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Production technology for entomopathogenic nematodes and their bacterial symbionts

DI Shapiro-Ilan[1], and R Gaugler[2]

[1]*USDA-ARS, SAA, 21 Dunbar Road, Byron, GA 31008, USA;* [2]*Rutgers University, New Brunswick, NJ, USA*

Entomopathogenic nematodes (genera *Steinernema* and *Heterorhabditis*) kill insects with the aid of mutualistic bacteria. The nematode–bacteria complex is mass produced for use as biopesticides using *in vivo* or *in vitro* methods, i.e., solid or liquid fermentation. *In vivo* production (culture in live insect hosts) is low technology, has low startup costs, and resulting nematode quality is high, yet cost efficiency is low. *In vitro* solid culture, i.e., growing the nematodes and bacteria on crumbled polyurethane foam, offers an intermediate level of technology and costs. *In vivo* production and solid culture may be improved through innovations in mechanization and streamlining. *In vitro* liquid culture is the most cost-efficient production method but requires the largest startup capital and nematode quality may be reduced. Liquid culture may be improved through progress in media development, nematode recovery, and bioreactor design. A variety of formulations is available to facilitate nematode storage and application.
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Introduction

Entomopathogenic nematodes (genera *Steinernema* and *Heterorhabditis*) are obligate parasites of insects that kill their hosts with the aid of bacteria carried in the nematode's alimentary canal [80]. The infective juvenile nematode, which is the only free-living stage, enters the host via natural openings, i.e., mouth, anus, spiracles [80], or occasionally through the insect cuticle [12]. The nematodes then release their symbiotic bacteria, which are the primary agents responsible for killing the host within 24 to72 h. After the nematodes complete one to three generations within the insect cadaver, infective juveniles exit to find new hosts [80]. The two nematode genera differ in their life cycles in that the steinernematids contain only amphimictic forms (males and females), whereas the first generation of heterorhabditids (arising from infective juveniles) contain only hermaphrodites, and subsequent generations may contain amphimictic and hermaphroditic forms [67,99].

The nematodes and bacteria have a mutualistic relationship. The bacteria provide nutrients to the nematodes, produce antibiotics that inhibit competing microbes, and kill the host through septicemia [5,6]. Although the nematodes may also contribute to host death through suppression of the immune system and toxin production [6], the most important role they play in the mutualism is serving as vectors for the bacteria. Without the nematode the bacteria cannot survive well in the natural environment and are generally not pathogenic when ingested by a host [6,75].

The relationship between nematode and bacteria is highly specific [6,15]. Steinernematids are associated with *Xenorhabdus* spp. and heterorhabditids are associated with *Photorhabdus* spp. [29,80]. Each nematode species is primarily associated with a single bacterial species although each bacterial species can be associated with more than one nematode [6]. Further, the suitability of particular bacteria for growth and compatibility with nematodes can vary on a strain level [43].

The bacteria can occur in two phase variants: primary and secondary, which differ in dye absorption, response to biochemical tests, and antibiotic production [4,5]. Although entomopathogenic nematodes have been reported to grow on secondary phase symbionts or nonsymbiotic bacteria, the primary phase is most conducive to growth and infective juveniles tend to retain only the primary phase symbiont [5,25].

Entomopathogenic nematodes and their endosymbiotic bacteria are potent bioinsecticides that can control a wide variety of economically important agricultural pests [92]. These nematodes possess a number of attractive qualities as biocontrol agents including a durable infective stage, host-seeking ability, suitability to mass production, and safety to mammals and other nontarget organisms, which allows exemption from US federal pesticide registration. Due to their sensitivity to ultraviolet light and desiccation [40], entomopathogenic nematodes have been most successful at suppressing populations of ground-dwelling pests or pests in other protected environments (e.g., greenhouses). Successful pest control with nematodes requires a proper match of the nematode to the host species and favorable economics relative to the value of the commodity and the cost of competing pest control strategies [92]. To be effective, entomopathogenic nematodes must generally be applied at rates of 2.5×10^9/ha or higher [41,42]. Some of the pests that have been targeted commercially with entomopathogenic nematodes are listed in Table 1. In addition to controlling harmful insect pests, new frontiers are opening by using entomopathogenic nematodes, and more so, their symbiotic bacteria or associated metabolites to suppress plant parasitic nematodes [47,51,52], and as antimicrobial agents in pesticide and pharmaceutical applications [69]. Furthermore, toxins produced by the bacteria are being investigated for their suitability as alternatives to other

Correspondence: Dr DI Shapiro-Ilan, USDA-ARS, SAA, 21 Dunbar Road, Byron, GA 31008, USA
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Table 1 Examples of important pests that have been successfully targeted commercially with entomopathogenic nematodes

