Commercialization of Biopesticides Based on Entomopathogenic Nematodes

Mahmoud M. E. Saleh, Hala M. S. Metwally and Mokhtar Abonaem

Abstract Entomopathogenic nematodes (EPN) are microscopic organisms existing in the soil and kill insects with the aid of their symbiotic bacteria. EPNs are considered safe to mammals, environment, and non-target organisms. The importance in the commercial developments of EPNs is due to its ease of mass production and exemption from registration. The importance in the commercial developments of EPNs is due to its ease of mass production and exemption from registration. These nematodes are mass produced worldwide using in vivo or in vitro techniques. In vivo culture (culture in live insect host) is low technology, has low establish costs and the quality of these nematodes is high. In vitro solid production, i.e. growing the nematode and bacteria on two-dimensional arenas containing different media or on three-dimensional rearing system (crumbled polyurethane foam). This technique offers an intermediate level of technology and costs. In vitro liquid technique requires the largest establish funds and the nematode quality is decreased. Efficiency in EPN applications can be supported through improved formulations. Recently, extensive progress has been made in developing EPN formulations, particularly for foliar applications. Efficacy of nematodes can also be increased through discovery of new strains and species, strain improvement and developed application equipment or approaches.

Keywords Entomopathogenic nematodes · In vivo production · In vitro production · Formulation · Foliar application

1 Introduction

Entomopathogenic nematodes (EPNs) are non-segmented round worms that carry bacteria inside their bodies and inhabit the soil. EPN species classified under two genera *Steinernema* and *Heterorhabditis* and are symbiotically associated with certain enterobacteria [1, 2]. Both steinernematids and heterorhabditids pass through four

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juvenile instars before the maturing. Only the third-instar infective juvenile can survive outside their host and move searching for a host insect. Infective juveniles (IJs) carry symbiotic bacteria (*Xenorhabdus* spp. for steinernematids and *Photorhabdus* spp. for heterorhabditids) in their intestines and use it to kill their hosts by releasing them in the insect blood [3]. Nematodes locate and invade suitable insect hosts through the natural openings (spiracles, mouth, or anus) or in case of heterorhabditids through the cuticle of certain insects [4].

After the nematode entry to the host's hemocoel, it releases the bacteria in the insect blood. Bacteria develop and cause a septicemia that kill the insect host within 48 h. After the bacteria break down the haemolymph, the nematodes start feeding and develop and complete their life cycle. At 18–28 °C, the life cycle lasted 6–18 days depending on the insect host and the nematode species. Invading IJs belonging to *Steinernema* develop into females or males and *Heterorhabditis* IJs develop into hermaphrodites and this only the first generation. Up to three progeny generations develop inside one host and the nematode reproduction continues until host nutrients are depleted. Nematodes become third-stage IJs that emerge from the cadaver, survive in the environment and search for new insect hosts. The IJs are eligible to tolerate stresses fatal to other developmental stages. Therefore, they can be formulated and maintained for several months.

The attributes that EPNs have made them excellent insect biocontrol agents as they can kill their insect host within 48 h; have a wide host range; have the ability to move searching for hosts; they can be in vivo or in vitro mass produced; and present no hazard vertebrates and most non-target invertebrates [5–7]. The mutualistic relationship between the nematodes and the bacteria make them act together as a potent insecticidal complex against a wide range of insect species [8]. Therefore, EPNs are used as biological control agents in many agroecosystems in many countries [9, 10]. From a commercial standpoint, the production of a durable IJ stage and the symbiotic association with lethal bacteria are the most attractive features of steinernematid and heterorhabditid nematodes. The nematodes mass production plays a key role in the commercially development of insect pests management. The recent development in the nematodes mass production and formulation has increased the interest in these biocontrol agents. Therefore, there are many nematode products commercially available and used successfully in many crop systems against soil-borne insect pests. Nowadays, foliar application against insect pests on leaf foliage is possible in suitable formulations and has been elaborated in a number of cases.

The present chapter focuses on isolation, mass production, formulation, as well as field application of EPNs for the management of insect pests in different crops, vegetables and fruit orchards.

2 Entomopathogenic Nematodes Isolation

Nematode isolation is the first step to start establishing EPNs culture. Nematodes can be isolated from the soil where they naturally inhabit. The nematodes occur

naturally in most of the soil types. There are some factors increases the nematodes availability like the moisture and the presence of insects which are the elements that accompany the existence of plants. Here is the simplest and the most popular technique for collecting EPNs from soil. This technique based on two main steps, firstly collecting soil samples, and secondly isolating the nematodes from the soil samples.

2.1 Soil Samples Collection

Soil samples collected from under cultivated plants should be at the fine roots area and at a depth of 15–30 cm (Fig. 1a). If the cultivated plants are trees, the proper place to collect the samples is at last point of the tree shade at noon and from two sides of the tree. The recommended sample size is 750–1000 g soil. The soil samples could be collected in paper or plastic bags and should be marked with location details, date, and the associated vegetation (Fig. 1b). The collected samples should be kept at temperature between 10 and 20 °C during the transporting to the laboratory.

