Entomopathogenic Nematodes as Biopesticides

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Atwa A. Atwa

Abstract

Entomopathogenic nematodes (EPNs) are microbial control agents which have become important in biological control or integrated pest management of insect pests as biopesticides. EPNs are widespread all over the world and are found in almost all places where there is agricultural land and forests, and in the desert where there are desert plants. Where insects are present in the environment, they may help the spread of EPNs of a number of species of the genera *Steinernema* (more than 61 species) and *Heterorhabditis* (more than 14 species). The factors responsible for aggregated distribution of EPNs may include behavior and the spatial and temporal variability of the nematodes' natural enemies, such as nematode trapping fungus. Nematodes also have limited dispersal ability. Many infective juveniles are produced from a single host, which can also produce aggregates. Patchy EPN distributions may also reflect the uneven distribution of the host and nutrients in the soil. The metapopulation as a whole can persist as long as the rate of colonization is greater than or equal to the rate of population extinction.

EPNs infect only insects and live inside the body of their insect host, so they are designated endoparasitic. EPNs infect many different types of soil insects, including the larval and pupal forms of butterflies, moths, beetles, and flies, as well as adult crickets and grasshoppers. EPNs have been found in all inhabited continents and a range of ecologically diverse habitats, from cultivated fields to deserts, yet are safe for plants and animals. Most biopesticides require days or weeks to kill their host, yet nematodes, working with their symbiotic bacteria (*Xenorhabdus* for the family Stienernematidae and *Photorhabdus* for the family

A.A. Atwa (✉)
Deanship of Scientific Research, King Abdul-Aziz University, Jeddah, KSA., P.O. Box 80230, Jeddah 21589, Egypt
e-mail: atwaradwan@yahoo.com

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Heterorhabditidae), can kill their insect hosts within 24–48 h. Dozens of different insect pests are susceptible to infection, yet no adverse effects have been shown against beneficial insects or other nontarget organisms in field study experiments. Nematodes are amenable to mass production and do not require specialized application equipment as they are compatible with standard agrochemical equipment, including various sprayers and irrigation systems. Although the biological control industry has acknowledged EPNs since the 1980s, today thousands of researchers representing more than 50 countries are working to develop nematodes as biological insecticides. Nematodes have been marketed on every continent except Antarctica for control of insect pests in high-value horticulture, agriculture, home gardens and garden niche markets. In this chapter, we focus on EPNs as biopesticides in insect control. Isolation and distribution, application techniques, and field application models of EPNs as biopesticides throughout the world are discussed. The chapter closes with a discussion of mass production of EPNs, the safety of EPNs, and quality control of EPN production.

Keywords
Entomopathogenic nematodes • Insect host • Mass production • Safety • Quality control

5.1 Introduction

Entomopathogenic nematodes (EPNs) of the families Steinernematidae and Heterorhabditidae possess impressive attributes for biological control. EPNs are recognized as insect-parasitic nematodes, beneficial nematodes, biocontrol agents, biological control agents, biological insecticides, or biopesticides. These nematodes are also recognized as pathogens or microbial control agents because of their symbiotic association with bacteria (*Xenorhabdus* spp. and *Photorhabdus* spp.) that are mainly pathogenic to insects. Because of a mutualistic relationship with pathogenic bacteria, these nematodes are named "entomopathogenic nematodes" (EPNs). They have a worldwide distribution as they have been isolated from every inhabited continent and many islands (Hominick et al. 1995). They have been isolated from different soil types, from sea level to high altitudes, and from natural habitats of disturbed agroecosystems. Because EPNs are obligate parasites in nature, they need to recycle in their hosts to maintain their presence in the environment. The distribution of the nematode population is patchy at any given site (Campbell et al. 1997) and may depend on various abiotic and biotic factors, including their seasonal variations and foraging strategies. From a practical point of view, after inundative release of the nematode, recycling is a highly desirable attribute because it can provide additional and prolonged control of the pest and avoid or reduce the need for further applications. Numerous studies have shown that nematode recycling in the soil environment occurs after inundative release (Kaya 1990), but factors that influence survival and infectivity also affect nematode recycling. Until we understand nematode behavior thoroughly, the practical approach will be to use these nematodes as biological insecticides.

EPNs contribute to the regulation of natural populations of insects. However, the population of naturally occurring EPNs is normally not high enough to manage soil-dwelling plant pests. Therefore, during the last four to five decades, these live nematodes have been commercially

mass-produced and inundatively applied to control many garden insects, turfgrass insects, nursery insects, greenhouse insects, and insects that feed on different field crops. These biopesticides (EPNs and their symbiotic bacteria) are safe to produce and are not harmful to humans, other mammals, most beneficial insects, or plants. EPNs do not pose any health risk to consumers of nematode-treated agricultural produce and do not cause any damage to the environment, and they are exempt from registration requirements in most countries. EPNs also have no detrimental effect on other beneficial nematodes, including bacterial feeders, some fungal feeders (*Aphelenchus* sp.), predatory nematodes, and other soil microbial communities. But EPNs can be detrimental to plant-parasitic nematodes that are responsible for causing a tremendous economic loss to the agriculture industry throughout the world. EPNs can suppress the populations of many economically important plant-parasitic nematodes, including foliar nematodes, potato cyst nematodes, ring nematodes, root-knot nematodes, root-lesion nematodes, sting nematodes, stubby root nematodes, and stunt nematodes.

Most recent publications on EPNs have focused on their potential use as biocontrol agents, but little is known about the structure and dynamics of their natural populations. Accordingly, a soil survey is conducted to assess the occurrence of EPNs and to find new isolates, across seasons, habitats, and geographic regions. Although the results from many laboratory tests with EPNs have been promising in regard to controlling insect pests, field evaluation results have often been highly variable, particularly in regard to well-hidden insects of cryptic habitats such as soil (scarabs) and tunnel-living (leopard moth and red palm weevil) insects. They are well protected from chemical insecticides, with a high rate of survival. Thus, these insect hosts are capable of producing large populations and new generations that subsequently disperse or migrate or both to more susceptible plant hosts, where more control measures are required. Therefore, field trials have been conducted to validate laboratory findings.

However, one species, *Steinernema scapterisci*, has been successfully introduced as a classic biological control agent against mole crickets in Florida (Parkman and Smart 1996), suggesting that suitable conditions prevail for this nematode to recycle. In this respect, EPNs belonging to the families Heterorhabditidae and Steinernematidae have already been successfully used throughout the world for the control of important agricultural insect pests. The qualities that make EPNs excellent biocontrol agents are their broad host range, their ability to search actively for their hosts, and to kill them relatively quickly, their economic mass-production, and their being noninjurious to vertebrates, easily applied, compatible with most chemical insecticides, and environmentally safe.

The main goal of this chapter is to illustrate the use of EPNs as bioinsecticides. This goal will be achieved through five main sections related to each other as follows: (1) isolation and distribution of EPNs, (2) techniques for application of EPNs as biopesticides, (3) field application models of EPNs as biopesticides throughout the world, (4) mass production of EPNs, and (5) safety of EPNs and quality control of EPN production.

5.2 Isolation and Distribution

EPNs from the families Heterorhabditidae (Poinar 1976) and Steinernematidae (Travassos 1927) are obligate insect parasites which can infect and kill a broad range of insect hosts (Kaya and Gaugler 1993). These nematodes are symbiotically associated with entomopathogenic bacteria of the genera *Photorhabdus* and *Xenorhabdus* (Boemare et al. 1993). These nematodes have been used successfully as bioinsecticides against insect pests. EPNs have a global distribution; the only continent where they have not been found is Antarctica. However, biotic and abiotic factors cause the distribution of EPNs to differ across different regions. Factors such as soil texture, moisture content, temperature, ultraviolet (UV) light, seasonal variation, dominating vegetation, host-finding ability, and dispersal agents are thought to be important in determining their distribution (Griffin et al.

1991). The goal of this section is to discuss the survey of EPNs and factors affecting the natural occurrence and distribution of EPNs around the world. The isolation of EPNs is the first step to establish EPNs as bioinsecticides for controlling insect pests.

5.2.1 Survey and Taxonomy

Nematodes belonging to the families Heterorhabditidae and Steinernematidae (Nematoda: Rhabditida) that are entomopathogens have been isolated from soil-inhabiting insects throughout many parts of the world (Poinar 1990). Several taxonomical publications (Liu and Berry 1996) have indicated that if some morphological characters of the infective juvenile (such as the body length and the distance from the head to the base of the esophagus) are combined with some other characters of the male (the shape of spicules, bursa, and genital papillae), most EPNs can be separated. However, other diagnostic methods, such as starch gel electrophoresis (Akhurst 1987), DNA restriction fragment length polymorphisms, restriction enzyme analysis (Smits et al. 1991), cross-mating, isoelectric focusing (Joyce et al. 1994), and randomly amplified polymorphic DNA PCR methods (Liu and Berry 1996), have been used to identify species and strains of EPNs. Also, the use of both molecular and classical methods can overcome the difficulties of extensive overlap in morphometric characters among EPN species and/or strains (Waturu et al. 1997).

The identification of EPNs by standard morphological criteria alone is rarely straightforward (Liu et al. 1999). Attempts to characterize these nematodes reliably have involved a range of techniques, including allozyme electrophoresis for analyzing DNA. The family Heterorhabditidae is monotypic, represented by the genus *Heterorhabditis*. The systematic problems encountered in this group when applying the phonetic approach arise because the adult nematodes feed and reproduce in the protected environment of the insect hemocoel (Liu et al. 1999). Such specialized, but essentially similar modi operandi imply a considerable degree of similarity in morphological features expressed in combination with excessive morphometric variability attributable to density-dependent nutritional factors (Liu et al. 1999). Classical techniques have therefore concentrated on the free-living infective stage, which, although lacking considerable gross morphological variation as a result of being a nonfeeding stage, does show enhanced morphometric consistency. These problems have resulted in considerable confusion as to the status of the nominal species, a confusion that has enormous practical importance now that the nematodes have attracted commercial interest as potential biological control agents. In addition, accurate identification is often demanded by quarantine regulations stipulating that only indigenous species/isolates can be released as part of a biological control program (Hunt 1997). Taxonomic relationships of both heterorhabditid and steinernematid nematodes are usually based on morphological characters; sometimes crossbreeding is used with members of the genus *Steinernema*. Morphological characters cannot be used unambiguously to place new isolates into a particular species. Hashmi et al. (1996) reported that the feasibility of using heterorhabditid and steinernematid nematodes as biological control agents depends on the resources required for a rapid and accurate means to determine the genetic diversity among existing populations of EPN species. These methods can also be used for the development of identification tools.

5.2.2 Life Cycle and Host Relationship

The general life cycle of heterorhabditid and steinernematid nematodes involves a free-living infective third-stage juvenile or dauer stage that carries species-specific bacterial symbionts, *Xenorhabdus* or *Photorhabdus*, along its gut or in a pouch off the gut depending on the nematode family (Akhurst 1986). The only life cycle difference between *Heterorhabditis* and *Steinernema* is in the first generation. *Steinernema* species are amphimictic; this means that for successful reproduction, male and female infective juveniles must

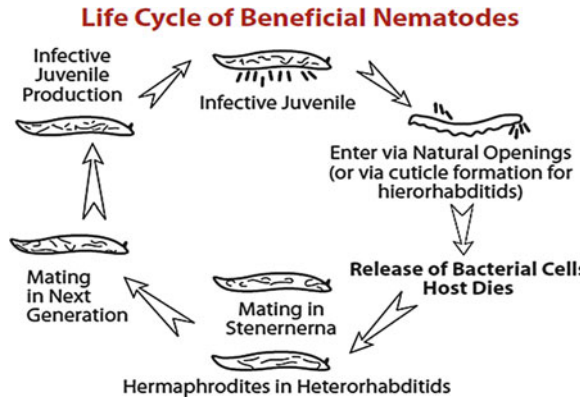

Fig. 5.1 The generalized life cycle of *Steinernema* and *Heterorhabditis* nematodes. (After Grewal 1999)

enter the host, whereas *Heterorhabditis* species are hermaphroditic, and only one infective juvenile in the host is sufficient for successful reproduction. In the second generation of both nematode genera, reproduction is amphimictic (Poinar 1990). The infective juveniles of both nematodes commonly seek out and enter a suitable insect host through a natural opening such as the spiracles, mouth, and anus, or in the case of *Heterorhabditids*, additionally by penetration of the cuticle by use of a tooth. Once the infective juveniles have penetrated into the host's hemocoel, the nematode releases the bacterial symbiont, which propagates and causes a rapid and fatal septicemia. The bacteria digest the contents of the cadaver, and the nematode feeds on the bacterial culture. The bacteria turn the freshly killed insect larvae a reddish color, and the tissue takes on a characteristic gummy consistency. Undoubtedly, the host is killed by multiplication of the bacteria associated with the nematodes (Poinar 1990). The heterorhabditid infective juvenile grows to become a self-fertile adult inside the invaded insect and reproduces hermaphroditically, whereas the steinernematid infective juvenile becomes either a male or a female and reproduces amphimictically (Fig. 5.1). Interestingly, Grewal et al. (1993) reported that the male infective juveniles of *Steinernema* spp. migrate and penetrate hosts earlier than do females. The females then seek out and penetrate the male-occupied insects. Later, each of the two nematodes passes through two or three dioecious generations before they produce new dauer larvae (infective juveniles), which emerge from the depleted host cadaver (Fig. 5.1) into the soil within 2–3 weeks depending on the conditions (Grewal 1999).

