



## Formulation of two native entomopathogenic nematodes at room temperature

Mona A.Hussein\* and M. A. Abdel-Aty

### ABSTRACT

In many commercial EPNs-based biopesticide companies, formulations ranging from the impregnation of EPNs on artificial sponge to highly advanced granular formulations have been developed. Major challenges have included the development of room-temperature shelf stability, ease of use, and contamination control. The aim of this work was to evaluate the suitability of two indigenous EPNs, *Heterorhabditis bacteriophora* (BA1) and *Steinernema carpocapsae* (BA2), for formulation and storage on Hydrogel, Kaolinite and Calcium Alginate at room temperature. The survival and virulence of both nematodes in the three formulations were discussed. Comparing the two nematodes, it was found that the storage potential of BA2 juveniles was only superior to that of BA1 in case of formulation with Calcium alginate. The pathogenicity of the two EPNs in the three formulations was tested against the wax moth, *Galleria mellonella*. It was concluded that BA2 was more virulent than BA1 to the larvae of the wax moth, *G. mellonella* in all tested formulae.

**Key words:** biopesticides, calcium alginate, entomopathogenic nematodes, formulation, Hydrogel, Kaoline,

### INTRODUCTION

Entomopathogenic nematodes (EPNs) in the genera *Steinernema* and *Heterorhabditis* are commercially available for the control of soil-inhabiting insects (Grewal *et al.*, 2005). They are safe for non-target vertebrates and to the environment (Ehlers, 2003). EPNs live in mutualistic symbioses with bacteria from the genera *Xenorhabdus* and *Photorhabdus*, respectively (Boemare, 2002). Since they are produced in large scale liquid culture, production costs have been significantly reduced and the application in horticulture, agriculture and forestry is increasing (Ehlers, 2001, Grewal *et al.*, 2005). Several companies in Europe, Asia and North America mass-produce nematodes either on a small scale *in vivo* or in large scale *in vitro* using bioreactors (Shapiro-Ilan and Gaugler, 2002).

Formulation technology of EPNs has made significant progress in the past 15 years. Once produced the nematodes can either be shipped in an active form on sponge or in liquids, but more usually they are formulated on to inert solid carriers (e.g. clays, vermiculite) in which the nematodes are partially dehydrated (Bedding, 1988; Grewal, 2000). In many commercial EPNs-based biopesticide companies, formulations ranging from the impregnation of EPNs on artificial sponge to highly advanced granular formulations have been developed and recently a novel formulation of tape-enclosed cadavers has potential for use in application

of EPNs was developed (Shapiro-Ilan *et al.*, 2010). The infective stage nematodes (IJs) need to be formulated in materials which guarantee survival for a period necessary to market the nematode product such as chitosan which has been used recently to coat *Steinernema carpocapsae* against Red Palm Weevil (Llacer *et al.*, 2009). Stable formulations have been achieved by immobilizing and/or partially desiccating IJs. These formulations allowed introduction of EPNs products with acceptable shelf-life into various market segments (Grewal, 1999). Therefore, the aim of this work was to evaluate the suitability of two Egyptian strains of EPNs, *H. bacteriophora* (BA1) and *S. carpocapsae* (BA2), for formulation and storage on hydrogel, kaolinite and calcium alginate.

### MATERIAL AND METHODS

#### Tested nematodes

Prior to experiments, the nematodes, *H. bacteriophora* (strain BA1, Hussein and Abo el-Souod, 2006) and *S. carpocapsae* (strain BA2) were cultured in *G. mellonella* at 25°C (Pests and Plant Protection Dept., NRC, Cairo) (Kaya and Stock, 1997). For all experiments, BA1 and BA2 were produced on filter paper (Whatmann No. 1) based on procedures described by Shapiro-Ilan *et al.* (2003); insects were inoculated with 200 IJs per insect. All infective juveniles (IJs) that emerged from the cadavers and moved to the water layer of the white traps (Woodring and Kaya, 1988) were collected daily for 6 days. Harvested IJs

were mixed and stored at, 15°C in aerated tissue culture flask with 35 ml of non-sterile tap water at densities of 5000 IJs/ml. Experiments were performed with 2-week-old IJs. Before being used in the experiments, the IJs were rinsed with tap water and allowed to pass through a sieve lined with a coarse paper filter into tap water and left at room temperature for 30 min.

