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Review Article

BIOLOGICAL CONTROL TECHNOLOGY UTILIZING *HETERORHABDITIS BACTERIOPHORA* AND *STEINERNEMA CARPOCAPSAE*

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ABSTRACT

Entomopathogenic nematodes (in the genus *Steinernema* and *Heterorhabditis*) have been studied and successfully commercialized as biological control agents. These organisms are highly virulent and safe for the non-target environment, animals and humans. For at least 200 target species, the nematode-bacteria complex has the potential to become a mass-marketed agricultural biopesticide. However, before nematodes can be successfully integrated into the agricultural system as a regular-use, "go-to" biopesticide, it is necessary to develop economical manufacturing processes. There are several manufacturing platforms: *in vitro* solid fermentation; *in vitro* liquid fermentation; and *in vivo* production. This review presents an analysis of each approach and discusses the advantages and disadvantages relative to the cost of production, technical expertise required, and quality of the final product.

Keywords: *Heterorhabditis bacteriophora*, *Steinernema carpocapsae*, beneficial nematodes, fermentation technology.

INTRODUCTION

Biological control is the systematic addition of natural microorganisms to an area with the intent to mitigate pests and pest effects in that area. To be most effective at controlling a pest, a biological agent should have the ability to adopt to habitat changes over time (Follett *et al.*, 2000; Holmes *et al.*, 2016). The most effective biological agents maintain the population in the absence of the target species. There are three basic strategies of biological control: (a) classical (importation), where a natural enemy of a pest is introduced in to a target area in hopes of eventually controlling the pest; (b) indicative augmentative control, which consist of the addition of natural enemies to an environment in which they are either not present or may be present but in small numbers; and (c) augmentation. Augmentation can be divided into two sub-categories: (a) inundative release, which consists of large numbers of the control pest being released in hopes of rapidly reducing a damaging pest population and (b) seasonal inoculative release (conservation) during which the control organism is released at specific intervals throughout a growing season and is expected to produce progeny that control the pest throughout the growing season (Bale *et al.*, 2008).

Biological control can affect pest biodiversity through predation, parasitism, pathogenicity, and competition (Ghosh, 2011). Entomopathogenic nematodes (EPNs), are highly virulent, kill their hosts quickly, are safe for the environment, humans, and other non-target vertebrates making them an attractive alternative to commercial pesticides (Mahmoud, 2016; Kooliyottil *et al.*, 2013). Both *Steinernema* and *Heterorhabditis* nematodes possess three biological traits which render them useful as commercial pesticides: 1) a mutualistic relationship with highly virulent bacterium 2) a broad insect host range, and 3) their safety towards non-target organisms such as plants, livestock, and humans (Gerdes, 2015). Their bacterial symbionts, *Xenorhabdus* and *Photorhabdus* bacterial genera undergo phase variation in which they shift from an unstable, pathogenic state (Phase I) to a

stable, less pathogenic form (Phase II) (O'Campo *et al.*, 2017; Gulley *et al.*, 2015). Phase I bacterial symbionts are necessary to kill hosts because they secrete a battery of toxins and enzymes that convert the insect host into nutrients for the nematode vector. Furthermore, Phase I variants also produce a wide range of antimicrobials that prevent other organisms from invading the insect cadaver. By doing so, the bacterial symbionts produce an ideal breeding ground for its nematode partner (Upadhyay, 2015).

EPNs inhibit pests through a symbiotic nematode-bacterium mechanism. The nematode-bacteria association is complex but necessary for their success as a viable biological control agent (Patterson, 2015). The process is as follows: after an insect is sensed, the nematode ambushes it, sheds its outer cuticle, and enters the insect's hemocoel through openings such as the anus or mouth (Noosidum *et al.*, 2010). Upon invasion, the infective stage of EPNs release witnesses of the virulent bacteria symbiont within the host which proliferate in the insect hemolymph. The bacteria contribute to killing the insect, providing infective juveniles (IJs) with nutrients, protection, and favourable growth conditions for the nematodes (Johnson *et al.*, 2016). When the resources are depleted, EPN progeny emerge from the carcass in search of another host and begin the cycle again (see Figure 1).

