# Formulation of Entomopathogenic Nematodes for Crop Pest Control – a Review

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**Abstract**

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The different materials and methods used to develop biopesticides with entomopathogenic nematodes (EPNs) still limit the quality of the final product, reducing field efficacy and complicating application strategies. Therefore, the objective of this review is to identify priority areas to improve the EPN formulation process based on the scientific and technological research developed so far. The results show great progress in the EPN survival time, from 7 days to 180 days, through two types of formulations: water dispersible granules and calcium alginate capsules. Also, EPNs formulated and applied as insect cadavers showed higher efficacy for the pest control than the EPNs applied in an aqueous solution. We consider that the priority areas of research are: automated massive formulation and exploration of new additives with better properties that may lead to increase the infectivity in the field. It is concluded that the study of these aspects requires a systemic approach with greater involvement of the invertebrate physiology and systems modelling disciplines.

**Keywords**: *Steinernema* spp.; *Heterorhabditis*; insect cadavers; pellets, survival time

The excessive use of chemical pesticides in agriculture causes serious damage to soil, air, water, flora, fauna and human beings (Georgis 1992; Vos *et al.* 2000). Therefore, it is necessary to develop environmentally friendly alternatives to control soil pests, such as entomopathogenic nematodes (EPNs). The EPNs of the families Heterorhabditidae and Steinernematidae are obligate parasites of insects and have demonstrated to pose a high potential for the agricultural pest control (Ehlers 1996; Georgis *et al.* 2006; Lacey & Shapiro-Ilan 2008; San-Blas 2013; Tofangsazi *et al.* 2014; van Zyl & Malan 2014; Půža 2015). Also, they have been exempted from registration in the USA, are compatible with many chemical pesticides and amenable to genetic selection (Kaya & Gaugler 1993). These EPNs possess a symbiotic association with pathogenic bacteria of the genera *Xenorhabdus* and *Photorhabdus*, which are associated with *Steinernema* and *Heterorhabditis*, respectively (Poinar 1990). The infective juveniles (IJs) are the only free-living stage and serve three main functions: dispersal, host finding, and survival under environmental detrimental conditions (Grewal *et al.* 2006). When the IJs enter the body cavity of a host-insect, the symbiotic bacteria are released, multiply and the host death occurs within two days (Poinar & Grewal 2012). The IJs complete their development and reproduce for two or three generations inside their host. When food is depleted, a new generation of IJs exits from the host cadaver

to search new hosts (Grewal & Georgis 1999; Shapiro-Ilan *et al*. 2012).

Currently, the factors that affect the application of EPNs in the field include: markets, crops and target insects, formulation and shelf life, usage directions, technical support, cost and others (Georgis *et al*. 2006; Lacey & Georgis 2012). However, the formulation is one of the most important factors for the successful use of EPNs as microbial insecticides (Grewal 2002). The EPN formulation is a process of the transformation of living entities into a product that can be applied by practical methods. The EPNs are formulated to facilitate their storage, transport and application (Grewal 2002). The formulations are based on the fundamental principle of energy conservation: restricting their movements (physical restraint) or reducing oxygen consumption to induce an anhydrobiotic partial state (Grewal & Georgis 1999). An optimal formulation should show high and consistent quality, easy handling and transport, high effectiveness and an easy way to be applied in the field.

In the last 15 years, several reviews have pointed out the progress of the EPN formulation and application technology (Grewal 2002; Grewal & Peters 2005; Shapiro-Ilan *et al*. 2006, 2012). However, the most detailed review on the EPN formulation was conducted by Grewal (2002). Therefore, we consider necessary to perform a new review of the latest developments in EPN formulations.

In this review, we present a critical analysis of the progress and perspective of the EPN formulations in the last years, emphasising a systemic approach to include disciplines such as invertebrate physiology, materials science, and systems modelling.

