International Journal of Advanced Research in Biological Sciences

Review Article

Entomopathogenic nematode as a biocontrol agent – Recent trends – A Review

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Abstract

Safety and environmentalcal insecticide issues surrounding the use of chemical insecticides has led to an emphasis on developing alternative control measures such as entomopathogens and their products. Entomopathogenic nematodeare effective biopesticide which can be incorporated in IPM programs because they are considered non-toxic to humans, relatively specific to their target pests and can be applied with standard pesticide equipment. Entomopthogenic nematodes have proven to be the most effective as biological control organisms. Entomopathogenic nematodes have been released extensively in crop fields with negligible effects on non target insects and are regarded as exceptionally safe to the environment. Our focus in this paper was to review mechanism and pathogencity of nematode, phylogeny of nematode for Steinernematidae and Heterorhabditidae. Steinernematidae is represented by the genera *Steinernema* and *Neosteinernema* and Heterorhabditidae is represented by the genus *Heterorhabditis*. They are associated with mutualistic bacteria in the genus *Xenorhabdus* for *Steinernema* and *Photorhabdus* for *Heterorhabditis*. Thus, it is a nematode bacterium complex that works together as a biological control unit to kill an insect host by penetrating the host through natural opening and there by releasing the bacterial symbiont which spread and multiply in the haemolymph of the insect pest and kill them by septicemia. Infective juvenile entomopathogenic nematode locate their hosts in soil by means of two strategies-ambusing and crusing. Nematode employs different foraging strategies to locate and infect hosts. Genetic diversity may be lost, or genetic variation may have been limited during collection or lost during importation and rearing. A serious problem for EPNs is founder effect because only a limited number of insect cadavers are collected at single geographical sites, resulting in reduced genetic variance. EPNs have been most efficacious in habitats that provide protection from environmental extremes, especially in soil, which is their natural habitat and in cryptic habitats. Excellent control has been archived against plant-boring insects because their cryptic habitats are favorable for nematode survival and infectivity. In developing biocontrol programs using EPNs, one mechanism to increase the chance of success is to screen novel nematode species or strains for potential efficacy against particular target pests.

Introduction

Nematodes are non-segmented, elongated roundworms that are colorless, without appendages, and usually microscopic. There are non-beneficial and beneficial nematodes. Non-beneficial nematodes are also called "plant parasitic nematodes" and cause damage to crops and other types of plants. Beneficial nematodes attack soil borne insect pests, yet are not harmful to humans, animals, plants, or earthworms, and can therefore be used as biological control organisms (Denno *et al.,* 2008). Beneficial nematodes that cause disease within an insect are referred to as "entomopathogenic" and have the ability to kill insects.

Entomopathogenic nematodes from the families Steinernematidae and Heterorhabditidae have proven to be the most effective as biological control organisms (Kaya and Gaugler, 1993). They are soil-inhabiting organisms and can be used effectively to control soilborne insect pests, but are generally not effective when applied to control insects in the leaf canopy. When considered as a group of nearly 30 species, each with its own suite of preferred hosts, entomopathogenic nematodes can be used to control a wide range of insect pests, including a variety of caterpillars, cutworms, crown borers, grubs, corn root worm, cranefly, thrips, fungus gnat, and beetles Entomopathogenic nematodes have been released extensively in crop fields with negligible effects on non target insects and are regarded as exceptionally safe to the environment.

The keys to success with are understanding their life cycles and functions; matching the correct nematode species with the pest species; applying them during appropriate environmental conditions (soil temperature, soil moisture, sunlight); and applying them only with compatible pesticides. Because EPNs are living organisms, they require careful handling to survive shipment and storage as well as appropriate environmental conditions to survive in the soil after application (Berry, 2007).