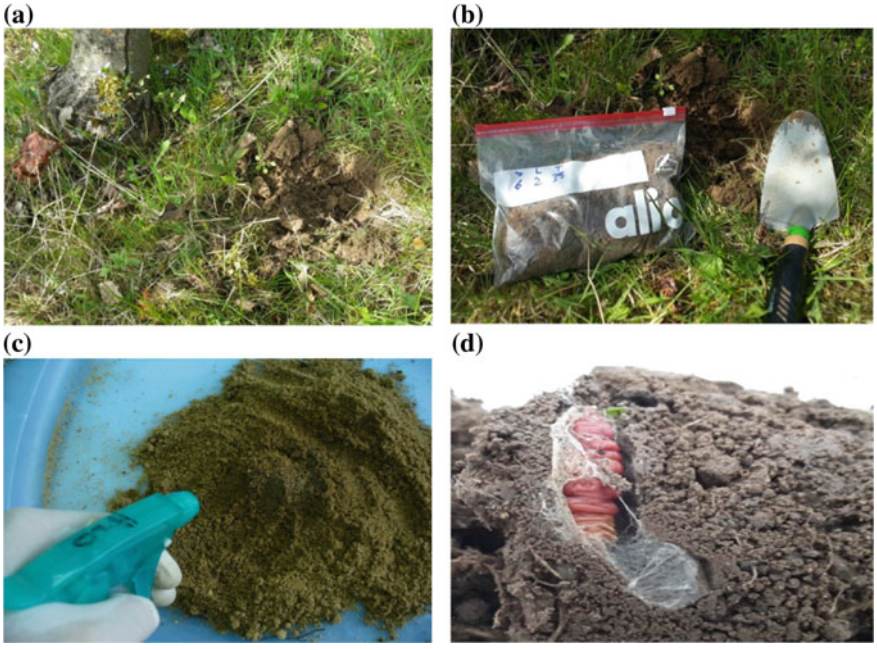

Fig. 1 Procedure of soil samples collection and isolation EPNs from the collected samples. **a** The fine root area where the soil sample should be collected and at depth of 15–30 cm. **b** Soil sample in a plastic bag and a hand shovel used for collecting the sample. **c** Increasing the moisture in the soil sample by adding water using a hand-sprayer. **d** Infected *Galleria mellonella* larva after 72 h in soil sample using insect-baiting technique

2.2 The Nematode Isolation from the Collected Soil Samples

This step based on the insect-baiting technique described by Bedding and Akhurst [11]. The collected soil should be moistened with water using a Hand-sprayer and be mixed gently (Fig. 1c). After that, the soil could be divided and added to plastic cups containing 7–10 *Galleria mellonella* last instar larvae. Then the cups should be covered by their lids after making small ventilation holes. The cups could be stored at room temperature or at 22–26 °C. The cups could be checked after 3–5 days to collect the dead larvae (Fig. 1d). The cadavers should be rinsed in water and placed individually in White-traps to collect the nematode progeny. Emerged nematodes (infective juveniles IJs) would be collected after 8–10 days. The infective juveniles (IJs) could be reproduced in vivo in *G. mellonella* larvae (see procedure below in this chapter).

3 Mass Production Approaches

Production process is vital for the success of EPNs in biological control. Production techniques comprise in vivo, and in vitro techniques (solid or liquid culture). In vivo production of EPNs is the suitable method for small scale field experiments. In vivo production is also suitable for niche markets and small farmers where a lack of capital and scientific expertise. In vitro approach is used when large scale production is required at reasonable quality and cost.

3.1 In Vivo Production of EPNs

Most EPNs intended for commercial application are produced in artificial media via solid or liquid fermentation. However, for laboratory research and small greenhouse or field trials, in vivo production of EPNS is the common method of propagation. This technique has managed to maintain itself as a cottage industry. In vivo production is probable to continue as small business projects for niche markets or in developing countries where labour is reasonably priced. Advances in this mechanization and host production have led to improvements in efficiency. In vivo culture is a two dimensional system that relies on production in trays and shelves [12, 13].

3.1.1 The Host Insect

The most common insect host used for EPNs in vivo production is the greater wax moth *Galleria mellonella* last instar larvae. Also, the yellow mealworm, *Tenebrio*

molitor, has been used for in vivo nematode production [14]. Here we focus on mass rearing of *G. mellonella*.

The greater wax moth *Galleria mellonella* L. is a serious pest attacking bee hives and stored bee wax. However, it is widely used as a model to study the interactions between pathogens and their hosts [15]. *G. mellonella* last instar larvae are the most used as insect hosts for producing EPNs in vivo. They have some advantages make them widely mass reared to use them for the previous purposes. The main advantages of this insect include their fast life cycle, the ease of rearing on artificial diets, the high susceptibility EPNs. There are many authors studied different proposed diets for rearing *G. mellonella* in laboratory conditions [16–19]. They studied the proposed diets effects on larval weight, longevity and/or fecundity of *G. mellonella*. Their studies results showed that no significant difference between the bee wax and artificial diets of main constituents: wheat bran, corn flour, wheat flour, rice bran, yeast, milk powder, honey, glycerol, malt, pollen, bee wax, and sucrose. The weight of full grown larva ranged between 0.2 and 0.25 g. Eischen and Dietz [20] found that adding 5% pollen; bee wax or honey significantly increased the longevity of *G. mellonella* moths.

