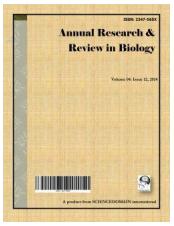

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Formulation of Insecticidal Nematode

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Authors' contributions

This work was carried out in collaboration between both authors. Author GD designed the study, wrote the protocol, and wrote the first draft of the manuscript. Author JG managed the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Insecticidal nematodes or entomopathogenic nematodes (EPNs) in the genera *Heterorhabditis* and *Steinernema* are lethal obligate parasites of insect pests. These nematodes carry specific pathogenic bacteria which are released into the insect hemocoel after penetration of the insect host. The economic importance of entomopathogenic nematodes (EPNs) is increasing as the nematodes are amenable for mass production, formulation, handling and application on a large scale and is currently marketed worldwide for use. The objective of the review is to discuss the principles of formulation and quality control, latest development and future perspectives of the EPNs formulation for the successful use of EPNs as bio insecticides.

Keywords: Bioinsecticides; insecticidal nematodes; symbiotic bacteria; mass production; formulation; quality control.

1. INTRODUCTION

Entomopathogenic nematodes (EPNs) are considered good candidates as biocontrol agent in integrated pest management programme. EPNs, also known as insecticidal nematodes have received the most attention because they have been utilized as inundatively applied

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augmentative biological control agent [1]. As a biocontrol agent they offer an ecologically safe alternative to chemical pesticides. Therefore, United States Environmental Protection Agency (EPA) has exempted them from all registration requirement and related regulation [2]. Entomopathogenic nematodes are ubiquitously distributed and comprise the families Heterorhabditidae and Steinernematidae. The families are not closely related phylogenetically but share similar life histories. These nematodes carry specific pathogenic bacteria, *Photorhabdus* spp. by Heterorhabditidae and *Xenorhabdus* spp. by Steinernematidae, which are released into the insect hemocoel after penetration of the insect host. The free-living, infective juvenile stage is able to infect the target pest. When a juvenile nematode locates a host insect, the juvenile enters via a natural opening; or, it may penetrate into the insect's cuticle. Once inside the host, the bacteria are released into the host, rapidly multiply, and produce antibiotics that kill the host insect generally within 48 hours. The bacteria protect the dead insect from invasion by other soil microbes. The infective nematodes complete one to several generations inside the host, feeding on the bacteria and nutrients within the dying host. Only when all the host tissues have been consumed, a new generation of juveniles emerges, all carrying the symbiotic bacteria with them in search of new hosts. One generation from egg to egg typically takes from 4 to 7 days. There are 2-3 two generations inside a host before the new juveniles emerge seeking a new host, so from the time of first infection by juveniles to the time new juveniles emerge may be from 8 to 14 days. Laboratory bioassays and semi-natural trial shows high virulence of the nematodes against many insect pests, but encouraging results can be obtained under field condition in convenient environmental conditions. This is due to their sensitivity to temperature gradient, ultraviolet radiation as well as low relative humidity.

According to Jones and Burges [3], formulation can be defined as an aid in preserving organisms, which helps to deliver them to their targets and, once there, assists in improving their mode of action. Even though nematodes can be produced in high numbers, it is necessary to keep them stable during storage, transport and application to ensure high efficacy in the field. Major challenges have included the development of room-temperature shelf stability, ease of use, and contamination control. Differences in storage stability among nematode species can be attributed to their thermal and behavioral adaptations. Each nematode species has a well defined thermal niche and an optimum temperature for the longest storage stability [4]. For instance, *S. feltiae* stores better at 5°C whereas *S. scapterisci*, *S. riobrave* and *H. bacteriophora* are more stable at 10°C. *S. carpocapsae* and *S. scapterisci* that adopt a quiescent posture during storage generally store better than the more active species such as *H. bacteriophora* and *S. riobrave.*

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 [5,6].

Formulation technology of EPNs has made significant progress with improved shelf life, scalability, and ease of use. This review emphasizes latest development and perspectives of the EPNs formulation for the successful use of EPNs as microbial insecticides.

2. BELOW GROUND APPLICATION

Though the adult stage of some insect pests also is susceptible, entomopathogenic nematodes generally are used for controlling the soil-borne larval or pupal stages of a pest. Therefore, entomopathogenic nematodes most often are applied by drench or band application or broadcast application. Selection of application method with proper formulated product may impact greatly the success of host location, infection, and control by the entomopathogenic nematodes. All of the formulations are intended to be mixed with water to release the nematodes through common application equipment such as small pressurized sprayers, mist blowers, electrostatic sprayers, along with irrigation systems or even aerial application.

