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In-vivo and *In-vitro* rearing of Entomopathogenic Nematode (EPN) by Fermentation technology

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Abstract:

Entomopathogenic nematodes (EPNs) have been utilized in classical, conservation, and augmentative biological control programs. The vast majority of applied research has focused on their potential as inundatively applied augmentative biological control agents. Extensive research over the past three decades has demonstrated both their successes and failures for control of insect pests of crops, ornamental plants, trees and lawn and turf. So Production and application technology is critical for the success of entomopathogenic nematodes (EPNs) in biological control. Production approaches include *in vivo* and *in vitro* methods (solid or liquid fermentation). For laboratory use and small scale field experiments, *in vivo* production of EPNs appears to be the appropriate method. *In vivo* production is also appropriate for niche markets and small growers where a lack of capital, scientific expertise or infrastructure cannot justify large investments into *in vitro* culture technology. *In vitro* technology is used when large scale production is needed at reasonable quality and cost. Infective juveniles of entomopathogenic nematodes are usually applied using various spray equipment and standard irrigation systems. Enhanced efficacy in EPN

applications can be facilitated through improved delivery mechanisms (e.g., cadaver application) or optimization of spray equipment. Substantial progress has been made in developing EPN formulations, particularly for above ground applications, e.g., mixing EPNs with Talc, kaolinite and cocopeat. Bait formulations and insect host cadavers can enhance EPN persistence and reduce the quantity of nematodes required per unit area. This study demonstrated successful control of several other insects that affect production and application of EPNs and offers insights for their future in biological insect suppression by increasing the shell life.

Keywords: Entomopathogenic nematode, *heterorhabditis*, production, *steinernema,* biological control, commercialization.

1. INTRODUCTION:

Nematodes that parasitize insects, known as entomopathogenic nematodes (EPNs), have been described from 23 nematode families (Koppenhofer, 2007, 2020). Of all of the nematodes studied for biological control of insects, the Steinernematidae and Heterorhabditidae have received the most attention because they possess many of the attributes of effective biological control agents (Kaya and Gaugler, 1993; Grewal et al., 2005a; Koppenhofer, 2007) and have been utilized as classical, conservational, and augmentative biological control agents. Entomopathogenic nematodes (EPNs) of the families Heterorhabditidae and Steinernematidae are obligate parasites of insects and are used as biological control agents of economically important insect pests. The vast majority of applied research has focused on their potential as inundatively applied augmentative biological control agents (Grewal et al., 2005a).The two major genera are Heterorhabditis (Poinar, 1976), and Steinernema (Travassos, 1927), with 85 species described to date. These nematodes possess a symbiotic association with pathogenic bacteria from the Xenorhabdus and Photorhabdus genera, associated with Steinernema and Heterorhabditis respectively (Poinar, 1990). EPNs are parasites of arthropods in nature that are only horizontally transmitted and possess an infective juvenile (IJ) stage that actively invades the insect host. They are always associated with symbiotic bacteria that play an important role in host infection. The biology of the EPN-symbiotic bacteria complex has been extensively reviewed in Burnell and Stock (2000), Griffin et al. (2005), Lewis and Clarke (2012), Stock (2015), and Shapiro-Ilan et al. (2017, 2018). Infective juveniles (IJs), considered the only free-living stage of EPNs, enter the host insect through its natural apertures (oral cavity, anus and spiracles) or in some cases through the cuticle (Dowds and Peters, 2002). After penetrating the insect's hemocoel, IJs release their symbiotic bacteria, which are the primary agents responsible for host death and also provide the nematodes with nutrition and defense against secondary invaders (Poinar, 1990). The nematodes complete their development and live for two or three generations inside their host. When food is depleted, IJs exit from host cadaver searching for new hosts (Grewal and Georgis, 1999, Koppenho¨fer, 2020). The host range of most known EPN species remains mostly unknown to date (Peters, 1996; Shapiro-Ilan et al., 2017, 2018) because most species have been isolated from

soil samples using the highly susceptible wax moth, *Galleria mellonella*, larvae as a bait insect. Many EPN species may infect a wide range of insect species in laboratory assays (e.g., *S. carpocapsae* > 200 insects across 10 orders). But after field applications and especially in nature, the host range is much narrower due to the ecology of the nematodes and its potential hosts. Some species that have been isolated from natural hosts in the field are particularly well adapted to a narrow group of hosts species but show poor infection of other hosts (i.e., *S. scapterisci* is adapted to Orthoptera and *S. kushidai* and *S. scarabaei* to larvae of Scarabaeidae).