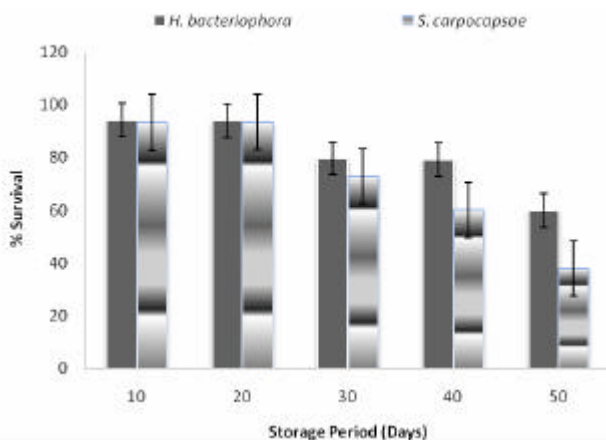
### Tested Formulae

Three materials were selected to test their viability as carriers for the nematodes as follows: hydrogel which is a colloidal superabsorbent polymer (SAP) that can absorb and retain extremely large amounts of a liquid relative to their own mass (Horie *et al.*, 2002). The second is kaolinite or kaolin which is a soft, earthy, usually white dioctahedral phyllosilicate clay mineral (part of the group of industrial minerals) with the chemical composition  $Al_2Si_2O_5(OH)_4$ . It has a low shrink-swell capacity and a low cation exchange capacity. The third carrier is calcium alginate, which is a water-insoluble, gelatinous, cream coloured substance. It is also used for entrapment of enzymes and forming artificial seeds in plant tissue culture.

### Preparation of the nematode formulae

A matrix of nematode gel was prepared as per Kaya and Nelsen (1985). The matrix was prepared by dissolving 2g of hydrogel, kaoline and calcium alginate in 100 ml water and blended for 4-5 minutes. Drops of nematode suspension (2000 IJs/ml) were mixed with the carrying material (hydrogel, kaoline and calcium alginate) in 4 x 9 cm plastic bags. An antifungal agent (0.05mg Streptomycin sulphate) was added to prevent the growth of microbes and pH was adjusted to 7.0. The bags were sealed with a plastic sealing machine and left at room temperature ( $25 \pm 2$  °C). Ten replicates for each treatment were done. Results

**Figure 1.** Viability of *H. bacteriophora* BA1 and *S. carpocapsae* BA2 on hydrogel as a storage carrier



were recorded at every 10 days and number of dead and live nematodes was recorded.

### Pathogenicity of formulated nematodes

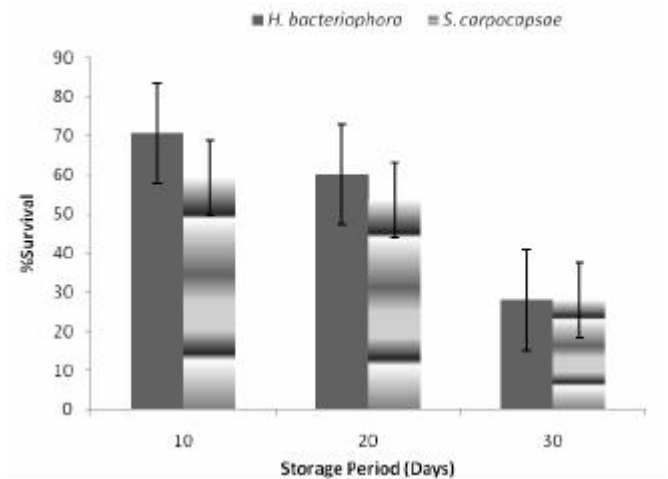
The experiments were conducted in earthen pots. One-hundred grams of sterilized soil was put in each pot and 15% moisture maintained according to the field capacity of the soil. IJs of BA1 and BA2 harvested from the three carriers were sterilized with 0.1% hyamine and three concentrations (1000, 500 and 250 IJs/ml) were tested against 10 last instar larvae of the greater wax moth, *G. mellonella*, which were reared on an artificial diet (Woodring and Kaya, 1988). All experiments were conducted at room temperature and replicated 15 times along with control. Observations on mortality were done at 24 intervals for three days. Data were analyzed using Microsoft Excel 2010 version.

## RESULTS

### Hydrogel

When BA1 was formulated with Hydrogel, the survival percent reached 94.3% after 10 days post-storage at 25 °C (Fig.1). No change recorded as the storage period expanded to 20 days. As the time proceeded, the survival percent of BA1 decreased to 79.8% and 79.2% after 30 and 40 days storage period, respectively. The lowest survival percent recorded for BA1 was after 7 weeks post formulation with Hydrogel. Moreover, in case of BA2, the survival ranged from 93.4 to 37.9% for the experiment storage periods 10 days and 50 days. No difference was recorded between the viability of the nematodes after 10 and 20 days post formulation with Hydrogel at 25 °C. For longer storage of tested EPNs, it seems from the results that the formulation of BA1 with hydrogel is more preferable than that of BA2.