Life Cycle of Entomopathogenic Nematodes (EPNs)

The life cycle of most nematodes includes an egg stage, four juvenile stages, and an adult stage. The third juvenile stage of EPNs is referred to as the "infective juvenile" or "dauer" stage and is the only free-living stage. The infective juvenile is capable of surviving in the soil, where it locates, attacks, and infects a pest insect (Poinar, 1990). Under optimal conditions, it takes 3-7 days for *Steinernematids* and *Heterorhabditids* to complete one life-cycle inside a host from egg to egg. Emergence of infective juveniles from the host requires about 6–11 days for *Seinernematids* and 12–14 days for *Heterorhabditids* (Kaya and Koppenhöfer, 1999). Entomopathogenic nematodes are a group of nematodes causing death to insects. EPNs have found in all over the world and a range of ecologically diverse habitats. They are highly diverse, complex and specialized. Thus, the highlight of the biopesticide is that these nematode/bacterium complex that works together as a biological control unit to kill an insect host. (Sujatha *et al.,* (2016)

Mechanism of EPNs Pathogencity

The term entomopathogenic first appeared in Nematology literature in reference to the bacterial symbionts of *Steinernema* and *Heterorhabditis* (Thomas, 1979). Bacteria are considered entomopathogenic when their LD_{50} is $< 10,000$ cells injected into the hemocoel (Bucher, 1960). Some pathogens associated with *Steinernema* and *Heterorhabditis* have LD_{50} is <10 cells when injected, but this varies with different hosts and these bacteria are not known to infect insects without the aid of their nematode *partners* (Forst, 1996). The term "Entomopathogenic" was applied to nematodes in 1981 and again in 1986 (Akhurst, 1986), a use that gained momentum in 1988 (Gaugler, 1988). This gradual, social use of the term entomopathogenic without formal definition complicates its application to emerging nematode-bacteria partnerships. Indeed, the convenience of this descriptor is that it currently, applies to both partners as a complex, rather than only the nematodes or bacteria. The only clearly identifiable EPN definition that we are aware of was proposed informally (Onstad *et al*., 2006 and Grewal, 2005). This definition focuses on mutualism with bacteria and on the exclusivity of the IJ as the free-living stage. We find the use of these criteria incomplete since they do not consider rapid death, which is necessary to differentiate EPNs from phoretic, necromenic, or other less virulent forms of parasitism, and the inclusion of a stage-specific requirement in defining EPNs is unnecessary. Since convention provides no standard to assess classification of EPNs, and because "entomopathogenic" was meant to differentiate insect-parasitic nematodes that serve as vectors of bacteria and to reinforce the link between nematology and insect pathology (Gaugler and Gaya, 1990), we formally suggest two criteria: 1) the nematodes use a symbiotic relationship with bacteria to facilitate pathogenesis, which implies that the association is non-transient, though not necessarily obligate, and 2) insect death is sufficiently rapid that it can be unequivocally distinguished from phoretic, necromenic, and other parasitic associations (i.e., <120 h), a time frame that also implies efficient release of the pathogen by the nematode vector. These criteria are based on early investigations of EPNs and what we consider the fundamental principles of the EPN lifestyle.

Pathogenicity of Nematode – Bacterium Complex

Koch's postulates can be used to establish pathogenicity of the nematode-bacterium complex or either partner alone, and we suggest that partner association across generations is particularly important in this evaluation (Lacey, 1997). To establish genetic heritability, genes must be passed through the F1 generation to the F2 generation; for example, a mule inherits, but does not pass on, traits inherited from its paternal donkey and maternal horse parents. Similarly, we argue that for an EPN association to be stable, nematodes must not only infect and kill an insect and produce progeny, but must also produce progeny that depart the carcass carrying the pathogenic bacteria. This does not require that the association be obligate as subsequent generations that thrive in non-insect environments may lose the symbiotic bacteria. However we believe it is crucial that symbiotic transmission from the infecting parental generation to emerging nematodes from at least two subsequent insect infections be clearly established to distinguish nematode carriage of the bacteria or bonafide association from transient cuticle hitchhiking. Also, in associating, each partner must benefit from the association. At a minimum, the bacteria should increase overall nematode fitness by assisting in insect killing, nutrient liberation, or scavenger deterrence, and the nematodes should provide the bacteria with access to the insect host either by delivery to otherwise inaccessible host cavities or tissues, or by increasing dispersal range through direct carriage. Though EPNs must be capable of infecting and killing insect hosts, this does not preclude them from also, opportunistically, acting as scavengers or from competing with other EPNs for already killed insects (San-Blas and Gowen, 2008). An additional cautionary point here is that the symbiont transmission rate and the stability of nematode-bacterium associations themselves have been well characterized in representative taxa (Poinar, 1979, and Han, 2000), but these details are unclear in most of the 75 EPN species reported to date (Nguyen, 2007).