Common name	Scientific name	Commodity	Nematode[a]	Reference(s)[b]
Artichoke plume moth	*Platyptilia carduidactyla*	Artichoke	Sc	[7]
Black vine weevil	*Otiorhynchus sulcatus*	Cranberries, ornamentals	Hb, Hm, Hmar	[11,85]
Billbugs	*Sphenophorus* spp.	Turf	Sc	[92]
Blue green weevils	*Pachnaeus* spp.	Citrus	Hb, Hi, Sr	[22,41]
Black cutworm	*Agrotis ipsilon*	Turf, vegetables	Sc	[41,68]
Cranberry girdler	*Chrysoteuchia topiaria*	Cranberries	Sc	[41,82]
Diaprepes root weevil	*Diaprepes abbreviatus*	Citrus	Hb, Hi, Sr	[92]
Fleas	*Ctenocephalides felis*	Household yard	Sc	[94]
Fungus gnats	Sciaridae	Mushrooms, Greenhouse	Sf	[49]
Mole crickets	*Scapteriscus* spp.	Turf	Sr, Ss	[92]
White grubs	Scarabaeidae	Turf	Hb, Sg, Sk	[92]

[a]Hb=*H. bacteriophora*, Hi=*H. indica*, Hm=*H. megidis*, Hmar=*H. marelatus*, Sc=*S. carpocapsae*, Sf=*S. feltiae*, Sg=*S. glaseri*, Sk=*S. kushidai*, Sr=*S. riobrave*.
[b]References indicate just some reports of efficacious control of these pests. For further evidence of efficacy and commercial development we suggest the reader consult Georgis and Hague [41], Grewal and Georgis [49], Kaya and Gaugler [65], and Shapiro-Ilan *et al* [92].

orally active insecticides such as toxins produced by *Bacillus thuringiensis* [16].

A key factor in the success of entomopathogenic nematodes as biopesticides is their amenability to mass production. These nematodes were first cultured more than 70 years ago [44], and currently they are commercially produced using three culture methods: *in vivo* and *in vitro* solid and liquid culture [31,36]. Each approach has advantages and disadvantages relative to cost of production, capital outlay, technical expertise required, economy of scale, and product quality, and each approach has the potential to be improved. Following production, a variety of formulation options are available [42]. This paper provides an up-to-date review and analysis of the production technology for entomopathogenic nematodes and their bacterial symbionts for commercial application as biopesticides.

In vivo culture

Method

In vivo culture is a two-dimensional system that relies on production in trays and shelves [31]. Production methods for culturing entomopathogenic nematodes in insect hosts have been reported by various authors [24,30,66,71,79,107]. All of these references describe (with some variation) a system based on the White trap [105], which takes advantage of the infective juvenile's natural migration away from the host cadaver upon emergence. The methods described consist of inoculation, harvest, concentration, and (if necessary) decontamination. Insects are inoculated with nematodes on a dish or tray lined with absorbent paper (e.g., filter paper) or another substrate conducive to nematode infection such as soil or plaster of Paris. After 2–5 days, infected insects are transferred to the White traps; if infections are allowed to progress too long before transfer, harm to nematode reproductive stages may occur, and the cadavers will be more likely to rupture [93]. White traps consist of a dish on which the cadavers rest surrounded by water, which is contained by a larger dish or tray (Figure 1). The central dish (containing the cadavers) provides a moist substrate for the nematodes to move upon, e.g., an inverted petri dish lid lined with filter paper (Figure 1) or filled with plaster of Paris. The progeny infective juveniles that emerge migrate to the surrounding water where they are trapped and subsequently harvested.

For commercial purposes harvested nematodes have to be concentrated prior to formulation. This can be accomplished by gravity settling [24], but prolonged periods of settling may be detrimental to the nematodes due to oxygen deprivation [18]. The process can be accelerated by vacuum filtration [71]. Centrifugation is also feasible [66], but, for commercial *in vivo* operations, the capital outlay for a centrifuge of sufficient capacity may be excessive. Prior to formulation, entomopathogenic nematodes (produced *in vivo* or *in vitro*) can be stored in aerated holding tanks for up to 3 months [42].

In the White trap method, contamination is minimized because infective juveniles migrate away from the cadaver leaving most potential contaminants behind. However, some host material or microbial contamination is possible and can be reduced by repeatedly washing the harvested nematodes using the concentration methods described previously. Additionally, decontamination can be accomplished by use of antimicrobial compounds [24,107] such as streptomycin sulfate, Hyamine® (methylbenzethonium chloride), merthiolate, NaOCl, or $HgCl_2$ [72], but the effects of these compounds on nematodes for commercial application has not been reported.

Factors affecting yield

In vivo production yields vary greatly among different insect hosts and nematode species. The most common insect host used for laboratory and commercial entomopathogenic nematode culture is the last instar of the greater waxmoth, *Galleria mellonella*, because of its high susceptibility to most nematodes, wide availability (at least in the US where it is sold commonly for fish bait or pet food), ease in rearing, and its ability to produce high yields [107]. There are only a couple of entomopathogenic nematodes not amenable to culture in *G. mellonella* (due to extremes in host specificity): *Steinernema kushidai* is most amenable to culture in scarab beetle larvae (Coleoptera: Scarabaeidae) [66,73], and *Steinernema scapterisci* is most amenable to mole crickets (*Scapteriscus* spp.) [53,77]. Other hosts in which *in vivo* production has been studied include the navel orangeworm (*Amyelois transitella*), tobacco budworm (*Heliothis virescens*), cabbage looper (*Trichoplusia ni*), pink bollworm (*Pectinophora gossypiella*), beet armyworm (*Spodoptera exigua*), corn earworm (*Helicoverpa zea*), gypsy moth (*Lymantria dispar*), house cricket (*Acheta domesticus*) and various beetles (Coleoptera) including the yellow meal worm (*Tenebrio molitor*) [13,19,27,53,70,89].