For improving native EPNs in vivo production in *G. mellonella* larvae, Metwally et al. [21] studied the differences among the natural food (bee-wax) and four low cost different diets for mass rearing *G. mellonella*. The evaluation among these diets was based on the diet cost, the food consumption, the larvae number and weight, the larval lipid content and the nematode productivity of produced larvae. They found two diets that were costly lower than the bee wax and had no adverse effects on the studied parameters. The first suggested diet consists of wheat flour (350 g), corn bran (200 g), milk powder (130 g), yeast powder (70 g), honey (100 mL) and glycerol (150 mL). The second diet was the same previous diet, but with adding sorbitol (150 mL) instead of glycerol.

Diet preparing

In our laboratory, *G. mellonella* is permanently mass-reared using an artificial diet consists of Wheat flour 30%, Wheatgerm 30%, Corn grits 10%, Brewer's yeast 5%, Milk powder 5%, Honey 5%, and Glycerol 15%. The diet preparing could be fulfilled by firstly, mixing the dry ingredients together in one container and thereafter adding the liquid components to the mixture and manually mixing. The resulting mixture (diet) could be stored in a refrigerator till use for feeding *G. mellonella* larvae in the rearing containers.

Rearing protocol

G. mellonella culture could be started by collecting contaminated bee wax from any hives. After that, the contaminated wax could be placed in glass jars or plastic boxes as rearing containers. The rearing containers could be closed by their lids which have a handmade metal screen window for allowing gaseous exchange (Fig. 2a). As oviposition sites, Filter paper or tissue strips could be placed under the lid on the container edges (Fig. 2b). The containers should be incubated in the dark at 28–30 °C.

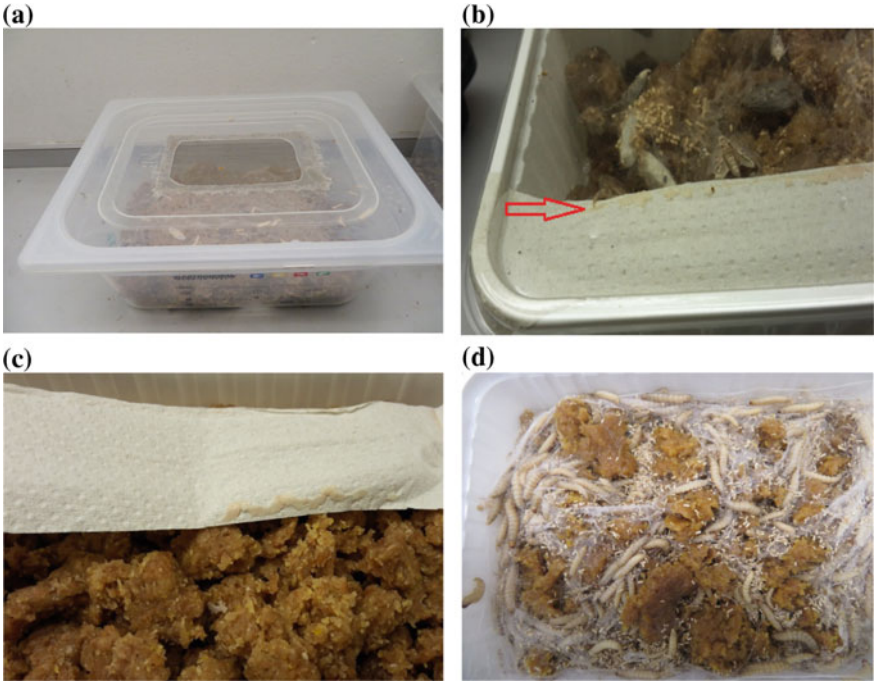

Fig. 2 Rearing protocol of host insect *G. mellonella*. **a** *G. mellonella* larvae inside a rearing box. **b** *G. mellonella* female moth lay eggs on paper tissue as an oviposition site. **c** *G. mellonella* deposited eggs on paper strip, then places on a fresh diet. **d** *G. mellonella* last instar larvae feed on artificial diet

The larvae feed and develop to pupae and then adults. After the mating take place, the female moths lay eggs on the paper stripes (Fig. 2b). The deposited eggs on the paper strips could be collected daily or every 48 h and placed in new rearing containers with a proper amount of the prepared diet (Fig. 2c). The eggs hatch and the emerged larvae feed on the diet and develop through six larval instars before pupations (Fig. 2d). The last instar larvae could be collected to be used in producing the nematodes in vivo. Some larvae should be left in the rearing containers to maintain the colony.

3.1.2 Culture Technique

In the first of many studies of this approach [22–26], they described systems for culturing EPNs based on the White trap that acquire advantage of the infective juveniles (IJs) usual migration away from the cadaver upon emergence (Fig. 3). These methods consist of inoculation, harvest, concentration and (if necessary) decontamination. Insects are inoculated with nematodes on dishes or trays lined with an absorbent