Insect host killing within five days of infection is an appropriate requirement and implies selection for virulence or at least selection against avirulence, differentiating entomopathogeny from other forms of parasitism such as those used by mermithids and allantonematids. "Potentially pathogenic" bacteria that cause septicemia at low inoculation when in the hemocoel but that lack mechanisms for actively invading the hemocoel, usually cause death within two to four days in common laboratory larvae such as *Galleria mellonella*, though larger or adult insect hosts, such as mole crickets or *Manduca sexta*, take longer to succumb, depending on the size of the nematode founding population and which pathogenic bacterium is used. Rapid death caused by EPNs reflects pathogenicity of the bacterial partner with possible contributions from the nematode and relies on efficient release of the bacteria into the hemolymph (Dillman *et al.,* 2012)

Xenorhabdus and Photorhabdus isolates were obtained from the EPNs infected *Galleria Mellonella* larvae. Primary phase of the isolates of Photorhabdus sp. and xenorhabdus sp. were identified by the colony morphology. The colonies were granulated conver, opaque and circular colonies with irregular margins. The growth rate of Photorhabdus isolates 143 and f18 and Xenorhabdus isolates 230 and 238 maintained at 15, 20, 25, 30 and 35° C respectively. In all the isolates the maximum growth was observed at 25° C when compared to other temperature conditions (Vani and Sujatha 2005).

Phylogeny of EPNs

According to the standards we propose above, *C. briggsae* may not be an EPN. IJs recovered from dead insects seem able to reinfect new hosts but are less virulent in *G. mellonella* as a complex than injection of the bacteria alone, suggesting either inefficient release of the pathogen or some antagonism by the nematode vector. This may reflect that *C. briggsae* is somewhere between necronemic and entomopathogenic, that it is a nascent entomopathogen and not yet efficient, or that *G. mellonella* is a poor host. However, symbiont heritability has not been demonstrated, and the nature of *C. briggsae*'s bacterial association remains unresolved (Abebe, 2011). As *C. briggsae* has not met the suggested criteria, it should not be considered an EPN, facultative or otherwise, until heritability of the pathogenic bacteria is demonstrated and more is known about bacterial release and speed of host death. Our suggested criteria have been tested and met for both *O. chongmingensis* and *O. carolinensis* (Zhang *et al.,* 2008 and Ye, 2010). Therefore, these taxa should be considered EPNs even though further research is required to determine the nature and heritability of their bacterial associations, and whether they are obligate or facultative EPNs.

Characteristics of EPNs

When considering appropriate criteria that define EPNs, it is tempting to use the particular details that are known for only a few representative taxa. Instead, we avoided specifics in favor of fundamental principles that underlie the associations, and observed that many interesting and often dogmatic EPN characteristics are less wide spread than expected. Specialization with particular bacteria is a hallmark EPN characteristic, and mono specificity between one nematode and one genus of bacteria or even one symbiont species is commonly observed among these taxa. However, growing evidence of promiscuous relationships between EPNs and their bacterial symbionts suggest that this may not be as common as originally thought (Kim *et al.,* 2009 and Babic, 2000). Although most *Heterorhabditis* and *Steinernema* symbionts localize to the nematode intestine, there are excellent examples of nematode-bacteria symbioses in other body sites (Ploz *et al*., 2000). Of note, *Paenibacillus nematophilus* associates on the cuticle of *Heterorhabditis* spp., and, relevant to this discussion, *O. carolinensis* is associated with insect pathogenic *Serratia marcescens* on its exterior cuticle. Also, dogma dictates that these associations are obligate, since *Steinernema* and *Heterorhabditis* symbionts are generally not free-living, and *S. carpocapsae*'s symbiont is auxotrophic for nicotinic acid, which is not available in the environment (Orchard, 2000). However, *Photorhabdus asymbiotica* may be free-living (Gerrard *et al.,* 2006). Also most nematodes require their symbionts for growth and reproduction, but exceptions have been observed (Akhurst, 1983 and Sicard, 2005). There are also differences between biological characteristics of the two nematode taxa. *Heterorhabditis* maternally transmit symbionts by a sophisticated multistep process, while *Steinernema* have specialized host structures within which they carry their symbionts (Enright, 2004). Also, some *Steinernema* infect and kill insect hosts even in the absence of pathogenic bacteria, at least in laboratory conditions, but *Heterorhabditis* nematodes have not been reported to have this behavior. Finally, as we mentioned above, symbiont transmission to new generations varies widely in the few taxa where it has been studied from > 95% to 10% (Cowles, 2008). Together, these findings reveal that *Steinernema* and *Heterorhabditis* are highly adapted to entomopathogeny and showcase adaptations likely to emerge as a result of long-term commitment to the entomopathogenic lifestyle, even though the biological basis for their symbiotic association with bacteria differs significantly (Chaston, 2010 and Goodrich-Blair, 2007). The exceptions and differences that have been observed for these entire hallmark characteristics highlight why specializations should not be used to exclude newly described associations, and emphasize that applying observations from a few representative members to whole clades can be problematic. Indeed, few species in either genus have been thoroughly explored, and we caution against assuming a priori these specializations to be true of all or even most *Steinernematids* or *Heterorhabditids* (Blaxter, 1998).