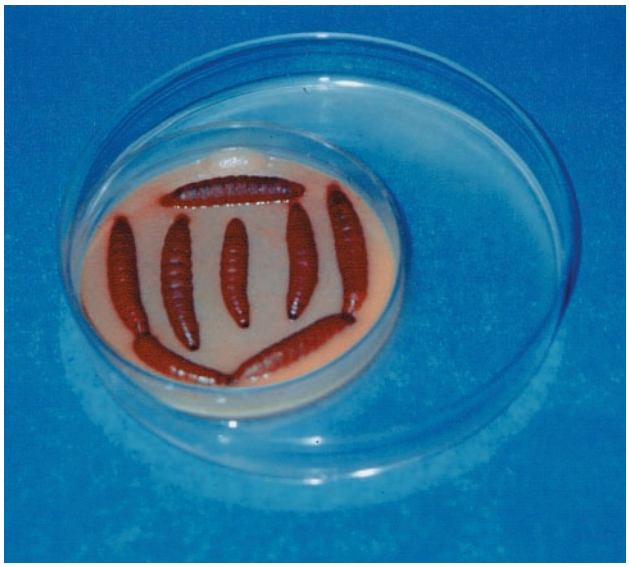

Figure 1 An example of a modified White trap. Insect larvae (*G. mellonella*) infected with entomopathogenic nematodes (*H. bacteriophora*) are placed on moist filter paper in an inverted petri dish lid (60 mm). As infective juvenile nematodes emerge from the insect cadaver they migrate into water, which is held in a larger petri dish (100 mm), and surrounds the central dish.

Other than *G. mellonella*, the most commonly used host for *in vivo* culture is *T. molitor*, but little research has been reported for production in this host. In response, we have compared relative yields in *T. molitor* for a number of entomopathogenic nematodes. Four *T. molitor* larvae (ca. 80±20 mg) were placed in a 90-mm petri dish lined with moist filter paper and exposed to nematodes at a rate of 800 infective juveniles per insect. After 4 days, the infected insects were transferred to White traps and yield was assessed according to procedures described by Shapiro *et al* [91]. There were three replicate (dishes) for each nematode strain and the experiment was repeated once. Average yields were compared among nematodes using analysis of variance and Duncan's multiple range test ($P<0.05$) [83]. The average infective juveniles produced were 115538a, 61786bc, 79292abc, 95667ab, 58250b, and 51875c for the nematodes *H. bacteriophora* (TF strain), *H. bacteriophora* (Hb strain), *H. bacteriophora* (Lewiston strain), *H. bacteriophora* (Oswego strain), *H. indica* (Hom1 strain), and *H. marelatus* (Point Reyes strain), respectively (numbers followed by different letters are significantly different, $P<0.05$). Clearly, nematode yield in *T. molitor* varies among nematode strains and species, e.g., *H. bacteriophora* (TF strain) produced approximately twice the progeny as *H. indica* (Hom1 strain) and *H. marelatus* (Point Reyes strain). Higher reproductive potential of one nematode relative to another (e.g., as observed in the TF strain) may result from a closer natural association to the host or its relatives [27,91].

In general, nematode yield is proportional to host size [13,30] yet yield per milligram insect (within host species) and susceptibility to infection is often inversely proportional to host size or age [13,24,91]. Ease of culture and infection are important factors when choosing a host, e.g., the long-horned beetle (Cerambycidae) can produce more than twice the number of nematodes as *G. mellonella* but (as with many of the insects listed above) difficulty or cost of rearing, and inconsistency of infection, precludes these insects from being suitable hosts [13]. Among nematode species yield is generally inversely proportional to size (see 50 and 59).

The choice of host species and nematode for *in vivo* production should ultimately rest on nematode yield per cost of insect and the suitability of the nematode for the pest target. Cost analysis among different host species has rarely been addressed. In a crude approach to the problem (i.e., without statistical analysis) Blinova and Ivanova [13] reported *T. molitor* to be superior in cost efficiency compared with *G. mellonella* and *T. ni* for producing *S. carpocapsae*. A hastened life cycle within the host might affect cost by allowing for faster production cycles; recently *S. abbasi* was reported to produce a roughly equivalent number of progeny in one half the time of other entomopathogenic nematodes (first emergence beginning after only 3.5 days) (Ref. [27] compared with Ref. [50]).

Another issue that has rarely been addressed in choice of nematode and host is the resulting quality of product. Nematode quality appears to be greater when cultured in hosts that are within the nematode's natural host range [2,3]. Furthermore, nematodes can adapt to the host they are reared on [101], which could reduce field efficacy if that host is not related to the target. Therefore, although *G. mellonella* may often be the most efficient host to use, it may not be the most appropriate ''medium'' for maximizing efficacy versus a particular target pest.