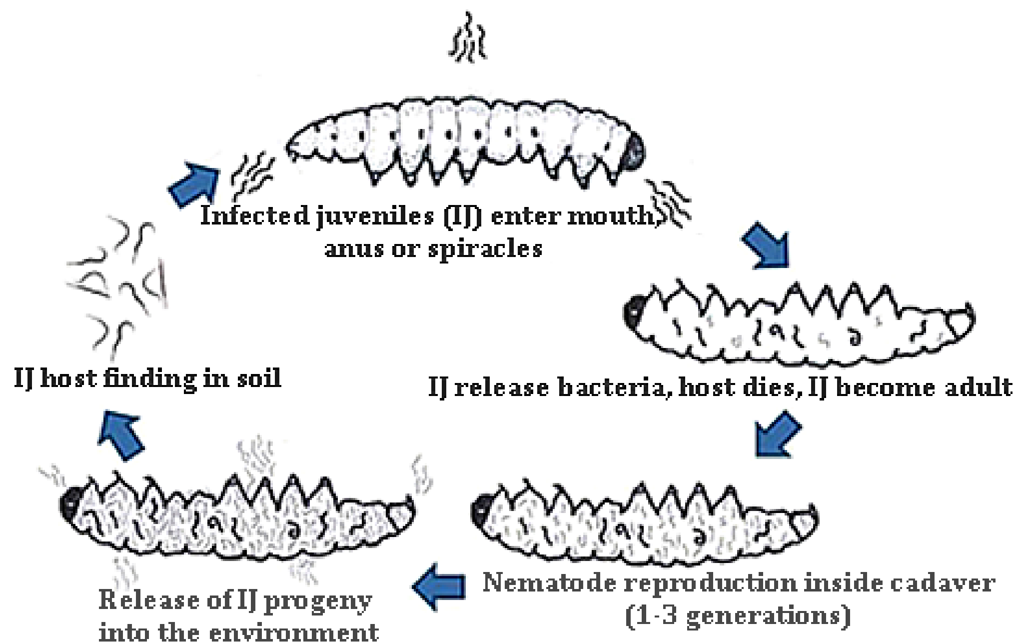

Figure 1. Entomopathogenic nematode lifecycle (Tofangsazi *et al.*, 2012).

Although there are many different species of nematodes, *Steinernema carpocapsae* and *Heterorhabditis bacteriophora* are uniquely useful to agricultural pest control. *Steinernema carpocapsae* is the most studied of all entomopathogenic nematodes because of ease of mass production and ability to survive in dry conditions which increases their "shelf-life" (Shapiro-Ilan *et al.*, 2012; Gerdes *et al.*, 2016). *Heterorhabditis bacteriophora* is considered to be one of the most effective species of EPNs and is used worldwide as a biopesticide (Johnson *et al.*, 2016). *Steinernema* and *Heterorhabditis* nematodes are used to control more than two hundred insect species. They have been shown to be effective in varying agricultural settings such as farms, orchards, nurseries, and greenhouses (Table 1) (Mahmoud, 2016). Because of this, the majority of research has focused on their potential as inundatively applied augmentative biological control agents (Kergunteuil *et al.*, 2016).

Table 1. Selected Examples of Biocontrol Nematodes Use, describes the success of several nematode species on various pests and target areas. (Shapiro-Ilan *et al.*, 2012).