Symbiosis and Entomopathogeny

Nematode-bacterium partnerships that do not explicitly fulfill the requirements to be classified as EPNs are still of extraordinary interest since they may represent developing, nascent partnerships, but they should not be considered entomopathogens. Our understanding of parasitism and its evolution is continually refined as biodiversity is explored and ecology and evolution become increasingly emphasized among established and satellite model systems. We have suggested specific and restricted use of the term entomopathogenic in nematology, which will facilitate unambiguous communication. Among the 20 or more parasitic lineages of nematodes, entomopathogeny is a unique type of insect parasitism not found among vertebrate or plant-parasitic nematodes. Recent work indicates that entomopathogeny has arisen at least three times within Nematoda, and that recently described species (*O. chongmingensis* and *O. carolinensis*) may represent nascent stages of EPN evolution. These developments emphasize the tremendous specialization exhibited by *Heterorhabditis* and *Steinernema* and increase their usefulness as models for the evolution of symbiosis and parasitism (Dillman *et al.,* 2012)

EPNs – Host finding strategies

An understanding of host-finding strategies will help you properly match entomopathogenic nematode species to pest insects to ensure infection and control (Gaugler 1999). Only entomopathogenic nematodes in the infective juvenile stage will survive in the soil and find and penetrate insect pests. Infective juvenile entomopathogenic nematodes locate their hosts in soil by means of two strategies-ambushing and cruising (Gaugler *et al.,* 1989). Ambusher species include *Steinernema carpocapsae* and *S. scapterisici*; cruisers include *Heterorhabditis bacteriophora* and *S. glaseri*.

S. riobrave and *S. feltiae* do a bit of both ambushing and cruising (Campbell and Gaugler, 1997).

Ambushing is EPNs that use the ambushing strategy tend to remain stationary at or near the soil surface and locate host insects by direct contact (Campbell et al. 1996). An ambusher searches by standing on its tail so that most of its body is in the air, referred to as "nictation." The nictating nematode attaches to and attacks passing insect hosts. Ambusher entomopathogenic nematodes most effectively control insect pests that are highly mobile at the soil surface, such as cutworms, armyworms and mole crickets.

Cruising is EPNs that use the cruising strategy are highly mobile and able to move throughout the soil profile. Cruisers locate their host by sensing carbon dioxide or other volatiles released by the host. Cruiser EPNs are most effective against sedentary and slow-moving insect pests at various soil depths, such as white grubs and root weevils.

Infection

Generally, several EPNs will infect a single insect host. Infective juvenile nematodes penetrate the insect's body cavity either through natural body openings (such as the mouth, anus, genital pore or breathing pore or by breaking the outer cuticle of the insect *Heterorhabditids* do this using a dorsal "tooth" or hook. Once inside the body cavity of the host, the infective juveniles release bacteria that live symbiotically within the EPNs gut but do not harm the nematode. The nematode-bacterium relationship is highly specific only *Xenorhabdus* spp. bacteria co-exist with *Steinernematids* and only *Photorhabdus* bacteria co-exist with *heterorhabditids.* Once released into the host, the bacteria multiply quickly and under optimal conditions cause the host to die within 24 to 48 hours.