In vivo production yields are dependent on nematode dosage [14,111]. A dosage that is too low results in low host mortality, and a dosage that is too high often results in a high level of failed infections due to competition with secondary invaders [107]. These outcomes reduce production efficiency due to the need to remove live or poorly infected insects. The number of nematodes that invade a host is proportional to the exposure concentration [84,87]. Selvan *et al* [84] found that optimization of initial nematode density within the host (e.g., at 100 *H. bacteriophora* or *S. carpocapsae* per *G. mellonella*) maximizes nematode survival and fecundity. Thus, intermediate dosages maximize yield [14]. Similarly, host density per unit area affects nematode invasion [28] and thus may affect yield. Epsky and Capinera [28] reported that the percentage of nematodes invading a host increases with the insects per unit area of substrate. Flanders *et al* [30] did not detect significant effects of nematode or host density, but this may have been due to a limited range of densities tested or to a peculiarity of the particular strain that was tested (*H. bacteriophora* Oswego strain).

Environmental factors such as temperature, aeration, and moisture can affect yield. Rearing temperature affects both yield and lifecycle duration (time to emergence) [50]. Generally the optimum culture temperature is related to the nematode's climate of origin [50,74]. Grewal *et al* [50] determined the optimum rearing temperature and time to emergence in *G. mellonella* for 12 species and strains of entomopathogenic nematodes; optimum temperatures varied from 18 to 28°C. Adequate aeration is necessary for nematode development [18,31]. Moisture level is another essential component for *in vivo* culture. High levels of humidity must be maintained throughout the production cycle [107]. In the White trap method, the substrate must remain moist to prevent cadaver desiccation and allow emerging infective juveniles to migrate, but too much water will prevent movement [103] and interfere with oxygen exchange.

Inoculation method can affect infection efficiency and thus the yield potential. Inoculation for *in vivo* production can be accomplished by pipetting or spraying nematodes onto a substrate,

immersion of hosts in a nematode suspension, or (for some hosts) applying the nematodes to the insect's food. Comparison of methods has rarely been addressed. Immersion of hosts is more time efficient but requires more nematodes than other procedures (unpublished data). Additionally, some host–nematode combinations may not be suitable to the immersion method, e.g., it appears *H. bacteriophora* cannot infect *T. molitor* at levels required for mass production (90% or higher) using the immersion method, but can do so when applied by feeding or pipette (unpublished data). Blinova and Ivanova [13] reported that infectivity of *S. carpocapsae* in *T. molitor* was increased using the feeding method relative to other methods. Feeding, however, would require an additional step of removing infected cadavers from food remnants (which may cause contamination); thus inoculation procedure must be included in a cost efficiency analysis before a method is decided upon.

A concern for both *in vivo* and *in vitro* production is strain deterioration. When a biological control agent is isolated from nature and reared in the laboratory, or mass produced for commercial purposes, it may lose beneficial traits due to genetic processes including drift, inbreeding, or inadvertent selection [60]. Thus, repeated culturing of nematodes can result in reduction of quality and fitness characters such as virulence, environmental tolerance, or reproductive capacity [90,101]. Therefore, precautions against strain deterioration should be employed, e.g., cryopreservation of stock cultures [21], minimization of serial passages, and introduction of fresh genetic material [36,38].

Analysis

In vivo production of entomopathogenic nematodes offers several advantages and disadvantages relative to *in vitro* culture. *In vivo* production requires the least capital outlay and technical expertise, e.g., expertise in fermentation technology is not required [31,36]. Additionally, the quality of *in vivo* produced nematodes tends to be equal to or greater than nematodes produced with other approaches [35,109]. On the other hand, the costs of labor and insects tend to make *in vivo* culture the least cost efficient approach.

Economy of scale results from cost decreases associated with increases in production volume. Friedman [31] proposed that *in vivo* nematode production lacks any economy of scale because costs of the central components remain constant with increases in scale, i.e., space, labor, and insects. We propose that, although *in vivo* production may not offer the same degree of economy of scale as *in vitro* approaches (particularly liquid culture), some economy of scale can be obtained. Cost of space generally does not remain constant but decreases in relation to amount utilized. Additionally, labor can be reduced through mechanization or streamlining the process. Likewise insect costs may be reduced if the hosts are produced on-site and if the rearing process is mechanized.

The LOTEK system [34] is one approach to increasing *in vivo* production efficiency and scalability. The White trap method requires two trays, one for inoculation and one for harvest; the transfer from inoculation to harvest tray requires labor. In contrast, the LOTEK method requires one perforated tray and an automated harvest system in which infective juveniles are misted downward and pumped to a collection tank (Figure 2). In the White trap method, yield is reduced when infective juveniles fail to reach the water trap, a problem that increases with scale. The misting system in LOTEK collects practically all infective juveniles and thus increases yield (Gaugler *et al*, unpublished data). Furthermore, the LOTEK process reduces space requirements and decreases the time required for harvest, thus increasing turnaround time.