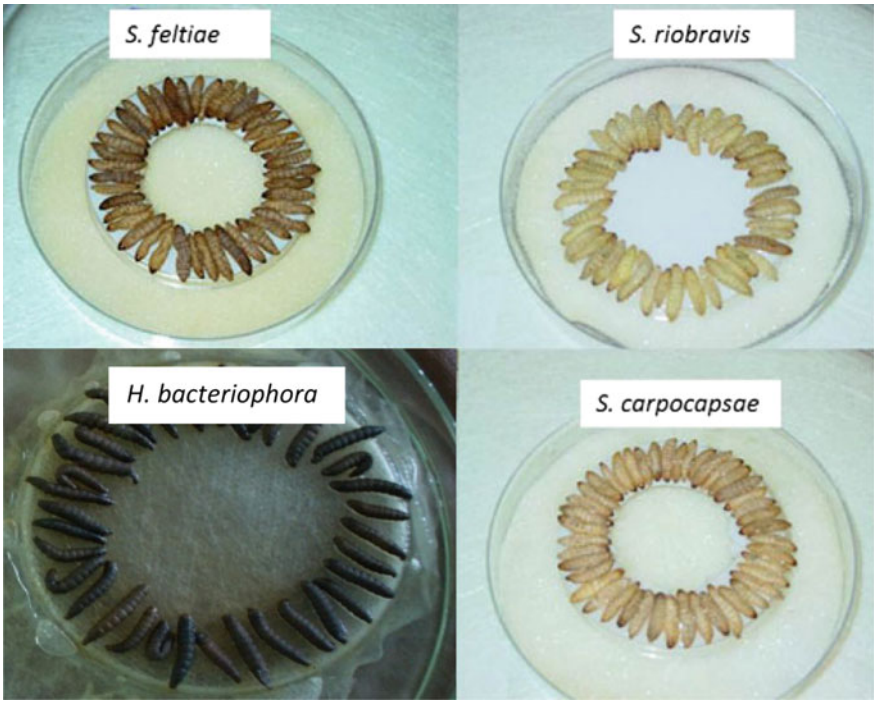

Fig. 3 White-trap: different EPN species (Nematode IJ) emerge from cadavers of *G. mellonella* larvae into water. Modified http://entnemdept.ufl.edu/creatures/nematode/entomopathogenic_nematode.htm

substrate. After 2–5 days, infected insects are transferred to the White traps i.e. harvest dishes. Following harvest, concentration of nematodes can be accomplished by gravity settling and/or vacuum filtration.

Based on the simple White trap methods indicated earlier, the process can be optimized and scaled-up to suit the needs of small field trials or cottage scale commercial ventures. Aspects that can be optimized and scaled-up include nematode species or strain as well as host species, inoculation rate and approach, host density and tray size, harvest, storage, and environmental conditions.

EPNs in vivo production protocol:

EPNs could be maintained as in vivo cultures in *G. mellonella* larvae by following production protocol:

1. Prepare nematode suspension in a concentration of 2000 IJs/mL water.
2. Apply 5 mL water to a Petri-dish (20 cm diameter) padded with two filter paper discs or paper tissue. The volume of water applied should make the discs moistened but not wetted.
3. Place about 200 *G. mellonella* last instar larvae in the previous Petri-dish contaminated with nematodes.

4. Transfer the dead larvae within 48–72 h to White traps.
5. Prepare modified White traps as described by Kaya and Stock [22]. Place an inverted Petri-dish (10 cm diameter) in another bigger Petri-dish (20 cm diameter). Fill the outer Petri-dish with 100 mL water. Drape a piece of muslin over the inverted Petri-dish with its edges touching the water. Place the cadavers were on the muslin (35–40 cadavers/dish) and cover it with the Petri-lid (Fig. 3).
6. After 10 days, new IJs could be emerged from the cadavers migrating over the muslin to the water.
7. Collect the migrated IJs in the water every 1–2 days.
8. Wash the collected EPNs by adding water and leave it for 10 min till the nematodes settle and remove the excess water. Repeat the previous step three times to remove the non-infective stages and the host tissues.
9. The washed IJs could be stored in water or special formulations at 12–14 °C.

3.1.3 Factors Affecting Yields

Production of EPNs differed among insect hosts [27]. Host diet that is improved for insect host production translates into improved efficiency in the overall process. The nutritional quality of insect host diet also impacts the quality and fitness of EPNs that are reared on those insects and thus improved efficiency and lowered costs for the entire process [28–30].

Nematode species is critical and can make a vast difference in IJs yields. Nevertheless, the nematode choice depends greatly on which insect pest one may be targeting (as efficiency will differ by species and strain). Nematode yield is generally proportional to insect host size, yet IJ yield per mg insect (within host species), and susceptibility to infection, is usually inversely proportional to host size or age. In addition to yield, ease of insect culturing and susceptibility to IJs are important factors when choosing a host. Finally, the choice of host species and nematode for in vivo production should depends on nematode yield per cost of insect, and the suitability of the nematode to the target pest [31, 32].

The technique of inoculation can be significant and may be optimized depending on nematode species and insect host [33]. Choices include pipetting, applying nematodes to insect food, or immersion the hosts in a nematode suspension. The inoculation rate (concentration of IJs and amount applied) should be optimized for each particular host and nematode species. In vivo production yields depend on nematode dosage. A dosage that is too low results in low host mortality and a dosage that is too high may result in failed infections due to competition with secondary invaders [33, 34].

Throughout the process, environmental conditions should be optimized such as for temperature, aeration, and relative humidity. Optimum production temperatures lie between 18 and 28 °C for different species [35–37]. It is also crucial to maintain adequate aeration and humidity throughout the production process [12]. To minimize overcrowding effects leading to oxygen deprivation, a pass-through HEPA filter

system is implemented. Advances in mechanization and production geared toward application of nematodes through infected host cadavers can improve efficiency and economy of scale [38].