## What is an EPN formulation?

Generally, the components of the formulations are: an active ingredient, a carrier and additives. Active ingredients in the formulations are EPNs, whereas the carriers used are solids, liquids, gels, and cadavers. The additives are various substances with different functions, such as absorbents, adsorbents, emulsifiers, surfactants, thickeners, humectants, dispersants, antimicrobials, and UV-ray protectors (Grewal 2002). The main purpose of the additives used in the formulations has been to increase the survival and maintain the virulence of the EPNs. Below, we present the EPN formulations currently developed.

## Formulations for storage and transport

*Aqueous suspension*. The most common EPN formulation is an aqueous suspension. It has been used mainly for storage, transportation, and application (Chen & Glazer 2005). Storage temperatures between 4 and 15°C have produced survival times of 6–12 months for *Steinernema* spp. and 3–6 months for *Heterorhabditis* spp. (Hazir *et al.* 2003). However, there are many factors that affect their survival time: sedimentation, high oxygen demand, decreased response of some species at low temperatures, susceptibility to microbial contamination, special storage conditions and appropriate concentration for each species (Grewal & Peters 2005). Also, the refrigeration requirements increase costs, hinder the transport (Grewal 2002) and involve the use of application equipment with specific requirements (Toepfer *et al.* 2010; Brusselman *et al.* 2012; Lacey & Georgis 2012; Beck *et al.* 2014; Shapiro-Ilan *et al.* 2015).

*Synthetic sponges*. The formulation in polyurethane sponges is accomplished by applying an aqueous suspension of 500–1000 IJs/cm$^2$, which results in an amount of 5–25 million IJs per sponge, which is subsequently placed in a plastic bag for storage. The EPNs formulated in sponges achieve a survival time of 1–3 months at 5–10°C (Grewal 2002) and for their release, the sponges are dipped in a bowl with water. This formulation is not appropriate for mass distribution because it needs refrigeration for storage, the release of the EPNs is time and labour demanding and great amounts of sponge waste are generated. For these reasons, this formulation is only used for storage and transport of small quantities of EPNs for the biological control of pests in home gardens in Europe and North America.

*Gels*. Yukawa and Pitt (1985) described a system for nematode storage and transport. The EPNs were homogeneously mixed with absorbent materials, such as activated carbon powder, to form a cream, but this formulation has presented the drawbacks of high cost, unpleasant handling and low stability at room temperature (Grewal 1998). Subsequently, Bedding and Butler (1994) and Bedding *et al.* (2000) developed an aggregate using polyacrylamide, where the EPNs were partially desiccated, but the survival time at room temperature was low and had difficulties to dissolve (Grewal 2002). On the other hand, Georgis (1990) reached a considerable improvement of this formulation through the use of calcium alginate sheets distributed on plastic

screens for EPN storage. Chang and Gehert (1991) encapsulated *Steinernema carpocapsae* in a matrix of macrogels, a partially hydrogenated vegetable oil paste containing mono- and diglycerides, which significantly prolonged the viability of the EPNs. The same authors (Chang & Gehert 1992) also developed a paste formulation in which the EPNs were mixed in hydrogenated oil and acrylamide, achieving 80% survival of *S. carpocapsae* after storage for 35 days at 24–35°C. However, this survival time is considered commercially unacceptable.

Alginate capsules represented an important advance in the EPN formulation. Chen and Glazer (2005) encapsulated *Steinernema feltiae* in this material, which was exposed to osmotic treatment before formulation, with 99.8% survival after 6 months at 23°C and 100% relative humidity. Goud *et al.* (2010) encapsulated *Heterorhabditis indica* with different concentration of EPNs and at different temperatures. In the research, the best combination of temperature, population density, and storage was 10°C, up to 1000 IJs per capsule and 90 days of storage. However, better understanding of nematode behaviour, physiology and biochemical process could lead to the further improvement of this formulation. Hussein and Abdel-Aty (2012) encapsulated nematodes of *Heterorhabditis bacteriophora* and *S. carpocapsae* in calcium alginate with survival values higher than 50% after 40 days. In general, with alginate capsules survival times up to 6 months at 25°C have been reached (Chen & Glazer 2005). Nevertheless, the extraction steps are time-consuming and expensive because a large number of screens and plastic containers are required, which makes this formulation unsuitable for large-scale applications (Grewal 2002). Also, in agricultural areas where there is not spray equipment availability, the use of this formulation presents serious difficulties.