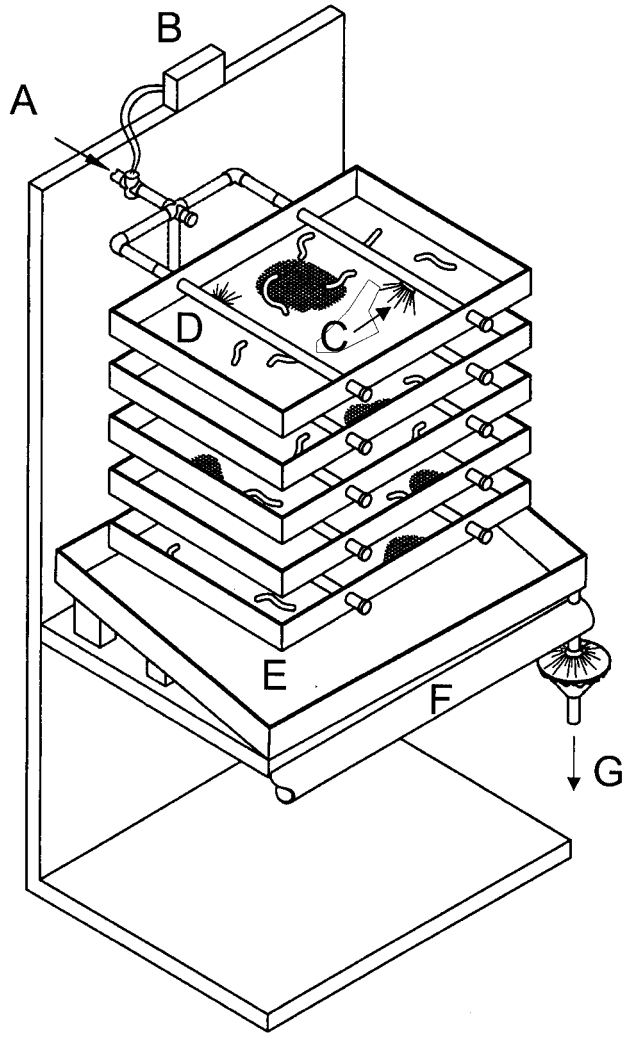

Figure 2 LOTEK system harvesting apparatus. Water, provided by mist nozzles, induces infective juvenile nematode emergence and rinses the nematodes via gravity flow to storage. (A) water supply, (B) timer, (C) mist nozzle, (D) holding tray, (E) collection pan, (F) collection pipe, (G) to storage tank.

Another approach to increasing *in vivo* production efficiency may be through production and application of entomopathogenic nematodes in infected hosts. Using this method, infected-host cadavers are applied to the target site and pest suppression is subsequently obtained by the emerging progeny infective juveniles. This approach is likely to reduce production costs substantially because labor-intensive steps of harvest and concentration are avoided [93]. Application of nematodes in infected-host cadavers provides significant pest control [62]. Additionally, studies indicate that nematodes emerging into soil directly from the host cadaver can be more infective and disperse more than nematodes applied in aqueous formulations/suspensions [86,87]. Application of nematode-infected hosts has not been commercialized possibly due to problems in storage, transport, and application of the fragile cadavers. To overcome these hindrances, Shapiro-Ilan *et al* [93] found that infected hosts can be formulated with a coating (e.g., starch and clay) thereby increasing desiccation tolerance and

storability, and preventing cadaver rupture or adhesion during handling.

Despite limitations in cost efficiency and scale, *in vivo* production is a cottage industry that has managed to sustain itself throughout the development of numerous larger companies producing nematodes *in vitro* [36,38]. *In vivo* production is likely to continue as small business ventures for niche markets where competition by *in vitro* producers is limited, and in developing countries where labor is inexpensive. Another arena for which *in vivo* production may be highly suitable is local production by grower cooperatives [33]. We anticipate that innovations to improve efficiency will enable *in vivo* production to play an expanded role in pest management programs.

In vitro: solid culture

Method

Entomopathogenic nematodes were first grown *in vitro* on a solid medium axenically [45]. Thereafter it was realized that growth increased with the presence of bacteria [46,61]. The importance of the natural symbiont was recognized [81] and monoxenic culture has been the basis for *in vitro* culture since [9,58,108]. To create monoxenic cultures surface-sterilized nematodes were added to a lawn of bacterial symbionts [9,108]. Lunau *et al* [72] suggested that surface sterilization of infective juveniles is insufficient to establish monoxenicity because contaminating bacteria survive beneath the nematode's cuticle. Therefore, an improved method has been developed where nematode eggs (which are axenic), obtained by rupturing gravid females in an alkaline solution, are placed on a pure culture of the symbiont [72].

Solid culture was first accomplished in two-dimensional arenas, e.g., petri dishes, containing various media such as those based on dog food, pork kidney, cattle blood, and other animal products [58]. Wouts [108] developed an improved medium (less expensive and more consistent from batch to batch) that included yeast extract, nutrient broth, vegetable oil, and soy flour.

In vitro solid culture advanced considerably with the invention of a three-dimensional rearing system involving nematode culture on crumbled polyether polyurethane foam [9]. A liquid medium is mixed with foam and autoclaved. Bacteria are inoculated first followed by the nematodes ca. 3 days later. Nematodes can be harvested within 2–5 weeks [9,10] by placing the foam onto sieves, which are immersed in water. Infective juveniles migrate out of the foam, settle downward, and are pumped to a collection tank; the product is cleaned through repeated washing with water, i.e., sedimentation and decanting [9,10]. As in petri dishes, media for this approach were initially animal product based (e.g., pork kidney or chicken offal) but was later improved (for cost and consistency) and may include various ingredients including peptone, yeast extract, eggs, soy flour, and lard [55,56].

The method developed by Bedding [9] was first accomplished in Erlenmeyer flasks (Figure 3) and then expanded to autoclavable bags with filtered air pumped in through a makeshift port [9,10]. The bacteria were inoculated first followed by the nematodes several days after [9,10]. Later it was realized that the two organisms could be added simultaneously if a large concentration of bacteria is used [36]. The potential for large-scale production was further advanced through several measures including using bags with a gas permeable Tyvac® strip for ventilation (rather than forced air), automated mixing and autoclaving, and harvest through centrifugal sifters [36].