Gaugler et al. [39] and Brown et al. [40] tried a scalable system for in vivo nematode mass production. Unlike the White trap, the LOTEK system of tools and procedures provides process technology for low-cost, high-efficiency mass production. The system consists of: (1) perforated holding trays to secure insect hosts during inoculation, conditioning (synchronizing nematode emergence), and harvesting, (2) an automated, self-cleaning harvester with misting nozzles that trigger IJ emergence and rinse the nematodes through the holding trays to a central bulk storage tank, and (3) a continuous deflection separator for washing and concentrating nematodes. The separator removes 97.5% of the wastewater in three passes, while nematode concentration increased 81-fold. The rearing system offers an increase in efficiency relative to the conventional White trap method with reduced laboratory and space. Morales et al. [41, 42] and Shapiro-Ilan et al. [43] improved the process of in vivo production of EPNs by automated separation of insect from media as well as automated inoculation and harvest (Fig. 4).

3.2 In Vitro Production of EPNs

In vitro culturing of EPNs is based on introducing nematodes to a pure culture of their symbiont in a nutritive medium. Such media must use sterile ingredients to avoid unwanted bacterial contamination, retain the nematode's specific symbiotic bacterium and provide all the necessary nutrients.

3.2.1 Solid Culture

EPNs were reared in vitro for the first time on a solid medium axenically [44, 45]. Thereafter it was realized that growth increased with the presence of bacteria. Then, the importance of the natural bacterial symbiont, and monoxenic culture was recognized [46] and has been the basis for in vitro culture. House et al. [47] formulate a dog food based medium to produce *Neoaplectana carpocapsae* on a commercial scale. Hara et al. [48] and Wouts [49] who stressed on monoxenicity, reported that solid culture was first accomplished in two-dimensional arenas e.g., Petri dishes, containing various media based on dog food, pork kidney, cattle blood, and other animal products at a cost of $0.28 per million.

Bedding [50, 51] reported practical solid culture technology that was a seminal step in nematode production because it leapt from two- to three- dimensional substrates. Bedding flask cultures involved thinly coating crumbed polyurethane foam sponge with poultry offal homogenate. Sterilizing the medium in large autoclavable bags and adding the appropriate bacterium and nematode and was able to produce about 50,000 million IJs of in a week. In Pakistan, different species of EPNs were

Fig. 4 Larval tray system. **A** Stacks of modified type 3 trays sitting on top of one unmodified type 3 tray and a dolly. **B** Open system showing larvae with food on a modified tray (**a**) and frass collected in the unmodified tray at the bottom (**b**). Cited from Shapiro-Ilan et al. [38]

mass produced using chicken offal media [52]. Nematodes can be harvested within 2–5 weeks by placing the foam onto sieves, which are immersed in water. IJs migrate out of the foam, settle downward, and are pumped to a collection tank; the product is cleaned through repeated washing with water. Media for this approach were later improved (for cost and consistency) and may include various ingredients as peptone, yeast extract, eggs, soy flour, and lard. Solid culture method is economically feasible up to a production level of approximately 10×10^{12} nematodes/month [53, 54].

The produced yield of in vitro culture of EPNs depends on different factors quality [55–58].

(1) Nematode inoculum size that affect yield in some strains but not others.
(2) Culture time is inversely related to temperature and should be optimized for maximum yield on a species or strain basis.
(3) Media composition can have a substantial effect on nematode yield. Increasing the quantity of lipids will increase nematode yield and quality. The greatest accumulation of lipids per dry weight was achieved by growing nematodes in *Popillia japonica* and solid culture [59]. It is cleared that level of polar lipids was higher in nematodes produced in artificial media. So, artificial media composition should be adjusted to meet the nutritional composition of a natural host. The optimum physical and chemical components of the medium for maximum production of EPNs were studied by Dunphy and Webster [55]. Tryptic soy broth and yeast extract and D-glucose enhanced the growth and yields of EPNs. Jewell and Dunphy [60] suggested that changes in medium total lipids, neutral and polar lipids, phosphatidylcholine and total protein did not affect nematode development however; changes in total medium carbohydrate did affect IJ yields. Tangchitsomkid et al. [61], Somwong and Petcharat [62] compared the growth of different EPNson different modified artificial media The lipid agar medium in the number of harvested nematodes was 60.8×10^6 IJs per one liter. The cost of nematode production by these modified media is 4.7 times less than that of the lipid agar medium. Yoo et al. [63], Abu Hatab and Gaugler [64] produced *H. bacteriophora* in media containing various lipid sources. They revealed that lipid source significantly affected lipid quantity and quality in *H. bacteriophora*. Media supplemented with extractable insect lipids produced yields 1.9 times higher than did beef fat- or lard-supplemented media. The modified dog biscuit medium recorded the positive result with respect to successful mass production of *H. indicus*. Ehlers [65] described the biology of the nematode-bacterium complex and advised that mass production of EPNs must be directed towards media development and cost reduction, as the bacteria are able to metabolize a variety of protein sources to provide optimum conditions for nematode reproduction. An inoculum level of 2000 IJs per flask yielded highest nematode of 60.11×10^6 IJs per flask which was significantly superior over other inoculum levels [66].

The inoculum size and the time are important for optimizing the final yields of IJs. The highest yield for *H. bacteriophora* was found with an inoculum of 10 IJs per flask, which was tenfold of the optimal inoculum for *S. carpocapsae*. Relationship between inoculum sizes, population development and the final IJ populations of these nematodes should improve the efficiency of commercial nematode production [58].

