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11. Mass Production Techniques of Entomopathogenic Nematodes

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Abstract:

Entomopathogenic nematodes (EPNs) have been identified as one of the most efficient biocontrol agents for insects that are damaging to many agricultural crops. Steinernema and Heterorabditis are the two most common EPNs genera. EPNs are an environmentally beneficial crop protection technology. EPNs use with mutualistic bacteria to kill insects, and they are readily mass manufactured. Methods of mass production of EPNs are in vivo and in vitro (solid or liquid fermentation). In vivo production is also suitable for niche markets and small-scale producers with a limited budget. Commercially in vivo manufacturing is used when market potential is limited/undeveloped or industrial production utilizing in-vitro technologies is not feasible or cost-effective. Currently, whenever expertise as well as starting funds are available, the in vitro approach is an economically viable technology. Currently, whenever expertise as well as starting funds are available, the in vitro approach is an economically viable technology. This chapter covers the biology, their bacterial symbionts and mass manufacturing of EPNs using in vivo and in vitro approaches.

Keywords:

Entomopathogenic nematodes, In vivo, In vitroand mutualistic bacteria.

11.1 Introduction:

Nematodes are a diverse collection of creatures that make up the phylum Nematoda and are also referred to as roundworms (Kiontke, 2013). Nematodes normally have a filiform, transparent body without segments and are bilaterally symmetrical, while certain plant-parasitic nematode females (such cyst and root-knot females) develop a globose morphology. They are the animal kingdom's most inclusive phylum because of their ability to adapt to living in a wide variety of environments. Nematodes are live in both free-living and parasitic forms of organisms such as animals and plants (Iqbal, 2016). Biopesticides are made from organic substances including plants, microbes, animals, and some minerals.

EPNs, which parasitize insects, have been explained from 23 nematode families (Koppenhofer, 2007). EPNs are parasitic microorganisms that parasitize, infect, and kill insects. Despite the fact that the group of EPNs is now being expanded to comprise othernematodes like as some species of the genus *Oscheius* (Dillman *et al.* 2012). Biopesticides have the potential to be efficient substitutes for chemical pesticides (Karthi *et al*., 2019). Developing nations have a significant opportunity to develop and marketing of biopesticides which reducing their dependency on chemical pesticides that are conventional (Senthil-Nathan, 2015). Entomopathogenic nematodes are biocontrol organisms that have the potential to infect and kill soil-dwelling and above-ground pests such as insects (Kaya and Gaugler, 1993; Laznik *et al*., 2010). EPNs pose no harm to human or animal health and are extremely specific (Boemare, Laumond, & Mauleon, 1996).

These nematodes are from the families Steinernematidae and Heterorhabditidae.The famil y Heterorhabditidae includes the genus *Heterorhabditis*, which has 19 species (Nguyen, 2 017) and Steinernematidae family contains the genera *Neosteinernema* (one documented species) and *Steinernema* (84 identified species) (Nguyen, 2017a). Entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabdztis* (Nematoda: *Rhabdltlda*) have emerged as effective biological control agents for insects. These Entomopathogenic nematodes are symbiotically connected to bacteria of the genus *Photorhabdus* and *Xenorhabdus* which belong to Enterobacteriaceae (Grewal, 2002). These Entomopathogenic nematodes enter the host insect body by the mouth, anus, spiracles, or integument, and then release their symbiont into the insect haemolymph, where the bacteria multiply. Infectious juveniles who have had developmental arrested recover to complete their growth cycle. The bacteria secrete poisons and antimicrobial substances that cause the insect host to die within 48 hours while creating favourable conditions for nematode growth and reproduction.

The nematodes eat the multiplying bacteria and the decaying insect carcass. These EPN grow, mate, and lay eggs there. A new generation of infective juvenile is subsequently produced, and when the host's supply of nutrients runs out, they go to look for fresh insect prey. Improved mass-produced research has also advanced (Shapiro-Ilan *et al*., 2012b). Currently, Entomopathogenic nematodes are produced *in vivo* or *in vitro* (solid and liquid culture) (Friedman, 1990; Ehlers and Shapiro-Ilan, 2005; Shapiro-Ilan *et al*., 2012b).