Infected cadavers: In this formulation, the insect cadaver, mostly larvae of the wax moth, *Galleria mellonella* serves as a reservoir to store the EPNs and then they are applied in the field from which the nematodes emerge to seek new hosts. 'Biotrol NCS-DD-136' was the first product in 1970. Another product 'Neocide' where *S. carpocapsae* was raised on crickets were used against carpenter worm during 1981. Various

tests have indicated that this method of application produces a good distribution of the EPNs in the soil with efficacy [7-18]. However, physical damage to the insect cadavers (stick together or rupture) during handling, transport and application as well as the potential detrimental impact of various soil biotic and abiotic factors could reduce the efficacy of cadaver applications [9]. To solve this problem, insect cadavers with coatings in a powdery substance such as Mirasperse (a starch) + clay (calcium silicate) were applied to facilitate storage and transportation. Ansari et al. [19] used a kaolin-starch mixture to the *Heterorhabditis bacteriophora* Poinar CLO51 infected cadavers. Emergence of IJs from cadavers as well as efficacy was significantly higher in formulated in kaolin than from non-formulated cadavers. Additionally, after 1 year, cadaver applications provided >90% *H. philanthus* control, while aqueous applications of *H. bacteriophora* gave only 55% control. Del Valle et al. [20] observed the effectiveness of various protective coverings(a commercial calcareous powder, a commercial talc powder, and gelatin capsules) applied to *Galleria mellonella* insect cadavers in terms of their potential impact on the emergence and virulence of infective juveniles of *Heterorhabditis baujardi* LPP7.An automatic packaging machine to wrap EPNs-infected cadavers in masking tape was developed for suppression of *Diaprepes abbreviates* and *Aethina tumida* by Shapiro-Ilan et al. [16] and Morales-Ramos et al. [21] and was evaluated with *Tenebrio molitor* L. cadavers with encouraging results. Further formulation can be done by desiccating cadavers, which can increase the longevity of the nematodes by decreasing their metabolism. Spence et al. [22] developed a technology that involved desiccation of *G. mellonella* and investigated the feasibility of applying EPNs within desiccated insect cadavers infected by *Heterorhabditis bacteriophora* 'HB1', *Steinernema carpocapsae* 'All', and *Steinernema riobrave*. Exposure of desiccated insect cadavers to water potentials greater than -2.75 kPa stimulated IJ emergence. Among the nematode species examined, H. bacteriophora exhibited lower proportions of desiccated insect cadavers producing IJs than the other two species. Zhu et al. [23] developed a mechanized system by modification of seed metering unit for the application of infected desiccated cadavers by *Heterorhabditis bacteriophora* Poinar strain GPS11 in the field. The desiccated cadaver delivery system performed satisfactorily in both laboratory and field tests, and delivered the cadavers at a rate of 1.6, 3.3, or 6.6 cadavers/m length in the soil and buried 5 to 7.5 cm beneath the soil surface. Wang et al. [24] observed that under desiccation, the IJ (*S. carpocapsae*) yield in cadavers (*G. mellonella*) increased gradually and reached a maximum on day 5, whereas cold storage at 6.7°C caused negative effects on IJ production in desiccated cadavers.

Aqueous suspension: The most common EPN formulation is an aqueous suspension. It has been used mainly for storage, transportation, and application [25,26]. 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. [27]. However, there are many factors that affect their survival time: sedimentation, high oxygen demand, and decreased response of some species at low temperatures, susceptibility to microbial contamination, special storage conditions and appropriate concentration for each species [28]. Also, the refrigeration requirements increase costs, hinder the transport [5] and involve the use of application equipment with specific requirements [29-33]. A cost-effective delivery system for highly concentrated nematode suspensions has been developed that allows transport at ambient temperatures. A metabolic inhibitor and an antimicrobial agent are added to the nematode suspension. Approximately 7-7.5 x 10^9 *S. carpocapsae* IJs can be stored for up to 6 days in a 10 L container at ambient temperature. This method can also be used for shipping liquid *S. riobrave*.

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 [5] 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.

Vermiculite formulation: It is a significant improvement over the sponges. The advantages

include a more concentrated nematode product, longer storage stability, and more convenient application. Normally, an aqueous nematode suspension is mixed homogeneously with micronized vermiculite. This mixture is placed in thin polythene bags for storage. *S. feltiae* could be stored for 4-5 months and *H. megidis* for up to 3 months at 3-5°C. The vermiculite nematode mixture is added to the spray tank directly, mixed in water, and applied either as spray or drench. The only drawback of this formulation is the lack of ambient stability. The higher water activity required enables the growth of bacterial and fungal contaminants in the formulation. Therefore, nematodes should be cleaned carefully and packed under aseptic conditions [28].