Bedding et al. [67] developed a culture vessel comprising a tray with side walls and overlapping lids that allowed gas exchange through a layer of polyether–polyurethane foam. These trays are particularly well suited for developing countries as forced aeration is not necessary, making this system independent from cuts in the power supply. Nematodes can be extracted from solid media with centrifugal sifters, or

by washing nematodes out of the sponge in simple washing machines and then separating the IJs by sedimentation or migration.

Gauglar and Han [68] stated that the approach was expanded to autoclavable bags with filtered air being pumped in Ehlers [69] reported that mass production relies on the scaling-up of culture volumes from flask cultures to volumes of several cubic meters. Stability of beneficial traits is a prerequisite for production of high quality insect control nematodes. Beneficial traits of nematodes are reproduction potential, longevity of the IJs, their host seeking ability and infectivity and tolerance to environmental stress factors.

In this context, Metwally [70] investigated the possibility of in vitro solid mass production of two Egyptian nematodes *S. carpocapsae* BA2 and *H. bacteriophora* BA1 on different agar media in comparison to two worldwide nematodes *S. riobrave* and *H. marilatus*. The Egyptian isolate *S. carpocapsae* BA2 can be successfully culture on all tested media. This was the first trial to propagate the Egyptian isolates *S. carpocapsae* BA2 and *H. bacteriophora* BA1 on an artificial medium (Fig. 5). The worldwide species *S. riobrave* and *H. marelatus* can be successfully culture on all tested media. Also, we determined the impact of inoculum sizes (bacteria and nematodes) on the growth and yield of *S. carpocapsae* BA2 and which inoculum size result in the greatest yield.

Also, the appropriateness of mass production of local and worldwide EPNs using Bedding flasks has been clarified in two solid media, modified Wouts medium and modified dog food medium. Neither *S. carpocapsae* BA2 nor *H. bacteriophora* BA1 could continue growth in the studied media in Bedding-flasks. However, the world wide species, *S. riobrave*, *S. scaptrisci*, and *H. marelatus* succeeded in propagation in Bedding-flasks (Fig. 6). Worldwide species *S. riobrave* achieved the highest offspring production scoring 45.54×10^6 IJs/flask.

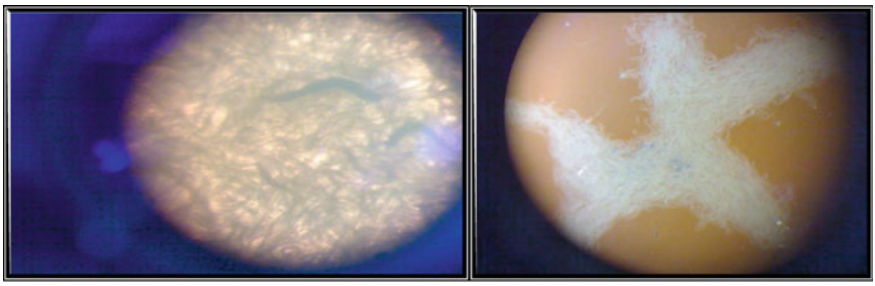

Fig. 5 Microscopic examination of *Steinernema carpocapsae* BA2 propagated on agar plates (x = 40). Reprinted from Metwally [70]

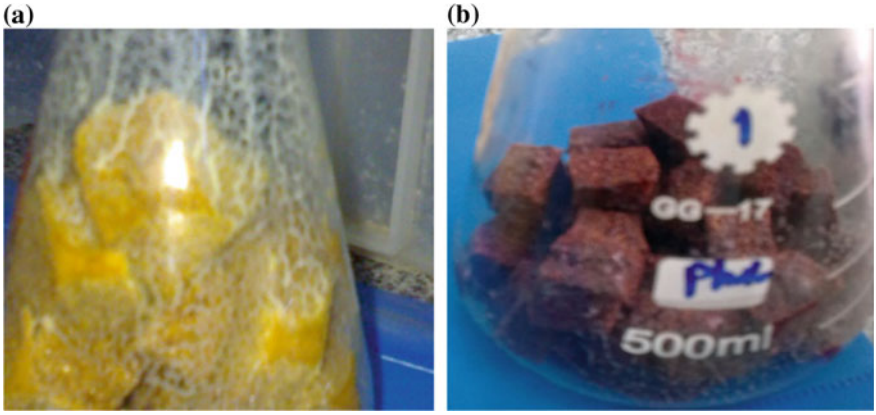

Fig. 6 Infective juveniles (IJs) started in emerging on the walls of the conical flasks. **a** *Steinernema riobrave*, **b** *Heterorhabditis marilatus*. Cited from Metwally [70]

3.2.2 Liquid Culture

Liquid rearing for EPNs was aimed first by Stoll [71]. Symbiotic bacteria are cultured in the shaker followed by the nematodes [72, 73]. The yield reaches approximately 400 IJs/mL at 21–25 °C and the yield was more in the dark.

Factors affecting yield for in vitro liquid culture:

1. The requirements of adequate aeration (without shearing).
2. The life cycles and reproductive biology of both genera. Males and females in Steinernematids are capable of mating in liquid culture [74] thus; maximization of mating is supreme and can be accomplished through regulation of ventilation [75]. However, mating in heterorhabditid is not applicable in liquid culture because the first generation is exclusively hermaphrodites and, although subsequent generations contain amphimictic forms, they cannot mate in liquid culture [74]. Thus, maximizing heterorhabditid yields in liquid culture depends on the degree of recovery. While levels of heterorhabditids recovery in vivo tend to be 100% [76], recovery in liquid culture may range from 0 to 85%.
3. Recovery can be affected by nutritional factors, aeration, CO_2, lipid content, and temperature [63, 76, 77].
4. Also, yield of EPNs from liquid culture may be affected by additional factors including nematode inoculum, species and media [77, 78]. The vital constituent of the liquid culture media is lipid source [63, 79]; glucose [80] and yeast extract content [81].
5. EPNs yield is inversely proportional to the size of the species [77]. Maximum yields reported include 300,000 and 320,000 IJs/mL for *H. bacteriophora* and *S. carpocapsae*, respectively [78].