***Clay and powder.*** Bedding (1988) encapsulated *S. feltiae*, *Steinernema bibionis*, *Steinernema glaseri*, and *Heterorhabditis heliothidis* in a hygroscopic attapulgite clay formulation with survival time of 8 weeks at 23°C. The formulation was called a "sandwich" type, because the EPNs are stored between two layers of clay. Products with this formulation were sold, but soon were discontinued due to poor storage stability, clogging of the spray nozzles, and a low nematode-clay proportion (Grewal 2002). Simultaneously, Capinera and Hibbard (1987) developed pellets consisting of a mixture of alfalfa meal, wheat flour, wheat bran, corn oil, and water.

They encapsulated *S. feltiae* and achieved a mortality of 78.1% on *Melanoplus* spp. under field conditions. Furthermore, Connick *et al.* (1993) developed wheat flour granules (Pesta) with *S. carpocapsae*, but achieved a low survival rate after 6 weeks of storage at 21°C. Besides it was observed that wheat flour and high relative humidity promoted the growth of fungi and bacteria. Therefore, Connick *et al.* (1994) added 0.2% formaldehyde to the mixture of wheat flour, bentonite, kaolin, and peat. They stored *S. carpocapsae* formulated as "improved" Pesta during 26 weeks at 21°C, obtaining 100% mortality of wax moth. These granules were evaluated by Nickle *et al.* (1994) in a greenhouse against corn rootworm and potato beetle larvae, achieving the 90% control. Silver *et al.* (1995) encapsulated the EPNs *S. carpocapsae*, *S. feltiae*, *Steinernema scapterisci,* and *Steinernema riobravis* in granules with diatomaceous earth, hydroxyethylcellulose, amorphous silica, fumed hydrophobic silica, lignosulfonate, starch, pregelatinised starch, and pregelled attapulgite clay achieving 90% survival after storage for 6 weeks at 25°C.

An increase of the survival time of *S. carpocapsae* was achieved with the dispersible granules (WG) developed by Grewal (2000a) with a survival rate greater than 80% and infectivity greater than 60% after 5 months storage at 25°C. Grewal (2000b) stored *S. carpocapsae*, *S. feltiae* and *S. riobravis* for 5 months achieving 90% survival at 25°C. Recently, Matadamas-Ortiz *et al.* (2014) encapsulated *S. glaseri* in diatomaceous earth pellets (Celite 209) by means of the downward vertical flow of hygroscopic clays in converging hoppers, with storage conditions at room temperature and ambient moisture, and reported 56% survival after 14.1 days. They proposed the mechanisation of the pellet production process. Finally, Cortés-Martínez *et al.* (2016) evaluated the effect of moisture evaporation from the diatomaceous earth pellets on the survival time and infectivity of *S. glaseri* stored at room temperature and high relative humidity. Also, they evaluated a moisture transfer model by diffusion and evaporation to describe the temporal behaviour of moisture content in diatomaceous earth pellets. It was found that the loss of moisture content by evaporation was related to the reduction of storage stability of the IJs in diatomaceous earth pellets.