Host defenses and immune reactions in response to EPN infection have been studied only in a few EPN species-insect species combinations (Lewis and Clarke, 2012; Shapiro-Ilan et al., 2017, 2018). Behavioral defenses may include intensive grooming behavior when in contact with IJs to prevent infection and evasion of areas with high numbers of IJs (scarabaeid white grubs). Physical barriers to prevent IJs from reaching the hemocoel may include reduced access to the hemocoel via the mouth through forward projecting hairs in the preoral cavity (elaterid wireworms) or a thick peritrophic membrane protecting the midgut epithelium (white grubs). Narrow, slit-like openings of the spiracles (wireworms) or fine sieve-like plates covering the spiracles (white grubs) may limit access to the hemocoel via the tracheal system.

Once inside the host, IJs may overcome or evade the host's immune response (reviewed in Lewis and Clarke, 2012; Shapiro-Ilan et al., 2017, 2018) by shedding of the second-stage-juvenile cuticle (sheath), depositing *de-novo* produced or host-sequestered immune factors as a camouflage, interfering with the host immune system by secreting putative proteins (*S. carpocapsae*), and releasing proteases (*Steinernema* spp.). Both *Xenorhabdus* and *Photorhabdus* symbionts play complimentary roles in overcoming the insect defenses and actively suppressing the immune response. They also produce and release several toxins lethal to the insect host as well as antibiotics to prevent secondary infections of the cadaver by other pathogens or scavengers.

Extensive research over the past three decades has demonstrated both their successes and failures for control of insect pests of crops, ornamental, and lawn and turf (Shapiro-Ilan et al., 2002; Georgis et al., 2006). They can be considered good candidates for integrated pest management and sustainable agriculture due to a variety of attributes. Some species can recycle and persist in the environment; they may have direct and/or indirect effects on populations of plant parasitic nematodes and plant pathogens; can play an indirect role in improving soil quality; and are compatible with a wide range of chemical and biological pesticides used in IPM programs.

Entomopathogenic nematodes are currently produced by different methods either *in vivo* or *in vitro* (solid and liquid culture) (Friedman, 1990). Each approach has its advantages and disadvantages relative to production cost, technical know-how required, economy of scale, and product quality, and each approach has the potential to be improved.

2. METHODOLOGY:

2.1. Source of entomopathogenic nematodes (EPNs):

Photorhabdus luminiscens was isolated from rhizosphere of citrus and mango respectively from the experimental farms in and around Tirupati, Andhra Pradesh. The nematodes were extracted using soil baiting technique using Galleria mellonella larvae as bait. All of these EPNs were cultured on G. mellonella larvae as per the procedure described by Woodring and Kaya [1988].

2.2. Isolation of symbiotic bacteria:

To isolate symbiotic bacteria, five final instar larvae of wax moth, Galleria mellonella, Linnaeus (Lepidoptera: Pyralidae), were inoculated with 500 IJs (40 -50 IJs per insect) of each of H. bacteriophora, in 100 µL distilled water placed in a Petri dish (65 mm) lined with double filter paper. Two days after inoculation, mutualistic bacteria Photorhabdus sp. from H. bacteriophora was isolated from the cadavers according to the procedure of Kaya and Stock [1997]. Cadavers were surface sterilized with 1% NaCl for 2 min & rinsed thrice with sterile water and by dipping in absolute ethanol for 30 seconds, and allowing the ethanol to evaporate. The cadaver was held upside down with forceps and the head was removed with sterile scissors. The first drop of haemolymph which oozed out was discarded, but the second drop was deposited onto a plate of MacConkey agar and NBTA agar medium [Akhurst, 1980]; 2.3% (w/v) nutrient agar, 0.025% (w/v) bromothymol blue, 0.004% (w/v) 2-3-5 triphenyl-tetrazolium chloride (TTC) and was streaked with an inoculation loop, to make primary, secondary and tertiary streaks to yield isolated colonies [Johnigk, 1999, Ulug et al., 2015] . Plates were incubated at 27 °C for 72 h & observed for appearance of bioluminescent colony.