4 Application and Formulation of EPNs

EPNs have used successfully against soil borne insect pests. But also foliar application against some insect pests has been studied in some cases. Also, formulations and application methods were investigated by many researchers.

Application technique is vital for the success of (EPNs) in biological control. Improved efficiency in applications can be supported through improved delivery means (e.g., optimization of spray equipment). Recently, essential advancement has been made in formulations of EPNs, particularly for foliar applications, e.g., mixing nematodes with surfactants or polymers. Insect host cadavers can improve nematode persistence and decrease the extent of nematodes desired per unit area.

EPNs can be applied with almost all agriculture ground tools, e.g., pressurized and electrostatic sprayers and as aerial sprays. The application tool used depends on the cropping system. The volume, agitation, nozzle type, pressure and recycling time must be taken into consideration [82–84]. Applicators may also be using other techniques such as through baits or subsurface injection. A range of formulations may be used for applying EPNs in aqueous suspension including water dispersible granules (WDG), activated charcoal, alginate and polyacrylamide gels, clay, peat, polyurethane sponge and vermiculite.

4.1 Factors Influencing Application Success

Mainly, the suitable nematode must be matched with the exacting target pest. The virulence of nematodes, persistence and environmental tolerance must be taken into consideration [85]. Furthermore of paramount significance, to be effective, entomopathogenic nematodes usually must be used to soil at minimum rates of 2.5×10^9 IJs/ha or higher [32]. Seasonal re-application is frequently essential where nematode populations will remain high enough to provide effective pest control for 2–8 weeks after application. Biotic agents can affect on nematode applications. EPNs have been announced to act synergistically with some entomopathogens such as *Bacillus thuringiensis* [86] and *Metarhizium anisopliae* Sorokin [87–89] however, other studies indicate antagonism, e.g., *Beauveria bassiana* [90].

Application of EPNs depends on some significant factors including temperature, protection from ultraviolet radiation and sufficient soil moisture [83, 91]. Certainly, entomopathogenic nematode applications for aboveground pests have been strictly limited due to environmental obstructions (e.g., UV radiation or desiccation) that reduce survival and efficacy [83, 92]. Also, Soil parameters can be vital for belowground or surface applications. Soil texture affects nematode movement and survival [91, 93]. Soil pH can affect entomopathogenic nematode distributions [94]. Chemical pesticides and fertilizers may be affected on entomopathogenic nematodes [95]. The relationship between chemical pesticides and EPNs fluctuates based on the nematode species or strain, dosages, and specific chemical and timing of application [96, 97].

Improved efficacy in nematode applications can be supported through enhanced formulation. Efficacy may also be accomplished on foliage with the addition of surfactants to increase leaf exposure [98, 99]. Nematode applications for control of the lesser peach tree borer were significantly enhanced by application of a sprayable gel [100]. Cadaver application technique increase nematode dispersal [101], infectivity [102], and survival [103]. This application may be facilitated through formulations that have been developed to protect cadavers from rupture and enhance ease of handling [104, 105] and progress of mechanized equipment for field application [106]. Also, advanced applications with EPNs can be accomplished through strain improvement or enhanced levels of various beneficial traits of EPN such as environmental tolerance, pathogenicity and progeny production capacity. Species discovery or genetic enhancement via selection, hybridization or molecular manipulation enhances entomopathogenic nematode application [9, 107].

4.2 Nematode Applications

There are many studies on nematodes utilization against insect pests in different crops, vegetables and fruit orchards. Here we present some studies performed using EPNs against some insect pests in different crop systems.

4.2.1 Apple

Sammour and Saleh [96] conducted a laboratory and field experiments to study the compatibility between two Egyptian entomopathogenic nematodes, *Heterorhabditis bacteriophora* (S1) and *Steinernema carpocapsae* (S2) and two well known organophosphate insecticides (Cidial 50% EC and Basudine 60% EC) to control *Zeuzera pyrina* larvae in fasting apple trees.

4.2.2 Sugarbeet

Saleh et al. [108] applied EPNs on sugar beet crop infested with larvae, pupae, and adults of the sugar beet beetle *Cassida vittata*. They applied *S. carpocapsae* at a concentration of 1000 IJs/mL. Within one week, the results were 65, 92, and 57.3% mortality in larvae, pupae, and the adults, respectively. Also, Saleh et al. [109] used entomopathogenic nematodes (EPNs) for the biological control of the sugar beet fly *Pegomyia mixta* in Egypt. They applied *S. feltiae* or *H. bacteriophora* against *P. mixta* on Sugar beet plants in field bioassay. The results were respectively 81.3 or 75.9% reduction in the larval population.

4.2.3 Peach

Saleh et al. [110] studied the efficacy of the new isolate *Heterorhabditis marelatus* D1 from Egypt against the peach fruit fly, *Bactrocera zonata* (Saunders). When the new isolate applied to the soil, resulted over 77% of *B. zonata* adults emerged from their pupae within 48 h after emergence.

