numbers of 12.0, 6.5 and 5.5, obtained for T7, T5 and T6, respectively. The number of IJ that escaped in week 2 did not differ significantly ($P = 0.73$) from that of week 3, and the results obtained in week 3 did not differ significantly ($P = 0.09$)

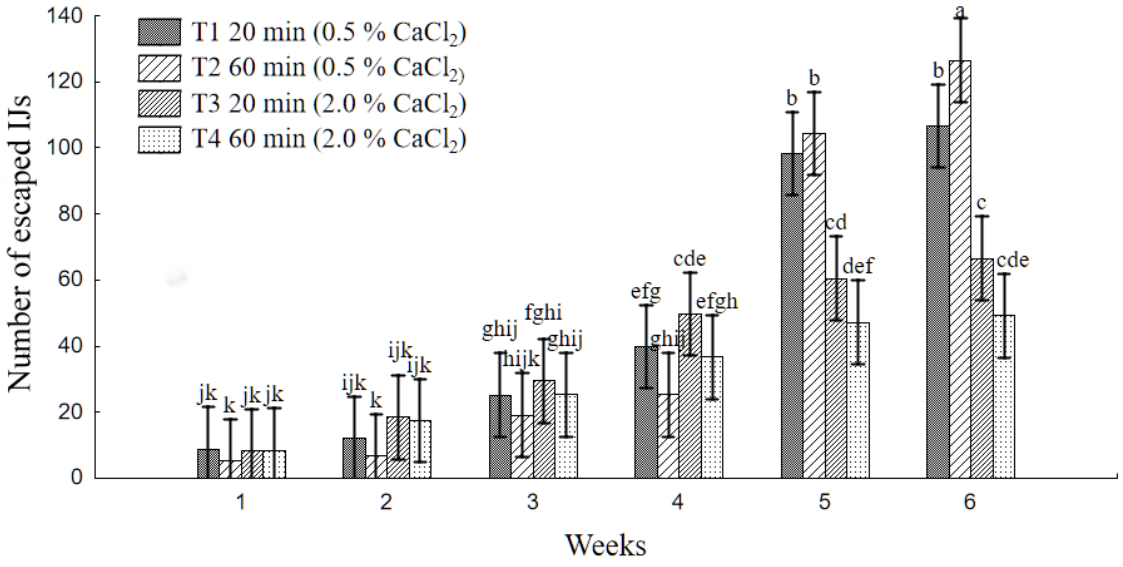

Fig. 2. Mean number of *Steinernema yirgalemense* infective juveniles (95% confidence level) that escaped from the alginate beads over a period of 6 weeks, using a concentration of 2% sodium alginate for all treatments, with 0.5% $CaCl_2$ for 20 min for treatment 1 (T1) and for 60 min for treatment 2 (T2) and with 2% $CaCl_2$ for 20 min for treatment 3 (T3) and for 60 min for treatment 4 (T4) ($F_{15, 120} = 8.715$, $P < 0.01$). Different letters above the bars indicate significant differences ($P < 0.05$) between the different weeks and the number of nematodes that escaped from the beads.

from those of week 4. Although the results obtained in week 4 did not differ significantly (P = 0.13) from those that were obtained in week 5, they did differ significantly (P = 0.0016) from those obtained in week 6. In the last week of the experiment (week 6), none of the treatments was found to differ significantly (P > 0.05) from another. The highest mean number of IJ to escape were recorded in T5