In review, we verified the microstructure of pellets produced mechanically by Matadamas-Ortiz *et al.* (2014) with scanning electron microscopy (Figure 1), where we observed that the particle form-size and
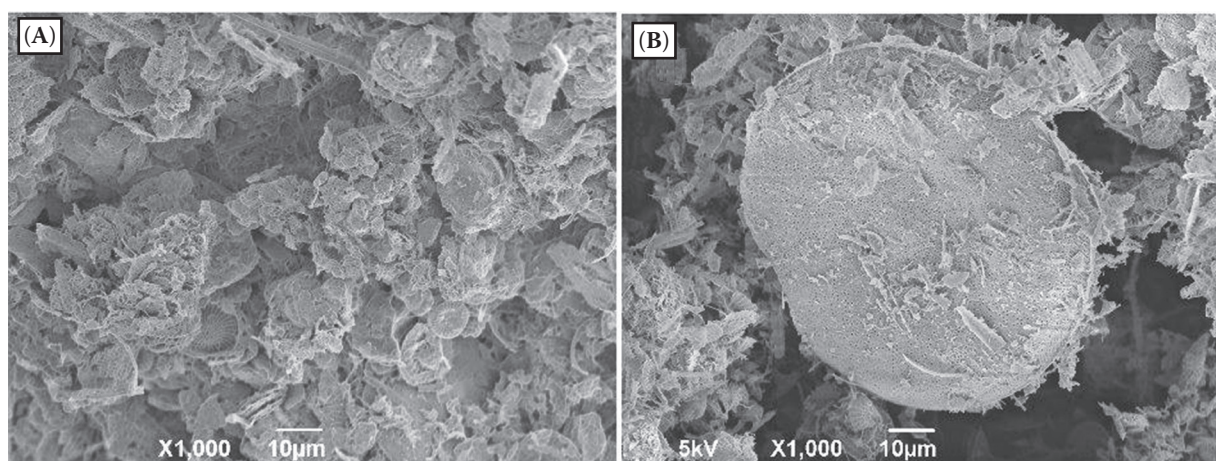
Figure 1. (**A**) Distribution of the particles and (**B**) variation of the particle size of the pellets. These were made with 100% diatomaceous earth

pore characteristics are not uniform. Thus, the survival time of the EPNs in granular reservoirs could be improved by controlling the moisture content of the granular structure. When the pellets have a lower rate of moisture loss, the EPNs can enter to a partial state of anhydrobiosis and therefore increase the survival time. Anhydrobiosis is considered as an important means of achieving the storage stability of entomopathogenic nematodes and is defined as a reversible and physiologically arrested state of dormancy induced by dehydration (Barrett 1991; Grewal 2000b; Grewal et al. 2006). Thus, moisture content and moisture loss rate of the pellets are essential research topics to enhance the storage stability of pellet formulations under conditions of room temperature and for retaining the biological control abilities of the EPNs (Grewal & Peters 2005; Perry et al. 2012; Cortés-Martínez et al. 2016).

In the pellet formulation, the factors extremely important for the survival time and the infectivity of the EPNs are: (a) properties of the liquid medium, especially the viscosity and pH of the suspension, (b) granular material properties: size and particle distribution, hygroscopicity, pH, and density, (c) physical phenomena present in the pellet: moisture and oxygen transfer, (d) storage conditions: temperature and relative humidity, and (e) elaboration method of the pellets (compactation and mechanical stress). All these factors contribute to design an encapsulation process that provides an adequate production rate, opportunity, accessibility, low cost and high quality. Therefore, it is necessary to involve disciplines of systems modelling that can predict the moisture content and moisture loss rate starting from structural properties (size and particle size distribution, hygroscopicity, pH of the material, and

density) and properties of their components (aqueous solution and additives) and based on the knowledge of the moisture requirements by EPNs to achieve a partial state of anhydrobiosis.

## Formulations for direct application in the field

*Gel*. With the aim of eliminating the disadvantages of releasing the EPNs from the alginate granules with sodium citrate, Kaya and Nelsen (1985) encapsulated the EPNs *S. feltiae* and *H. heliothidis* in calcium alginate granules coated by lipid membranes and fed to larvae of *Spodoptera exigua* Hübner. While feeding on the capsules, the larvae released the EPNs. When moisture was present, larval mortality was nearly 100%. To evaluate another possibility of applying directly the alginate granules, Kaya et al. (1987) developed sodium alginate granules with *S. feltiae* nematodes and a tomato seed in the same conglomerate; as the seed came in contact with moisture, the seed germinated and destroyed the granule structure, releasing the EPNs, and causing 100% mortality of *Galleria mellonella* L. larvae. Also, Navon et al. (1998) encapsulated *S. riobravis* in edible-to-insects calcium alginate gel and yeast extract used as a phagostimulant to improve the relative consumption rate and digestibility by *Spodoptera littoralis* Boisduval larvae, obtaining a 100% control. Afterwards, Navon et al. (2002) encapsulated *S. carpocapsae* in an edible-to-insects gel to control *Helicoverpa armigera* Hübner and *S. littoralis*, at a concentration of 1000 *S. carpocapsae* IJs/g, which caused 95% mortality in *H. armigera* and 100% in *S. littoralis* larvaes..