Pest Common name	Pest Scientific name	Key Crop(s) targeted	Efficacious Nematodes *
Artichoke plume moth	*Platyptilia carduidactyla*	Artichoke	Sc
Armyworms	*Lepidoptera: Noctuidae*	Vegetables	Sc, Sf, Sr
Banana moth	*Opogona sachari*	Ornamentals	Hb, Sc
Banana root borer	*Cosmopolites sordidus*	Banana	Sc, Sf, Sg
Billbug	*Sphenophorus spp. (Coleoptera: Curculionidae)*	Turf	Hb,Sc
Black cutworm	*Agrotis ipsilon*	Turf, vegetables	Sc
Black vine weevil	*Otiorhynchus sulcatus*	Berries, ornamentals	Hb, Hd, Hm, Hmeg, Sc, Sg
Borers	*Synanthedon spp. and other sesiids*	Fruit trees & ornamentals	Hb, Sc, Sf
Cat flea	*Ctenocephalides felis*	Home yard, turf	Sc
Citrus root weevil	*Pachnaeus spp. (Coleoptera: Curculionidae*	Citrus, ornamentals	Sr, Hb
Codling moth	*Cydia pomonella*	Pome fruit	Sc, Sf
Corn earworm	*Helicoverpa zea*	Vegetables	Sc, Sf, Sr
Corn rootworm	*Diabrotica spp.*	Vegetables	Hb, Sc
Cranberry girdler	*Chrysoteuchia topiaria*	Cranberries	Sc
Crane fly	*Diptera: Tipulidae*	Turf	Sc
Diaprepes root weevil	*Diaprepes abbreviatus*	Citrus, ornamentals	Hb, Sr
Fungus gnats	*Diptera: Sciaridae*	Mushrooms, greenhouse	Sf, Hb
Grape root borer	*Vitacea polistiformis*	Grapes	Hz, Hb
Iris borer	*Macronoctua onusta*	Iris	Hb, Sc
Large pine weevil	*Hylobius albietis*	Forest Plantings	Hd, Sc
Leafminers	*Liriomyza spp. (Diptera: Agromyzidae)*	Vegetables, ornamentals	Sc, Sf
Mole crickets	*Scapteriscus spp.*	Turf	Sc, Sr, Scap
Navel orangeworm	*Amyelois transitella*	Nut and fruit trees	Sc
Plum curculio	*Conotrachelus nenuphar*	Fruit trees	Sr
Scarab grubs**	*Coleoptera: Scarabaeidae*	Turf, ornamentals	Hb, Sc, Sg, Ss, Hz
Shore flies	*Scatella spp.*	Ornamentals	Sc, Sf
Strawberry root weevil	*Otiorhynchus ovatus*	Berries	Hm
Small hive beetle	*Aethina tumida*	Bee hives	Yes (Hi, Sr)
Sweet potato weevil	*Cylas formicarius*	Sweet potato	Hb, Sc, Sf

Nematodes species used are abbreviated as follows: Hb=*Heterorhabditis bacteriophora*, Hd = *H. downesi*, Hi = *H. indica*, Hm = *H. marelata*, Hmeg = *H. megidis*, Hz = *H. zealandica*, Sc = **Steinernema carpocapsae**, Sf = *S. feltiae*, Sg = *S. glaseri*, Sk = *S. kushidai*, Sr = *S. riobrave*, Sscap = *S. scapterisci*, Ss = *S. scarabaei*.
** Efficacy of various pest species within this group varies among nematode species.

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DISCUSSION

Beneficial nematodes are highly virulent, and are safe for the environment and humans, making them an attractive alternative for commercial pesticides (Mahmoud, 2016). There are currently three methods used for the production of nematodes: *in vitro* solid fermentation, *in vitro* liquid fermentation, and *in vivo* production. Each approach has advantages and disadvantages relative to production cost, technical expertise required, and product quality and consistency (Shapiro-Ilan *et al.*, 2012; Upadhyay *et al.*, 2013). Presently *in vitro* methods are best suited for large-scale commercialization. Most researchers agree that *in vitro* liquid fermentation technologies are better suited for commercial technologies because of superior cost efficiency (Shapiro-Ilan *et al.*, 2012).

In vitro culturing methods: *In vitro* liquid culturing is the most economical method of large scale commercial production. The quality of EPNs can be observed through measuring the difference in their foraging behaviour, establishment efficiency, and invasion rate (Converse and Miller, 1999). Conflicting reports indicate the reduced quality or efficacy of *in vitro* liquid produced EPNs when compared to those produced in solid culture or *in vivo* (Shapiro-Ilan *et al.*, 2012). Fortunately, the continuous optimization of media composition, inoculation procedures, cell density (Hirao and Ehlers, 2009), environmental conditions within fermenters and the overall down streaming process, (Chavarría-Hernández *et al.*, 2006) has significantly increased the quality of EPNs produced via liquid culturing methods.