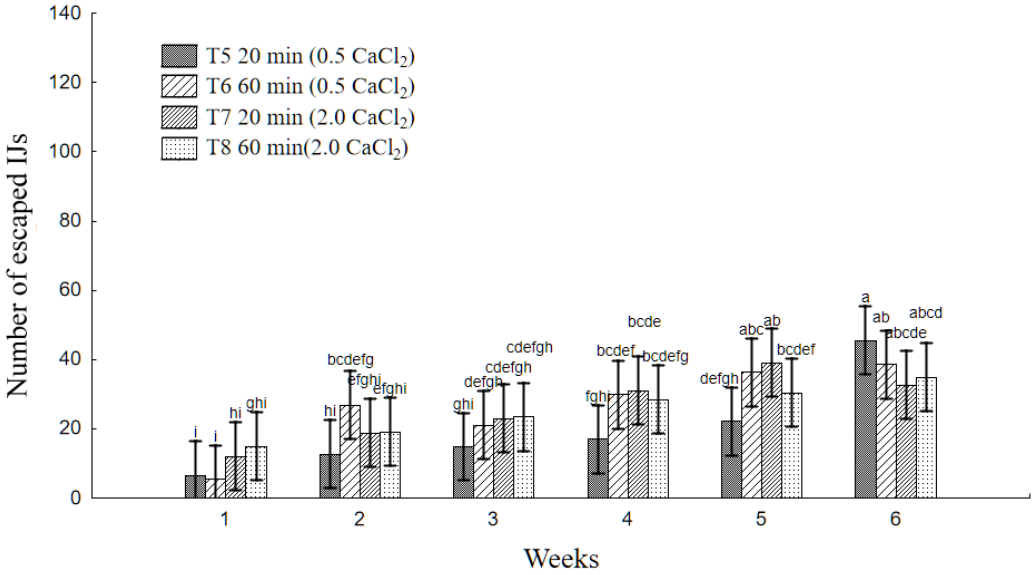

Fig. 1. Mean number of *Steinernema yirgalemense* infective juveniles (95% confidence level) that escaped from the alginate beads over a period of 6 weeks, using a concentration of 4% sodium alginate for all treatments, with 0.5% $CaCl_2$ for 20 min for treatment 5 (T5) and for 60 min for treatment 6 (T6) and with 2% $CaCl_2$ for treatment 7 (T7) for 20 min and for 60 min for treatment 4 ($F_{15, 120}$ = 1.159, P < 0.31). Different letters above the bars indicate significant differences between the different weeks and the number of nematodes that escaped from the beads.

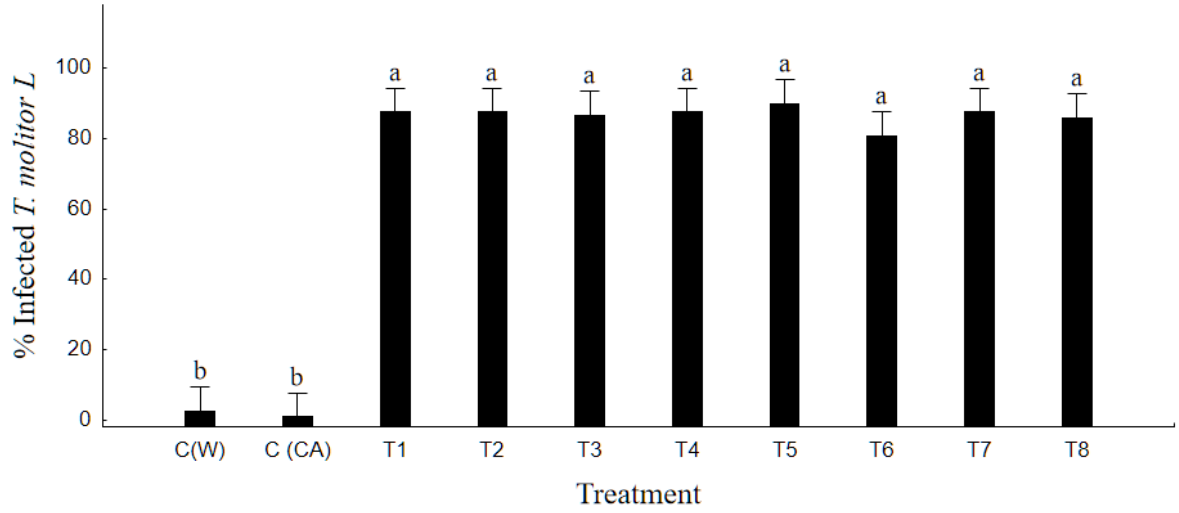

Fig. 2. Mean percentage (95% confidence level) of infected *Tenebrio molitor* L. inoculated with infective juveniles of *Steinernema yirgalemense*, formulated in alginate beads and stored at 25°C for 6 weeks ($F_{9, 90}$ = 107.72, P < 0.01). Means separated by Games-Howell post host; Error: Between MSE = 119.75, df 90.000. The letters above the bars indicate significant differences (P < 0.005). C(w) – distilled water, C(CA) – citric acid solution , T1 – 2% alginate 0.5% Ca 20 min , T2 – 2% alginate 0.5% Ca 60 min, T3 – 2% alginate 2% Ca 20 min, T4 – 2% alginate 2% Ca 60 min, T5 – 4% alginate 0.5% Ca 20 min, T6 – 4% alginate 0.5% Ca 60 min, T7 – 4% alginate 2% Ca 20 min, T8 – 4% alginate 2% Ca 60 min.

