

Entomopathogenic Nematode Production and Application Technology

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Abstract: 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 recent years in developing EPN formulations, particularly for above ground applications, e.g., mixing EPNs with surfactants or polymers or with sprayable gels. Bait formulations and insect host cadavers can enhance EPN persistence and reduce the quantity of nematodes required per unit area. This review provides a summary and analysis of factors that affect production and application of EPNs and offers insights for their future in biological insect suppression.

Key words: application technology, entomopathogenic nematode, *Heterorhabditis*, production, *Steinernema*.

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 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). 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).

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. Following production, a variety of formulation and application alternatives are also available (Grewal, 2002). The objectives of this review are to provide a summary and analysis of factors that affect

production and application of EPNs, and provide insight toward enhancing approaches to achieve greater success in biological control.

PRODUCTION METHODS

In vivo culture method: The general approach to *in vivo* culture is a two dimensional system that relies on production in trays and shelves (Friedman, 1990; Shapiro and Gaugler, 2002; Ehlers and Shapiro-Ilan, 2005). *In vivo* production methods for culturing EPNs have been reported by various authors (Dutky et al., 1964; Poinar, 1979; Woodring and Kaya, 1988; Lindegren et al., 1993; Flanders et al., 1996; Kaya and Stock, 1997; Shapiro-Ilan et al., 2002a). The systems described in all of these references (with some variation) entail a system that is based on the White trap (White, 1927), which takes advantage of the progeny IJ's natural migration away from the host-cadaver upon emergence. The approach consists of inoculation, harvest, concentration, and (if needed) decontamination. Insect hosts are inoculated on a dish or tray lined with absorbent paper or another substrate conducive to nematode infection such as soil or plaster of Paris. After approximately 2-5 days, infected insects are transferred to the White traps; if infections are allowed to progress too long before transfer the chance of the cadaver rupturing and harm to reproductive nematode stages is increased (Shapiro-Ilan et al., 2001). White traps consist of a dish or tray on which the cadavers rest surrounded by water, which is contained by a large arena. As IJs emerge they migrate to the surrounding water trap where they are harvested. The scale of the White trap in size and number can be expanded to commercial levels.

Factors affecting yield for in vivo culture: *In vivo* production yields depend on nematode dosage and host density (Zervos et al., 1991; Boff et al., 2000; Shapiro-Ilan et al., 2002a). A dosage that is too low results in low host mortality and a dosage that is too high may result in failed infections due to competition with secondary invaders (Woodring and Kaya, 1988). Thus, intermediate dosages

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can be used to maximize yield (Boff et al., 2000). For example, for infecting the greater wax moth *Galleria mellonella*, rates of approximately 25 to 200 IJs per insect are sufficient (depending on nematode species and method of inoculation) whereas higher rates are generally needed for the yellow mealworm, *Tenebrio molitor* (e.g., 100 to 600 IJs per insect). Crowding of hosts can lead to oxygen deprivation or buildup of ammonia (Shapiro-Ilan et al., 2000a; 2002a). In general, the number of hosts exhibiting patent signs of nematode infection increases with nematode concentration and decreases with host density per unit area (Shapiro-Ilan et al., 2002a). Thus, optimization of host density and inoculation rate for maximum yield is recommended (Shapiro-Ilan et al., 2002a).

In vivo production yields vary greatly among insect hosts and nematode species. Due to high susceptibility to most nematodes, wide availability, ease in rearing, and the ability to produce high yields, the most common insect host used for laboratory and commercial EPN culture is *G. mellonella* (Woodring and Kaya, 1988). Significant research (and commercial application) has also been achieved for production of EPNs in *T. molitor* (Blinova and Ivanova, 1987; Shapiro-Ilan et al., 2002a). Other hosts in which *in vivo* production has been studied include the navel orangeworm (*Ameylois 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) (Lindegren et al., 1979; Blinova and Ivanova, 1987; Cabanillas and Raulston, 1994; Grewal et al., 1999; Elawad et al., 2001).