EPNs feed on both bacteria they release and host insect tissue. After a few days inside the host, EPNs mature to the adult stage. These adult EPNs produce hundreds of thousands of new juveniles that may undergo several life cycles within a single host. When the host has been consumed, the infective juveniles, armed with a fresh supply of bacteria, emerge from the empty shell of the host, move into the soil, and begin the search for a new host. A protective exterior cuticle surrounds the infective juvenile, protecting it from the environment and predators. Under ideal conditions, *Steinernematids* emerge 6–11 days after initial infection and *Heterorhabditids* emerge 12–14 days after initial infection (Kaya and Koppenhofer, 1999).

The duration of infective juvenile survival in soil is unknown because they can become prey to invertebrates and microorganisms.

The polyphagous devastating pest *Spodoptera litura* was infected in with the bioinsecticide *Steinernema – Xenorhabdus* symbiont and the larvae were used to analyse the protein content in both infected and control larvae. The EPN – Entomopathogenic nematodes were isolated from Westernghats region of Marudhamalai area and used in this study. The infected larvae after 24 hours were taken along with non infected control S. litura with three replication revealed that the infected had less protein content compared to control. This may be due to the utilization of the protein by the *Steinernema – Xenorhabdus* symbiont for their growth, development and reproduction (Sujatha and Chitra 2014).

The enzyme analyzed in the *Helicoverpa armigera* and *Lucinodes orbonalis* infected with *Xenorhabdus* sp. of Munnar. After 24 hours a clean zone was observed. The lipase has insecticidal toxic activity which had degraded the lipid content of the pest *Helicoverpa armigera* and *Lucinodes orbonalis.* The protein profiling was done in *Steinernema* and *Xenorhabdus* sp. Infected *H.armigera* and *L.orbonalis*. The control had 32.0 to 41.0 kDa and infected showed 31.7 to 45.0 kDa in *Xenorhabdus* sp. and *Steinernema* sp. infected it was 20.0kDa to 43.0 kDa in *H.armigera* and *L.orbonalis*. The control showed 32kDa whereas the infected showed 31 kDa to 98 kDa in *Xenorhabdus* sp and the *Steinernema* infected showed 31.7 kDa. The protein sub unit is high molecular weight compare protein which has death of *H.armigera* and *L.Orbonalis*. (Chitra *et al.,* 2016).

The biochemical components namely protein, carbohydrates and lipids were analyzed. The symbiont of *Steinernema* sp. munnar samples *Xenorhabdus* sp. was cultured in liquid broth and 1 loop of inoculum was taken and infected to five larvae of *Helicoverpa armigera* and *Lucinodes orbonalis*. The death was observed in 24 hours after infection and Protein, Carbohydrates and Lipids were estimated. In *Helicoverpa armigera* the protein content of control showed 3.12 g/dl, infected were 2.18 g/dl. Carbohydrate in control was 42 mg/100mg, infected were 23 mg/100mg. Lipid in control was showed 18.75 mg/100mg, infected were 6.25 mg/100mg. *Lucinodes orbonalis* protein content of control showed 3.93 g/dl, infected was 0.77 g/dl. Carbohydrates in control were 27 mg/100mg, infected were 18 mg/100mg. Lipid in control was showed 12.5

mg/100mg, infected were 6.25 mg/100mg. This low content of protein, carbohydrates and lipid in infected may be used by the *Steinernema* sp. for its growth, development and reproduction and the pathogens have also destroyed the hosts for *Helicoverpa armigera* and *Lucinodes orbonalis* immune system for their development (Chitra *et al.,* 2016)

Employment of EPNs

Worldwide, over 80 species of EPNs have been identified and 11 commercialized (Kaya and Koppenhofer, 1999). The different species of EPNs vary in the range of insects they attack, environmental needs, and stability in commercial products (Gaugler, 1999). A given species of EPN may also control a particular pest more effectively than another species. Therefore, the insect pest must be identified before choosing the EPN species most appropriate for biological control.