(45.5 ± 18.9), followed by in T6 (38.5 ± 6.0), T8 (34.8 ± 11.0) and T7 (32.7 ± 15.9) (Fig. 3).

Quality control of beaded infected juveniles. As no significant difference was obtained between the two batches ($F_{9, 90} = 107.72$, $P < 0.01$) in terms of the main effects of the treatments and the time involved, the data obtained were pooled and analysed, using a one-way ANOVA. The analysis was done for the percentage infectivity obtained over the course of 6 weeks in terms of the number of encapsulated IJ against mealworms. The evaluation of the infectivity of mealworms indicated no significant difference among the treatments. All eight treatments tested, did not differ significantly from each other ($P > 0.05$). Although no differences occurred between the means, there was significant difference from the control (Fig. 4). Treatment 5 caused the highest mean mortality percentage of *T. molitor* of 90.0% ± 10.2%. The lowest mean mortality percentage within the treatments was obtained for T6, with 80.8% ± 11.2%.

Soil experiments. The bead hardness significantly affected both the release of the IJ and the disintegration of the beads in soil. Whereas the IJ from the treatments with 4% sodium alginate concentration started moving out of the beads after day 5, in the case of the treatments with 2% alginate sodium concentration, the release of IJ was noticeable from day 2 onwards. Mealworms in the soil were infected 48 h after the release of the IJ. In the control experiment, with water only and no beads added, mealworms were found to be all alive. The beads disintegrated after 2 weeks in the soil.

DISCUSSION

The biodegradability of alginate beads, and the fact that they are harmless to the environment, make their use suitable for applying EPN in the field. The short shelf life of EPN formulations has been a limiting factor since their initial commercialisation as the biological control agents of insect pests (Chen & Glazer, 2005). Many challenges have been encountered with formulating EPN through the encapsulating of IJ in the bead's face, including IJ escaping from, and moving out of, the soft beads (Hiltpold *et al.*, 2012). The fact that the alginate beads cannot retain IJ for prolonged periods of time limits their long-term storage possibilities (Kagimu & Malan, 2019). Kim *et al.* (2015) mention that the adjustment of bead properties, through the addition of Ca^{2+} as the post-treatment, improves the degree of hardness involved which prevents the IJ escaping the beads.

The current study shows that the sodium alginate percentage, which affects the hardness of the bead, has a significant effect on the number of IJ escaping from the beads. Of all the factors undergoing investigation (alginate, $CaCl_2$ and hardening time), the alginate concentration had a significant effect on the number of IJ that escaped from the beads, with the Ca^{2+} and the hardening time not significantly affecting the number of IJ moving out of the beads. The treatment that resulted in the highest number of IJ escaping from the beads consisted of 2% alginate, 0.5% $CaCL_2$, and 60 min with a mean of 126.5 ± 11.4. By contrast, the treatment that was recorded as resulting in the least number of nematodes escaping at 6 weeks consisted of 4% alginate, 2% $CaCl_2$, and 20 min with a mean of 32.7 ± 16.0. The results show that the best combination for retaining the IJ inside the beads was 4% alginate, 0.2% $CaCl_2$, with a 20 min hardening time. The adjustment of the alginate bead properties, by means of pairing different concentrations of sodium alginate with $CaCl_2$, together with different hardening times, allows for the release of the IJ from the beads to be as slow as possible over a period of time, thus overcoming the challenges experienced in the study conducted by Hiltpold *et al.* (2012) of the IJ escaping from the soft beads. The results of the current study support the findings of Lotfipour *et al.* (2012), with regard to the effect of alginate and $CaCl_2$ concentrations, as well as of the hardening times, on the extent of the encapsulation efficiency, size and morphology of the bacterium, *Lactobacillus acidophilus* (Lactobacillales: Lactobacillaceae). Lotfipour *et al.* (2012) found that, of all the variables tested, the one to play the most important role was the alginate concentration. They reported that the firm, hard beads contained an increased number of entrapped bacteria with relatively high encapsulation efficiency. Furthermore, they concluded that the effect of $CaCl_2$ concentration and hardening was not significant. Lastly, the increase in the percentage, or the concentration, of alginate tends to result in increased alginate viscosity and subsequent increased bead size, resulting in the formation of a firm protective layer (Chandramouli *et al.*, 2004), thus preventing a high number of IJ from escaping.