Generally nematode yield is proportional to host size (Blinova and Ivanova, 1987; Flanders et al. 1996). Yet yield per mg insect (within host species) and susceptibility to infection is often inversely proportional to host size or age (Dutky et al. 1964; Blinova and Ivanova 1987; Shapiro et al. 1999a; Dolinski et al., 2007; Dias et al., 2008). Yield is also generally inversely proportional to nematode size (Grewal et al., 1994; Hominick et al., 1997; Shapiro and Gaugler, 2002). 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 (Blinova and Ivanova, 1987; Shapiro-Ilan et al., 2002a).

Environmental factors including optimum temperature, and maintaining adequate aeration, and moisture can affect yield (Burman and Pye, 1980; Woodring and Kaya, 1988; Friedman, 1990; Grewal et al., 1994; Shapiro-Ilan et al., 2002a; Dolinski et al., 2007). Infection efficiency and yield can also be affected by inoculation method. *In vivo* can be accomplished by pipetting or spraying nematodes onto a substrate, immersion of insects in a nematode suspension, or (in some cases) applying the nematodes to the insect's food. Immersion of hosts is generally more time efficient but requires more

nematodes than other procedures (Shapiro-Ilan et al., 2002a). Blinova and Ivanova (1987) reported that infection of *T. molitor* by *S. carpocapsae* was increased using the feeding method relative to other methods. Feeding, however, requires an additional step of removing infected cadavers from food remnants (which may cause contamination); thus, before a method is decided upon, inoculation procedures must be included in a cost efficiency analysis.

In vitro solid culture method: *In vitro* culturing of EPNs is based on introducing nematodes to a pure culture of their symbiont in a nutritive medium. In earlier work, to create monoxenic cultures surface sterilized nematodes were added to a lawn of the bacterial symbionts (Akhurst, 1980; Wouts, 1981). However, Lunau et al. (1993) suggested that surface sterilization of IJs is insufficient to establish monoxenicity because contaminating bacteria survive beneath the nematode's cuticle. Thus, an improved method has been developed where axenic nematode eggs are placed on a pure culture of the symbiont (Lunau et al. 1993).

Solid culture was first accomplished in two dimensional arenas e.g. petri dishes, using various media (Hara et al., 1981; Wouts, 1981). Subsequently, *in vitro* solid culture advanced considerably with the invention of a three-dimensional rearing system involving nematode culture on crumbled polyether polyurethane foam (Bedding, 1981). A liquid medium is mixed with foam, autoclaved, and then inoculated with bacteria followed by the nematodes. Nematodes are then harvested within 2-5 weeks (Bedding, 1981; Bedding, 1984) by placing the foam onto sieves immersed in water. Media for this approach was initially animal product based (e.g., pork kidney or chicken offal) but was later improved and may include various ingredients including peptone, yeast extract, eggs, soy flour, and lard (Han et al., 1992; 1993). The approach developed by Bedding (1981) was expanded to autoclavable bags with filtered air being pumped in (Bedding, 1984). Large scale production was further advanced through several measures including using bags with gas permeable Tyvac® strips for ventilation, automated mixing and autoclaving, simultaneous inoculation of nematodes and bacteria, sterile room technology, and automated harvest through centrifugal sifters (Gaugler and Han, 2002).

Factors affecting yield for in vitro solid culture: Nematode inoculum rate (IJs per unit of media) can affect yield in some nematode strains but not others (Han et al. 1992, 1993; Wang and Bedding, 1998). Culture time is inversely related to temperature and thus should be optimized for maximum yield on a species or strain basis (Dunphy and Webster, 1989; Han et al. 1992, 1993). Increasing inoculum size can increase nematode growth and decrease culture time (Han et al., 1992). Longer culture times can provide higher yields yet nematode mortality may also increase with time (Han et al., 1992, 1993) and culture time must be weighed against the cost of space and diminishing returns.