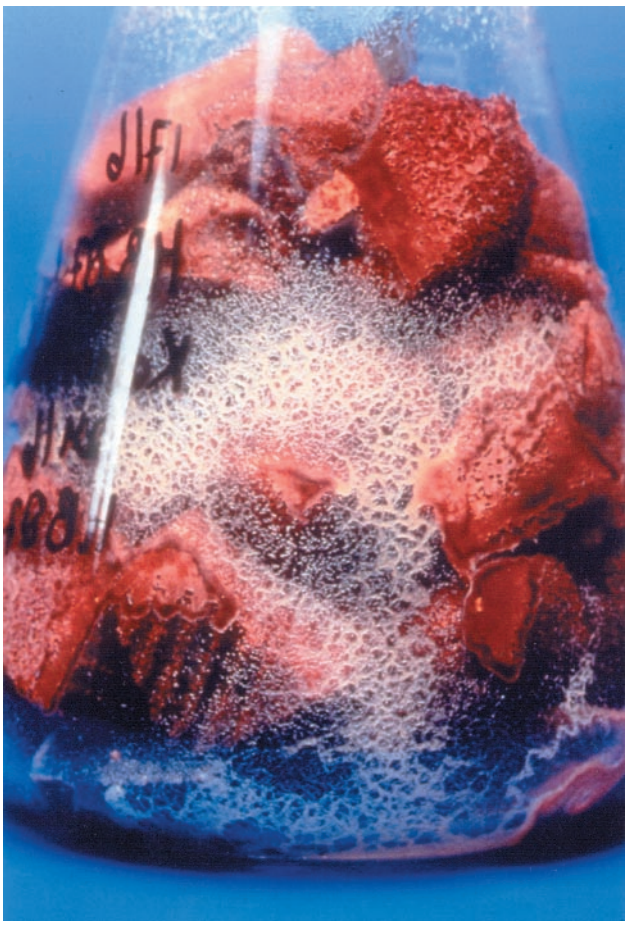

Figure 3 Solid culture (Bedding method) of entomopathogenic nematodes in an Erlenmeyer flask. Entomopathogenic nematodes and their bacterial symbionts are grown *in vitro* on crumbled polyurethane foam infused with growth medium. Infective juvenile nematodes that have completed the production cycle are evident on the interior surface of the flask. Photograph courtesy of LJM Gerritsen and PH Smits (Plant Research International, Wageningen, The Netherlands).

Factors affecting yield

Nematode inoculum size can affect yield in some strains but not others [55,56,104]. For example, *S. carpocapsae* (Agriotos strain) produced optimum yields at an intermediate inoculum size (2,000 infective juveniles per g medium) [56], whereas *S. carpocapsae* (CB2B strain) and *H. bacteriophora* (H06) were not affected by inoculum size [55]. Bacteria inoculum size does not appear to be important in yield determination [55,56].

Culture time is inversely related to temperature and should be optimized for maximum yield on a species or strain basis [23,55,56]. Increasing inoculum size may increase nematode growth rate and decrease culture time [55]. Longer culture times can provide higher yields but nematode mortality may also increase with time [55,56] and culture time must be weighed against the cost of space.

Media composition can have a substantial effect on nematode yield. Increasing the quantity and quality of lipids will increase nematode yield and quality [23,55]. Lipid components that reflect

the composition of the nematode's natural host are most suitable to production [2,3]. Other medium ingredients that may have a direct effect on nematode yield include protein source and salts [23].

Analysis

Advantages of *in vitro* solid culture lie in it being an intermediate between *in vivo* and liquid culture. The level of capital required for startup and level of expertise required for the process fall in between the other two methods. Scale of production can be limited by autoclave size (needed to sterilize bags) and sterile hood space (for inoculation), but these issues can be overcome if automated mixing/sterilization is used, and if clean room technology is implemented [36]. Friedman [31] suggested that mass production using *in vitro* solid methods is likely to produce higher quality nematodes than *in vivo* methods, but no reports have substantiated this claim. Several studies indicate quality to be similar in nematodes produced by solid and *in vivo* methods [1,3,35]. Contrarily, Yang *et al* [109] reported reduced quality in *S. carpocapsae* produced in solid culture compared with *in vivo* culture.

Production of nematodes in solid culture has not grown substantially in the last two decades, and appears to be used by only two companies (one in the US and one in China) [36]. Without sophisticated mechanization (e.g., bulk sterilization) solid culture may not offer substantial advantages in cost efficiency relative to *in vivo* production (a cost analysis is warranted). Yet large-scale mechanization for solid culture requires substantial capital [36]. If *in vitro* solid culture is to be adopted on wider scale, efficiency will have to be increased by finding less capital-intensive methods of mechanization.