Target Pests and Efficacy

Key target pests - EPNs have been tested against a large number of insect pest species with results varying from poor to excellent control (koppenhofer, 2000). Many factors can influence the successful use of nematodes as biological insecticides, but matching the biology and ecology of both the nematode and the target pest is a crucial step towards successful application. The foraging behavior and temperature requirements of a nematode species and to the accessibility and suitability of the pest to the nematode have to be consideration. EPNs have been most efficacious in habitats that provide protection from environmental extremes, especially in soil, which is their natural habitat and in cryptic habitats. Excellent control has been achieved against plant-boring insects because their cryptic habitats are favorable for nematode survival and infectivity (e.g. no natural enemies of the nematodes and adequate moisture). Low or highly variable control has been achieved in manure because of high temperatures in animal rearing facilities and toxic effects of manure contents (ammonia) on the infective juveniles. Control of aquatic insects has been unsuccessful because the nematodes are not adapted to directed motility (host finding) in this environment. The infective juveniles face harsh conditions on foliage and other exposed habitats that can be only marginally remedied by adjuvants.

Availability and Procurement of EPNs

Perhaps the biggest challenge to the use of EPNs as effective biological control organisms is the variable quantity and quality of nematodes in commercial products (Gaugler *et al*, 2000). EPNs are cultured on a large scale in laboratories and are available from many commercial suppliers in North America and Europe. In past assessments of cottage industry commercial products, most contained lower numbers of EPNs than the suppliers claimed. In addition, in some cases the species of EPNs in the product were mixed and therefore inconsistent with the product label. The industry has made progress, however, in increasing the quality of its products.

Foraging Strategies

A major factor restricting the EPN host range is the foraging behavior of the infective juveniles. These nematodes employ different foraging strategies to locate and infect hosts, which range from one extreme of sit-and-wait to the other of widely foraging strategy (Campbell, 2002 and Lewis, 2002). Most nematode species are situated somewhere along a continuum between these 2 extremes, placing them as intermediate foraging strategists (e.g. *S. riobrave and S. feltiae*) (Campbell, 1999b). These intermediate strategists are adapted to infecting insects that occur just below the soil surface, such as pre pupae of lipid opterous insects, fungus gnats, or weevil larvae. The sit-and-wait strategists or ambushers (e.g. *S. carpocapsae and S. scapterisci*) are characterized by low motility and a tendency to stay near the soil surface. They tend not to respond to volatile and contact host cues unless presented in an appropriate sequence and efficiently infect mobile host species such as the codling moth, cutworms and mole crickets near the soil surface. At the other extreme, the widely foraging strategists or cruisers (e.g. *S. glaseri* and *H. bacteriophora*) are characterized by high motility and are distributed throughout the soil profile. They orient to volatile host cues and switch to a localized search after host contact and are well adapted to infecting sedentary hosts such as scarab and lepidopterous prepupae and pupae. Another behavior of infective juveniles is their typical body-waving where 30-95% of their body is raised off the substrate for a few seconds. Most nematode species that have an ambush or intermediate foraging strategy can body-wave by raising >95% of their body off the substrate, standing on a bend in their tail and assuming a straight posture or alternating periods of no motion and active waving (Campbell, 1999a). Cruisers can body-wave but

cannot stand on their tails. Infective juveniles that can stand on their tails and body-wave (i.e. ambushers and some intermediate foragers) can also jump. This jumping behavior can be used for host attachment or be nondirected where it may play a role in dispersal.

Recycling of nematodes

Recycling is desirable after an application of EPNs because it can provide additional and prolonged control of a pest. The abiotic and biotic factors that affect persistence, infectivity, and motility of infective juveniles influence nematode recycling. Because they are obligate pathogens, the availability of suitable hosts is a key to recycling of the nematodes. Recycling is rather common (Klein 1993) after nematode application but is probably not sufficient for prolonged host suppression, and the nematodes have to be reapplied to maintain adequate control of soil insect pests. In natural populations of EPNs, recycling occurs in their insect hosts, but only a few studies have examined the dynamics of nematode populations and the factors affecting them. Within-site distribution of nematode populations is patchy (Stuart, 1994 and Strong, 1996) and biotic and abiotic factors such as seasonal fluctuations, foraging strategy of the infective juveniles, host population dynamics and alternate hosts play a key role in nematode recycling.