5.2.3 Detection of EPNs in Soil

To detect the presence of EPNs in soil, a search for infected insect cadavers should be conducted. Since infected cadavers disintegrate within about 2 weeks, finding these is at best haphazard. Also, this method is unsatisfactory when host insect or nematode densities in nature are low or at low soil temperatures. Bedding and Akhurst (1975) found that the last instar larvae of the greater wax moth, *Galleria mellonella* L., when buried in soil are more susceptible to parasitism by EPNs than are the usual hosts. Normally, *Galleria* larvae live in beehives and are not exposed to nematodes, whereas soil-inhabiting insects have been exposed to EPNs for millions of generations and are expected to have evolved some immune protection. Thus, the *Galleria*-bait method of extracting EPNs from soil samples has become the standard in soil surveys (Fig. 5.2). In addition, laboratory cultures of nematodes can be initiated by force-feeding the infective stages, as they emerge from field-infected insects, to the last instar larvae of the greater wax moth. Until now, larvae of *G. mellonella* have been used as universal hosts for all species and strains of EPNs. However, *G. mellonella* is neither a good host for *S. scapterisci* nor does it reproduce in it (Nguyen and Smart 1990).

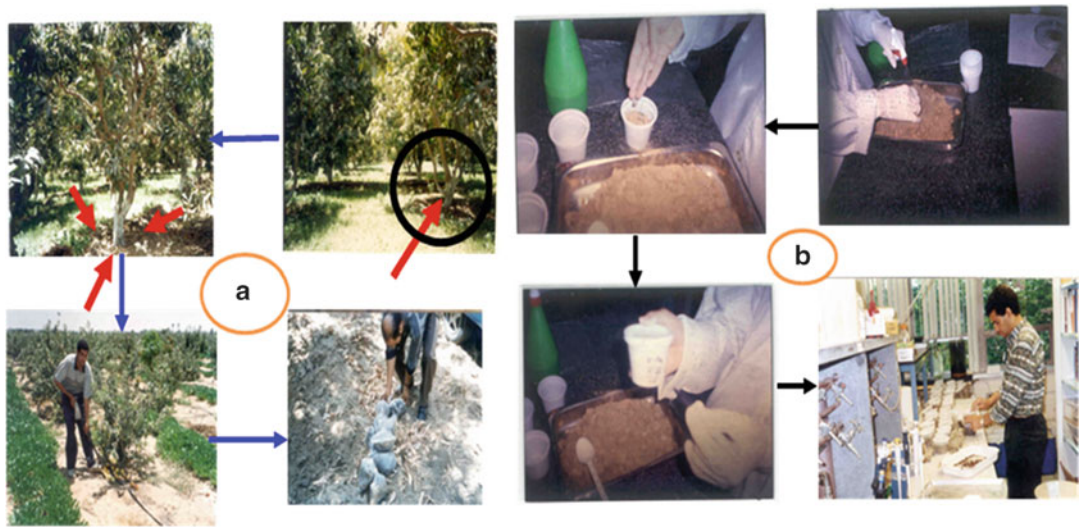

Fig. 5.2 Isolation of entomopathogenic nematodes from soil. **a** Sequence and locations of soil sampling in the field; **b** the preparation of soil samples in the laboratory to isolate the nematodes. (After Atwa 2002)

5.2.4 Factors Affecting the Distribution of the Surveyed Nematodes

Great understanding of the abiotic and biotic factors governing the natural occurrence and abundance of EPNs is of importance in determining the distribution of these species in any survey.

5.2.4.1 Soil Type

Many EPNs have been isolated from different soil types. These nematodes have been associated with humus and organomineral soil layers in Czechoslovakia (Mrácek 1982), humus and sandy soils in Sweden (Burman et al. 1986), sandy loam and loam soils in Ireland (Blackshaw 1988), calcareous soils in England (Hominick and Briscoe 1990), a coral sand in Hawaii (Lindegren et al. 1990), sandy soil restricted to ocean beach areas in the Hawaiian Islands (Hara et al. 1991), sandy and loamy soils in Egypt (Shamseldean and Abd-Elgawad 1994), and sandy soils rather than clay soil in Pakistan (Shahina et al. 1998). Apparently, EPNs travel less well through soils with a small pore space (Molyneux and Bedding 1984). In contrast, the occurrence of EPNs was not influenced by soil or vegetation type in Italy (Deseö et al. 1988). Therefore, Akhurst and Brooks (1984) speculated that the difference in the distribution of nematodes in various countries may reflect the availability of suitable host insects, although environmental influences such as soil type may also determine their distribution. Shapiro-Ilan et al. (2012) indicated that *Steinernema carpocapsae*'s response to electrical fields diminishes with infective juvenile age. Conceivably, the importance of a directional response in foraging strategies may be most important early in the nematode's life cycle. Alternatively, sensitivity to electrical fields may simply degenerate with age. Additionally, in a broader sense, differing substrates may affect EPN response in different soil types.

5.2.5 Moisture Content

The infective juvenile or dauer stage carries, initially at least, the unshed second-stage cuticle as a sheath (Nguyen 1993). These infective juveniles can survive the stress of desiccation, particularly if dehydration occurs very slowly (Womersley 1990). This indicates that, under natural conditions, infective juveniles can

survive slow drying, perhaps by aggregating alone or in association with soil colloids, plant root gels, or cadavers (Downes and Griffin 1996). In contrast, Hominick and Briscoe (1990) indicated that the temperate and moist climate of Britain provides conditions suitable for the year-round presence of steinernematids. Also, Garcia Del Pino and Palomo (1996) stated that the greater frequency of occurrence of EPNs in surveyed areas in Spain was associated with medium temperatures and higher rainfalls. They suggest that these climatic conditions are more favorable to nematode survival in the western Mediterranean area. EPNs are frequently found in sites adjacent to the sea (Griffin et al. 1994; Hara et al. 1991) in associations that are intriguing and unexplained. However, it has been shown that infective juveniles are capable of surviving in seawater for several weeks. They suggested that postglacial recolonization by EPNs may have been aided by the migration of coastal sand dune systems under the influence of a rising sea.

5.2.5.1 Temperature

Steinernematids are widely distributed in temperate and cool areas, for example, Czechoslovakia (Mrácek 1980), Sweden (Burman et al. 1986), Britain (Hominick and Briscoe 1990), Germany (Ehlers et al. 1991), Ireland (Griffin et al. 1991; Downes and Griffin 1991), Scotland (Boag et al. 1992), and Norway (Haukeland 1993). These observations seem to suggest that steinernematids prevail in cool and temperate climates because they are better adapted to low temperature (Hominick et al. 1995). Similarly, steinernematids are prevalent, but there is a greater or lesser presence of heterorhabditids, in temperate areas of North America (Akhurst and Booker 1984), Australia (Akhurst and Bedding 1986), and Canada (Mrácek and Webster 1993).

Heterorhabditis seems to be commoner in tropical and subtropical climates such as those of Puerto Rico (Roman and Beavers 1982), Hawaii (Hara et al. 1991), Israel (Glazer et al. 1996), Egypt (Shamseldean and Abd-Elgawad 1994), and Pakistan (Shahina et al.1998). These findings may indicate that heterorhabditids are better adapted to warm and hot weather since they need higher temperatures than steinernematids (Molyneux 1986). Recent publications have reported the occurrence of steinernematids in warm and tropical countries, for example, Puerto Rico (Roman and Figueroa 1994), Spain (Garcia Del Pino and Palomo 1996), Portugal (Rosa et al. 1994), Argentina (Stock 1994), Korea (Stock et al. 1997), and Kenya (Waturu et al. 1997). These findings support the view that the broad generalization which holds that steinernematids are temperate species whereas heterorhabditids are tropical species must be questioned (Garcia Del Pino and Palomo 1996).

5.2.5.2 UV Light

EPNs have very poor UV tolerance, indicating that the UV hazard is rarely encountered by natural EPN populations. However, the superior tolerance of *S. carpocapsae* over *Heterorhabditis bacteriophora* may be related to the tendency of that species to nictate at the soil surface (Gaugler et al. 1992).

5.2.5.3 Seasonal Variation

There is some evidence of seasonality in the occurrence of the EPNs in different surveys (Griffin et al. 1991). This may presumably be due to the different climates and/or localities (Akhurst and Bedding 1986) where nematode infectivity is affected by many environmental conditions, including temperature (Grewal et al. 1994) and moisture (Kung et al. 1991), or both factors (Shahina et al. 1998). In contrast, there was no apparent seasonality to the EPN population densities throughout many surveys (Campbell et al. 1995). This indicates that EPNs are present during periods when pest insects are also present and/or suitable climatic conditions for nematode infection and reproduction prevail throughout the year (Hominick and Briscoe 1990). Kanga et al. (2012) illustrated that the diversity of the EPNs found in Cameroonian soils was low, with only three species detected, viz., *Heterorhabditis baujardi*, *Steinernema* sp. A, and *Steinernema* sp. B. *H. baujardi* was much more frequently isolated than the other

species. This suggests a wide range of susceptible hosts for the species.

5.2.5.4 Dominating Vegetation

The literature on the habitat preference of EPNs is contradictory. In Tasmania, Akhurst and Bedding (1986) stated that there were no differences between forests and pasture regarding the presence of EPNs. In Britain, Hominick and Briscoe (1990) pointed out that vegetation had little effect on nematode persistence, similar to results of surveys in Ireland (Griffin et al. 1991) and Spain (Garcia Del Pino and Palomo 1996). Other surveys assessed habitat preferences of these nematodes. In Czechoslovakia, nematodes were commoner in forest than in cultivated fields and were not found in meadows (Mrácek 1980). In North Carolina, woodlands were less suitable than cultivated soils or pastures (Akhurst and Brooks 1984). Nematodes were commoner in Scottish pastures than in forests or croplands (Boag et al. 1992). In New Jersey, nematodes were more abundant in a weedy area than in nearby turf, but across some sites, nematodes appeared to be equally abundant in turf and weedy habitats (Stuart and Gaugler 1994). In this respect, Akhurst and Bedding (1986) suggested that these differences in nematode distribution are related to differences in the distribution of suitable insect hosts and to differences in the species or nematode involved.

5.2.5.5 Host-Finding Ability

Different EPN species and strains exhibit differences in searching behavior which make them more or less suitable for insect pest infectivity; for example, *Steinernema glaseri* dispersed up to 90 cm in a sandy soil (Kaya 1990), whereas some species of *Heterorhabditis* migrate very actively through the soil (Smits et al. 1991), and other species such as *S. carpocapsae* migrate less and may nictate on a solid surface when relative humidities are high (Ishibashi et al. 1994) but become inactive in soil in the absence of hosts (Ishibashi and Kondo 1986). Generally, heterorhabditid infective juveniles are more migratory than those of steinernematids (Downes and Griffin 1996).

In seeking new hosts, EPNs that search by moving throughout their environment to find hosts are termed "cruisers," whereas those that wait for hosts to come to them are termed "ambushers" (Lewis et al. 1992). *S. glaseri* is a cruiser that actively moves in the soil (Schroeder and Beavers 1987), responds strongly to host cues, and is adapted to infect sedentary hosts (Campbell and Gaugler 1993). In contrast, *S. carpocapsae* is an ambusher that stays near the soil surface and does not disperse into the soil, is unresponsive to host cues, and is adapted to infect mobile hosts on the soil surface (Moyle and Kaya 1981). However, cruiser and ambusher behaviors reflect different balances of advantage for the species that display them. Movement increases the probability of encounter with a stationary host, but also with the nematode's natural enemies (Downes and Griffin 1996). Furthermore, an active nematode undoubtedly uses up its limited reserves more quickly. Regarding the attraction of nematodes to insect hosts, EPNs have been shown to respond positively to a chemical gradient around the host (Schmidt and All 1979), carbon dioxide and thermal gradients (Burman and Pye 1980), and materials from hosts or their feces (Kondo and Ishibashi 1986). Further, they can be activated by thermal or mechanical shock, and by certain chemicals (Gaugler and Campbell 1991).

5.2.5.6 Dispersal Agents

Since the infective juveniles are adversely affected by desiccation and UV light, aerial dispersal over great distances is not likely (Downes and Griffin 1996). On the other hand, many adult insect hosts are capable of flying after infection over a period of at least 1 or 2 days after inoculation, and for longer if survival factors are suboptimal for the development of the infection. For example, the infected adults of coleopteran (Glaser and Farrell 1935) and lepidopteran (Timper et al. 1988) species serve as dispersal agents for EPNs. Although such internal infection or external phoresis may be a common method of dispersal in EPNs over a relatively short distance, wind-transported insects are capable of traveling up to 2,000 miles.

Humans are the most effective dispersal agents for nematodes (Ferris et al. 1976). Akhurst and Bedding (1986) speculated that nematodes were introduced into Australia during the immigration of Europeans, probably in soil introduced with exotic plants or ship ballast, or both. Also, EPNs are more frequently found in areas such as parks, lawns, seashores, and nurseries, where human impact has been substantial, rather than in natural habitats (Mrácek and Webster 1993). In addition, EPNs may be imported by researchers for laboratory and limited field testing (Hara et al. 1989). Reasonably, many countries have quarantine laws concerning importation of exotic organisms to protect the natural fauna and flora and local agriculture.

5.2.5.7 Nematode Antagonists

Potential interactions between EPNs and predatory mites, nematodes, and pathogenic fungi in soil fields might have been at least partially responsible for an extended period of infectivity, lack of infectivity, and discontinuities in the temporal pattern of infectivity (Kaya 1990). Accordingly, the persistence of EPNs in sterilized soil was greater than that in unsterilized soil (Curran and Heng 1992). In addition, Fan and Hominick (1991) reported that only 30–40 % of EPNs present in the soil are capable of establishing themselves in *G. mellonella* larvae although all environmental conditions are optimal.

Abiotic stress factors negatively influence the persistence of EPNs. Mrácek and Webster (1993) reported that the absence of EPNs from forest nursery tree beds in Canada may be due to the use of chemical insecticides against root weevils in those tree beds. Similarly, the absence of nematodes from a British Columbian forest, where western spruce budworm larvae and pupae were present, may be due to the unsuitability of the forest litter for nematode survival (Mrácek and Webster 1993).