Media composition can have considerable effects on yield in solid culture. Increasing the lipid quantity and quality leads to increases in nematode yield (Dunphy and Webster, 1989; Han et al., 1992). Lipid components reflecting the nematode's natural host composition are most suitable (Abu Hatab and Gaugler, 2001; Abu Hatab et al., 1998). Other media ingredients that may have an effect on nematode yield include proteins and salts (Dunphy and Webster, 1989).

In vitro liquid culture method: The development of monoxenic liquid culture of EPNs faces the opposing challenges of supplying enough oxygen while preventing excessive shearing of nematodes (Pace et al., 1986; Buecher and Popiel, 1989; Friedman et al., 1989, 1990). The issue was initially addressed in various ways such as relying on bubbling, e.g., with a downward sparger, coupled with limited agitation (Pace et al., 1986), or using an airlift fermenter coupled with a variable agitation regime (Friedman et al., 1989). Various innovations in mixing and aeration have been subsequently introduced including internal (Strauch and Ehlers, 2000) and external (Neves et al., 2001) bioreactors; internal loop vessels have baffles placed inside the single vessel, which creates the channels required for the circulation, whereas in external loop vessels circulation takes place through separate conduits.

Generally, in liquid culture, symbiotic bacteria are first introduced followed by the nematodes (Buecher and Popiel, 1989; Surrey and Davies, 1996; Strauch and Ehlers, 2000). Various ingredients for liquid culture media have been reported including soy flour, yeast extract, canola oil, corn oil, thistle oil, egg yolk, casein peptone, milk powder, liver extract and cholesterol (Surrey and Davies, 1996; Ehlers et al., 2000; Yoo et al., 2000). Culture times vary depending on media and species, and may be as long as three weeks (Surrey and Davies, 1996; Chavarria-Hernandez and de la Torre, 2001) though many species can reach maximum IJ production in two weeks or less (Friedman 1990; Ehlers et al., 2000; Neves et al., 2001; Strauch and Ehlers, 2000; Yoo et al., 2000). Once the culture is completed, nematodes can be harvested from media via centrifugation (Surrey and Davies, 1996).

Factors affecting yield for in vitro liquid culture: Both steinernematids and heterorhabditids share the requirements of adequate aeration (without shearing).

Otherwise, the strategies for maximizing yield of the two genera in liquid culture differ due to their life cycles and reproductive biology. Steinernematids (except one species) occur only as males and females and are capable of mating in liquid culture (Strauch et al., 1994); thus, maximization of mating is paramount and can be achieved through bioreactor design and regulation of aeration (Neves et al., 2001). Maximization of mating, however, is not applicable for heterorhabditid production in liquid culture because the first generation is exclusively hermaphrodites and, although subsequent generations contain amphimictic forms, they cannot mate in liquid culture (Strauch et al., 1994). Thus, maximizing heterorhabditid yields in liquid culture depends on the degree of recovery (the developmental step when IJs molt to initiate completion of their life cycle). While levels of heterorhabditids recovery *in vivo* tend to be 100% (Strauch and Ehlers, 1998), recovery in liquid culture may range from 0-85% (Ehlers et al., 2000; Jessen et al., 2000; Strauch and Ehlers, 1998, 2000; Yoo et al. 2000). Recovery can be affected by nutritional factors, aeration, CO₂, lipid content, and temperature (Ehlers et al., 2000; Jessen et al., 2000; Strauch and Ehlers, 1998, 2000; Yoo et al., 2000).

Yield from liquid culture may also be affected by other factors including media, nematode inoculum, and nematode species (Han, 1996; Ehlers et al., 2000). The central component of the liquid culture media is lipid source and quantity (Abu Hatab et al., 1998; Yoo et al., 2000). Other nutrients that have been reported to affect yield positively include the content of glucose (Jeffke et al., 2000) and yeast extract content (Chavarria-Hernandez and de la Torre, 2001). Generally nematode yield is inversely proportional to the size of the species (Ehlers et al., 2000). Maximum average yields reported include 300,000 and 320,000 IJs per ml for *H. bacteriophora* and *S. carpocapsae*, respectively (Han, 1996), 138,000 per ml for *H. megidis* (Strauch and Ehlers, 2000), 71,470 IJs per ml for *S. feltiae* (Chavarria-Hernandez and de la Torre, 2001), and 450,000 IJs per ml for *H. indica* (Ehlers et al., 2000).