2.3. Mass production:

Mass production of EPN *Heterorhabditis bacteriophora* was done by three different methods in the following ways ie; Gallaria as host, solid state fermentation and liquid state fermentation.

2.3.1 Solid State Fermentation:

To determine yield, 25 g of each production media was absorbed in 1 g of sponge cubes (measuring 0.5 cm2 each) in 100 mL Erlenmeyer flasks and autoclaved at 121 °C (20 lb pressure per square inch) for 15min. The media were inoculated with 2 mL of 48 hour old bacterial suspension and incubated at 27 °C for 72 h. Infective juveniles (IJs) emerged from the G. mellonella larvae were surface sterilized with 0.1% NaCl for 15 min and washed with sterilized distilled water thrice before inoculation. The IJs were inoculated aseptically into the flasks @ 1000 IJs /flask under laminar flow chamber. The sealed flasks were incubated at room temperature (28 ^{0}C ± 2 ^{0}C) for 30 days. Care was taken not to shake the flasks after inoculation of nematodes. The colonies of nematodes started appearing on the walls of the flasks after 22 days of post inoculation.

Four different media's are used in this solid state fermentation ie:

a) Modified Wout's medium
b) Modified egg yolk medium
c) Modified wheat flour medium
d) Modified dog biscuit medium

2.3.2 Extraction of infective juveniles from different media:

After 30 days of inoculation of nematodes into the media, they were extracted from the media by transferring the foam chips from the flasks into a facial tissue paper which was spread over a 20 mesh aluminum wire mesh support. The flasks were thoroughly washed and washings were also added to the foam chips. The wire mesh support was then kept over a Petri dish filled with water, so that a thin film of water touched the material over the filter paper. The nematodes settled in the Petri dish were collected at frequent intervals and transferred to one liter beaker. The nematode-bacterial suspension in the flasks was allowed to settle and the supernatant was decanted. This process was repeated several times until a clear suspension of nematode was obtained.

2.3.3 Liquid state fermentation:

In liquid culture, symbiotic bacteria are first introduced followed by the nematodes. Various ingredients for liquid culture media have been used including soy flour, yeast extract, canola oil, corn oil, sunflower oil, coconut oil, egg yolk, milk powder, liver extract and cholesterol for stable emulsion. The different oils used in the process as carriers are

<u>Oil carriers</u>

- **Coconut oil,**
- **Sunflower oil,**
- **Ground nut oil**
- **Mineral oil**

Then Bacterial cells along with nematode solution (100-5000 nematodes / ml) where added to the medium. While processing, pressure, dissolved oxygen and the RPM (150) are maintained. Once the culture is completed, nematodes can be harvested from media via centrifugation.

2.4 FORMULATION:

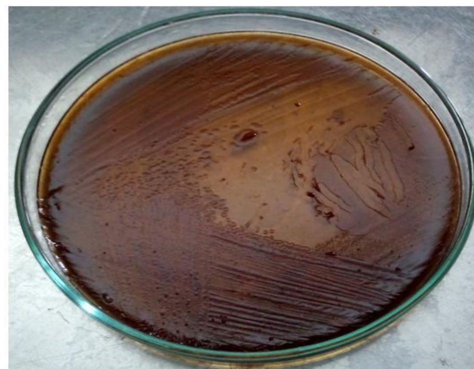
After harvesting the nematodes as aqueous suspension in normal water, the nematode concentration per ml is counted under the stereo zoom microscope. IJs may either be delivered inside the infected cadavers, or they are suspended in water and separated from the growing medium via sedimentation, centrifugation, or sieving techniques. The size and the density of the nematodes are mainly explored in separation techniques at a commercial scale. Techniques based on the migration activity of nematodes, while often used to harvest pure IJ suspensions in