5.3 Techniques for Application of EPNs as Biopesticides

EPNs have received increasing attention because of their potential as bioinsecticides aganist soil insect pests easily found in soil. Poinar and Lindhardt (1971) found that bibionid fly larvae and pupae (*Bibio hortulanus*) in Denmark are probably continuously associated with steinernematids; hence, they may reduce host numbers in barley fields. Poinar (1975) reported that *H. bacteriophora* appeared to be an important pathogen of *Heliothis punctigera* in alfalfa fields in South Australia. Cabanillas and Raulston (1994) stated that *Steinernema riobravis* appears to be endemic in Texas, where it was found parasitizing prepupae and pupae of both corn earworm (*Helicoverpa zea*) and fall armyworm (*Spodoptera frugiperda*).

EPNs possess many attributes of an ideal bioinsecticide: they have a wide host spectrum, are environmentally safe, can be produced in large-scale bioreactors, are easily applied, are compatible with most chemical pesticides, are applied in diverse climatic conditions, and are capable of finding hosts in soil (Garcia Del Pino and Palomo 1996). In addition, the use of naturally occurring nematodes in a particular area as biological control agents may also reduce the risk to nontarget organisms when compared with the use of exotic isolates (Blackshaw 1988).

Selection of appropriate EPNs as bioinsecticides includes bioassays in the laboratory to identify virulent strains and evaluating efficacy under simulated field conditions (Jansson et al. 1993). Gray and Webster (1986) demonstrated that differences in virulence among nematode strains were influenced by temperature. It affects their motility, infectivity, pathogenicity, survival, and reproduction (Glazer et al. 1996). For example, Grewal et al. (1993) stated that *H. bacteriophora* adapted to cold or warm temperature by improving reproduction, but not virulence, whereas *Steinernema anomali* improved virulence, but not reproduction. Additionally, co-inhabiting nematode species may reduce competition in their niche by having different thermal optima (Freckman and Caswell 1985). For the above-mentioned reasons, temperature may be one of the most important factors limiting the success of *Heterorhabditis* ssp. and *Steinernema* ssp. in biological control of insect pests as bioinsecticides.

Application techniques were summarized by Atwa (2011), who reported that the application of EPN studies indicated that *S. carpocapsae* applied to soil may survive relatively longer than when applied foliarly. Soil applications should include the insecticide acephate or permethrin to maintain nematode activity for a long time without having a detrimental effect on these nematodes. For controlling insect borers, the injection technique achieved better control than the spray technique in separate applications of either *Heterorhabditis* sp. or *Steinernema* sp. (1,000 nematodes per milliliter) or the chemical insecticides Cidial 50 % EC and Basudin 60 % EC (3,000 ppm). The best results were obtained by injecting Basudin at 750 ppm with 500 infective juveniles of *Heterorhabditis* sp. per milliliter (64.74 % mortality) or by injecting it at 1,500 ppm with 500 infective juveniles of *Heterorhabditis* sp. per milliliter (63.89 % mortality). Atwa and Shamseldean (2008) found that *Steinernema* sp. (EGB20) was superior to *H. bacteriophora* (EGB13) and *Heterorhabditis indica* (EBN16) when applied for control of *Zeuzera pyrina* with 1,000 infective juveniles of EPNs per milliliter.

The effects of different application technologies were evaluated on the concentration, viability, and efficacy of infective juveniles of *H. indica* and *Steinernema* sp. (IBCB-n6) to control *S. frugiperda* Smith on corn plants by Garcia et al. (2008). Two hundred eighty infective juveniles of *Steinernema* sp. were required to kill 100 % of third-instar fall armyworms in petri dishes, as compared with 400 infective juveniles of *H. indica* to acheive 75 % fall armyworm control. It is possible to spray EPNs without significant loss of their concentration and viability with equipment that produces electrically charges to the spraying mix, and with equipment using hydraulic and rotary nozzle tips. The concentrations of infective juveniles of *H. indica* and *Steinernema* sp. were reduced by 28 and 53 %, respectively, when hydraulic spraying nozzles that require 100-mesh filtrating elements were used (Garcia et al. 2008). Tensoactive agents of the organosilicone and ethoxylate groups did not affect the viability of infective juveniles of *Steinernema* sp. Spraying corn plants (V6 growth stage) with up to 288 million infective juveniles of *Steinernema* sp. per hectare, diluted in the spraying mix to 800 L ha^{-1}, with 0.01 % ethoxylate tensoactive agent, or at the same volume followed by artificial rain (6-mm water depth), was not sufficient to control *S. frugiperda* in a controlled environment (Garcia et al. 2008).

5.4 Field Application of EPNs

5.4.1 Efficacy of EPNs

The efficacy of biopesticides is determined by the biological characters of the agent and the intended target, the physical aspects of the site to which they are applied, and the interactions of the biopesticide and the environment. For biopesticides that are applied to manage soil pests, the opaque, patchy, and complex milieu of soil presents an especially challenging suite of environmental characteristics to consider when trying to predict efficacy. EPNs are used to control insect pests primarily in soil, and can serve as part of a model system to study the interaction of soil processes with soilborne biological control organisms. EPNs in the families Steinernematidae and Heterorhabditidae use symbiotic bacteria (in the genera *Xenorhabdus* and *Photorhabdus*, respectively) to kill and develop inside their hosts (Kaya and Gaugler 1993). On finding a host, infective juveniles penetrate the hemocoel, usually via natural openings, and release symbiotic bacteria which kill the host usually within 24–48 h and provide essential nutrients for nematode development (Fig. 5.3). The nematodes generally complete two to three generations within the host's cadaver and emerge as infective juveniles, which forage for new hosts (Poinar 1990).

Infective juveniles, the only stage existing outside the insect, locate their host by responding to cues such as CO_2, temperature, feces, cuticle, electromagnetic fields, and vibration. They can also find their host via indirect cues from plants damaged by insect feeding. The foraging strategy varies with the species; some cruise through the

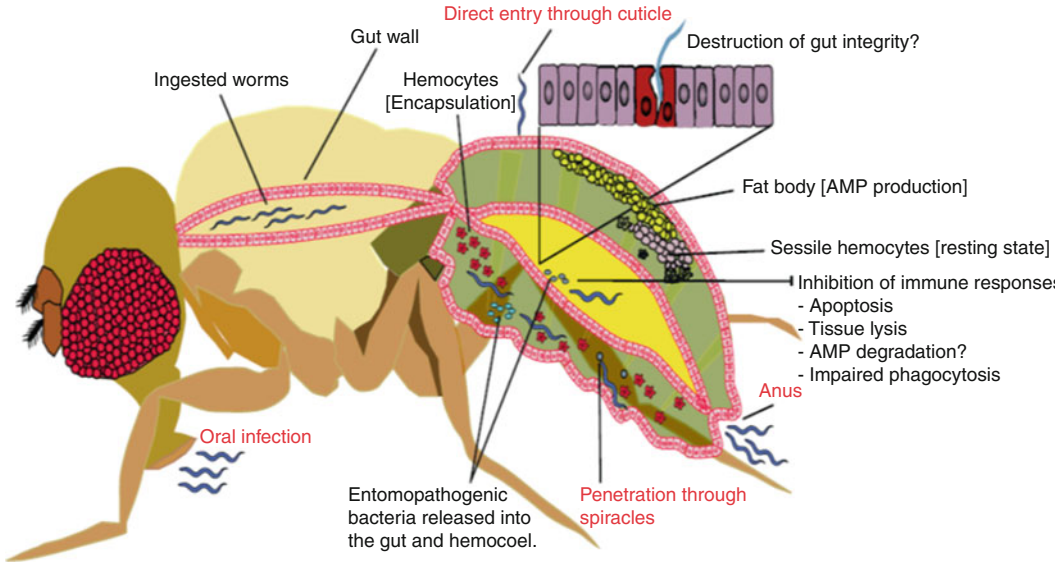

Fig. 5.3 The routes most commonly used by entomopathogenic (or insect-pathogenic) nematodes to infect their insect hosts. Infective juveniles enter the insect body cavity through the mouth, anus, or spiracles. Once nematodes have gained access to the hemocoel (the insect open circulatory system), they may physically damage various insect tissues and organs, such as the gut and fat body. In the case of entomopathogenic nematodes of the genera *Heterorhabditis* and *Steinernema*, the release of symbiotic bacteria (*Photorhabdus* and *Xenorhabdus*, respectively) into the insect host leads to suppression of the insect immune response as the bacteria are able to inhibit key cellular immune mechanisms (e.g., phagocytosis). In turn, this leads to a pathological state within the insect (septicemia) that results in rapid insect death. Nematodes are potentially able to cross the disrupted midgut epithelium. The nematodes and their symbiotic bacteria replicate within the insect, where they complete their life cycles before they emerge as a complex from the insect carcass in search of new suitable hosts. The main insect immune-related tissues (circulating, sessile hemocytes, and fat body) are shown. *AMP* antimicrobial peptide. (After Castillo et al. 2011)

soil following cues associated with hosts, others wait to ambush hosts near the soil surface, and many use intermediate foraging strategies (Atwa 2011). Compared with ambushers, cruisers spend more time moving and actively following host-associated cues in the soil, increasing the probability of locating sedentary and cryptic insect hosts.

The efficacy of aboveground applications of EPNs can be limited by the harmful effects of UV radiation and desiccation. Nonetheless, a number of studies indicate aboveground applications of EPNs can result in high levels of control for a variety of pests, including several *Synanthedon* spp. In the case of *Synanthedon pictipes*, however, our initial studies indicated that aboveground field applications with *S. carpocapsae* failed to cause significant *S. pictipes* mortality. Conceivably, improved formulations or application techniques may improve the efficacy of aboveground applications of EPNs. For example, addition of antidesiccants or other adjuvants has been reported to provide improved aboveground control of various foliar pests, including the diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), the sweet potato whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae), and the Egyptian cotton leafworm, *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae). Compared with foliar applications, relatively little attention has been devoted to improvement of EPN formulations for application to borer pests.

5.4.2 Virulence of EPNs

Selection of appropriate EPNs as biological control agents includes bioassays in the laboratory to identify virulent strains and evaluating efficacy under simulated field conditions

(Jansson et al. 1993). Gray and Webster (1986) demonstrated that differences in virulence among nematode strains were influenced by temperature. It affects their motility, infectivity, pathogenicity, survival, and reproduction (Selvan et al. 1992). For example, Grewal et al. (1993) stated that *H. bacteriophora* adapted to cold or warm temperature by improving reproduction, but not virulence, whereas *S. anomali* improved virulence, but not reproduction. Additionally, co-inhabiting nematode species may reduce competition in their niche by having different thermal optima. For the above-mentioned reasons, temperature may be one of the most important factors limiting the success of *Heterorhabditis* ssp. and *Steinernema* ssp. in biological control of insect pests.

5.4.2.1 Temperature and Infectivity

Soil temperature may be a limiting factor in the ability of nematodes to attack a host. For example, *S. carpocapsae* and *Heterorhabditis* spp. are less adapted to controlling pests at 6 °C (Steiner 1996). Grewal et al. (1994) revealed that differences in thermal adaptation may result in host specialization among EPN species that are adapted to cool-temperature reproduction; for example, *Steinernema feltiae* would be effective against insects that are more active during winter seasons, whereas species that are adapted to warm-temperature reproduction, for example, *S. riobravis* and *Steinernema scapteriscae*, would parasitize insects that are more prevalent during summer. However, Molyneux (1986) and Wright (1992) reported that the Australian and New Zealand strains of *S. feltiae* were virulent at low temperatures, even though they were isolated from warmer climates. Also, Jaworaska (1992) reported that the Polish local strain of *H. bacteriophora* was virulent at a lower temperature of 10 °C although heterorhabditids are endemic to warmer climates. In addition, Grewal et al. (1994) found that the strains of *S. feltiae* isolated from France and Argentina had cool-temperature activities as they infected insects between 8 and 30 °C and reproduced between 10 and 25 °C. They propose that nematodes may have colonized diverse climatic regions without alterations in thermal niche breadth. In this context, the relationship between insect mortality and the number of infective juveniles seems to be density-dependent under certain temperature. Koppenhöfer and Kaya (1997) stated that increasing densities of *S. glaseri* infective juveniles in soil affected the penetration efficiency and reproduction of the nematodes in larvae of *G. mellonella*.

5.4.2.2 Temperature and Activity

Heat may affect "short-range" attraction of nematodes over a few millimeters, as well as host arrest (Burman and Pye 1980). Byers and Poinar (1981) indicated that EPNs aggregate in response to temperature gradients even less than 0.3 °C above ambient temperature, which was the temperature of *G. mellonella* larvae. Apparently, insects in the soil lose very little heat by evaporation. Therefore, their body temperature may rise a "few degrees above ambient" owing to metabolic processes. However, EPNs are attracted not only to the insect body temperature, but also to various stimuli; for example, aqueous surface washes of *G. mellonella* larvae (Schmidt and All 1978), CO_2 (Gaugler et al. 1980), the symbiotic bacterium *Xenorhabdus nematophilus* (Ishibashi and Kondo 1990), and the components of insect feces (Schmidt and All 1979). Heat may also stimulate nematode entry through insect orifices (Byers and Poinar 1981).

Khlibsuwan et al. (1992) indicated that nematode migration toward the source of attraction was impaired at 35 and 37 °C. In addition, Steiner (1996) stated that failure of *S. feltiae* nematodes to parasitize *G. mellonella* larvae depends on their poor ability to move at low temperatures (6 °C). These examples illustrate that the nematode's searching strategy depends on temperature, which has important consequences for biological control under field conditions. Nevertheless, host-finding ability appears to be related also to nematode body length, or more likely to the amount of food reserves. Steiner (1996) found that *Steinernema kraussei* traveled a significantly longer distance than *S. feltiae*, and the smallest species of *Heterorhabditis* ssp. and *S. carpocapsae* dispersed only a short distance.