4.2.4 Potato

Moawad et al. [111] studied treatments of EPNs *S. carpocapsae* and *H. bacteriophora* for the biological control of the potato tuber moth, *Phthorimaea operculella* (Zell.) infesting potato tubers in the soil. Their work included protective (against insects outside the tubers) and curative (against insects inside the tubers). In the protective treatments the nematodes decreased up to 100% of the pest population.

4.2.5 Maize

Saleh and El-Kifl [112] applied *H. bacteriophora* on the hibernating larvae of corn borer, *Ostrinia nubilalis* inside their tunnels in stored corn stalks. They found that the nematodes were able to kill the hibernating larvae inside their tunnels.

Saleh et al. [113] treated *H. taysearae* and *H. bacteriophora* on corn plants infested with *Sesamia cretica* larvae. Within one week, the applied nematode species resulted 67.8 and 40.6% larval mortality, respectively. El-Wakeil and Hussein [114] evaluated the efficacy of two EPN species against *Sesamia cretica* larvae infesting corn hearts. One week post spraying *H. bacteriophora* and *S. carpocapsae*, the results were 97% and 100% larval mortality, respectively. After two weeks, the larval mortality was 100% due to both EPN species.

4.2.6 Date Palm

Saleh et al. [115] applied the nematodes on soil around the palm trunks targeting the adults of red palm weevil, *Rhynchophorus ferrugineus* in soil or the pupae inside their cocoons aggregated in the palm leaf petioles (Fig. 7). The applications of *H. bacteriophora*, *H. indica*, and *S. carpocapsae* caused mortality in pre-pupae and pupae inside their cocoons reached 98.3%, 90.4%, and 60.3%, respectively. *Steinernema* or *Heterorhabditis* on soil resulted up to 90% mortality in the insect adults.

5 Conclusion

Entomopathogenic nematodes are important biological control agents against soil pests as well as plant-boring pests. Nematodes have been commercially developed by several companies in North America, Europe and Australia and for the control of

Fig. 7 Nematodes application on soil around the palm trunks targeting the adults of red palm weevil, *Rhynchophorus ferrugineus*

a vast range of pests. Progress in developing large-scale production and application technology has led to the expanded use of EPNs. In vivo nematode production is the suitable technique for niche markets and small-scale field-testing. This method requires the least capital outlay and the least amount of technological proficiency, but is blocked by the costs of insect host media. Thus, producing the insect hosts "in-house" in low-cost culture and mechanizing the process is a must for large scale production of EPNs especially in a developing country like Egypt. Also, technical enhancement and reducing the cost of the ingredients of the artificial media will develop efficiency and large-scale production of in vitro solid culture. Also, EPNs have been commercially produced by numerous companies in large liquid fermentation tanks in different industrialized countries. However, this technique requires greater funds investment and an advanced level of technical proficiency. There are different biotic and abiotic factors can affect efficiency of EPN application. Nematodes can be suppressed a diversity of economically significant insect pests.

Our case study deals with the biological control of beet fly *Pegomyia mixta*, the most significant pest of strategic crop of sugar industry in Egypt, using different species of entomopathogenic nematodes. All tested nematodes killed the larvae inside their mines in the sugar beet leaves and pupae in the soil and developed in their bodies. In Egypt, as in many other parts of the world, the peach fruit fly, *Bactrocera zonata* has ever been considered a major orchard pest attacking several fruits. A particularly inviting approach to selective control employs EPNs that enter into the host through its mouth opening, spiracles and anus and kill their hosts through the association with their bacteria when auspiciously applied on both guava fruits infested with *B. zonata* eggs and the soil under these fruits or spraying soil containing old pupae of this pest. The potato tuber moth (PTM), *Phthorimaea operculella* is one of the more serious potato pests in the world. Our work indicated that an integrated control program including EPNs would be valuable against the PTM immature stages outside

or inside the potato tubers and may reduce the reliance on chemical insecticides in controlling the PTM. Formulation of nematodes is destined to develop absorption, storage stability, activity, delivery and ease-of-use. Application of nematodes for above ground pests is an advance in the field of bio-insecticides. Our work adds evidence that some formulation adjuvants are valuable in improving field efficiency of EPNs in controlling the larvae of the cotton leaf worm *Spodoptera littoralis* infesting leaves of corn seedlings.

6 Future Prospects

Additional technological advancements are desired to expand and develop the market potential of the nematode-based biopesticides. Recent progress in mass production involves using cheap materials for the nutrient diets of the insect host of in vivo technique and also economic solid media for in vitro production. Isolation of additional species and selective breeding are required for proper classification, for biodiversity studies. This will also contribute to enhance the economic value of EPNs in biological control. Improved efficiency in nematode applications can be supported through improved formulation. Recently, the trend towards improving the aboveground application of nematodes, e.g., mixing EPNs with a polymer and surfactants to increase leaf coverage. Also, efficacy of EPNs can be increased through improved application equipments, e.g., optimizing spray systems (e.g., nozzles, pumps, spray distribution) for increased nematode dispersion and survival. Advanced EPN applications can also be accomplished through strain enhancement. Enhanced strains may possess different valuable traits such as reproductive capacity and environmental tolerance. Genetic enhancement via selection, hybridization or molecular manipulation is a way to improve applications of nematodes.

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