Genetic Diversity of EPNs

EPNs are obligate pathogens in the field, but in the laboratory they can be maintained *in vivo* or *in vitro.* During their laboratory maintenance, the genetic diversity may be lost, or genetic variation may have been limited during collection or lost during importation and rearing. On the other hand, preservation of genetic variation for nematodes is affected by founder effect, inbreeding, and inadvertent selection. A serious problem for EPNs is founder effect because only a limited number of insect cadavers are collected at single geographical sites, resulting in reduced genetic variance. To maintain or enhance genetic diversity, the same nematode species should be collected from as many geographical sites as possible and the isolates should be hybridized. If laboratory-adaptation occurs or is suspected, the nematodes can be out crossed with new field isolates or with other sources to maintain or infuse genetic diversity. EPNs may benefit from genetic improvement through selective breeding or genetic engineering. Examples of successful selective breeding are selection for cold tolerance (Grewal, 1996 and Griffin 1994), improved control efficacy

(Tomalak, 1994), and nematicide resistance. In addition, genetic engineering to improve beneficial traits of EPNs and their associated bacteria has been done on a limited scale (Hashmi *et al.,* 1995b) incorporated a plasmid containing heat-shock protein genes from the free-living nematode *Caenorhabditis elegans* into *H. bacteriophora* and the resulting transgenic strain had a higher tolerance to short temperature spikes than did the wild type. Field trials showed no increased persistence of the transgenic strain compared to the wild-type nematode indicating that the transgenic form has no advantage over the wild type. Thus, the transgenic nematode had an advantage over the wild type in storage and application because of its higher tolerance to short temperature spikes. However, regulatory issues in various countries may affect the commercialization and eventual field release of transgenic nematodes. For the mutualistic bacteria, some of the main targets for genetic improvement include pathogenicity, host specificity, symbiont specificity, resistance to environmental extremes, and control of phase variation (Burnell, 1996). A number of genes from these bacteria such as outer membrane protein genes, low-temperature induced genes, lux genes, extracellular enzyme genes, and crystalline protein genes have been cloned (Forst, 2002). Proteins with insecticidal activities have been isolated and the genes identified, and they show potential to be incorporated into plants for insect control (Bowen, 1998).

Shelf Life of EPNs

In general, EPNs do not have a long shelf life. Many microbial insecticides, including *Bacillus thuringiensis*, have a resting stage facilitating long-term storage. The infective juvenile EPNs stage is not a resting stage; juveniles are metabolically active and use energy reserves while in formulation (Lewis, 1999). For this reason, it is advisable to order EPNs only 3-4 days prior to application. EPNs should be shipped by overnight delivery in their infective juvenile stage and used within 1–2 days after arrival.
The EPNs should be examined upon receipt to make sure they arrived alive. The shipment container should not feel warm or hot. Open the container and check the color and odor of the nematodes. To the naked eye, the nematodes on a sponge formulation will appear as a light tan or gray paste, while nematodes in vermiculite or liquid suspension will not be discernible from the carrier material. The container should have a mild odor; if there is a strong smell, like ammonia, then it is likely the nematodes are dead. If the formulation is a sponge or vermiculite, remove a tiny

portion of the product with tweezers and place in a teaspoon of cool water (approximately 60ºF) for six hours. If the formulation is a liquid suspension, swirl the liquid to ensure distribution of the nematodes and remove a small droplet (about 0.05 ml).

Place the soaked nematode sample (from the sponge or vermiculite) or the droplet from the liquid suspension on a slide or in a small, clear glass bowl. View the samples with a hand lens (15X) or microscope. Live entomopathogenic nematodes will be mobile and have a bend to their shape. *S. carpocapsae* has a resting "J" shape and will move only when prodded with a pin or needle. All other nematodes will move in an "S" pattern (Lewis, 1999). If the nematodes are straight and not moving, it is likely they are dead. A mortality rate of 10% is typical. If more than 20% of the nematodes are dead, inform your supplier immediately.

EPNs should be stored in their shipment containers under refrigeration until ready for use. The storage life of EPNs is species and formulation-dependent. Specific storage instructions will be included with the EPN shipment and should be carefully followed. Storing nematodes under refrigeration will increase their shelf life, but their infectivity will still decrease the longer they are in storage. When the storage life has expired, expect 70–100% mortality of the nematodes (Grewal, 2000).

Quality control of EPNs

The quality of the nematodes should be checked before and after formulation. At a minimum, their viability and infectivity should be monitored. Several bioassay protocols are available, but assays using many nematodes are considered inappropriate for quality control purposes due to host parasite interactions such as recruitment (Grewal, 2002) advocates the use of a one-on-one (one nematode to one Galleria larva) sand-well assay as a standard quality control tool. The one-on-one assay works well for steinernematids and five-on-one assay works well for heterorhabditids (Gaugler, 2000). Additional quality control parameters include assessment of energy reserves (dry weight or total lipid content) as a predictor of longevity.