In vitro: liquid culture

Method

Entomopathogenic nematodes were first grown in liquid culture axenically [45,57]. In the development of monoxenic liquid culture in a bioreactor, it was recognized that the major barrier would be the opposing challenges of supplying enough oxygen while preventing excessive shearing of the nematodes [17,31,32,78]. The problem can be exacerbated by media viscosity [31]. Pace *et al* [78] addressed the dilemma by relying on bubbling, e.g., with a downward sparger, coupled with limited agitation. Friedman *et al* [32] used an airlift fermenter coupled with a variable agitation regime based on the determination that nematode susceptibility to shearing varies with developmental stage. Various innovations in mixing and aeration have been introduced since the work of Friedman *et al* [32] and Pace *et al* [78] including internal [98] and external [76] bioreactors. Foaming, another problem observed during agitation, can be reduced by consideration of bioreactor design [98] and antifoam or defoaming agents [36].

In liquid culture, symbiotic bacteria are introduced first followed by the nematode infective juveniles [17,98,102]. Various ingredients for liquid culture media have been reported including soy flour, yeast extract, canola, corn oil, thistle oil, egg yolk, casein peptone, milk powder, liver extract, and cholesterol [26,102,110]. Culture times, which can vary depending on media [102] and species, may be as long as 3 weeks [20,102] but many species reach maximum production in 2 weeks or less [26,31,76,98,110]. Once the culture is completed, nematodes can be removed from the medium through centrifugation [102].

Factors affecting yield

Although the steinernematids and heterorhabditids both have the basic requirement of tolerating adequate aeration without shearing, the strategies for maximizing yield of the two genera in liquid culture diverge due to differing life cycles and reproductive biology. Steinernematids are amphimictic and are capable of mating in liquid culture [99] and thus, maximization of mating can have a profound effect on yield [76]. Maximization of encounters between male and female steinernematids (which differ significantly in mass) can be achieved through bioreactor design and regulation of aeration, e.g., optimum mating was observed at an aeration rate of 0.05 vessel volumes per minute (vvm) when a range of 0.01–0.15 vvm was tested [76]. Maximization of mating, however, is not an issue for production of heterorhabditids in liquid culture because the first generation is exclusively hermaphrodites and, although subsequent generations can contain amphimictic forms, male and female heterorhabditids cannot mate in liquid culture [99]. Thus, optimization of liquid heterorhabditid production must focus on the first generation in which all the nematodes are capable of reproducing (by selfing).

Maximizing heterorhabditid yields in liquid culture is dependant on the degree of recovery. Recovery is a term used to describe the development step when the developmentally arrested infective juvenile molts to initiate growth [64]. Obviously, infective juveniles that do not recover will not contribute to yield (only adult nematodes can reproduce). Whereas levels of heterorhabditid recovery *in vivo* tend to be 100% [97], recovery in liquid culture ranges from 0% to 85% [26,64,97,98,110]. An undescribed food signal has been found to be a key in initiating recovery [97]. Additionally, recovery can be positively affected by increased levels of aeration [98], CO_2 [64], and lipid content in media [110], and negatively affected by temperature increases [26].

The central component of liquid culture media is lipid content and composition. Yoo *et al* [110] reported that increases in media lipid content from 2.5% to 8% caused significant yield increases. Yield and quality of nematodes in liquid culture are also affected by the lipid source [3,110] and, similar to solid culture, lipid components are recommended to mimic natural host lipids [3]. Other nutrients that have been reported to affect yield positively include the concentration of glucose [63] and yeast extract [20].

Other factors that affect entomopathogenic nematode yield in liquid culture include nematode inoculum size and species effects. Han [54] reported optimal *H. bacteriophora* yields with intermediate inoculum sizes, whereas a positive relationship between inoculum size and yield exists for *S. carpocapsae*. Ehlers *et al* [26] found no effect of inoculum size on *H. indica* yields. Entomopathogenic nematodes vary greatly in their potential yields in liquid culture; generally yield is inversely proportional to nematode size [26]. The greatest production in liquid culture thus far was for the relatively small and recently discovered nematode, *H. indica*, which provided an average yield over 450,000 infective juveniles per ml [26]. Maximum average yields reported for other species include 300,000 and 320,000 infective juveniles per milliliter for *H. bacteriophora* and *S. carpocapsae*, respectively [54], 138,000 for *H. megidis* [98], and 71,470 infective juveniles per milliliter for *S. feltiae* [20].

Analysis

In vitro liquid culture is the most cost efficient process for producing entomopathogenic nematodes. The cost of producing

1 million *S. carpocapsae* in a bioreactor can be as low as US$0.012, which is one-tenth the cost of the insects alone (*G. mellonella*) required to produce an equivalent amount of *S. carpocapsae in vivo* [36]. The efficiency of the liquid approach lies in its economy of scale; as scale increases, production cost per unit decreases [31]; liquid culture of entomopathogenic nematodes has been accomplished in bioreactors of up to 80,000 l [42]. Although liquid culture offers increased cost efficiency relative to other production methods, it also demands greater capital investment and a higher level of technical expertise.