Analysis of production methods and potential for improvement (the road to the future): A comparison of production methods is summarized in Table 1. *In vivo* EPN production offers several advantages relative to *in vitro* culture including requiring the least capital outlay and

TABLE 1. A comparison of entomopathogenic nematode production approaches.

Factor	Production Approach		
	<i>In Vivo</i>	<i>In Vitro - Solid</i>	<i>In Vitro - Liquid</i>
Initial costs/capital outlay	Low	Intermediate	High
Expertise required	Nominal	Intermediate	Extensive
Ease of achieving quality	Easy	Difficult	Difficult
Adoption of technique to new nematode species	Easy	Difficult	Difficult
Labor requirements	High	Intermediate	Low
Economy of Scale	Low	Intermediate	High

the least amount of technical expertise for start-up (Shapiro-Ilan and Gaugler, 2002); additionally, the quality of *in vivo* produced nematodes tends to be equal to or greater than EPNs produced with other approaches (Gaugler and Georgis, 1991; Yang et al., 1997). Furthermore, adapting production techniques to new or additional nematode species or strains is straightforward with *in vivo* production, whereas *in vitro* methods can require substantial adjustments in media or processing parameters. The primary challenge for *in vivo* EPN culture relative to *in vitro* methods is the costs of insects, which tends to make *in vivo* culture the least cost efficient approach.

The economics of *in vivo* production can be improved substantially by producing the insect hosts “in-house” and mechanizing the process thereby reducing labor. Several approaches to mechanization of nematode inoculation (Shapiro-Ilan et al., 2009) and harvest (Gaugler et al., 2002; Shapiro-Ilan et al., 2011a) have been introduced. Additionally, optimization of insect diets can lead to improved efficiency in insect host production and quality of nematodes (via tri-trophic interactions) (Morales et al., 2011a; Shapiro-Ilan et al., 2008, 2011c). Insect host production can also be mechanized, e.g., through automated sifting of insects for separation (Morales et al., 2011b). Despite shortcomings in economy of scale, *in vivo* production has managed to sustain itself as a cottage industry throughout the commercial development of *in vitro* enterprises (Shapiro-Ilan and Gaugler, 2002), and it is likely to continue and perhaps expand based upon advancements in mechanization described above.

In terms of capital outlay and economy of scale, the merits of *in vitro* solid culture are generally considered to be intermediate between *in vivo* and liquid culture. Similar to *in vivo* production, efficiency can be increased through mechanization (labor reduction) and media enhancement (Gaugler and Han, 2002; Shapiro and Gaugler, 2002). Several studies indicate quality to be similar to nematodes produced by *in vivo* methods (Abu Hatab et al., 1998; Abu Hatab and Gaugler, 1999; Gaugler and Georgis, 1991). Contrarily, Yang et al. (1997) reported reduced fitness in *S. carpocapsae* produced in solid culture compared with *in vivo* culture. Recently, advances have been made in expanding *in vitro* solid production of EPNs. For example, in China, a pilot factory was established for solid production of several EPN species based on the lower labor cost, improved media and mechanization process. The factors influencing the production efficiency were explored, including medium development, optimization of the culture parameters, recovery of the IJ inocula, formation of the IJs, extraction and harvest of IJs (Han et al., 1995, 1997). A company called Century Horse Development Ltd, under the guidance of Guangdong Entomological Institute, is currently in commercial production; products from the solid culture system are provided for field trials in China and for internal and international markets.

In vitro liquid culture is considered to be the most cost efficient process for producing entomopathogenic nematodes and thus accounts for the bulk of the world market in EPNs. Liquid culture of entomopathogenic nematodes has been accomplished in bioreactors of up to 80,000 liters (Georgis et al., 1995). 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.