There has been scant information published concerning quality control of EPN-based products; ten articles in the last three decades (Ramakuwela *et al.*, 2016). In February 1999, a quality assessment study of commercially produced EPN's was conducted to determine how much the inconsistencies varied between different "cottage" (home based) producers. It focused on the following: product packaging, instructions/ease of use, product availability, and pathogenicity. The study concluded that although manufacturing has made substantial progress, it was not uncommon for sellers to market nematode strains with inferior pathogenicity and that some packages contained more dead than living nematodes. These inconsistencies were determined to be caused by improper product storage, shipping and handling. As a result, research continues to develop better storage/shipping technologies. Moreover, conflicting reports also indicate reduced quality or efficacy *in vitro* liquid produced EPNs relative to those produced in solid culture or *in vivo* (Shapiro-Ilan *et al.*, 2012).

Mass culturing of EPNs through *in vitro* methods requires in-depth knowledge of nematode biology and the technologies involved in the production (Vashisth *et al.*, 2013). A production medium must be formulated that provides an optimal chemical environment for the nematodes as well as the symbiont bacteria (Inman *et al.*, 2012; Alsaidi *et al.*, 2017). The general *in vitro* liquid state process is as follows: a nutrient-rich liquid medium is created and mixed with antifoam, autoclaved, inoculated with bacteria and then IJ3 nematodes. Once inoculated, a sample is examined for health, stages, and nematode count. Separation of IJs from the fermentation broth, waste products, and other non-IJ life stages is arguably the most challenging part of commercialization. When only IJ's are observed in the sample, harvesting procedures begin. The general harvesting protocol is as follows: 1) the culturing media is collected and centrifuged at low speed to separate the adult nematodes from juvenile nematodes (adult nematodes are found in pellet, while juveniles stay in the supernatant). 2) The pellet containing adult nematodes is washed multiple times with sterile distilled water and incorporated back into the production media. 3) While the supernatant (containing the IJ's) is centrifuged at medium speed to separate the IJ's from leftover media, and waste products. 4) The resulting infective juveniles are either packaged or used to inoculate another bioreactor (Upadhyay *et al.*, 2013). Although some companies are producing IJ stages through submerged liquid culture technology, results remain inconsistent due to lack of quality control measures taken during production and the fragility of the nematode species.

Entomopathogenic nematodes have been grown *in vitro* on a solid medium axenically (pure culture) (Shapiro-Ilan *et al.*, 2012), however early attempts to grow EPNs in axenic culture showed low yield. Additionally, the media was too expensive to be useful for mass production (Ehlers, 2001). *In vitro,* solid culture advanced considerably with the invention of a three-dimensional system involving culturing nematodes on crumbled polyether polyurethane foam (Bedding, 1981). In this system, a liquid medium is mixed with foam, autoclaved, and then inoculated with bacteria and nematodes. Within 2–5 weeks nematodes are harvested by placing the foam onto sieves, which are immersed in water. Once the infective juveniles migrate from the foam and settle, they

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are pumped to a collection tank where they are washed repeatedly and then packaged. Another significant development for solid fermentation technology involves improved medium. But these were found to be too expensive and complicated to reproduce and use for commercialized production. Wouts developed a medium that included easily accessible and inexpensive ingredients such as yeast extract, nutrient broth, vegetable oil, and soy flour (Wouts, 1981). In this case, agar is not a suitable binding agent because it liquifies and drips out of the sponge during sterilization. Instead, soy flour or ground millet are used and serve as binding agents. Wheat germ or fine cornmeal have also been used in solid culturing. Agar works well when employing a plate rearing system as long as the media contain less than one percent oil. If more is used, a continuous layer of oil will form in the surface of the plate inhibiting bacterial growth (Wouts, 1981).

In vivo culturing methods: *In vivo* culturing, methods involve the use of a live insect host for production. The host used in *in vivo* culturing methods is typically the larvae of the wax moth, *Galleria mellonella*. The technology required for culturing is minimal; employing the use of trays and shelves for production (Brown *et al.*, 2006). A highly referenced method of *in vivo* culturing is based on the 'White Trap' technique which takes advantage of the progeny IJ's natural migration away from the host-cadaver upon emergence (Wouts, 1981).