5.4.2.3 Temperature and Reproduction

The influence of ambient temperatures on the development, maturation, and reproduction of EPNs is well documented in the literature. Dutky et al. (1964) and Kaya (1977) confirmed that the most favorable temperature for the growth and reproduction of the DD-136 strain of *Steinernema (=Neoaplectana) carpocapsae* is between 23 and 28 °C, whereas no development was observed at 10 and 33 °C. However, this nematode developed to the adult stage at 30 °C but did not reproduce. Members of the genus *Heterorhabditis* generally have a wider host range than most steinernematid species, but their activity and reproduction are restricted by cool temperatures (Wright et al. 1989). Molyneux (1983) found that *H. bacteriophora* strain V16 and *Heterorhabditis zealandica* strain HNZ were unable to reproduce at 10 °C. In contrast, Wright (1992) stated that the two nematode strains CA and AKLD of *S. feltiae* could produce infective juveniles within *G. mellonella* larvae at 10 °C and the rate of reproduction was directly correlated to the rate of growth of their associated *Xenorhabdus* clones at 10 °C. Grewal et al. (1994) indicated that the thermal niche breadth for reproduction was wide for *S. glaseri* (12–32 °C) and *Steinernema* sp. (20–32 °C). They were more adapted to warm temperatures, whereas *S. feltiae* was more adapted to cooler temperatures (10–25 °C). The inability of the other steinernematid and heterorhabditid species to reproduce at 10 °C may result from the lack of viable sperms or ova or from the mating behavior of the nematodes (Kaya 1977) or may be correlated with the lack of a hot-temperature-active *Xenorhabdus* clone (Wright 1992). In addition, Zervos et al. (1991) observed that the reproduction rate of *S. glaseri* in wax moth larvae was affected by inoculum levels as well as ambient temperatures.

5.4.2.4 Temperature and Survival

Infective juveniles of EPNs may have mechanisms to survive under adverse thermal environments. The nematodes may survive in soil in a quiescent state (Ishibashi and Kondo 1986; Womersley 1990), migrating downward to avoid adverse conditions (Kaya 1990), remaining in the host cadaver for extended periods, lowering the nematode and bacterial metabolic rates and oxygen demands (Brown and Gaugler 1997), or synthesizing trehalose, which prevents freezing, in response to cold environmental stresses. Also, survival may be partly density dependent; hence, no surviving infective juveniles of *S. carpocapsae* were found in heavily infected cadavers (Brown and Gaugler 1997). The relationship between temperature and survival has been studied in many nematode species. Infective juveniles of steinernematids such as *S. carpocapsae*, *S. feltia*e, and *S. glaseri* can survive prolonged storage at 1–5 °C (Bedding 1984), and an Arkansas isolate of *S. carpocapsae* survived for 2 weeks in soil at 40 °C (Gray and Johnson 1983). The contrast among these results could be due to differences in heat tolerance of the strains used. Nevertheless, heat shock treatment for 2 h at 37 °C before exposure to 40 °C enhanced the survival of *Heterorhabditis* sp. IS-5 juveniles to 43 % as compared with a non-heat-shocked control (Glazer et al. 1996). Ogura and Nakashima (1997) indicated that storage of *Steinernema kushidai* at 5 °C caused 90 % mortality within 10 days, but when these nematodes were preconditioned at 10 °C for more than 8 days, a survival rate exceeding 50 % was recorded 100 days after storage at 5 °C.

5.4.2.5 Temperature and Pathogenicity

Pathogenicity of the EPN–bacterium associations of *Steinernema* and *Xenorhabdus* and *Heterorhabditis* and *Photorhabdus* was investigated as a promising means of biological control, including broad host range, high virulence, and host-seeking capability (Poinar 1990). The bacteria converted the insect into a suitable environment for development and reproduction of the nematode's feeding stages (Poinar 1990).

Temperature may be directly related to the growth rate of bacteria, and nematode biology and virulence. Milstead (1981) indicated that development of *H. bacteriophora* was inhibited at 12 and 30 °C, whereas *Xenorhabdus* bacteria

can grow and cause mortality at 12–33 °C, and the length of the incubation period depended on the bacterial growth rate. Furthermore, bacterial dose–mortality responses in *G. mellonella* were similar for all temperatures (15, 20, 25, 28.5, 30 °C), except at 12 °C, where a larger dose was required to kill that host. Wright (1992) stated that the reproduction capacity of different *S. feltiae* strains was related to the growth rate of their associated *Xenorhabdus* clone. Grewal et al. (1993) demonstrated that improvement in nematode virulence and establishment and extension of the thermal infection niche breadths may be fully or partially due to improvements in the growth rate of symbiotic bacteria, *Xenorhabdus* sp.

5.4.3 Field Trials

EPNs have been field-tested against numerous agricultural insect pests; forest, vegetable, corn, and turf insect pests (soil, cryptic habitat, or foliar insects) are the targets of EPNs to be controlled. Comprehensive reviews have recently been published on the efficacy of EPNs against insects inhabiting soil and other habitats. Field application showed that EPNs of the genera *Steinernema* and *Heterorhabditis* are effective biopesticides against a wide variety of soil insect pests and for various cropping systems (Atwa 2011), such as the black vine weevil, *Otiorhynchus sulcatus* (F.), the citrus weevil, *Diaprepes abbreviatus* (L.), fungus gnats (Diptera; Sciaridae), various white grubs (Coleoptera; Scarabaeidae) (Atwa 2003), and some lepidopterous insects—the leopard moth, *Z. pyrina*, the Egyptian cotton leafworm, *S. littoralis*, and the cabbage looper, *Pieris brassica* (Atwa 1999). The inoculate release of nematode-based biopesticides is thought to succeed when (1) the pest is present throughout most of the year, (2) the pest has a high economic threshold, and (3) soil conditions are favorable to nematode survival (Atwa 2009). All these criteria can be met in a turf system in which the scarab's larvae are present in the soil for most of the year and the turf is irrigated during dry conditions favorable to nematodes (Atwa 2009). In this section, we will focus on some models of EPNs used under field conditions.

A promising and highly successful use of EPNs as bioinsecticides has been achieved against the soil stage of the fruit borer *Carposina nipponensis* in apple orchards in China and the strawberry scarab *Temnorhynchus baal* in Egypt (Atwa 2003). *Carposina* larvae overwinter in the soil at the base of the trees and emerge in the spring when the temperature reaches 19 °C. Invectives of *S. carpocapsae* are applied to the soil at the time of emergence. In trials performed for 4 years in succession, *Carposina* larval mortality was more than 90 % and fruit damage was below 3 %, values superior to those achieved with chemical insecticides. Inoculate release of *S. glaseri* is applied annually to achieved more than 95 % reduction of the scarab population. Such dramatic success resulted from an extensive systematic effort by Chinese and Australian scientists, and depended on detailed knowledge of the biology of the insect collected over many years by the Chinese. EPN species were screened for effectiveness in the laboratory and in small-scale trials. Extensive field trials with the most appropriate nematodes were then performed. Currently, trials are being conducted over hundreds of hectares of apple orchard. This effort will stand as an exemplary model for the development of an insect control strategy using EPNs.

In Europe, Australia, and North America, the most successful use of nematodes has been against several species of weevils (Fig. 5.4). Applications of *Heterorhahditis* sp. against *O. sulcatus*, the black vine weevil, in containerized soil repeatedly reduced insect densities by 90 %. Other weevils successfully controlled by nematodes include *D. abbreviatus*, the citrus weevil (Schroeder 1990; Tomalak 2005) ,and *Hyalohius ahiefis*, the large pine weevil. The excellent control of weevils that is usually achieved is probably due to a combination of their susceptibility to EPNs and favorable conditions for EPN survival and infection (Fig. 5.4).

In the USA, extensive efforts have been made to control *Popillia japonica*, the Japanese beetle, a major pest of turfgrass. Beetle larvae emerge to feed on grass roots in the spring and autumn.

Fig. 5.4 Efficacy of the entomopathogenic nematode *Heterorhabditis megidis* on the pupal stage of weevil. Healthy pupae (*right*) and infected pupa (*left*). (After Tomalak 2005)

EPNs are applied in the autumn because temperatures in the spring are usually too low for the EPNs to be effective. *S. carpocapsae* and *Heterorhabditis* sp. have been field-tested the most, simply because of availability. Heterorhabditids have been generally more effective, although their performance has not been consistent. Although approximately 100 field trials against *P. japonica* have been performed, some notable gaps in the knowledge of the interactions among EPNs, insects, and the environment remain. Published data from laboratory screening of different EPN species and strains are scanty, little is known about the ability of different nematode species and strains to pass through the thatch layer (a dense layer of dead roots and organic matter that accumulates above the living root zone) to the root zone where the insects occur, few experiments have been performed to identify the physical factors which limit nematode effectiveness in turf, and the effect of biotic factors is unknown. Consequently, low efficacy in field trials often goes unexplained. Improvements in efficacy may come from subsurface injection of EPNs (Berg et al. 1987), which delivers them directly to the zone of insect activity, and spring applications of strains that are infective at low temperatures, for example, *S. feltiae*. However, what is most required is a redirection of effort from repetitive field trials to the acquisition of more knowledge of the interactions between different nematode species and strains with the target insect and the turf environment. The results of attempts to control the corn rootworm, *Diabrotica* sp., a major pest in the USA, have also been variable. Results of field tests with various strains of *S. carpocapsae* have ranged from no control to control superior to that achieved with chemicals. Once again, the factors contributing to success and failure were not always identified, and the use of nematodes in this application remains unpredictable.

Cryptic habitats within plants, although not the natural habitat of EPNs, provide ideal conditions for their survival and infectivity. Indeed, some of the most reliable results have been achieved against plant-boring insect pests. The blackcurrant borer, *Synanthedon tipuliformis*, was successfully controlled by applying *S. feltiae* to blackcurrant cuttings. In China, the tree-boring cossid moth, *Holcocercus insularis*, has been successfully controlled by manual application of EPNs to the uppermost entry and exit holes on the tree. This species of borer produces interconnecting galleries,

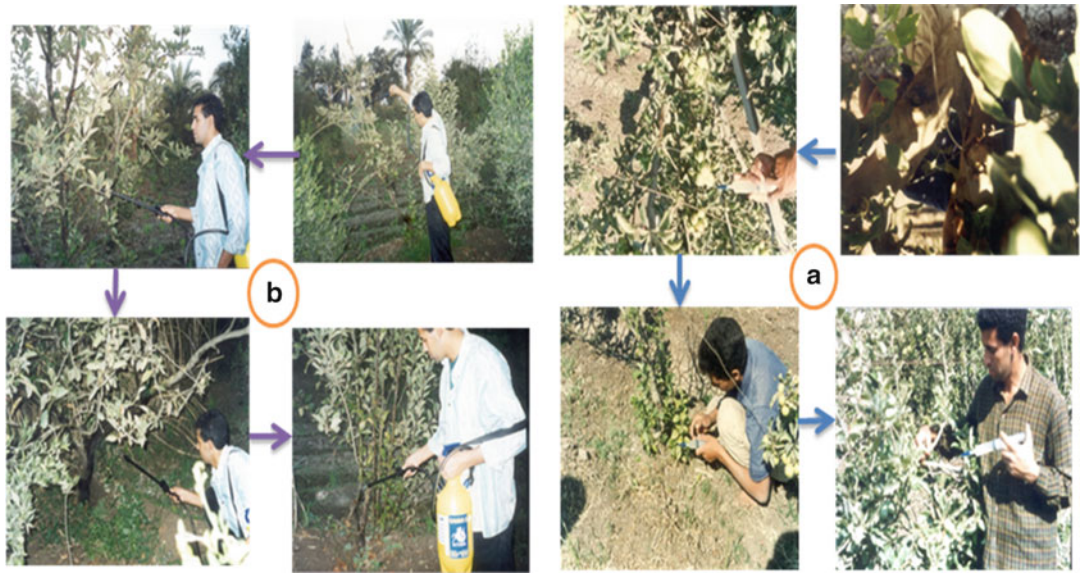

Fig. 5.5 Control of the leopard moth, *Zeuzera pyrina*, with entomopathogenic nematodes in Egypt. **a** Injection technique for controlling larvae in infected tunnels; **b** direct spraying technique. (After Atwa 1999)

which facilitate EPN recycling; insect mortalities in excess of 90 % are common. In developed countries, lack of a cost-effective method of delivery to gallery openings, which are often difficult to find, is a major limitation to the use of EPNs against boring insects. The injection technique achieved better control than the spray technique (Atwa 1999) in separate applications of either *Heterorhabditis* sp. or *Steinernema* sp. (1,000 nematodes per milliliter). Atwa and Shamseldean (2008) found that *Steinernema* sp. (EGB20) was superior to *H. bacteriophora* (EGB13) and *H. indica* (EBN16) when applied for control of *Z. pyrina* with 1,000 infective juveniles of EPNs per milliliter (Atwa 1999). Injection of the tested nematode suspension into the insect galleries of *Z. pyrina* was more effective than the spray technique (Fig. 5.5). The addition of an evaporation retardant and sticker agent was associated with efficient insect control. Moreover, *S. glaseri* (NJ strains) was tested in the field against *T. baal* infestation on strawberry plants, with the population reduction ranging from 89.2 to 96.8 % after four field applications. The overall population reduction after eight field applications was 96.3–99.1 % (Atwa 2009). The results also showed that both *H. bacteriophora* (EGB13) and *Steinernema* sp. (EGB20) nematode isolates were more effective in reducing the larval population of *S. littoralis* and *P. brassica* on cabbage plants than *H. indica* (EBN16) (Atwa and Shamseldean 2008). Application of *S. carpocapsae* to artichoke plume moth larvae infesting artichoke leaf stalks has been successful. This part of the plant provides conditions suited to EPN survival, as does the cool foggy climate of the artichoke growing area. In contrast to the use of EPNs in cryptic habitats, attempts to use EPNs for insect control in foliar, manure, and aquatic habitats have met with little success, largely because the environmental conditions are not suitable for EPN survival and/or infectivity.