Due to the interest in direct application of EPNs in the field, Hiltpold *et al.* (2012) encapsulated EPNs in an alginate shell based on reverse spherification principles. First, a solution with a high concentration of $Ca^{2+}$ ions is dripped in an alginate bath, resulting in the formation of a spherical shell surrounding a liquid core. The liquid core was obtained by mixing demineralised water with gluconolactate where the nematodes *H. bacteriophora* were poured. Addition of xanthan gum increased the viscosity of the solution to help in the formation of spheres. After the formation of the spheres, the shell of the capsules was allowed to polymerise. In the field tests, the encapsulated *H. bacteriophora* nematodes were more effective to control *Diabrotica virgifera virgifera* LeConte than water sprays over the soil surface. However, EPNs were found to escape readily out of the capsules within a few days after encapsulation especially when stored at room temperature, which is inappropriate for commercialization. To overcome this drawback, Kim *et al.* (2015) modified the capsule properties by changing the reaction temperature for the capsule formation and by adopting the post-treatment of alginate capsules with excessive $Ca^{2+}$, and then evaluated the capacity of the EPNs to escape from the capsules manufactured under different conditions.

This formulation represented a step forward to direct applications in the field. In order to obtain a marketable product, we consider necessary to retain EPNs inside the capsule until needed, maintain EPN infectivity during storage, increase the survival time of the EPNs in the granules, investigate methods for the mass production of granules, evaluate methods to release the EPNs in the soil and the possibility of increasing the number of EPNs per granule.

*Infected cadavers.* The cadavers are another way to apply EPNs in the field (Shapiro-Ilan *et al.* 2001, 2008; Shapiro *et al.* 2003; Bruck *et al.* 2005; Del Valle *et al.* 2008a, b; Lacey *et al.* 2010; Raja *et al.* 2015). In this formulation, the insect cadaver serves as a reservoir to store the EPNs and then they are applied in the field. Laboratory tests have indicated that this method of application produces a better distribution of the EPNs in the soil than that obtained with the aqueous solution (Shapiro & Glazer 1996), increases infectivity (Shapiro & Lewis 1999) and is more effective (Shapiro-Ilan *et al.* 2003). However, it presents a drawback that during storage and transport the larvae can stick or break, which affects the effectiveness of the EPNs (Shapiro-Ilan *et al.* 2001).