Although significant differences were found in the number of IJ that moved out of the beads undergoing the different treatments, such variation did not affect the infectivity percentage developed against mealworms. High mortality against mealworms was reported, with the mean mortality percentages ranging from 81% to 90% being observed in all treatments. The high mortality caused by *S. yirgalemense* recorded in the current study correlates with the findings made in Kagimu & Malan's (2019) study, where the 4-week old alginate beads

encapsulating *S. yirgalemense* stored at 25°C attained a mean percentage mortality of 98% against *Galleria mellonella* L. (Lepidoptera: Tortricidae). The survival of *S. yirgalemense* under such a temperature does not support the general statement made by Grewal & Peters (2005) that low temperatures, ranging from 4°C to 15°C, are best suited to prolonging the survival rate of IJ in storage.

Kagimu & Malan (2019), mention that the ability of beads stored at 25°C to retain more IJ than can beads at lower temperatures (6°C and 14°C) is due to the fact that they are less well adapted to the colder temperatures, as they were originally isolated from warmer regions. Furthermore, the treatment with 4% alginate and 2% $CaCl_2$, for a period of 20 min, with a mean of 33% over a 6-week period, indicates that the slow release of nematodes in alginate beads at relatively slow rates can improve and prolong the shelf life of such a formulation. Regarding the important role that EPN play in the agricultural crop protection industry, their slow-release advantages can benefit production under cover, as in glasshouses. For example, Katumanyane *et al.* (2018) report a significant reduction in the number of fungus gnats, *Bradysia impatients* (Johannsen) (Diptera: Scaridae), in a cucumber glasshouse 21 days post the application of *S. yirgalemense*. An inherent storage tolerance for relatively high temperatures was clearly shown for South African species by Hill *et al.* (2015) and Kagimu & Malan (2019).

The properties of encapsulated IJ in sodium alginate could improve their effectivity against pest insects when broadcast in the field, as the layered film of the alginate protects against UV light. In relation to the above, Dlamini *et al.* (2020) mention how the temperature affected the results of two trial experiments, which did not differ significantly in terms of *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) reduction in blueberry tunnels, despite the low and high concentrations of *S. yirgalemense* used in the first and second trial, respectively. The recorded mean temperatures of the experiment were higher in the first trial than were those in the second trial.

In conclusion, the current study shows that the use of workable percentages of sodium alginate, together with $CaCl_2$, as well as the hardening bead time, should, in future, lead to the overcoming of the challenge set by the escape of the IJ from the beads, and to achieving their long-term storage and slow release into the soil. In the present study, the beads that were stored at 25°C, which is approximately the same as the optimal room temperature, were able to survive for weeks, which shows potential in terms of reducing the degree of refrigeration required during transportation, as well as in terms of reducing the storage-related costs. Considering the above recorded results, it would be interesting to know how the beads would release the IJ in the field, and how they would cause mortality against the targeted insect pest. Further glasshouse and field experiments are, therefore, recommended to provide additional insight.

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A. Nxitywa and A.P. Malan. Подготовка препарата на основе *Steinernema yirgalemense* посредством заключения их в альгинатные шарики.

Резюме. Широкое применение и коммерческий успех использования энтомопатогенных нематод существенно осложняются короткими сроками хранения готовой продукции. Разработка методов сохранения этих нематод, обеспечивающих их хранение без снижения эффективности против насекомых-вредителей имеют ключевое значение. Для удержания и сохранения инвазионных личинок *Steinernema yirgalemense* заключали в шарики на основе альгината натрия. Оценивали воздействие на личинок двух концентраций альгината натрия (2% и 4%), двух концентраций хлорида кальция (0.5% и 2%), а также времени отверждения шариков – 20 и 60 мин. Шарики альгината с нематодами хранили при 25°C и еженедельно, на протяжении 6 недель, оценивали число выходивших из них инвазионных личинок. Разрушение шариков в почве и инвазионность личинок исследовали также в течение 6 недель, используя в качестве экспериментального хозяина мучных хрущаков *Tenebrio molitor*. Наивысший выход личинок из шариков (127 ± 11 вышедших нематод) наблюдали при использовании 2% альгината натрия и 0.5% $CaCl_2$, при времени отверждения 60 мин. Лишь 33 ± 16 вышедших личинок было получено через 6 недель при обработке 4% альгинатом натрия и 2% $CaCl_2$ при времени отверждения 20 мин. Различия в характере обработки не влияли на инвазионность личинок для *T. molitor*. В садовой почве инвазионные личинки из альгинатных шариков успешно заражали мучных хрущаков, распадаясь поностью через 2 недели после внесения. Именно концентрация альгината оказалась самым существенным фактором, определяющим выход личинок из шариков.