11.2 Biology and Life Cycle Entomopathogenic Nematodes:

EPNs life stages are divided into the following phases: eggs, juvenile, and adult (Fig. 1). The infective juvenile (IJ) or dauer stage is a free-living, parasitic third juvenile stage that enters the host by natural openings such as the mouth, anus, spiracles, or infrequently through the insect cuticle (Shapiro-Ilan *et al*., 2014). The nematode's symbiotic bacteria are discharged after entering the host's hemocoel. Using an anterior tooth, several *Heterorhabditid* species get entry through inter-segmental membranes of insect cuticles. These bacteria proliferate quickly in nutrient-rich insect hemolymph and secrete toxins that cause septicemia disease, causing the host to die within 24 - 48 hours (Bedding and Molyneux, 1982). The carcass is digested by the bacteria and becomes food for the EPNs. Furthermore, the antibiotics and other toxic compounds they release and protect the host carcass from other microorganisms (Strong *et al*., 1996). Once within the insect, IJs moult

and the nematodes reproduce in 1-3 generations, whereas Entomopathogenic nematode bacteria multiply by mass production (Lewis and Clarke, 2012). When nutrients are exhausted, new IJs develop and escape from the insect carcass in search of new susceptible prey in the environment.

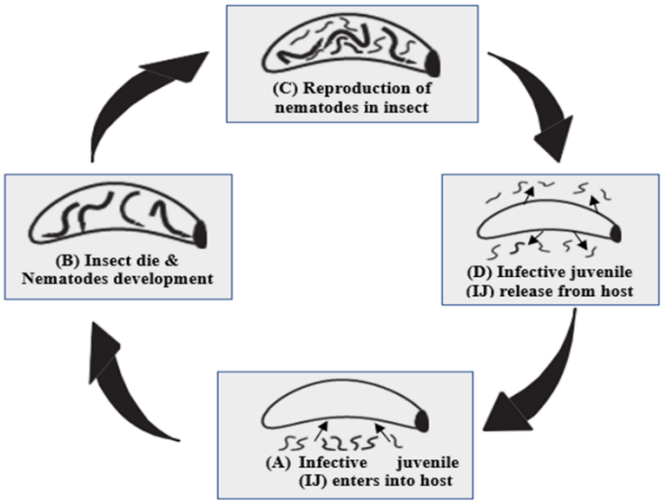

Figure 11.1: Lifecycle of Entomopathogenic Nematodes

11.3 Mass Production Techniques of Entomopathogenic Nematodes:

The manufacturing of EPNs on a wide scale at a competitive price within a short period of time is the primary condition for their effective and economically sensible use in crop protection (Ehlers, 2001). Nematodes that are entomopathogenic to insects may be easily cultivated in the lab using *in vivo* and *in vitro* techniques. These nematodes were initially cultivated more than 70 years ago and are now commercially produced utilising by *in vivo* and *in vitro* (solid and liquid culture) techniques. In the *in vivo* approach, an insect serve as a bioreactor, whereas in the *in vitro* approach, artificial medium is employed.

11.3.1 *In-Vivo* Culturing Entomopathogenic Nematode (Epns):

For in vivo mass rearing of EPNs, the White trap technique—which White invented in 1927 and later improved and rebuilt—is employed (Dutky *et al*., 1964). The Baermann gadget was initially used to extract IJs from cultures. Based on the characteristics of parasite nematodes migrating in the third larval stage, a novel technique and apparatus for extracting parasitic nematodes from the charcoal faecal mixture sample were developed (White, 1927). He created a trap for migratory IJs that contained water in a big Petri plate or tray, a dead larvae resting on it, and a Petri plate. This trap was made by the inventor using plates with a diameter of 125–150 mm, test tubes with a size of 20–150 mm, filter sheets 9–12 cm in diameter, a bladed spatula, a test tube rack, a boiler with a lid, animal charcoal, and sterile water. In the watch glasses, he first combines the charcoal and the waste before transferring it to the half-Petri plate with the wetted filter paper wrapped at the bottom. The culture is put in the half Petri dish after adding sterile water to the crystallising dish to fill the bottom.