5.4.4 Ecological Considerations

In more than three decades there has an explosion of activity in the use of EPNs for insect control, yet, with a few exceptions, their efficacy has generally been lower than that of chemicals, and the effects of nematode application have been less predictable. In this section, a number of important principles to be followed to obtain the best possible field results are described, and some areas for research which could lead to

better exploitation of the nematodes are recommended. Although EPNs are not host-specific, each nematode species and strain has a number of preferred hosts rather than being equally efficient at infecting all insects. There are significant differences in pathogenicity toward sheep blowfly (*Lucilia cuprina*) larvae between *Heterorhabditis* sp. (median lethal dose 18 IJs) and *S. feltiae* (median lethal dose 53,490 IJs). Differences in median lethal time as great as 50-fold were also observed between strains of the same species (Grewal et al. 1993). It is now generally accepted that a number of nematode species and strains should be tested against a particular insect prior to field testing. The median lethal time should preliminarily be determined for individual insects in sand. Two or three EPN species which are the most effective should then be evaluated in pot tests using appropriate soil and plants, followed by small-scale field trials. Although this is possible in theory, in practice few EPN species and strains are available in large enough numbers for field trials, making it impossible to field-test some EPN strains which show most promise in laboratory tests. For example, *S. glaseri* and *Heterorhabditi megidis* were the most effective species against *P. japonica* larvae in laboratory tests (Klein and Georgis 1992), but they have yet to be produced in sufficient numbers for field testing. Thus, for many insect pests, acceptable control with nematodes will not be achieved until an appropriate production method has been developed.

Although strain variability of EPNs is a recognized phenomenon, the possibility of strain variability of the hosts has been neglected. This is no doubt a complicating factor, which will play a part in affecting the efficacy of nematodes. It is necessary to time EPN applications to coincide with or slightly precede the peak occurrence of the most susceptible stage of the insect's life cycle. This is especially critical where the life span or accessibility of the target stage is short, for example, in root maggots. More than one application may be required when insects feed on plants for longer than 2 months, for example, root weevils and mole crickets, or in cases in which there is more than one generation of insects per year, for example, Japanese beetle. Applications are best done at dusk to allow the EPNs time to disperse to cryptic habitats and avoid the lethal effects of UV light and desiccation. For turf and soil applications, irrigation before and after application is recommended for EPN movement and persistence. However, in soils close to their saturation points, EPNs are less effective (Molyneux and Bedding 1984), so moisture levels are critical. In general, temperatures above 30 °C and below 18 °C are held to be outside the optimum for EPN effectiveness. However, temperatures in this range are rare in the UK, and the EPNs are widely distributed (Hominick and Briscoe 1990), so temperature optima should be investigated for species and strains. Applications of at least one billion nematodes per acre are recommended for adequate control, but spot application in containers and greenhouses can lower this density.

Even when all of the above-mentioned factors are considered, unsuccessful field trials are often unexplained. Hundreds of field trials have been performed, yet few have included investigations of the dispersal and persistence of the EPNs or the environmental barriers to infection using appropriate controls. This huge information void is discussed at length by Gaugler (1988), who suggests that further knowledge of nematode soil ecology could be gained from a critical analysis of the differences between successful and unsuccessful trials. Indeed, researchers are increasingly reporting a list of field test parameters, which include the method, the time of application, air and soil temperatures, cloud cover, soil type, soil moisture, stage of pest and pest density, irrigation, and rainfall, all of which help interpretation of field trials. Clearly, the analysis of the results of multiple field trials is valuable. However, when trials are unsuccessful or the results are variable, this approach is a poor second best to performing ecological experiments designed to evaluate the important variables. More emphasis must be placed on this experimental approach for better understanding of the ecological issues of each pest problem. It is often claimed that infective juveniles actively seek out their hosts, but there is little evidence to support this. In a laboratory

assessment of the host-finding capability of S. *curpocupsue*, although a small proportion of infective juveniles moved toward the host, most remained inactive. The available data indicate that EPNs tend to remain at the point of application (Moyle and Kaya 1981). Information on nematode movement in the soil is important because dispersal ability may affect interactions with soil antagonists and strategies with respect to the most advantageous placement of infective juveniles during application. A major focus of research should be on application techniques to determine how best to obtain the optimum distribution of EPNs for a given pest. For example, an approach advocated by Ishibashi et al. (1987) is to use chemicals to activate nematodes to overcome poor nematode mobility in soil. Various agents, including dilute oxamyl (an insecticide/nematicide) and kale and aloe extracts, were shown to stimulate EPN activity. When these agents were applied with EPNs in field trials, higher insect mortalities were achieved.

5.5 Mass Culture of EPNs

Many insect antagonists are found within the phylum Nematoda, but only members of the genera *Steinernema* and *Heterorhabditis* have gained major importance as bioinsecticides. These genera are closely related to *Caenorhabditis* (Ehlers 2001). Furthermore, *Caenorhabditis elegans*, the genome sequence of which has been obtained, is the current model organism for studying animal development and genetics. *Steinernema* and *Heterorhabditis* have a symbiotic relationship with bacteria of the genera *Xenorhabdus* and *Photorhabdus*, respectively, and the nematode–bacteria complexes are used in the biological control of insects. In the field, EPNs are mobile and persistent in soil; furthermore, they are highly effective as bioinsecticides and often render better results than those obtained by means of chemical compounds used also for control.

Since EPNs are safe for humans and are environmentally friendly, the commercialization of these nematodes and their associated bacteria is, in many cases, exempt from legislative hurdles and requirements in many countries. Nowadays, EPNs are mainly used in environments in which chemical compounds fail (soil, galleries of boring insects, etc.), or in cases where resistance to insecticides has developed (Ehlers 2001); however, they are used primarily against insects that occur in high-value crops. The main commercial production of EPNs occurs in Asia, Europe, and the USA, but only very few companies produce them in liquid culture using bioreactors. The price of EPNs is still too high to permit their application on low-priced crops. Therefore, the successful commercialization of nematode products depends on the ability to produce sufficient quantities of the product containing infective juvenile forms with the symbiotic bacteria at convenient prices for a full pest-control program. Major problems related to mass production of nematodes in submerged culture remain unsolved. In vivo mass culture can be used for small-scale production of EPNs used with soil insects or cryptic habitat insects, and in vitro mass culture can be used for large-scale production of EPNs for insect control.

5.5.1 In Vivo Mass Culture of EPNs

In vivo production methods have been used in the past to produce relatively large numbers of EPNs. Bedding (1981) developed a solid culture technology using flask cultures involving coating crumbed polyurethane foam sponge with poultry offal homogenate. The porous foam afforded a very high surface area to volume ratio for growth while providing adequate gas exchange. The next advance was the adoption of large autoclavable plastic bags to replace flasks as rearing vessels. Medium and shredded foam were hand-blended and sealed in bags before sterilization by an autoclave. Bacterial inoculum was injected into the bags, the contents of the bags were mixed manually, and the bags were placed on racks in an incubation room. After 24 h of bacterial growth, the bags were inoculated with nematode infective juveniles. The bags were kept on racks equipped with a small air compressor and able to deliver air

to each bag over the 2-week incubation period. A conventional medium based on an animal protein and lipid was used. A scaled-up version of the Bedding process would require expensive automated equipment, would have difficulty to maintain an aseptic state, and would present difficulties in medium preparation and nematode harvesting (Gaugler et al. 2002).

Since the early 1980s, EPN liquid culture has been actively researched. One of the great difficulties in optimizing a liquid monoxenic culture is to provide sufficient aeration for both the bacteria and the EPNs without exposing the nematodes to excessive shear forces. Oxygen transfer is not a limiting factor for cultures in shaker flasks, but it is the main problem for the bacterial symbiont growth in commercial bioreactors; in contrast, nematodes have a comparatively low oxygen demand. Further, it has long been recognized that intense agitation can inhibit nematode reproduction. One of the approaches to overcome these problems was to use a bubble column bioreactor for commercial production. These reactors use only air injected at the base for mixing. This bioreactor type proved satisfactory until product demand increased and the need for a more scalable, widely available, conventional stirred tank reactor became evident. Others used low-shear paddle impellers to gently mix the medium and a downward-pointing air sparger. Another bioreactor type for nematode mass production was a stirred-tank bioreactor with an internal draft tube or central cylinder, using a marina impeller that improves circulation and oxygen transfer, reducing shear forces.

In vivo culture is a two-dimensional system that relies on production in trays and shelves (Friedman 1990; Gaugler et al. 2002). Production methods for culturing EPNs in insect hosts have been reported by various authors (Poinar 1990; Woodring and Kaya 1988). All of these references describe (with some variation) a system based on the White trap (White 1927), which takes advantage of the infective juvenile's natural migration away from the host cadaver on emergence.

For commercial purposes, harvested nematodes have to be concentrated prior to formulation. This can be accomplished by gravity settling (Dutky et al. 1964), but prolonged periods of settling may be detrimental to the nematodes because of oxygen deprivation (Burman and Pye 1980). The process can be accelerated by vacuum filtration (Lindegren et al. 1993). Centrifugation is also feasible, but, for commercial in vivo operations, the capital outlay for a centrifuge of sufficient capacity may be excessive. Prior to formulation, EPNs (produced in vivo or in vitro) can be stored in aerated holding tanks for up to 3 months (Georgis et al. 1995). 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 (Dutky et al. 1964; Woodring and Kaya 1988) such as streptomycin sulfate, Hyamine® (methylbenzethonium chloride), merthiolate, NaOCl, and $HgCl_2$ (Lunau et al. 1993), but the effects of these compounds on nematodes for commercial application have not been reported.

5.5.2 Factors Affecting In Vivo Yield of EPNs

In vivo production yields differ greatly among different insect hosts and nematode species. The insect host most commonly used for laboratory and commercial EPN culture is the last instar of the larvae of the greater wax moth, *G. mellonella*, because of its high susceptibility to most nematodes, wide availability, ease of rearing, and ability to produce high yields (Woodring and Kaya 1988). There are only a couple of EPNs not amenable to culture in *G. mellonella* (due to extremes in host specificity): *S. kushidai* is most amenable to culture in scarab beetle larvae (Coleoptera: Scarabaeidae), and *S. scapterisci* is most amenable to culture in mole crickets (*Scapteriscus* spp.) (Nguyen and Smart 1990). 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 (*H. zea*), gypsy moth (*Lymantria dispar*), house cricket (*Acheta domesticus*), and various beetles (Coleoptera), including the yellow mealworm (*Tenebrio molitor*) (Lindegren et al. 1979). Other than *G. mellonella*, the host most commonly used for in vivo culture is *T. molitor*, but little research has been reported for production in this host. In response, Gaugler et al. (2002) compared relative yields in *T. molitor* for a number of EPNs. Clearly, nematode yield in *T. molitor* differs among nematode strains and species; for example, *H. bacteriophora* (TF strain) produced approximately twice the progeny of *H. indica* (Hom1 strain) and *Heterorhabditis 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 with the host or its relatives (Shapiro et al. 1999).

In general, nematode yield is proportional to host size (Flanders et al. 1996), yet yield per milligram of insect (within the host species) and susceptibility to infection are often inversely proportional to host size or age (Shapiro et al. 1999). Ease of culture and ease of infection are important factors when choosing a host; for example, 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. Among nematode species, yield is generally inversely proportional to size (Grewal et al. 1994; Hominick et al. 1997).

The choice of the host species and the 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 (1987) reported *T. molitor* to be more cost-efficient than *G. mellonella* and *T. ni* for producing *S. carpocapsae*. A hastened life cycle within the host might affect the cost by allowing faster production cycles; recently, *Steinernema abbasi* was reported to produce a roughly equivalent number of progeny in half the time of other EPNs (first emergence beginning after only 3.5 days) (Atwa 1999; Grewal et al. 1994). Another issue that has rarely been addressed in the choice of nematode and host is the resulting quality of the product. Nematode quality appears to be greater when the nematode is cultured in hosts that are within the nematode's natural host range (Abu Hatab and Gaugler 2001). Furthermore, nematodes can adapt to the host on which they are reared (Stuart and Gaugler 1996), 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 with regard to a particular target pest.

In vivo production yields are dependent on nematode dose (Boff et al. 2000). A dose that is too low results in low host mortality, and a dose that is too high often results in a high level of failed infections owing to competition with secondary invaders (Woodring and Kaya 1988). These outcomes reduce production efficiency owing to the need to remove live or poorly infected insects. The number of nematodes that invade a host is proportional to the exposure concentration. Selvan et al. (1993) found that optimization of the initial nematode density within the host (e.g., at 100 *H. bacteriophora* and *S. carpocapsae* nematodes per *G. mellonella* moth) maximizes nematode survival and fecundity. Thus, intermediate doses maximize yield (Boff et al. 2000). Similarly, host density per unit area affects nematode invasion and thus may affect yield.

Environmental factors such as temperature, aeration, and moisture can affect the yield of infective juveniles produced. The rearing temperature affects both the yield and the life-cycle duration (time to emergence) (Grewal et al. 1994). Generally, the optimum culture temperature is related to the nematode's climate of origin (Grewal et al. 1994; Molyneux 1986). Grewal et al. (1994) determined the optimum rearing temperature and time to emergence in *G. mellonella* for 12 species and strains of EPNs; the optimum temperatures ranged from 18 to 28 °C. Adequate aeration is necessary for nematode development (Friedman 1990). The moisture level is another

essential component for in vivo culture. High levels of humidity must be maintained throughout the production cycle (Woodring and Kaya 1988). 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 and interfere with oxygen exchange.