Wettable powder formulation: Wettable powder formulation has been developed that allows storage of *S. feltiae*, *S. carpocapsae* and *H. megidis* product for 2-3.5 months at room temperature [29]. This formulation is very easy to apply due to its high dispersibility in water. This formulation will be suitable for commercialization of heterorhabditid nematodes.

Clay formulation: Bedding [30] 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 [31].

Pellet formulation: Capinera and Hibbard [32] 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. Connick et al. [33] developed wheat flour granules (Pesta) with *S. carpocapsae*, but achieved a low survival rate after 6 weeks of storage at 21°C. This formulation was called 'pesta' and also included filler and humectants to enhance nematodes survival. The process involved drying of granules to low moisture to prevent nematode migration and reduce risk of contamination. However, granules become very hard due to drying, and are difficult to dissolve. Besides it was observed that wheat flour and high relative humidity promoted the growth of fungi and bacteria. Therefore, Connick et al. [34] added 0.2% formaldehyde to the mixture of wheat flour, bentonite, kaolin, and peat. They stored *S. carpocapsae* formulated as 'Pesta' during 26 weeks at 21°C, obtaining 100% mortality of wax moth. These granules were evaluated by Nickle et al. [35] in a green house against corn rootworm and potato beetle larvae, achieving the 90% control. Silver et al. [36] encapsulated the EPNs *S. carpocapsae*, *S. feltiae*, *Steinernema scapterisci,* and *Steinernema riobravis* in granules with diatomaceous earth, hydroxyethyl cellulose, amorphous silica, fumed hydrophobic silica, lingo sulfonate, starch, pregelatinised starch and pregelled attapulgite clay achieving 90% survival after storage for 6 weeks at 25°C. The infective stage of *S. carpocapsae* was formulated in materials such as chitosan which guarantee survival for a period necessary to market the nematode product against Red Palm weevils [36].

Activated charcoal formulation: Yukawa and Pitt [37] described a system for nematode storage to restrict nematode movement wherein nematodes are homogeneously mixed with absorbent materials such as powdered activated charcoal. This formulation had several disadvantages including high cost, unpleasant to handle and no ambient storage stability [29].

Water dispersible granular formulation: Infective juveniles of *Steinernema* and *Heterorhabditis* species were survived up to 2-3 months at room temperature in water dispersible granular formulation (WDG) [38]. Infective juveniles are encased in 10-20 mm diameter granules consisting of mixtures of various types of silica, clays, cellulose, lignin and starches [36,39]. Grewal, [40] reported the commercial formulation that enabled storage of *S. carpocapsae* for 4-5 months and *S. feltiae* and *S. riobrave* for 2-3 months at 25°C. The water dispersible granules also enhanced nematode tolerance to temperature extremes [41]. Matadamas-Ortiz et al. [42] 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. Cortes-Martínez et al. [43] evaluated the effect of the diatomaceous earth pellets on the survival time and infectivity of *S. glaseri* stored at room temperature and high relative humidity which allows access of oxygen to nematodes during storage and shipping. WDG

enables easier and less expensive transport, improved ease of use of the nematodes by eliminating time and labour intensive preparation steps, a decreased container size and coverage ratio and reduced disposal material (i.e. screens and containers). However, this formulation was found to be prone to microbial contamination when stored at room temperature. Therefore, antimicrobial and antifungal agents are added to suppress the growth of microbes.