In the water of the Petri plates, the migratory IJs from the culture were trapped. The half Petri plates containing the charcoal culture are taken out with forceps once the watch-glass cover has been removed. Test tubes are filled with the IJ-containing water that has been removed from the crystallising plates. Gravity causes the IJs to fall to the bottom, and once the supernatant is pipetted away, the IJs concentration is what remains. The culture was cultured at room temperature with a high humidity level after he used steam to disinfect the apparatus. This method, which had the advantage of collecting worms entirely in their infectious phases with little contamination, allowed him to isolate eight different species of nematodes from four genera. This strategy has been altered and rewritten about by other scholars (Poinar, 1979; Woodering and Kaya, 1988; Kaya and Gaugler, 1993; Lindegren *et al*., 1993; Abdel- Razek and Abd-Elgawad, 2007). All strategies are used to track and gather IJs that naturally depart from the infected body, producing high-quality EPN. Some researchers have described the process of generating EPNs in the bigger wax moth (Galleria mellonella L.) and the yellow mealworm (*Tenebrio molitor* L.) (Shapiro-Ilan *et al.*, 2002b; Shapiro-Ilan and Gaugler, 2002a).

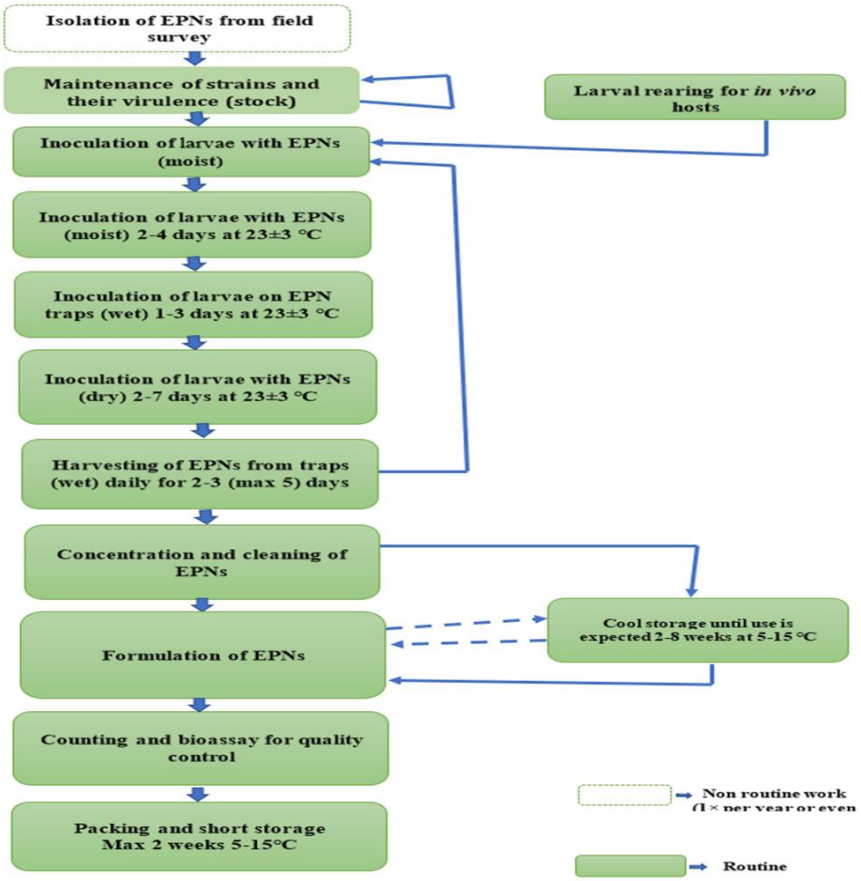

Figure 11.2: A Schematic Flow Chart Of The *In Vivo* Mass Production Technique For Epns Source: Holmes *Et Al.* (2015)

For laboratory and small-scale field testing, EPNs have been more heavily mass multiplied in vivo. Commercial in vivo manufacture is employed when there is a small or underdeveloped market or when using in-vitro technology for industrial production is not technically or financially possible (Ehlers and Shapiro Ilan, 2005a). Figure 2 shows a schematic flow diagram of the in vivo manufacturing method. Another schematic EPN production process employing an in vivo technique is shown in Figure 3 in a small unit.