The inoculation method can affect infection efficiency and thus 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. Additionally, some host–nematode combinations may not be suitable for the immersion method; for example, 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. Blinova and Ivanova (1987) 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, the inoculation procedure must be included in a cost-efficiency analysis before a method is decided on.

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 because of genetic processes, including drift, inbreeding, and inadvertent selection (Hopper et al. 1993). Thus, repeated culturing of nematodes can result in reduction of quality and fitness characters such as virulence, environmental tolerance, and reproductive capacity (Stuart and Gaugler 1996). Therefore, precautions against strain deterioration should be taken; for example, cryopreservation of stock cultures, minimization of serial passages, and introduction of fresh genetic material (Gaugler et al. 2000).

5.5.3 In Vitro Mass Culture of EPNs

In vitro technology requires substantial capital investment in sterilization equipment, as well as considerable technical expertise. However, these disadvantages are offset by production costs as low as US$12 for *S. carpocapsae* (Gaugler and Han 2002). In contrast, in vivo production has low requirements for capital or expertise, but is difficult to scale up and hence it is difficult to achieve economies of scale. Lindegren et al. (1993) estimated in vivo production costs of US$150 per billion EPNs. Consequently, nematode producers reliant on in vivo methods form a cottage industry of low-volume producers (Gaugler et al. 2000). In vivo production is based on the adaptation of the White trap (White 1927) by Dutky et al. (1964), albeit with some modifications (e.g., Lindegren et al. 1993), in which nematode-killed hosts are placed above a water reservoir. The method exploits the tendency of infective nematodes to migrate from depleted host cadavers into the reservoir, which is decanted to collect infective juveniles. This system is appropriate for laboratory bench-scale production of inoculum for experiments, but its labor-intensive nature makes it inefficient for large-scale production.

Scale-up of in vivo production has consisted of providing larger White traps, reducing the extraction efficiency by increasing the migration distance to the reservoir. Apart from Carne and Reed (1964),who described a harvest system that was conceptually similar to the Baermann funnel, no further ideas for mechanizing in vivo production surfaced over the intervening more than 50 years until now. We report the first scalable in vivo system for mass production of EPNs.

5.5.4 Overview of Mass Production of EPNs

Mass production on artificial media was realized 30 years before Dutky et al. (1964) established effective in vivo methods. Solid culture was pioneered by Rudolf Glaser, who was the first to artificially culture a parasitic nematode. Glaser and his coworkers, in one of the most ambitious

and least known experiments in biological control, produced and released billions of *S. glaseri* throughout New Jersey to attack Japanese beetles from 1939 to 1942 (Fleming 1968). Regrettably, early workers were unaware of the nematode's bacterial partner. Nematode mass production was conducted in shallow trays of veal-pulp medium with salicylic acid and formaldehyde to repress contaminating microbes (McCoy and Girth 1938), including apparently the natural symbiont, *Xenorhabdus poinarii*. Today, the need for monoxenicity is universally recognized as one of the cornerstones of nematode in vitro culture.

Others extended Glaser's accomplishment by developing other media as alternatives to costly animal tissue homogenates, such as the dog food medium of House et al. (1965). Regardless of the growth medium, cultures were produced on the substrate surface because of the need for adequate gas exchange. That is, cultures were two-dimensional, perfectly suited for laboratory cultures, but a limitation that precluded commercial-scale production. The development by Bedding (1981, 1984) of practical solid culture technology was a seminal step in nematode production because it made the leap from two-to three-dimensional substrates. Bedding flask cultures involved thinly coating crumbed polyurethane foam sponge with poultry offal homogenate. The porous foam afforded an outstanding surface area to volume ratio for growth while providing adequate gas exchange. A primer for preparing Bedding flasks is found in Woodring and Kaya (1988). The next advance was the adoption of large autoclavable plastic bags to replace flasks as rearing vessels (Bedding 1984). Medium and shredded foam were hand-blended and sealed in bags before sterilization by an autoclave. Bacterial inoculum was injected into the bags, the contents of the bags were mixed manually, and the bags were placed on racks in an incubation room. After 24 h of bacterial growth, nematode inoculum was injected into the bags (e.g., *S. scapterisci* was introduced at 2,000 infective juveniles per gram of medium), and the contents were mixed again. Holding racks were equipped with a small air compressor and gang valve leading to a network of hoses delivering air to each bag over the 2-week incubation period.

A conventional medium was also developed because poultry entrails cannot be standardized and so provide unreliable results. Spurred by the development by Wouts (1981) of a practical yeast extract, corn oil, and soy flour medium for the Bedding flask, a new medium based on yeast extract, corn oil, corn starch, and dried egg solids was developed. Scaling up Bedding's advances to commercial production was undertaken in collaboration with Biotech Australia, which licensed the technology. The use of bags and an improved medium permitted commercial-scale nematode production, but shortcomings were encountered that reduced the effectiveness. Most troublesome was that each bag required a laborious custom fitting of costly inlet and outlet microbial filters. The compressors increased the air-conditioning load in the incubation room, which became problematic for *S. scapterisci* as this species produced metabolic heat within the bag to the point that growth could be retarded. Condensation (metabolic water) sometimes saturated bag edges and was associated with poor growth. These limitations contributed to inconsistent yield.

Bedding et al. (1996) addressed the gas-exchange issue in developing a stainless steel box system. The key innovation was a foam sponge gasket lining the inside edge of the box lid that provided passive ventilation. Nevertheless, Biotech Australia judged there was insufficient improvement to justify the expense of constructing the boxes, and the new system was not implemented. Nematode extraction from the foam medium was accomplished using active migration and sedimentation as in a Baermann funnel apparatus. Harvest trays were constructed, several square meters in size, with a bottom support screen of aluminum. The water level in the trays was adjusted to the same height as the screen, and a cloth fabric was placed over the screen. The bags were emptied onto the cloth, and nematodes migrated through the cloth and into the water reservoir. Trays were hinged so

they could be decanted after migration to remove bacteria and medium residue. The collected nematodes were then pumped to a chilled holding tank with a bacteriostat to await formulation. This manufacturing process worked moderately well for highly mobile species such as *H. bacteriophora*. By contrast, migration rates for sedentary nematodes such as *S. scapterisci* ranged from 50 to 75 %, often with excessive numbers of noninfective stages that stimulated microbial activity and reduced shelf life.

5.6 Safety of EPNs and Quality Control of EPN Production

5.6.1 Safety of EPNs

EPNs are exceptionally safe biological control agents; they are certainly more specific and are less of a threat to the environment than chemical insecticides. Since the first use of the EPN *S. glaseri* against the white grub *P. japonica* in New Jersey (USA) (Glaser and Farrell 1935), not even inferior damage or hazards caused by the use of EPNs to the environment have been recorded. The use of EPNs is safe for the user. EPNs and their associated bacteria have detrimental effect on mammals or plants (Akhurst and Smith 2002). A joint workshop supported by EU COST Action 819 "Entomopathogenic Nematodes" and the OECD research program "Biological Resource Management for Sustainable Agriculture Systems," which met in 1995 to discuss potential risks related to the use of EPNs in biological control, concluded that EPNs are safe for production and application personnel and consumers of agriculture products treated with EPNs. The expert group could not identify any risk to the general public related to the use of EPNs.

No reports exist documenting any effect on humans caused by the symbiotic bacteria. A related nonsymbiotic species, *Photorhabdus asymbiotica*, was reported five times from humans in the USA (Farmer et al. 1989). Another group of nonsymbiotic *Photorhabdus* was reported from five patients in Australia (Peel et al. 1999). From most of the patients, other human-pathogenic bacteria were also recorded; for example, *Photorhabdus* spp. were opportunistic. The route of the infections was not established. Three infections might have been related to spider bites. Both clinical groups lack symbiotic relations with nematodes, and strains within each group have a high level of within-group relatedness but do not cluster in groups containing the nematode symbionts (Akhurst and Smith 2002). The existence of bacterial species with and without pathogenic effects on humans within one genus is common (e.g., *Bacillus*). No action is therefore required and no conclusions should be drawn from the reports of pathogenic effects on humans caused by nonsymbiotic *Photorhabdus* spp. or potential risks related to the use of EPNs and their symbiotic bacteria.

Naturally occurring nematode populations cause sustainable effects on pest populations. These effects have not been very well exploited because we understand little of EPN population dynamics and the possibilities to enhance EPN populations by culture methods (Fischer and Führer 1990). At present, we cannot evaluate the economic benefits of sustainable effects. The economic effect of introducing an exotic species is easier to assess. In the case of a pest population surpassing the economic threshold, the use of an exotic nematode might be economically reasonable. It is often argued that prior to the release of exotic species, it should be tested whether an endemic population might also be the solution to a problem. However, the naturally occurring species, even if superior in its control potential, might not be commercially available. Waiting until the endemic population has increased and reached an even distribution to significantly reduce the pest population will result in economic losses. The benefit from introducing the exotic species will overwhelm the damage caused by a reduction of the population of the endemic EPN species. Should the exotic species persist, we will have a case of "biological pollution." However, is this

damage or a benefit for the farmer? As exotic species have not been recorded to eliminate the endemic EPN species, no real hazard has yet been identified with the introduction of the exotic species and the "biological pollution."

5.6.2 Quality Control of EPN Production

The quality of EPNs, when applied to steinernematids and heterorhabditids and their associated bacteria from a technical perspective, is usually defined as a set of linked parameters to be monitored and evaluated, such as nematode viability or percent viable, total viable nematodes per unit of product, nematode virulence (as indicated via bioassay), nematode age (after harvest, formulation, shelf life, etc.), and morphological measurements, and demonstrated performance of all these parameters should be available on the product label. Until now and according to different points of view for most farmers and end users of chemical products, they believe that the liquid formulation is the best among all types of formulations. Concurrently, most nematologists believe that the liquid formulation of nematodes is the best one to be used in the field. However, to obtain a promising EPN as a bioinsecticide, some points should be taken into consideration, such as storage, packaging, transportation, and field application methods (injection and/or spraying). The quality of the nematode products was determined under field conditions and after field transportation. The quality test was accomplished on nematodes before field trips, in the laboratory, and after transportation to the field. Viability (total viable nematodes), nematode virulence, storage ability of nematodes, morphological examination, and field efficacy were recorded. The data showed that, with optimal conditions, the nematode quality did not change (Atwa 2003). All tested parameters have indicated that, despite the low nematode numbers obtained from the in vivo methods, the nematodes have better quality, virulence, and better performance in the field compared with the nematodes produced by in vitro mass culture methods. The low numbers of nematodes produced through in vivo mass culture methods is not a great problem yet with the use of automated in vivo mass culture such as the LOTEK system (Gaugler et al. 2002).

5.7 Future Focus of EPNs as Bioinsecticides

This chapter has provided information on the applied aspects of EPNs as biopesticides against insect pests. These EPNs are already applied on ornamentals and vegetables in greenhouses and container crops in tree nurseries in Europe and the USA. EPNs as biopesticides can make an important contribution to the development of sustainable agriculture, but relatively few EPN biopesticides have been commercialized. EPNs as biopesticides can make important contributions to integrated control management and help reduce reliance on chemical pesticides. Hence, they have a major role to play in the development of sustainable farming. There are a range of definitions of what constitutes EPNs as biopesticides, and the terms used can be confusing at times. Essentially, we are dealing with a broad group of agents. We have defined and illustrated EPNs as biopesticides as mass-produced, biologically based agents used for the control of plant insect pests. This definition encompasses not only the active ingredient of a biopesticide, but also how it is used. Nowadays, the only bioinsecticide manufactured on an industrial scale and available on the market at prices which farmers can afford is the bacterium *Bacillus thuringiensis*. However, *B. thuringiensis* is not effective with the soil insect while EPNs are ideal to control soil insect. The great potential of EPNs for effective insect pest control, the possibility to apply them with conventional sprayers, and the possibility to produce them in liquid culture bioreactors make them a good candidate for large-scale manufacturing processes at reasonable cost. Two research and development strategies will provide the necessary progress to achieve this goal; further formulation improvement to stabilize the quality of nematode-based products and reduction of nematode application concentration. The information in this chapter on isolation of

EPNs to mass culture and product safety and quality control will allow the necessary progress to be made to achieve the main goals of this chapter to scale up the use of EPNs as bioinsecticides. In general, this chapter will have a significant economic and technical impact on the overall progress in agriculture practice and will stimulate further development and application of biotechnology and bioengineering within India and some countries in Asia and the Middle East.