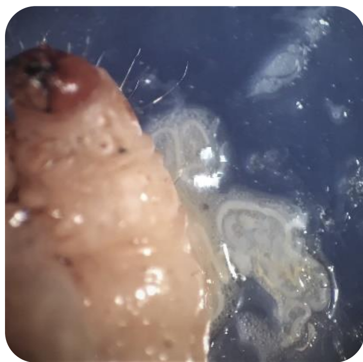

Figure 2. *In vivo* production of the beneficial nematode *Steinernema carpocapsae* using *Galleria mellonella.*

White Traps require a large dish or tray as well as a small plate, usually placed on an inverted petri dish, where the cadavers lay. The large dish is filled with water and the small dish containing the nematode infected cadavers is placed in the center of it. The infective juveniles that emerge then migrate to the surrounding water where they are trapped and subsequently harvested (Shapiro-Ilan *et al.*, 2012).

The production of EPNs via *in vivo* culturing techniques depends on successful infection during inoculation. A change of colour (from yellow to tan for *S. carpocapsae* and reddish for *H. bacteriophora*) is characteristic of successful EPN infection. Hosts that do not exhibit these characteristic colour changes and instead turn black, mold, or begin to rot, should be removed because they may contaminate the rest of the batch (Kaur, 2013). If host infection is insufficient or inconsistent, the low yield will result (Brown *et al.*, 2006). Because this production requires a low level of technology, has low startup costs, and resulting nematode quality is generally high, *in vivo* production is often used for grower cooperatives, university research and for developing countries (Abu Hatab and Gaugler, 1999). However, *in vivo* production methods are not cost-effective for commercialization. Production costs may be lower in the long run if the nematode progeny can continue recycling throughout an entire growing season.

Distinct advantages and Disadvantages: EPNs have distinct advantages over other biological control agents because, under optimal environmental conditions they live and remain in the infective stage for several months (Lewis *et al.*, 2006). Unlike chemicals and other biological pesticides, EPN applications are safe for workers. Moreover, residues, groundwater contamination, chemical trespass, and pollinators are not issues as they are with commercial chemical products (Vashisth *et al.*, 2013).

In 1991, it was estimated that it costs as much as 60% more to control insects with nematode-based products than with chemical insecticides. Fortunately, as new technologies create improvements in production, formulation, packaging, and shelf life, the production and market prices of nematode products continue to decrease, making nematodes more useful to larger agricultural markets (Smart Jr, 1995). EPN's can be stored for two to five months depending on the species and storage conditions. Unlike other microbial control agents such as fungi and bacteria, EPNs do not have a fully dormant resting stage lose viability during storage (Tofangsazi *et al.*, 2012), and must be immobilized to prevent depletion of their lipid and glycogen reserves. Improved formulation techniques are continuously being developed to facilitate prolonged storage and application. Current formulations include activated charcoal, alginate polyacrylamide gels, clay, peat, polyurethane sponge and

vermiculite. Shelf-life now ranges from one to seven months compared to the previous expectation of two to five weeks. (Vashisth *et al.*, 2013). Another challenge in EPN research is consistency. Published yields of IJs vary considerably with average counts between 20,000 to 40,000 per mL (Upadhyay *et al.*, 2013; Upadhyay, 2015). The quality of infective juveniles (IJs) also depends on the method of production, media composition, and successful infection during inoculation. Various resources suggest that the quality of nematodes produced *in vitro* solid culture is similar to that produced *in vivo*. However, the quality of EPN juveniles produced in artificial liquid media may be less than that of nematodes produced *in vivo* (Abu Hatab and Gaugler, 1999). Since nematode products are safe to apply and do not contaminate the environment, some growers will choose a biological control method even at a higher cost. Also, at least in some situations, the nematodes become established, recycle, and their offspring continue to control the target insect. Thus, the higher short-term cost may be lower in the long run when continued control by the recycling nematode is obtained (Smart Jr, 1995).

CONCLUSION

The successful integration of EPN's as regular-use biological control agents requires specific knowledge and understanding of the adaptation and establishment of applied biological control agents in agricultural ecosystems as well as an efficient production and recovery process. Fortunately, technological advancements continue to improve mass-production techniques, lessen manufacturing costs, increase efficiency and ease of use. The future use of EPN's as a regular use biopesticide is promising.

AUTHOR CONTRIBUTIONS

All the authors contributed equally.

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CONFLICTS OF INTEREST

The authors have no conflict of interests.

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