laboratory scale (Dutky et al., 1964). Ultimately, the IJs are stored in water and amended with salts (Strauch et al., 2000). They need to be stirred and aerated in these aqueous suspensions and therefore cannot be shipped this way.

The number of nematodes per gram of wettable powder formulation is controlled by adjusting the concentration of nematodes in the aqueous suspension (Stock). Formulations for EPNs must allow sufficient gas exchange and humidity (water activity >0.96). To produce such formulations, excess water is first removed via various sieving techniques. The resulting nematode paste is then mixed with suitable binders (Talc, kaolinite and cocopeat at (1:1:0.25) to turn the slurry into a moist powder. This moist powder allows for sufficient gas exchange and at the same time buffers the water content.

3. RESULTS:

The isolated symbiotic bacterium, *Photorhabdus luminiscens* associated with entomopathogenic nematode, *Heterorhabditis indica* gives Bright-pink to red-colored bacterial colonies on MacConkey agar medium.



***Photorhabdus* like colonies on MacConkey agar**

The bacterium was identified as *Photorhabdus luminiscens based on molecular characterization and designated as EPN-2.*

3.1 Solid State Fermentation

S. No.	Inoculum size (Per ml)
1	500IJs
2	1000 IJs
3	1500 IJs
4	2000 IJs
	CD@1%

The maximum number of Nematode growth observed only in modified dog biscuit medium after 30days of incubation when compared with other media. An inoculum level of 2000 IJs per ml yielded significantly highest nematode concentration of 4.05 lakh IJs per ml.Thus an inoculum level of 2000 IJs per ml was considered as optimum dosage for the maximum production of *H. indica* on modified dog biscuit medium which is then used for formulation.

Table 2. Mass production of *H. indica* on modified dog biscuit medium

S. No.	Inoculum size (Per ml)	IJs/ml (in lakhs)
1	500IJs	1.32[c]
2	1000 IJs	2.62[b]
3	1500 IJs	3.14[b]
4	2000 IJs	4.15[a]
	CD@1%	0.68

3.2 Liqiud Fermentation: The nematode growth was checked with different carrier oils. Among the four oil carr ers tested, nematode growth was observed more in Sun flower oil medium after 15- 16days of incubation where as less growth with other oils . The yield was 4.03 lakh IJs per ml with an inoculum size of 2000 IJs per ml.

3.3 Powder formulation of *H. indica:*

Powder formulation was done to increase the shell life of nematodes by mixing Talc, kaolinite and cocopeat at (1:1:0.25) ratio with a Nematode concentration of 50000 per g of powder. Here the moisture was maintained at 23-25% per 230-250 ml per kg of the powder mix.

4. CONCLUSION:

The development and the increase in scale of the liquid culture resulted in a reduction of production costs by more than 75% in the past 30 years, and this process has not finished yet. As a result, the use of EPNs in low-value crops is now at hand. Another important prerequisite for the success of EPNs as biocontrol agents is the low level of regulation. Nematodes are macro-organisms and, like the equally widely used beneficial arthropods, do not need to be register as plant protection agents in most countries. It is probably due to this liberal legislation that the

nematode production business is versatile and various culture techniques coexist. In vivo and solid-state production is likely to continue as local businesses for niche markets where competition by in vitro producers is limited, and in developing countries where labor is inexpensive. A significant benefit of a more local production is the avoidance of long delivery chains at controlled temperature, which is a major cost driver for worldwide delivery of centrally produced EPNs. This study anticipates in the innovations to improve efficiency will enable in vivo production to play an expanded role in pest management programs.

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