5.8 Summary

Data were collected from field studies of EPNs to discuss isolation techniques and nematode distribution in different soil types all over the world. Environmental factors affecting nematode distribution were also discussed. EPNs have received increasing attention because of their potential as bioinsecticides against soil insect pests easily found in soil. Selection of appropriate EPNs as bioinsecticides includes bioassays in the laboratory to identify virulent strains and evaluating efficacy under simulated field conditions. EPN studies indicated the differences between EPN species, isolates, and/or strains applied to soil or applied foliarly. The effects of different application technologies were evaluated with regard to the concentration, viability, and efficacy of infective juveniles of the nematode species, isolates, and/or strains, and factors affecting field efficacy and reproduction were discussed. The efficacy of biopesticides is determined by biological characters of the agent and the intended target, the physical aspects of the site to which they are applied, and the interactions of the biopesticide and the environment. EPNs are used to control insect pests primarily in soil, and can serve as part of a model system to study the interaction of soil processes with soilborne biological control organisms. The effects of temperature and sunlight on EPN virulence were discussed. The mass production of EPNs depends on in vivo production in the greater wax moth, *G. mellonella*, and/or the beetle *T. molitor*. Novel devices (LOTEK) have been developed for in vivo mass production. The number of infective juveniles of EPNs produced from one unit has reached 50×10^7–75×10^7. One unit consists of ten racks with 500 cadavers of insects per rack, with a total of 5,000 insect larvae per unit, with a total weight ranging from 750 to 900 g, which may be enough for a small-scale experiment. This chapter closed with discussion of the safety of EPNs and quality control. EPNs are exceptionally safe biological control agents: they are certainly more specific and are less of a threat to the environment than chemical insecticides. Since the first use of the EPN *S. glaseri* against the white grub *P. japonica* in New Jersey (USA), not even inferior damage or hazards caused by the use of EPNs to the environment or humans have been recorded. The use of EPNs is safe for the user. Quality control of EPN production is very important. Nematode viability or percent viable, total viable nematodes per unit of product, nematode virulence (as indicated via bioassay), nematode age (after harvest, formulation, shelf life, etc.), morphological measurements, and demonstrated performance are all parameters that should be available on the product label. Finally, we conclude that EPNs are very promising for future use as bioinsecticides, mass production of EPNs can be conducted, and the nematodes produced can be easily applied in the field by any farmer. Field releases of EPNs by farmers can be applied through the spread of insect cadavers infected with the nematodes in fields infested with insect pests, where emerged nematode infective juveniles will infect other insect pests. EPNs can be used exclusively to control insect pests which live in cryptic habitats such as tree borers and soil insects, for example, the red palm weevil and white grubs of scarab pests of strawberries.

References

Abu Hatab M, Gaugler R (2001) Diet composition and lipids of *in vitro*-produced *Heterorhabditis bacteriophora*. Biol Control 20:1–7

Akhurst RJ (1986) *Xenorhabdus nematophilus* spp. *poinarii*: its interaction with insect pathogenic nematodes. Syst Appl Microbiol 8:142–147

Akhurst RJ (1987) Use of starch gel electrophoresis in taxonomy of the genus *Heterorhabditis* (Nematoda : Heterorhabditidae). Nematologica 33:1–9

Akhurst RJ, Bedding RA (1986) Natural occurrence of insect pathogenic nematodes (Steinernematidae and Heterorhabditidae) in soil in Australia. J Aust Entomol Soc 25:241–244

Akhurst RJ, Brooks WM (1984) The distribution of entomophilic nematodes (Heterorhabditidae and Steinernematidae) in North Carolina. J Invertebr Pathol 44:140–145

Akhurst R, Smith K (2002) Regulation and safety. In: Gaugler R (ed) Entomopathogenic nematology. CABI Publishing, Oxford, pp 311–332

Atwa AA (1999) Interaction of certain insecticides and entomopathogenic nematodes in controlling some insect pests on fruit and vegetable crops. MSc thesis, Faculty of Agriculture, University of Ain Shams at Shobra El-Khaima, Cairo, p 161

Atwa AA (2002) Isolation and identification of entomopathogenic nematodes in Egypt. In: Proceedings of the first international workshop on entomopathogenic nematodes "Developing an insecticidal nematode industry in Egypt: opportunities and constraints", Sharm El Sheikh. Egypt J Biol Pest Control, pp 83–87

Atwa AA (2003) Identification, mass culture, and utilization of entomopathogenic nematodes against insect pests. PhD thesis, Faculty of Agriculture, University of Cairo at Giza, p 172

Atwa AA (2009) Comparison between inoculative and inundative release for controlling scarab beetles in strawberry using entomopathogenic nematodes under field conditions. Bull Fac Agric Cairo Univ 60:197–205

Atwa AA (2011) Mode of actions and field applications of entomopathogenic nematodes. In: Borgio JF, Sahayaraj K, Susurluk IA (eds) Microbial insecticides: principles and applications. Nova, New York, pp 211–236

Atwa AA, Shamseldean MM (2008) Entomopathogenic nematodes as a model of field application against some important insect pest in Egypt. Alex J Agric Res 53(2):41–47

Bedding RA (1981) Low cost in vitro mass production of *Neoaplectana* and *Heterorhabditis* species (Nematoda) for field control of insect pests. Nematologica 27:109–114

Bedding RA (1984) Large scale production, storage and transport of the insect parasitic nematodes *Neoaplectana* spp. and *Heterorhabditis* spp. Ann Appl Biol 104:117–120

Bedding RA, Akurst RJ (1975) A simple technique for the detection of insect parasitic rhabditid nematodes in soil. Nematologica 21:109–110

Bedding RA, Stanfield MA, Crompton GW (1996) Apparatus and methods for rearing nematodes, fungi, tissue cultures and the like, and for harvesting nematodes. US Patent No 5,554,533 960, 910.

Berg GN, Willian P, Bedding RA, Akhurst RJ (1987) A commercial method of application of entomopathogenic nematodes to pasture for controlling subterranean insect pests. Plant Prot 4:174–178

Blackshaw RP (1988) A survey of insect parasitic nematodes in Northern Ireland. Ann Appl Biol 113:561–565

Blinova SL, Ivanova E (1987) Culturing the nematode-bacterial complex of *Neoaplectana carpocapsae* in insects. In: Sonin MD (ed) Helminths of insects. American Publishing, New Delhi, pp 22–26

Boag B, Neilson R, Gordon SC (1992) Distribution and prevalence of the entomopathogenic nematodes *Steinernema feltiae* in Scotland. Ann Appl Biol 121:355–360

Boemare NE, Akhurst RJ, Mourant RG (1993) DNA relatedness between *Xenorhabdus* spp. (Enterobacteriacae) symbiotic bacteria of entomopathogenic nematodes, and a proposal to transfer *Xenorhabdus luminescens* to a new genus, *Photorhabdus* gen. nov. Int J Syst Bacteriol 43:249–255

Boff M, Wiegers GL, Gerritsen LJM, Smits PH (2000) Development of the entomopathogenic nematode *Heterorhabditis megidis* strain NLH-E 87.3 in *Galleria mellonella*. Nematology 2:303–308

Brown I, Gaugler R (1997) Temperature and humidity influence emergence and survival of entomopathogenic nematodes. Nematologica 43:363–375

Burman M, Pye AE (1980) *Neoaplectana carpocapsae*: respiration of infective juveniles. Nematologica 26:214–219

Burman M, Abrahamsson K, Ascard J, Sjoberg A, Erikson B (1986) Distribution of insect parasitic nematodes in Sweden. In: Samson RA, Vlak JM, Peters D (eds) Fundamental and applied aspects of invertebrate pathology. Foundation of the Fourth International Colloquium of Invertebrate Pathology, Wageningen, p 312

Byers JA, Poinar GO Jr (1981) Location of insect hosts by the nematode, *Neoaplectana carpocapsae,* in response to temperature. Behavior 79:1–10

Cabanillas HE, Raulston JR (1994) Evaluation of the spatial pattern of *Steinernema riobravis* in corn plots. J Nematol 26(1):25–30

Campbell JF, Gaugler R (1993) Nictation behavior and its ecological implications in the host search strategies of entomopathogenic nematodes (Heterorhabditidae and Steinernematidae). Behavior 126:155–169

Campbell JF, Lewis EE, Yoder F, Gaugler R (1995) Entomopathogenic nematodes (Heterorhabditidae and Steinernematidae) seasonal population dynamics and impact on insect population in turfgrass. Biol Control 5:598–606

Campbell JF, Lewis EE, Yoder F, Gaugler R (1997) Entomopathogenic nematode (Heterorhabditidae and Steinernematidae) spatial distribution in turfgrass. Parasitology 113:473–482

Carne PB, Reed EM (1964) A simple apparatus for harvesting infective stage nematodes emerging from their insect hosts. Parasitology 54:551–553

Castillo JC, Stuart ER, Eleftherianos I (2011) Insect immune responses to nematode parasites. Trends Parasitol 27(12):537–547

Curran J, Heng J (1992) Comparison of three methods for estimating the number of entomopathogenic nematodes present in soil samples. J Nematol 22(2):170–176

Deseö KV, Fantoni P, Lazzari GL (1988) Presenza di nematodi entomopatogeni (*Steinernema* spp., *Heterorhabditis* spp.) nei. terreni agricoli in Italia. Atti Giornate Fitopatol 2:269–280

Downes MJ, Griffin CT (1991) Recovery of heterorhabditid nematodes from Irish and Scottish soils. In: Smits PH (ed) Proceedings of the third European meeting microbial control of pests, IOCP/WPRS bulletin, Wageningen, The Netherlands, pp 216–218

Downes MJ, Griffin CT (1996) Dispersal behavior and transmission strategies of the entomopathogenic nematodes of *Heterorhabditis* and *Steinernema*. Biocontrol Sci Technol 6:447–356

Dutky SR, Thompson JV, Cantwell GE (1964) A technique for the mass propagation of the DD-136 nematode. J Insect Pathol 6:417–422

Ehlers RU (2001) Mass production of entomopathogenic nematodes for plant protection. Appl Microbiol Biotechnol 56:623–633

Ehlers RU, Deseö KV, Stackebrandet E (1991) Identification of *Steinernema* spp. (Nematoda) and symbiotic bacteria *Xenorhabdus* spp. from Italian and German soils. Nematologica 37:360–366

Fan X, Hominick WM (1991) Efficiency of the *Galleria* (wax moth) baiting technique for recovering infective stages of entomopathogenic rhabditids (Steinernematidae and Heterorhabditidae) from sand and soil. Rev Nematol 14:381–387

Farmer JJ III, Jörgensen JH, Grimont PAD, Akhurst RJ, Poinar GO Jr, Ageron E, Pierce GV, Smith JA, Carter GP, Wilson KL, Hickman-Brenner FW (1989) *Xenorhabdus luminescens* DNA hybridization group 5 from human clinical specimens. J Clin Microbiol 27:1594–1600

Ferris VR, Goseco CG, Ferris JM (1976) Biogeography of free-living soil nematodes from the perspective of plate tectonics. Science 193:508–510

Fischer P, Führer E (1990) Effect soil acidity on the entomophilic nematode *Steinernema kraussei* Steiner. Biol Fert Soils 9:174–177

Flanders KL, Miller JM, Shields EJ (1996) *In vivo* production of *Heterorhabditis bacteriophora* 'Oswego' (Rhabditida: Heterorhabditidae), a potential biological control agent for soil inhabiting insects in temperate regions. J Econ Entomol 89:373–380

Fleming WE (1968) Biological control of the Japanese beetle. US Department of Agriculture technical bulletin no 1383

Freckman DW, Caswell EP (1985) The ecology of nematodes in agroecosystems. Annu Rev Phytopathol 23:275–296

Friedman MJ (1990) Commercial production and development. In: Gaugler R, Kaya HK (eds) Entomopathogenic nematodes in biological control. CRC Press, Boca Raton, pp 153–172

Garcia Del Pino F, Palomo A (1996) Natural occurrence of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) in Spanish soils. J Invertebr Pathol 68:84–90

Garcia LC, Carlos GR, Luis GL (2008) Application technology for the entomopathogenic nematodes Heterorhabditis indica and Steinernema sp. (Rhabditida: Heterorhabditidae and Steinernematidae) to control Spodoptera frugiperda (Smith) (Lepidoptera: Noctuidae) in corn. Neotrop Entomol 37(3):305–311

Gaugler R (1988) Ecological considerations in the biological control of soil-inhabiting insects with entomopathogenic nematodes. Agric Ecosyst Environ 24:351–360

Gaugler R, Campbell J (1991) Behavioural response of the entomopathogenic nematodes *Steinernema carpocapsae* and *Heterorhabditis bacteriophora* to oxamyl. Ann Appl Biol 119:131–138

Gaugler R, Han R (2002) Production technology. In: Gaugler R (ed) Entomopathogenic nematology. CABI, Wallingford, UK, pp 289–310

Gaugler R, LeBeck L, Nakagaki B, Boush GM (1980) Orientation of the entomogenous nematode Neoaplectana carpocapsae to carbon dioxide. Environ Entomol 9:649–652

Gaugler R, Bednarek A, Campbell JF (1992) Ultaviolet inactivation of heterorhabditid and steinernematid nematodes. J Invertebr Pathol 59:155–160

Gaugler R, Grewal PS, Kaya HK, Smith-Fiola D (2000) Quality assessment of commercially produced entomopathogenic nematodes. Biol Control 17:100–109

Gaugler R, Brown I, Shapiro-Ilan D, Atwa AA (2002) Automated technology for *in vivo* mass production of entomopathogenic nematodes. Biol Control 24:199–206

Georgis R, Dunlop DB, Grewal PS (1995) Formulation of entomopathogenic nematodes. In: Hall FR, Barry JW (eds) Biorational pest control agents: formulation and delivery. American Chemical Society, Washington, pp 197–205

Glaser RW, Farrell CC (1935) Field experiments with the Japanese beetle and its nematode parasite. J N Y Entomol Soc 43:345–371

Glazer I, Kozodoi E, Hashmi G, Gaugler R (1996) Biological characteristics of the entomopathogenic nematode *Heterorhabditis* sp. IS-5: a heat tolerant isolate from Israel. Nematologica 24:481–492

Gray BD, Johnson DT (1983) Survival of the nematode *Neoaplectana carpocapsae* in relation to soil temperature, moisture and time. J Ga Entomol Soc 18:454–460

Gray BD, Webster JM (1986) Temperature effects on the growth and virulence of *Steinernema feltiae* strains and *Heterorhabditis heliothidis*. J Nemaotol 18(2):270–272

Grewal PS (1999) Insecticidal nematodes laboratory. In: Polavarapu S (ed) Workshop proceedings: optimal use of insecticidal nematodes in pestmanagment, Rutgers University, pp 15–24