Gel formulation: Alginate capsules represent an important EPN formulation. This type of formulation is beneficial in preserving the lipids of nematodes characterized by a cruising foraging behavior and thus extends shelf-life. Kaya and Nelsen [44] encapsulated the EPNs *S. feltiae* and *H. heliothidis* in calcium alginate granules coated by lipid membranes and fed to larvae of *Spodoptera exigua.* While feeding on the capsules, the larvae released the EPNs. When moisture was present, larval mortality was nearly 100%. Kaya et al. [45] 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 causing100% mortality of *Galleria mellonella* larvae. This discovery subsequently led to the development of a commercial nematode product, which used thin sheets of calcium alginate spread over a plastic screen to trap nematodes [46]. Chang and Gehert [47] encapsulated *S. carpocapsae* in a matrix of macrogels, a partially hydrogenated vegetable oil paste containing mono and diglycerides, which significantly prolonged the viability of the EPNs. Chang & Gehert [48] 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. Bedding and Butler [49] and Bedding et al. [50] 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. The alginate based *S. carpocapsae* products were the first to possess room temperature shelf life of about 3-4 months, and led to an increased acceptability of nematodes in the high and medium value niche markets [29]. Navon et al. [51] 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* larvae, obtaining a 100% control. Navon et al. [52] 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. In general, with alginate capsules survival times up to 6 months at 25°C have been reached [26]. Chen and Glazer [26] encapsulated *S. 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. [53] encapsulated *H. 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. Hussein and Abdel-Aty [54] encapsulated nematodes of *Heterorhabditis bacteriophora* and *S. carpocapsae* in calcium alginate with survival values higher than 50% after 40 days. Hiltpold et al. [55] encapsulated EPNs (*H. bacteriophora*) in an alginate shell based on reverse spherification principles. Addition of xanthan gum increased the viscosity of the solution. 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 [5]. Also, in agricultural areas where there is not spray equipment availability, the use of this formulation presents serious difficulties. To overcome this drawback, Kim et al. [56] modified the capsule properties by changing the reaction temperature for the capsule formation. This formulation represented a step forward to direct applications in the field. A flowable gel formulation was developed to improve the ease of use by the consumer. In this formulation the nematodes are suspended in a viscous flowable gel that can be squeezed out of the paper tubes directly into the spray tank. This development did improve the ease of application, but nematode shelf-life in the flowable gel [57] was shorter than the alginate gel. Bedding and Butler [49] developed another gel formulation in which nematode slurry is mixed in anhydrous polyacrylamide so that the resulting mixture attains a water activity of 0.80 to 0.995, thus facilitate partial anhydrobiosis of nematodes.

3. ABOVE GROUND APPLICATIONS

Substantial progress has been made in recent years in developing EPN formulations,

particularly for aboveground applications, 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 *S. carpocapsae* against insect pests living in cryptic habitats. The approach was tested using two model insect pests: a chestnut tree pest, the goat moth *Cossus cossus* L., and a lawn caterpillar, *Spodoptera cilium* Guenee. *C. cossus* is considered a pest hard-to-reach via aqueous spray and *S. cilium* is more openly exposed in the environment. This approach showed an immense potential to control insect pests living in hard-to-reach cryptic habitats [25]. EPN applications to apple tree trunks for control of codling moth, *Cydia pomonella* (L.), were enhanced when the treatments included the sprayable fire-gel or wood flour foam as a protecting agent [15]. *S. carpocapsae* applications for control of the peach tree borer, *Synanthedon exitiosa* were greatly improved by a follow-up application of a sprayable gel (the gel is commonly used for protecting structures from fire) [58]. *H. indica* IJs stored in slurry formulation with antidessicants A.V. gel (1% and 10%), CMC 1%, Glycerine 1% was found to be enhanced IJs survivability up to 88- 90 days than they stored in suspension and granular formulation at room temperature [59]. Improved efficacy may also be achieved by relying on leaf flooding with the addition of surfactants to increase leaf coverage [60,61] and mixing EPNs with a surfactant and polymer [62]. *S. carpocapsae* caused high levels of suppression (98% efficacy) in the red palm weevil, *Rhynchophorus ferrugineus*, when applied in a chitosan (as adjuvant) formulation [63,64]. 'Brighteners' which protect nematodes from harmful ultraviolet radiation [65,66] and antidesiccants may well be combined in the future to formulate nematode products to be used as sprays to control foliar insects.

4. QUALITY CONTROL

To ensure consistent performance of formulated nematode products in the field, quality control measures are implemented in the production process [65]. Standardized quality tests should be conducted during production and storage, after delivery, and both before and after application, in order to certify that nematodes are in an optimal condition. Quality control measures include: determination of the percentage of dead nematodes in a batch; establishment of the movement ability of IJs; heat shock assays; insect bioassays; and neutral lipids estimation [67]. However, field efficacy is considered to be the ultimate measure of EPN quality (nematode virulence) [68].

5. COST ANALYSIS

The cost of insect pest control using EPNs is 10-60% more than chemical insecticides as the production and storage costs associated with EPNs tend to be higher [69]. Even though the short term initial costs are high, recycling of nematodes in nearby susceptible hosts after application takes place, with the result that nematodes control the insect pest for a prolonged period. This could lead to lower costs in the long term [69]. Grewal and Georgis [70] stated that the price of some nematode products is comparable to that of standard insecticides in certain markets. The production costs of EPNs need to be further reduced to ensure branching out from the high-value crop market into the low-value crop market. However, the positive impact of nematodes on the control of pest insects without harming either the environment or humans cannot be measured in monetary terms only.

6. CONCLUSIONS

Although steady progress has been made, the development of a formulation that is having a long shelf-life has not yet been achieved. Improvement of the production process by enhancing formulation efficacy, through understanding of nematode biology, ecology and behavior as well as favorable regulation requirements, will help in achieving the stated goal [71-76,77,78]. Further technological advancements are needed to expand the market potential of the nematode-based products.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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