Several reports indicated reduced quality or efficacy in *in vitro* liquid produced EPNs relative to those produced in solid culture or *in vivo* (Gaugler and Georgis, 1991; Cottrell et al., 2011) whereas other comparisons did not detect any differences (Georgis and Gaugler, 1991; Shapiro and McCoy, 2000a). Thus, it is clear that high quality EPNs can be produced using *in vitro* liquid culture, but factors such as media composition and environmental conditions in the bioreactor or downstream processing can reduce quality. Some recent advancements in liquid culture technology serve to increase quality and efficiency of production e.g., optimizing media and bioprocess kinetics through modeling (Chavarria-Hernandez et al., 2006, 2010), as well as improvements in inoculum and bacterial cell density (Hirao and Ehlers, 2010), timing of inoculation (Johnigk et al., 2004) and downstream processing (Young et al., 2002). Future research and development in liquid culture (focusing on media optimization and bioreactor design) are expected to lead to additional benefits such as higher yields and reduced costs.

A concern for both *in vivo* and *in vitro* production is strain deterioration. Repeated culturing of nematodes can result in reduction of beneficial traits such as virulence, environmental tolerance or reproductive capacity (Shapiro et al., 1996a; Wang and Grewal, 2002; Bai et al., 2005; Bilgrami et al., 2006); therefore, precautions against strain deterioration should be employed, e.g., minimization of serial passages, introduction of fresh genetic material, improved cryopreservation methods of stock cultures (Bai et al., 2004), or creation of homozygous inbred lines which are resistant to trait deterioration (Bai et al., 2005; Chaston et al., 2011).

APPLICATION TECHNOLOGY AND FACTORS THAT AFFECT EFFICACY

Standard application approaches: Entomopathogenic nematodes can be applied with nearly all agronomic or horticultural ground equipment including pressurized sprayers, mist blowers, and electrostatic sprayers or as aerial sprays (Georgis, 1990; Wright et al., 2005; Shapiro-Ilan et al., 2006a). The application equipment used depends on the cropping system, and in each case there are a variety of handling considerations including volume, agitation, nozzle type, pressure and recycling time, system environmental conditions, and spray distribution pattern (Grewal, 2002; Fife et al., 2003, 2005;

Wright et al., 2005; Shapiro-Ilan et al., 2006a; Lara et al., 2008). It is important to ensure adequate agitation during application. For small plot applications, hand-held equipment (e.g., water cans) or back-pack sprayers may be appropriate. When nematodes are applied to larger plots, a suitable spraying apparatus such as a boom sprayer should be considered. Conceivably, applicators could also be using other methods such as through microjet irrigation systems, subsurface injection or baits (Wright et al., 2005; Lara et al., 2008). Various formulations for entomopathogenic nematodes may be used for applying EPNs in aqueous suspension including activated charcoal, alginate and polyacrylamide gels, clay, peat, polyurethane sponge, vermiculite, and water dispersible granules (WDG) (Georgis, 1990; Georgis et al., 1995).

Biotic factors affecting application success: A number of factors related to the nematode are critical for application success. Most importantly, the appropriate nematode must be matched with the particular target pest. Factors that must be considered in choosing the appropriate nematode include virulence, host finding, and environmental tolerance and in some cases persistence (Shapiro-Ilan et al., 2002b; Shapiro-Ilan et al., 2006b). Also of paramount importance, to be effective, EPNs usually must be applied to soil at minimum rates of 2.5×10^9 IJs/ha ($\approx 25/\text{cm}^2$) or higher (Georgis and Hague, 1991; Georgis et al., 1995; Shapiro-Ilan et al., 2002b). Depending on the target pests, a higher rate of application may be required (or in some rare cases lower rates may suffice) (Shapiro-Ilan et al., 2006a). Recycling potential should also be considered. Generally, as long as environmental conditions are conducive, nematode populations will remain high enough to provide effective pest control for 2 to 8 weeks after application (Kaya, 1990; Duncan and McCoy, 1996; Shapiro-Ilan et al., 2002b). Thus, seasonal re-application is often necessary. However, in some cases effective control has been reported over multiple seasons or years (Klein and Georgis, 1992; Parkman et al., 1994; Shields et al., 1999).