**Figure 2.** Viability of *H. bacteriophora* BA1 and *S. carpocapsae* BA2 on kaoline as a storage carrier



**Kaoline**

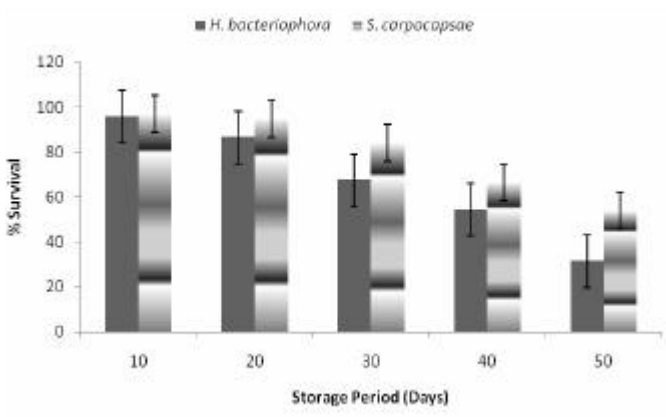
Results showed that using Kaoline as an individual material for nematodes storage is not preferred (Fig. 2). For all EPNs species, nematode numbers fell sharply during the following three weeks. Survival fell steadily over the first four weeks and then stabilized. For BA1 stored in kaoline, numbers of live nematodes declined sharply from 100% at the beginning of experiment to 70.7% after 10 days of storage. Meanwhile the reduction of BA2 reached over 41% after 10 days storage period in Kaoline at 25 °C. For both nematodes, the survival fell slowly after three weeks of storage with kaoline. The survival % recorded was 60.1 and 53.6 for BA1 and BA2, respectively. For both EPN species, alive IJs numbers dropped sharply (Fig. 2) during the following days and the survival

percent for BA1 and BA2 were recorded 28% each. The pH value was measured as 9.5.

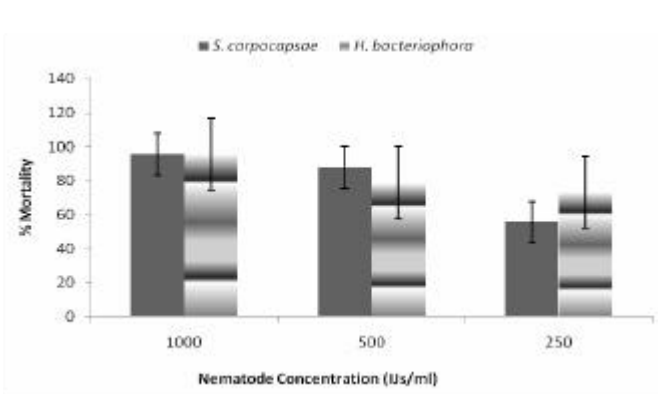
**Calcium alginate**

Based on data in Figure (3) and in contrast to hydrogel and kaoline, calcium alginate was preferable to the steinernematids formulation than the heterorhabditids. After 10 days post mixing with calcium alginate, *S. carpocapsae* recorded 97% survival compare to 95.9 for BA1. After 20 days, the nematode survival decreased to reach 95% and 86.9% for *S. carpocapsae* and *H. bacteriophora*, respectively. Four weeks after formulation with calcium alginate at 25 °C, the nematode *H. bacteriophora* recorded less survival than *S. carpocapsae*. After 6 weeks, numbers of live nematodes of BA1 fell sharply

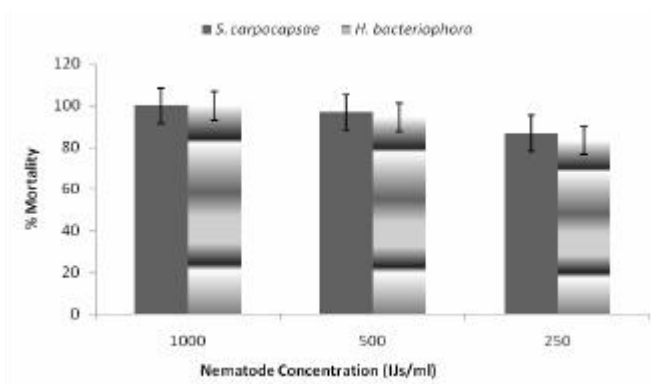
**Figure 3.** Viability of *H. bacteriophora* BA1 and *S. carpocapsae* BA2 on calcium alginate as a storage carrier