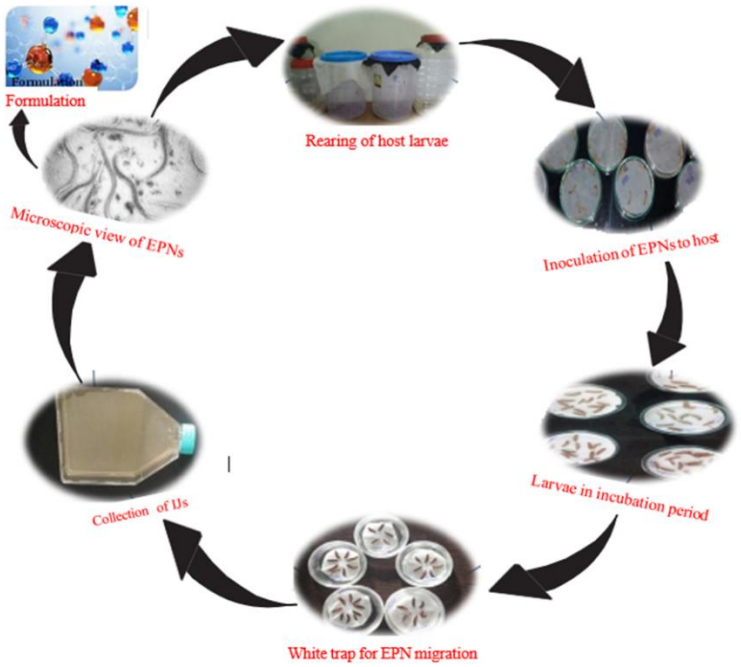

Figure 3: A Systemic Diagram of Epns Production Through *In-Vivo* Technology in A Mini Unit

11.3.2 *In-Vitro* Culturing Entomopathogenic Nematode (Epns):

A. Solid Culture Method: EPNs were initially produced in vitro using an axenic process on a solid media (Glaser 1932). After then, it was discovered that the presence of bacteria facilitated growth. Chicken offal or another protein-rich media were soaked in an inert carrier (sponge, polyurethane) by Bedding to create the first successful commercial scale monogenic culture, also known as a solid culture (Bedding, 1984). This method involves growing nematodes on a crumbed polyether polyurethane sponge that has been infused with symbiotic bacteria, emulsified beef fat, and pig's kidney. Between 66105 and 106105 IJs/g of material were produced using this method (Bedding 1984). Today, it is widely regarded as the foundation of nematode in vitro cultivation that monoxenicity be present (Poinar and Thomas 1966). In vitro solid culture greatly improved with the discovery of a three-dimensional rearing technique incorporating nematode culture on crumb polyether polyurethane foam (Bedding 1981). Foam and a liquid media are mixed before being autoclaved. First, nematodes are injected, then three days later, bacteria. Nematodes can be collected in 2 to 5 weeks by pouring the foam through water-soaked sieves. The product is

cleaned by repeated water washings, also known as sedimentation and decanting, as IJs migrate out of the foam, settle downhill, and then are pumped to a collecting tank. Similarly, to Petri dishes, the medium for this technique was initially based on animal products but was later adjusted for cost and uniformity. It can include a range of ingredients, including peptone, yeast extract, eggs, soy flour, and lard. The nematode can be introduced after the bacteria a few days later. The two species might potentially be injected simultaneously if a high quantity of bacteria is used. Several efforts were taken to increase the possibility for scale-up production, including employing bags with a gas permeable Tyvac strip for ventilation, automated mixing and autoclaving, and harvesting using centrifugal sifters.