Biotic agents can have positive, negative, or neutral effects on EPN applications. Antagonists include nematode pathogens or predators such as phages, bacteria, protozoans, nematophagous fungi, predacious mites and nematodes, etc. (Kaya, 2002). Phoretic relationships have been indicated with other soil organisms such as mites, earthworms, and isopods (Epsky et al., 1988; Shapiro et al., 1995; Eng et al., 2005). Entomopathogenic nematodes have been reported to act synergistically with other entomopathogens such as *Paenibacillus popilliae* (Thurston et al., 1994), *Bacillus thuringiensis* (Koppenhöfer and Kaya, 1997), and *Metarhizium anisopliae* Sorokin (Ansari et al., 2004, 2006; Anbesse et al., 2008), yet other studies indicate antagonism, e.g., with *Beauveria bassiana* (Balsamo) Vuillemin (Brinkman and Gardner, 2000) or *Isaria fumosorosea* (Shapiro-Ilan et al., 2004). The relationship between nematodes and other

entomopathogens (antagonism, additivity, synergism) can vary depending on the nematode species and relative timing or rate of application (Barbercheck and Kaya, 1990; Koppenhöfer and Kaya, 1997; Shapiro-Ilan et al., 2004).

Abiotic factors affecting application success: Successful application of EPNs depends on several critical factors including protection from ultraviolet radiation, adequate soil moisture/relative humidity, and temperature (Kaya, 1990; Shapiro-Ilan et al., 2006a). Indeed, EPN applications for aboveground pests have been severely limited due to environmental hindrances (e.g., UV radiation or desiccation) that reduce survival and efficacy (Begley, 1990; Grewal and Georgis, 1999; Arthurs et al., 2004; Shapiro-Ilan et al., 2006a), and thus, biocontrol success is most likely achieved when EPNs are applied to soil or cryptic habitats. Furthermore, because ultraviolet radiation is detrimental to nematodes (Gaugler and Boush, 1978), applications are best applied in the evening or early morning hours, or exposure to ultraviolet radiation avoided, through subsurface application (Cabanillas and Raulston, 1995). For soil applications, moisture for EPN survival and movement is required, but too much moisture may cause oxygen deprivation and restrict movement (Wallace, 1958; Kaya, 1990; Womersley, 1993; Koppenhöfer et al., 1995). Thus, irrigation is recommended for maintaining adequate moisture (Shetlar et al., 1988; Zimmerman and Cranshaw, 1991; Downing, 1994). Optimum moisture levels will vary by nematode species and soil type (Koppenhöfer et al., 1995). Optimum temperatures for infection and reproduction will also vary among nematode species and strains (Grewal et al., 1994). Some nematodes such as *H. indica*, *S. glaseri*, and *S. riobrave* are relatively heat tolerant whereas others, such as *H. megidis*, *S. feltiae*, and *Heterorhabditis* are more tolerant to cooler temperatures (Kung et al., 1991; Grewal et al., 1994; Berry et al., 1997; Shapiro and McCoy, 2000b).

Soil parameters can also be important for surface or below-ground applications. Soil texture affects nematode movement and survival (Kaya, 1990; Barbercheck, 1992). Generally, compared with lighter soils, soils with higher clay content restrict nematode movement and have potential for reduced aeration, which, in combination, can result in reduced nematode survival and efficacy (Georgis and Poinar, 1983; Molyneux and Bedding, 1984; Kung et al., 1990a). However, exceptions to this trend have been reported (Georgis and Gaugler, 1991; Shapiro et al., 2000b). Soil pH can affect natural EPN distributions (Kanga et al., 2012). A soil pH of 10 or higher is likely to be detrimental to EPN applications, whereas a range of 4-8, is not likely to have any significant effect on EPNs (Kung et al., 1990b).