To solve this problem, insect cadavers with coatings were applied to facilitate storage and transportation. Ansari *et al.* (2009) used a kaolin-starch mixture and Del Valle *et al.* (2009) used unflavoured gelatin; both authors demonstrated that the coating provided protection and promoted the conservation of the insect cadavers. Another alternative was developed by Shapiro-Ilan *et al.* (2010) and Morales-Ramos *et al.* (2013). They designed an automatic packaging machine to wrap EPNs-infected cadavers in masking tape. When it was evaluated with *Tenebrio molitor* L. cadavers, no damage was caused to the EPNs. This mechanical-electronic prototype facilitates the mass packaging of the IJs, reduces work in the process and standardizes the final product (Figure 2). A year later, Spence *et al.* (2011) developed a technology that involved desiccation of *G. mellonella* larvae to facilitate the transport and handling of EPNs-infected cadavers. Simultaneously, Deol *et al.* (2011) demonstrated the possibility of directly delivering EPN-infected cadavers through the commercial culture medium Miracle-Gro®. Also, Zhu *et al.* (2011) developed a mechanised system for the application of infected cadavers in the field (Figure 3). Wang *et al.* (2014) developed techniques for desiccation and cold storage to promote the mass production and application of EPN-infected cadavers in the field. Most recently, a new application approach was performed to apply EPNs to the field. This approach consists in the releasing of live insect hosts that were pre-infected with the EPN *S. carpocapsae* against insect pests living in cryptic habitats. The
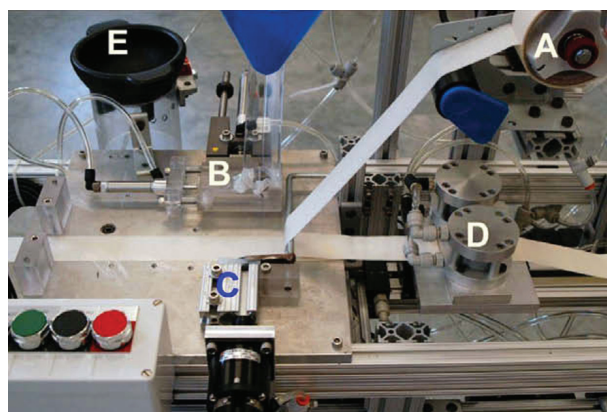
Figure 2. An automated formulation and packaging machine for enclosing nematode infected hosts in tape (Reprinted with permission from Elsevier)
The photograph is a close up with labeled parts including: (A) tape dispenser, (B) cadaver positioning device, (C) cadaver wrapper, (D) moving piston, and (E) cadaver holding bowl (Shapiro-Ilan *et al.* 2010)
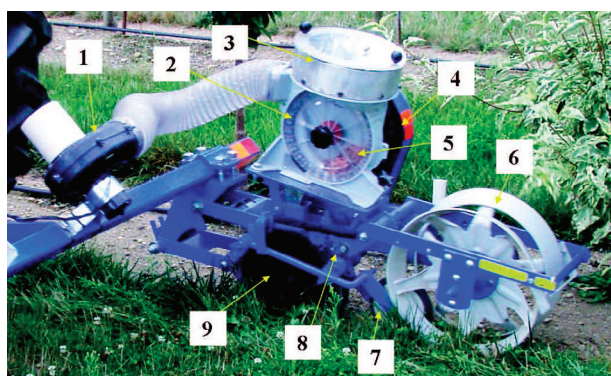
Figure 3. The complete delivery system mounted to one side of the tractor (Zhu *et al.* 2011) (Reprinted with permission of the first author Zhu H.)

(1) blower with air controller; (2) metering unit; (3) cadaver hopper; (4) roller chain; (5) nematode infected desiccated cadavers; (6) press-drive wheel; (7) packer wheel; (8) cadaver discharging tube; (9) double disk opener

approach was tested using two model insect pests: a chestnut tree pest, the goat moth *Cossus cossus* L., and a lawn caterpillar, *Spodoptera cilium* Guenée. *C. cossus* is considered a pest hard-to-reach via aqueous spray and *S. cilium* is more openly exposed in the environment. The percentage larval mortality of *C. cossus* was superior to the application in aqueous spray and the mortality of *S. cilium* was equal to the aqueous spray method. This approach showed an immense potential to control insect pests living in hard-to-reach cryptic habitats (Gumus *et al.* 2015).

EPN-infected cadavers have proved to be a good alternative to the direct application of EPNs for the control of soil pests, especially on the basis of the results of Ansari *et al.* (2009), Del Valle *et al.* (2009), Shapiro-Ilan *et al.* (2010), and Spence *et*

*al.* (2011) to solve the problem of the fragility of the cadavers. In order to obtain an optimal product, we consider necessary: increasing the survival time at room temperature, standardising the final product and evaluating these technologies with other nematode species of economic importance for crop protection.