B. Liquid Culture Method: The first liquid media for Steinernema glaseri axenic growth was created by the Glaser group (Glaser 1940) and was based on kidney extract. After then, the EPN was chemically created in liquid culture (Stoll 1952). On a shaker, he cultured the colonies in a liquid medium that contained raw liver extract. Axenic nematodes, on the other hand, were unable to be used for biocontrol because of low yields, expensive media, and—most importantly—a lack of symbiotic bacteria in the culture (Ehlers *et al*. 1997). In solid cultures, bedding (1984) showed that even mild movement (shear effect) decreased nematode development. Liquid culture components include soy flour, milk powder, yeast extract, maize oil, casein peptone, thistle oil, egg yolk, liver extract, and cholesterol (Friedman *et al*. 1989). Culture periods can vary according on medium and species, may be as long as three weeks Culture times can range from one day to three weeks depending on the medium and species, however many species reach their peak production in two weeks or less. When the culture is finished, nematodes can be extracted from the media by centrifugation. Lipid metabolism is receiving more attention than other dietary components since it generates 60% of the total energy for the non-feeding IJs (Hatab and Gaugler 1997). Furthermore, it has been demonstrated that yields from lipid sources with high saturated fatty acid contents are subpar (Hatab and Gaugler 2001). Wherever knowledge and startup capital are available, the in vitro liquid culture approach is currently a commercially viable technology. This technique has been implemented by companies including Microbio, USA, E-Nema GmbH, Germany, and SDS Biotech (Ehlers 2001; Gaugler and Han 2002; Maurya *et al*., 2023).

Table 11.1: Some EPNs using for management of target pests

Target Pests		**Entomopathogenic nematodes**
Common name	**Scientific Name**	
Peach fruit moth	*Carposina lipogenesis*	Sc
Cotton bollworm	*Helicoverpa armigera*	Hi, Sg
Rice gall midge	*Orseolia oryzae*	Hi
Corn rootworm	*Diabrotica* spp.	Hb, Sf
Corn earworm	*Helicoverpa zea*	Sc, Sf, Sr
Diamondback moth	*Plutella xyostella*	Sc, Hi
Cabbage maggot	*Delia radicum*	Sf
Red hairy caterpillar	*Amsacta albistriga*	Sc, Hb, Hi
Potato tuber moth	*Phthorimaea operculella*	Sb, Hi
Leaf miner	*Liriomyza* spp.	Sf, Sc
Turnip cutworm	*Agrotis segetum*	Sc, Sf

Target Pests		Entomopathogenic nematodes
Common name	**Scientific Name**	
Stem borer	*Chilo suppressalis*	Sc, Sg, Hb
Cat flea	*Ctenocephalides felis*	Sc, Hb
Tomato pinworm	*Tuta absoluta*	Sf, Sc, Hb
Borers	*Synanthedon* spp.	Sc, Hb, Sf

References: Gitanjali, 2018

Note abbreviation: ***H.* sp.**-*Heterorhabditis* species, **Hb**-*H. bacteriophora*, **Hi**-*H. indica*

***S.* sp.**-*Steinernema* species, **Sb**-*S. brazilense*, **Sc**-*S. carpocapsae*,

Sf-*S. feltiae*, **Sg**-*S. glaseri*, **Sr**-*S. riobrave*,

11.4 Conclusion:

Entomopathogenic nematodes have emerged as an important biocontrol tool against various kinds of agricultural pests. Growing interest in synthetic pesticide alternatives and organic agriculture creates prospects for Entomopathogenic nematodes, but they must be enhanced in terms of efficacy, cost reduction, and simplicity of application. Commercially, Entomopathogenic nematodes and their mutualistic bacteria are used as safe alternatives to chemical pesticides. Entomopathogenic nematode production technique, both *in vivo* and *invitro*, has enabled these organisms to become major biopesticides. *In vitro* liquid production is the most cost-effective method and is expected to continue to dominate the total amount of Entomopathogenic nematodes produced globally. On the other hand, although *in-vivo* manufacturing is the least cost-effective method, it will likely continue to be acceptable for some niche markets or small or startup companies; advances to *in vivo* production may boost cost efficiency. EPNs will continue to assist reduce agricultural dependency on chemical inputs and improve sustainability. We concluded that Entomopathogenic nematodes and their applications play an important role for pest management.

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