Biocontrol success can also be impacted by fertilizers and chemical pesticides, which can have positive, neutral, or negative effects on entomopathogenic nematodes. In general, fertilizers that are applied at recommended rates have little impact on EPN efficacy (Shapiro et al., 1996b;

Bednarek and Gaugler, 1997). However, fresh manure or high rates of chemical fertilizers (e.g., urea at 560 kg N per ha) can be detrimental to EPN persistence and efficacy (Shapiro et al., 1996b; Bednarek and Gaugler, 1997; Shapiro et al., 1999b). Some chemical pesticides are toxic to EPNs (e.g., abamectin, acephate, aldicarb, dodine, fenamiphos, methomyl, parathion, and Teflubenuron), whereas others tend to be compatible and in some cases may be synergistic when applied with EPNs (e.g., carbaryl, chlorpyrifos, dimethoate, endosulfan, fonofos, tefluthrin, imidicloprid) (Koppenhöfer and Kaya, 1998; Nishimatsu and Jackson, 1998; Alumai and Grewal, 2004; Koppenhöfer and Grewal, 2005; Koppenhöfer and Fuzy, 2008; Shapiro-Ilan et al., 2011b). Similar to interactions with other microbial agents, the relationship between chemical pesticides and EPNs varies based on the specific chemical and nematode species or strain, dosages, and timing of application (Benz, 1971; Koppenhöfer and Grewal, 2005); thus, combinations should be tested on a case by case basis.

Improved technology for EPN application (the road to the future): Enhanced efficacy in EPN applications can be facilitated through improved formulation. Substantial progress has been made in recent years in developing EPN formulations, particularly for aboveground applications, e.g., mixing EPNs with a surfactant and polymer (Schroer and Ehlers, 2005). Improved efficacy may also be achieved by relying on leaf flooding with the addition of surfactants to increase leaf coverage (Williams and Walters, 2000; Head et al., 2004). Additionally, *S. carpocapsae* applications for control of the lesser peachtree borer, *Synanthedon pictipes*, were greatly improved by a follow-up application of a sprayable gel (the gel is commonly used for protecting structures from fire) (Shapiro-Ilan et al., 2010a), and *S. carpocapsae* caused high levels of suppression (98% efficacy in a preventative treatment) in the red palm weevil, *Rhynchophorus ferrugineus*, when applied in a chitosan formulation (Llàcer et al., 2009). Furthermore, EPN applications to apple tree trunks for control of codling moth, *Cydia pomonella* (L.), were enhanced when the treatments included the sprayable fire-gel or wood flour foam as a protecting agent (Lacey et al., 2010).

Efficacy of EPN applications can also be enhanced through improved application equipment or approaches. Despite well-established procedures, equipment used for entomopathogen application can be improved further, e.g., optimizing spray systems (e.g., nozzles, pumps, spray distribution) for enhanced pathogen survival and dispersion (Shapiro-Ilan et al., 2006a; Brusselman et al., 2010). Bait formulations can enhance EPN persistence and reduce the quantity of microbial agents required per unit area (Grewal, 2002); though limited thus far, conceivably, baits can be developed further for wide applications. Another novel application approach that has gained attention is delivery of EPNs in their infected host cadavers (Jansson et al., 1993; Shapiro and Glazer, 1996;

Del Valle et al., 2008). Advantages to the cadaver application approach relative to standard application in aqueous suspension have been reported such as increased nematode dispersal (Shapiro and Glazer, 1996), infectivity (Shapiro and Lewis, 1999), survival (Perez et al., 2003), and efficacy (Shapiro-Ilan et al., 2003), whereas other studies did not detect a benefit in the cadaver approach (Bruck et al., 2005). Application of cadavers may be facilitated through formulations that have been developed to protect cadavers from rupture and improve ease of handling (Shapiro-Ilan et al., 2001, 2010b; Del Valle et al., 2009), and development of mechanized equipment for field distribution (Zhu et al., 2011). The time period of six to ten days between infection and application on soil of *Galleria mellonella* cadavers resulted in higher emergence of IJs and was thus recommended when using the cadaver application approach (Del Valle et al., 2011). Recently, nematodes applied in host cadavers were effective and persistent when added to bags of potting media for subsequent distribution to target pest sites (Deol et al., 2011).