Quality can be an issue for *in vitro* liquid culture of entomopathogenic nematodes. Gaugler and Georgis [35] reported reduced field efficacy of liquid culture produced *H. bacteriophora* relative to *in vivo* and solid culture produced nematodes, yet *S. carpocapsae* [35] and *S. riobrave* [88] were not affected by culture method. Media composition, particularly lipid content, which is important in determining nematode survival and virulence, is critical to predicting nematode quality [1-3,110]. Even the quality of live media (insects) can determine the relative quality of nematodes produced *in vivo* versus *in vitro*. For example, *H. bacteriophora* and *S. glaseri* produced in the Japanese beetle, *Popillia japonica* (which is a natural host for these nematodes), had a higher lipid content than nematodes produced in the factitious host *G. mellonella* or liquid culture, which did not differ from each other [1,3]. Similarly, Grewal *et al* [53] reported that *S. scapterisci* produced in the house cricket, *A. domesticus*, an insect that is not the nematode's natural host [77], had lower virulence than liquid culture produced nematodes. Again, the need to develop media that mimic the natural host is emphasized.

Future research and development in liquid culture are expected to lead to higher yields and reduced costs for capital investment. The greatest advances in yield are likely to result from increases in recovery rates for heterorhabditids. Reduction in startup costs may come from development of cheaper, chemically sterilized bioreactors [36] or from fermentation "kits" in which users are provided ingredients (media and inoculum) and grow their own nematodes in plastic bubble column minifermenters [37]. Such innovations may be quite successful because (unlike the large-scale bioreactor approach) they will enable nematode production to be directed to local marketing [33].

Formulation

Regardless of culture method, once entomopathogenic nematodes are commercially produced they must be formulated for delivery and application [39,42]. An effective formulation provides a suitable shelf life, stability of product from transport to application, and ease of handling [42]. Shelf life, in most entomopathogenic nematode formulations, is obtained by reducing nematode metabolism and immobilization, which may be accomplished through refrigeration and partial desiccation [39,42].

Optimum storage temperature for formulated nematodes varies according to species: generally, steinernematids tend to store best at temperatures near 4–8°C whereas heterorhabditids have longer shelf life at temperatures close to 10–15°C. The climate of origin is predictive of the optimum storage temperature, e.g., *H. indica*, a nematode originating only in warm climates, stores better at 15–20 than at 10°C [91,100]. Strauch *et al* [100] reported increased survival in various formulations with the addition of various preservatives, acids, and spice extracts.

Desiccation may aid in achieving useful nematode formulations. However, the process can reduce nematode fitness and longevity [48,106], and the potential for using desiccation for long-term storage is limited because entomopathogenic nematodes apparently cannot reach a true cryptobiotic state (fully arrested metabolism) upon desiccation [106]. Successful desiccation is dependant on rate; the nematodes cannot tolerate rapid desiccation [95]. Desiccation tolerance can be enhanced by preconditioning the nematodes, e.g., by exposing them to 97% RH for 72 h [96]. Levels of desiccation tolerance vary by nematode species [48] and strain [96].

Various formulations for entomopathogenic nematodes have been reported including activated charcoal, alginate and polyacrylamide gels, baits, clay, peat, polyurethane sponge, vermiculite, and water-dispersible granules (WDG) [39,42]. Due to cost, *in vivo* producers tend to use low-technology formulations such as sponge and paste. The nematodes are not desiccated and tend to retain high viability. However, these formulations cannot be packaged at high densities and are therefore not appropriate for large-scale usage because of labor requirements in application. Formulations used by most *in vitro* producers include clay, gels, vermiculite, and WDG. For example, a successful nondesiccated formulation has been developed for *in vitro* produced nematodes based on vermiculite, which allows a shelf life of at least 1 month for *H. megidis* and 2–3 months for steinernematids (Graeme Gowling, MicroBio, Cambridge, UK, personal communication).

A breakthrough in formulation technology was cited in the introduction of WDG, in which the steinernematids enter a partially anhydrobiotic state allowing them to survive up to 6 months at 4–25°C (substantially longer than previous formulations) [42]. Subsequent research, however, indicated reduced efficacy of WDG formulated *S. carpocapsae* relative to *in vivo* produced nematodes [8]. Contrarily, Shapiro and McCoy [88] and Grewal [48] reported no effect of WDG on steinernematid virulence; the reduced virulence observed by Baur *et al* [8] may have been due to culture method (and perhaps a poor culture medium). Formulations comparable to WDG have not been reported for heterorhabditids, which tend to be less desiccation tolerant than steinernematids.

Conclusions

Development of *in vivo* and *in vitro* entomopathogenic nematode production technology has enabled these organisms to become important biopesticides. However, the greatest barrier to wider application of these nematodes is cost. In many arenas, nematodes, products cannot compete with the relatively low cost of chemical insecticides. Thus, nematodes have, for the most part, been relegated for use in high value agricultural niche markets, or in home gardens and yards. Further advances in nematode production technology, coupled with increasing restrictions on chemical insecticide use, will likely narrow the gap between these two pest management strategies. The increasing interest in production by developing countries and progress in mechanization and streamlining, will lead to expansion of *in vivo* nematode production. *In vitro* solid culture will continue to offer an intermediate step in technology and cost between *in vivo* and liquid culture; innovations in mechanization will also likely lead to expansion of this approach. *In vitro* liquid culture can be enhanced through further development of media (for quality and yield), improvement of heterorhabditid recovery, and reductions in startup costs (e.g., cheaper bioreactors).

Production technology will also be advanced through the discovery of new entomopathogenic nematode strains or species (akin to the recent introduction of *H. indica* in liquid culture). Finally, interest in the antimicrobial properties of the symbiont, and associated metabolites, will inevitably lead to advances in nematode production, albeit indirectly.

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