Applications of EPNs

i)   Preparing for Application

EPNs should be prepared for field application no earlier than one hour ahead of time. If nematodes are

in a liquid suspension, shake the shipment container well and pour the liquid into the application container (e.g., tank, backpack sprayer, or watering can). Rinse the shipment container twice with cool water (approximately 60°F), and pour the rinse water into the application container. If nematodes are on a sponge, soak the sponge in one gallon of cool water for 10 minutes and then pour the water into the application container. Rinse the sponge several times, pouring the rinse water into the application container after each rinse. If nematodes are in vermiculite, add the vermiculite-nematode mixture directly to water in the application container and stir until dispersed. Once the nematodes have been mixed with water, agitate the mixture every five minutes to keep the nematodes in suspension and supplied with oxygen.

ii)   Application Rates

The application of any biological control agent including EPNs requires the reading of product label for specific application instructions. A broadcast application rate of 1 billion nematodes per acre is generally recommended to control most soil insects. For smaller areas, the recommended application rate is 250,000 nematodes per square meter. If nematodes are banded (applied in a band beside the crop row), a lower rate may be applied. Research at the University of Florida has demonstrated that a rate of up to 200 million nematodes per acre applied in a band provided effective control of root weevil in citrus orchards (Duncan *et al.*, 1999). More research is needed to determine specific rate responses for each species of EPNs in various cropping systems to control specific pests. An excellent overview of sprayer calibration is provided in the *Private Applicator Pesticide Education Manual,* EM020 (Ramsay *et al.,* 2009).

iii)   Evaluation of Nematode Applications

It can be difficult to be sure if the EPNs reached the soil and the target pests, as it is very laborious to recover the cadavers of the insects they have killed. There are two simple tests that can be used to assess the efficacy of all EPN species. Both tests employ *Galleria mellonella* waxworms (Berry, 2007), which are the caterpillar stage of a waxmoth species that are extremely susceptible to EPN infection. *Galleria* waxworms are readily available at fishing bait and pet supply stores.

For the first test, 2–3 *Galleria* waxworms are placed in a tea strainer and bury the strainer is buried 4 inches deep in the soil. The waxworms can be buried either

just before you apply the EPNs or anytime afterwards. It is best to place several baited strainers in the area where nematodes are being applied either 3-4 strainers for a garden area or approximately 10 strainers per acre. The strainers are removed from the soil after 2 days the waxworms are rinsed with distilled water and then stored on moistened filter paper or thick paper towel in a dark location at room temperature. The waxworms regularly over the next 7–10 days to look for nematode infection. Infected waxworms usually change color; *Steinernematid*-infected waxworm turn yellow, tan, or brown, while *Heterorhabditid*-infected waxworms will turn pink or purple. If the waxworms turn black, they are likely to be killed by other means. For the second test, collect EPN-treated soil was collected from the treated area at least one day after nematodes have been applied. Then 10 soil samples were collected from a garden area and 20 soil samples were collected per acre. Each soil sample should be approximately ¼ cup from a depth of 4 inches. Mix the soil together, place ¼ cup into a wax cup, and place a Galleria waxworm on top of the soil. Evaluate 2-3 wax cups for a garden area or approximately 10 wax cups per acre. Place the cups in a dark area at room temperature for 2 days. The waxworms were rinsed, stored and evaluated as described above.

Conclusion

The challenge we face is to determine which behaviors might be the most important to document. Given that it will not be possible to study all aspects of bio-control of EPN foe all species and strains, some list of priority bio-control and ecological attributes might be appropriate. Surely one aspect each of the main sections of this review should be given priority. A list of suggested behaviors might be useful to those who focus on surveying new areas for EPNs populations. Determining which behaviors would be best study would depend on both the aspect of EPN biology in question and the repeatability of the assay among all the various laboratories that would conduct it. This determination would also depend upon whether the focus of study developing biological control agents or understanding behavioral ecology of EPNs. What is important in each of these contexts could be quite different. Perhaps the biggest challenge will be to come to a consensus as a research community on what is important to know about Entomopathogenic Nematodes.

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