Finally, superior biocontrol applications with EPNs can also be achieved through strain improvement. Improved strains may include EPNs that possess enhanced levels of various beneficial traits such as environmental tolerance, virulence, reproductive capacity, etc. Methods to improve EPNs include strain or species discovery or genetic enhancement via selection, hybridization or molecular manipulation (Gaugler, 1987; Burnell, 2002; Grewal et al., 2005). Discovery of new strains and species that are superior to currently commercialized nematodes is a straightforward approach that can rapidly result in enhanced efficacy. The rate of EPN species discovery has been increasing considerably (Poinar, 1990; Adams and Nguyen, 2002; Lewis and Clarke, 2012). Since the time that the first EPN species was reported in 1923 (Steiner, 1923; Poinar, 1990) more than 85 EPN species have been described (Nguyen and Buss, 2011; Lewis and Clarke, 2012) and more than half the described species have been reported in the past 10 years. Additionally, the numerous new strains of existing species being discovered may offer enhanced virulence or other properties (e.g., Grewal et al., 2004; Stuart et al., 2004). The number of new strains and species discovered will likely continue to rise adding more potential for biocontrol applications. However, in order to leverage the advantages that strain/species offer, characterization of biocontrol potential in these new organisms must keep pace with the survey/discovery research. Currently, less than 20% of the species discovered since 2001 have been evaluated for biocontrol efficacy indicating there is substantial untapped potential.

If existing or newly discovered entomopathogen strains or species cannot achieve desired levels of biocontrol efficacy, strain enhancement might be achieved through genetic improvement approaches, which can include molecular or non-molecular methods. One non-molecular approach is selection for desired traits;

directed selection has been demonstrated to be effective for improving various traits in EPNs such as host-finding (Gaugler et al., 1989, 1990) and nematocidal resistance (Glazer et al., 1997). Hybridization (the transfer of beneficial traits from one strain to another) is another option for strain improvement that has shown promise (Shapiro et al., 1997; Shapiro-Ilan et al., 2005). Combination of the two non-molecular approaches (selection and hybridization) can also be effective for development of superior EPN strains (Mukaka et al., 2010). In addition to strain improvement approaches described above, molecular methods (e.g., transgenics) may offer potential for enhanced biocontrol (Gaugler et al., 1997), and we can expect that both molecular and non-molecular genetic approaches to strain improvement will be enhanced through the forthcoming advances in genomics (Bai and Grewal, 2007; Ciche, 2007; Bai et al., 2009).

CONCLUSION

Progress in developing large-scale production and application technology has led to the expanded use of EPNs. For laboratory use, small-scale field-testing, and niche markets, *in vivo* EPN production is the appropriate method requiring the least capital outlay and the least amount of technical expertise for start-up, but is hindered by the costs of labor and insect media. When it comes to commercial use for international markets, *in vitro* liquid culture is considered to be the most cost efficient process while *in vitro* solid culture is generally considered to be intermediate between *in vivo* and liquid culture. 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. Improvements in efficiency and scalability by producing the insect hosts “in-house” and mechanizing the process reduce labor, enabling *in vivo* production to play an expanded role in pest management programs. Similar to *in vivo* production, technical improvements will expand efficiency of *in vitro* solid production, but even so, neither approach may reach the scale-up potential of liquid culture technology.

EPNs are excellent biocontrol agents for insect pests. When an EPN is used against a pest insect, it is critical to match the right nematode species against the target pest. Biotic agents including nematode pathogens, predators and other soil organisms, as well as abiotic factors such as ultraviolet radiation, soil moisture/relative humidity, temperature, etc. can affect EPN application efficacy. Recently, improvement of nematode formulation, application equipment or approaches, and strain improvement have been made to enhance EPN application efficacy. Additional research toward lowering product costs, increasing product availability, enhancing ease-of-use, and improving efficacy and

carryover effect will stimulate the extensive use of EPNs in biocontrol. With these advances EPNs will serve to reduce chemical insecticide inputs and contribute to the stabilization of crop yields and the environment.

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