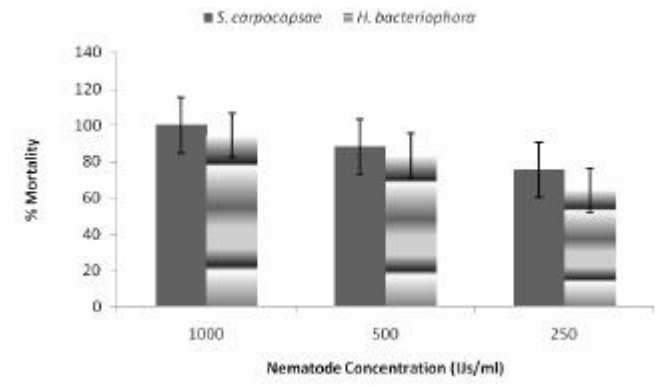
**Figure 4.** Pathogenicity of *S. carpocapsae* BA2 and *H. bacteriophora* BA1 formulated on hydrogel against *G. mellonella*



**Figure 5.** Pathogenicity of *Steinernema carpocapsae* BA2 and *Heterorhabditis bacteriophora* BA1 formulated on Kaoline against *Galleria mellonella*



**Figure 6.** Pathogenicity of *Steinernema carpocapsae* BA2 and *Heterorhabditis bacteriophora* BA1 formulated on Calcium Alginate against *Galleria mellonella*



than BA2. Similarly, the nematode numbers fell sharply during ten days and after 7 weeks of storage at 25°C.

#### Pathogenicity of formulated nematodes

The pathogenicity of IJs which were formulated in Kaolinite was more virulent against *G. mellonella* than the other two carriers (Fig. 4). At 1000 IJs/ml/10 larvae, *S. carpocapsae* caused more mortality. The mortality of the same species formulated in kaolinite recorded 100, 96.7 and 86.7% against *G. mellonella* at concentrations of 1000, 500 and 250 IJs/ml/10 larvae, respectively. Meanwhile, the heterorhabditis nematode formulated in Hydrogel induced mortality (Fig. 5). *S. carpocapsae* formulated in Calcium alginate (Fig. 6) caused high mortality than *H. bacteriophora* in *G. mellonella* larvae when treated with the same concentrations.

#### DISCUSSION

The methods of EPNs storage and formulation should meet two major criteria, a maximum survival of the IJs and a maximum conservation of their infectivity. Since EPNs entered commercial use for the biological control of insect pests, the problem of short shelf life has inhibited the expansion in the use of EPNs (Grewal, 2002). Since EPNs are aquatic multicellular organisms, their viability depends on retaining enough water inside and around their bodies (Chen and Glazer, 2005). To maintain survival and infectivity, it was necessary to keep a balance between reduction of metabolism and water availability. This investigation provides data on the influence of the storage stability of two Egyptian strains of EPNs, BA1 and BA2 IJs and compares the survival and infectivity of IJs formulated using hydrogel, kaoline and calcium alginate. This study was for the first time to use osmotically treated nematodes other than *S. feltiae* stored in calcium alginate granules at room temperature. The results have confirmed observations by Fan and Hominick (1991) who also recorded a positive influence of cold storage on the survival of nematode IJs. Energy reserves usually exhausted much faster as storage time increase (Jung, 1996; Georgis and Kaya, 1998). Low temperature can reduce the growth of these contaminants.

The results recorded in this investigation confirmed that recorded by Chen and Glazer (2005) who used calcium alginate to formulate *S. feltiae*. However, the granules preserved fresh *S. feltiae* for only 15–30 days at 22–25°C. Both *S. feltiae* and *S. riobrave* Cabanillas, Poinar, and Raulston exhibited desiccation avoidance behaviors as they rapidly migrated out of the granules, whereas *S. carpocapsae* exhibited no such migration out of granules (Grewal, 2002). In our study, IJs exited from the granules after 20 days at 25 °C, similar to the findings of Kaya and Nelson (1985). The formulation in clay cannot cope with the conditions likely to be encountered

during nematode transport. A loss of > 50% within two weeks is unacceptable. Further investigations are needed to improve the formulation in clay materials. Future activities will have to concentrate on defining an optimum humidity in the clay formulation which induces the quiescent state in the IJs and thus increases their viability. Comparing the two nematode species, it must be concluded that the storage potential of *S. carpocapsae* is inferior to that of *H. bacteriophora*. Future research will have to take some factors into consideration such as the storage potential and resistance to environmental extremes of different nematode strains, the production method and conditions, the environmental conditions in different formulation materials and packing technology to maintain optimum conditions during transportation.

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**Mona A. Hussein\* and M. A. Abdel-Aty**  
Safe Agriculture Group, Centre of Excellence for  
Advanced Sciences, Pests and Plant Protection  
Department of National Research Centre, El-Tahrir St.,  
Dokki-Cairo-Egypt, \*Email: monahussein4@yahoo.com

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