The formulations of EPNs have presented a great progress in the pest control in the field. However, the reality is that successful application of EPNs depends on several critical factors such as: ultraviolet radiation, adequate soil moisture/relative humidity and temperature (Grewal 2002; Shapiro-Ilan *et al.* 2012). Improved efficiency and survival of EPNs can be achieved through genetic improvement. Improved strains of EPNs may possess several beneficial traits such as environmental tolerance, virulence, reproductive capacity (Strauch *et al.* 2004; Mukuka *et al.* 2010; Anbesse *et al.* 2012; Shapiro-Ilan *et al.* 2012). Besides, the isolation of native strains resistant to specific conditions of temperature and humidity is an approach that can enhance efficacy in the field (Emelianoff *et al.* 2008; Malan *et al.* 2011). In the future, it will be important to develop massive characterisation and selection techniques to obtain the fittest EPNs for formulation.

## Conclusions and Remarks

The use of EPNs as biopesticides against insect pests has grown rapidly in recent years. Since the late 1970s, the formulations have focused on increasing the storage time and preserving EPN infectivity in the field. In this review, we identified two types of EPN formulations:

Table 1. Nematode producers in Europe and in the USA. The table was taken from Kaya *et al.* (2006). However, in this table we present only the producers that we ratified that are currently operating

| Country | Company | Formulation | Nematode species |
|---|---|---|---|
| Germany | BASF | polymer, clay | Ph, Sf, Sc, Hb, Sk, |
| | e-nema GmbH | clay, polymer | Sf, Hb, Sc, |
| Switzerland | Andermatt Biocontrol | clay | Hb, Sc, Sf |
| The Netherlands | Koppert | clay, Polymer | Sf, Hb, Sc, S sp. |
| USA | Asa Jung Laboratory | clay | Sc, Sf |
| | BioLogic | bulk, dispersable granule, sponge, granular | Sc, Sf, Hb |
| | Hydro-Gardens | sponge | Sc, Sf, S sp., H sp. |
| | M & R Durango | sponge | Sc, Sf, Hb |

Ph – *Phasmarhabditis hermaphrodita*; Sf – *S. feltiae*; Sc – *Steinernema carpocapsae*; Hb – *Heterorhabditis bacteriophora*; Sk – *S. kraussei*; S sp. – *Steinernema* species; H sp. – *Heterorhabditis* species

(1) Formulations for storage and transport, results show great progress in the EPN survival time, from 7 to 180 days with water dispersible granules and calcium alginate capsules. Commercial products are distributed for storage and transport of nematodes in Europe and North America (Kaya *et al.* 2006). In Table 1 the EPN producers in Europe and in the USA are listed, but several of them require refrigeration and spraying equipment for application in the field.

(2) Formulations for direct application in the field are the most promising and provide the ability to directly apply EPN-based biopesticides for the control of insect pests. EPN-infected cadavers have positioned as the most viable formulation to apply EPNs for the pest control in the field. Also, Hiltpold-alginate granulation begins to position itself as a great alternative to apply EPNs in the field. However, it is still possible to improve the granular (pellet with clay and powder) formulation to design an optimal microstructured reservoir for storage, transport, and direct application of EPNs. Also, it is necessary to study the materials taking into account their physical and chemical interaction with the other factors of the formulation by changing the interacting conditions. To identify materials that can gradually induce the nematodes into a partial state of anhydrobiosis. It will be necessary to involve materials science, systems modelling disciplines and mechanical standardised process.

From 2010 to 2015, several studies were developed to facilitate storage and transportation of the EPN-infected cadavers, such as: coating and desiccation of the EPN-infected cadavers and prototype design to wrap EPN-infected cadavers in masking tape. Also, a new formulation was developed (Hiltpold-alginate granulation) as a great alternative to apply EPNs in the field. In general, the future research should be focused on the study of the formulations through a systematic approach to identify new and safe materials that can increase survival time and retain infectivity, and thus obtain an optimal formulation (standardisation of the final product) for direct application in the field. Finally, to achieve the best efficiency in the application of EPNs in the field is necessary to have more knowledge of the nematode physiology, soil ecology, systems modelling disciplines, materials science and other factors related with the farmer, such as the technological and socio-economic context and cultural conditions which may hinder the adoption of EPN technology for the widespread control of agricultural pests.

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