Grewal PS, Gaugler R, Kaya HK, Wusaty M (1993) Infectivity of the entomopathogenic nematode *Steinernema scapterisci* (Nematoda: Steinernematidae). J Invertebr Pathol 62:22–28

Grewal PS, Selvan S, Gaugler R (1994) Thermal adaptation of entomopathogenic nematodes: niche breadth for infection, establishment, and reproduction. J Thermal Biol 19:245–253

Griffin CT, Moore JF, Downes MJ (1991) Occurrence of insect-parasitic nematodes (Steinernematidae, Heterorhabditidae) in the Republic of Ireland. Nematologica 37:92–100

Griffin CT, Finnegan MM, Downes MJ (1994) Environmental tolerances and dispersal of *Heterorhabditis*: survival and infectivity of European *Heterorhabditis* following prolonged immersion in seawater. Fundam Appl Nematol 17:415–421

Hara AH, Mello CL, Arita Tsutsumi LH, Kaya HK (1989) Laboratory susceptibility of some tropical pest species and a non-target organism to the entomopathogenic nematode, *Steinerneme carpocapsae*. J Hawaii Pac Agric 2:6–9

Hara AH, Gaugler R, Kaya HK, Lebeck LM (1991) Natural population of entomopathogenic nematodes (Rhabditidae: Heterorhabditidae, Steinernematidae) from the Hawaiian Islands. Environ Entomol 20:211–216

Hashmi G, Glazer I, Gaugler R (1996) Molecular comparisons of entomopathogenic nematodes using randomly amplified polymorphic DNA (RAPD) markers. Fundam Appl Nematol 19(4):339–406

Haukeland S (1993) Entomopathogenic nematodes found in Norway. Norw J Agric Sci 7:17–27

Hominick WM, Briscoe BR (1990) Survey of 15 sites over 28 months for entomopathogenic nematodes (Rhabditida: Steinernematidae). Parasitology 100(2):289–294

Hominick WM, Reid AP, Briscoe BR (1995) Prevalence and habitat specificity of steinernematid and heterohabditid nematodes isolated during soil surveys of the UK and the Netherlands. J Helminthol 69:27–32

Hominick WM, Briscoe BR, Garcia del Pino F, Heng J, Hunt DJ, Kozodoy E, Mrácek ZK, Nguyen B, Reid AP, Spiridonov S, Stock P, Sturhan D, Waturu C, Yoshida M (1997) Biosystematics of entomopathogenic nematodes: current status, protocols, and definitions. J Helminthol 71:271–298

Hopper KR, Roush RT, Powell W (1993) Management of genetics of biological-control introductions. Annu Rev Entomol 38:27–51

House HL, Welch HE, Cleugh TR (1965) Food medium of prepared dog biscuit for the mass-production of the nematode DD-136) (Nematoda: Steinernematidae). Nature 206:847

Hunt DJ (1997) Nematodes species: concepts and identification strategies exemplified by the longidoridate, Steinernematidae and Heterorhabditidae. In: Claridge MF, Wilson MR (eds) Species: the units of biodiversity. Chapman and Hall, London, pp 221–245

Ishibashi N, Kondo E (1986) A possible quiescence of the applied entomogenous nematode, *Steinernema feltiae*, in soil. Jpn J Nematol 16:66–67

Ishibashi N, Kondo E (1990) Behavior of infective juveniles. In: Gaugler R, Kaya HK (eds) Entomopathogenic nematodes in biological control. CRC, Boca Raton, pp 139–150

Ishibashi N, Choi DR, Kondo E (1987) Integrated control of insect nematodes by mixing application of steinernematid nematodes and chemicals. J Nematol 19:531

Ishibashi NN, Takii S, Kondo E (1994) Infectivity of nictating juveniles of *Steinernema carpocapsae* (Rhabditida: Steinernematidae). Jpn J Nematol 24:20–29

Jansson RK, Lecrone SH, Gaugler R (1993) Field efficacy and persistence of entomopathogenic nematodes (Rhabditida: Steinernematidae, Heterorhabditidae) for control of sweetpotato weevil (Coleoptera: Apionidae) in southern Florida. J Econ Entomol 86(1):1055–1063

Jaworaska M (1992) Effect of the soil texture and temperature on the activity of *Steinernema feltiae* and *Heterorhabditis bacteriophora* in the soil. Entomonematologica 1(5):31–37

Joyce SA, Burnell AM, Power TO (1994) Characterization of *Heterorhabditis* isolates by PCR amplification of segments of mtDNA and rDNA. J Nematol 26:260–270

Kanga FN, Waeyenberge L, Hauser S, Moens M (2012) Distribution of entomopathogenic nematodes in southern Cameroon. J Invertebr Pathol 109:41–51

Kaya HK (1977) Development of the DD-136 strain of Neoaplectana carpocapsae at constant temperatures. J Nematol 9(4):346–349

Kaya HK (1990) Soil ecology. In: Gaugler R, Kaya HK (eds) Entomopathogenic nematodes in biological control. CRC, Boca Raton, pp 93–115

Kaya HK, Gaugler R (1993) Entomopathogenic nematodes. Ann Rev Entomol 38:181–206

Khlibsuwan W, Ishibashi N, Kondo E (1992) Effects of enzymes, chemicals and temperature on *Steinernema carpocapsae* attraction to host plasma. J Nematol 24(4):482–488

Klein MG, Georgis R (1992) Persistence of control of Japanese beetle (Coleoptera: Scarabaeidae) larvae with steinernematid and heterorhabditid nematodes. J Econ Entomol 85:727–730

Kondo E, Ishibashi N (1986) Nictating behaviour and infectivity of entomogenous nematodes, *Steinernema* spp., to the larvae of common cutworm, *Spodoptera litura* (Lepidoptera: Noctuidae), on the soil surface. Appl Entomol Zool 21:553–560

Koppenhöfer AM, Kaya HK (1997) Additive and synergistic interaction between entomopathogenic

nematodes and *Bacillus thuringiesis* for scarab grub control. Biol Control 8:131–137
Kung SP, Gaugler R, Kaya HK (1991) Effect of soil temperature, moisture, and relative humidity on entomopathogenic nematode persistence. J Invertebr Pathol 57:242–249
Lewis EE, Gaugler R, Harrison R (1992) Entomopathogenic nematode host finding: response to host contact cues by cruise and ambush forgers. Parasitology 105:103–107
Lindegren JE, Hoffman DF, Collier SS, Fries RD (1979) Propagation and storage of *Neoaplectana carpocapsae* Weiser using *Amyelois transitella* (Walker) adults. USDA Adv Agric 3:1–5
Lindegren JE, Wonge TT, Melnnis DO (1990) Response of Mediterranean fruit fly (Diptera: Tephritidae) to the entomopathgenous nematode *Steinernema feltiae* in field tests in Hawaii. Environ Entomol 19:383–386
Lindegren JE, Valero KA, Mackey BE (1993) Simple *in vivo* production and storage methods for *Steinernema carpocapsae* infective juveniles. J Nematol 25:193–197
Liu J, Berry RE (1996) *Heterorhabditis marelatus* n. sp. (Rhabditida: Heterorhabditidae) from Oregon. J Invertebr Pathol 67:48–54
Liu J, Berry RE, Blouin MS (1999) Molecular differentiation and phylogny of entomopathogenic nematodes (Rhabditida : Heterorhabditidae) based on ND4 gene sequences of mitochondrial DNA. J Parasitol 85(4):709–715
Lunau S, Stoessel S, Schmidt-Peisker AJ, Ehlers RU (1993) Establishment of monoxenic inocula for scaling up *in vitro* culture of the entomopathogenic nematodes *Steinernema* spp. and *Heterorhabditis* spp. Nematologica 39:385–399
McCoy EE, Girth HB (1938) The culture of *Neoaplectana glaseri* on veal pulp. N J Agric 285
Milstead JE (1981) Influence of temperature and dosage on mortality of seventh instar larvae of *Galleria mellonela* (Insecta: Lepidoptera) caused by *Heterorhabditis bacteriophora* (Nematoda: Rhabditoidea) and it's bacterial associate *Xenorhabdus luminescens*. Nematologica 27:303–306
Molyneux AS (1983) The biology of entomopathogenic nematodes *Heterorhabditis spp*. (Heterorhabditidae) and *Steinernema spp*. (Steinernematidae). Master of agricultural science thesis, University of Tasmania, Hobart
Molyneux AS (1986) *Heterorhabditis* spp. and *Steinernema* (= *Neoaplectana*) spp.: temperature, and aspects of behavior and infectivity. Exp Parasitol 62:169–180
Molyneux AS, Bedding RA (1984) Influence of soil texture and moisture on the infectivity of *Heterorhabditis* sp. D1 and *Steinernema glaseri* for larvae of the sheep blowfly, Lucilia cuprina. Nematologica 30:358–365
Moyle PL, Kaya HK (1981) Dispersal and infectivity of the entomogenous nematode, *Neoaplectana carpocapsae* Weiser (Rhabditidae: Steinernematidae), in sand. J Nematol 13:295–300
Mrácek Z (1980) The use of "*Galeria* traps" for obtaining nematode parasites of insects in Czechoslovakia (Lepidoptera: Nematoda, Steinernematidae). Acta Entomol Bohemoslov 77:378–382
Mrácek Z (1982) Horizontal distribution in soil, and seasonal dynamics of the nematode *Steinernema kraussei,* a parasite of *Cephalcia abietis*. Z Angew Entomol 94:110–112
Mrácek Z, Webster JM (1993) Survey of Heterorhabditidae and Steinernematidae (Rhabditida: Nematoda) in western Canada. J Nematol 25(4):710–717
Nguyen KB (1993) Identification to entomopathogenic nematode species of the genus Steinernema. Nematol Circ 202:190–193
Nguyen KB, Smart JRGC (1990) *Steinernema scapterisci* n. sp. (Rhabditidae: Steinernematidae). J Nematol 22(2):187–199
Ogura N, Nakashima T (1997) Cold tolerance and preconditioning of infective juveniles of *Steinernema kushidai* (Nematoda: Steinernematidae). Nematologica 43:107–115
Parkman JP, Smart JRGC (1996) Entomopathogenic nematodes, a case study: introduction of *Steinernema scapterisci* in Florida. Biocontrol Sci Technol 6:413–419
Peel MM, Alfredson DA, Gerrad JG, Davis JM, Robson JM, McDougall RJ, Scullie BL, Akhurst RJ (1999) Isolation, identification and molecular characterization of strains of Photorhabdus luminescens from infected humans in Australia. J Clin Microbiol 37:3647–3653
Poinar JRGO (1975) Entomogenous nematodes, a manual and host list of insect-nematode associations. Brill, Leiden, p 254
Poinar JRGO (1976) Description and biology of a new insect parasitic rhabditoid, *Heterorhabditis bacteriophora* n. gen. n. sp. (Rhabditida: Heterorhabditidae n. fam). Nematologica 21:463–740
Poinar JRGO (1990) Taxonomy and biology of Steinernematidae and Heterorhabditidae. In: Gaugler R, Kaya HK (eds) Entomopathogenic nematodes in biological control. CRC, Boca Raton, pp 23–61
Poinar GO Jr, Lindhardt K (1971) The re-isolation of *Neoaplectana bibionis* Bovien (Nematodea) from Danish bibioids (Diptera) and their possible use as biological control agents. Entomol Scand 2:301–303
Roman J, Beavers JB (1982) A survey of Puerto Rico soils for entomogenous nematodes which attack *Diaprepes abbreviatus* (L.) (Coleoptera: Curculionidae). J Agric Univ P R 67:311–316
Roman J, Figueroa W (1994) *Steinernema puertoricensis* n. sp. (Rhabditidae: Steinernematidae), a new entomopathogenic nematode from Puerto Rico. J Agric Univ P R 78:167–175
Rosa JS, Martins A, Mendes C, Amaral JJ, Lacey AL, Simoes N (1994) Natural occurrence of soil entomopathogens in the Azores Islands. In: Proceedings of the VIth international colloquium on invertebrate pathology and microbial control, abstracts, Montpellier, France, vol 2, pp 275–276

Schmidt J, All JN (1978) Chemical attraction of *Neoaplectana carpocapsae* (Nematoda: Steinernematidae) to insect larvae. Environ Entomol 7:605–607

Schmidt J, All JN (1979) Attraction of *Neoaplectana carpocapsae* (Nematoda: Steinernematidae) to common excretory products of insects. Environ Entomol 8:55–61

Schroeder WJ (1990) Water-absorbent starch polymer: survival aid to nematodes for control of *Diaprepes abbreviatus* (Coleoptera: curculionidae) in citrus. Fla Entomol 73(1):129–132

Schroeder WJ, Beavers JB (1987) Movement of the entomogenous nematodes of the families Heterorhabditidae and Steinernematidae in soil. J Nematol 19:257–259

Selvan MS, Gaugler R, Lewis E (1992) Energy reserves of entomopathogenic nematodes. J Parasitol 79:167–172

Selvan S, Campbell JF, Gaugler R (1993) Density dependent effects on entomopathogenic nematodes (Heterorhabditidae and Steinernematidae) within an insect host. J Invertebr Pathol 62:278–284

Shahina F, Anis M, Zainab S, Maqbool MA (1998) Entomopathogenic nematodes in soil samples collected from Sindh, Pakistan. Pak J Nematol 16(1):41–50

Shamseldean MM, Abd-Elgawad MM (1994) Natural occurrence of insect pathogenic nematodes (Rhabditida: Heterorhabditidae) in Egyptian soils. Afro-Asian J Nematol 4(2):15–154

Shapiro D, Cate JR, Pena J, Hunsberger A, McCoy CW (1999) Effects of temperature and host range on suppression of *Diaprepes abbreviatus* (Coleoptera: Curculionidae) by entomopathogenic nematodes. J Econ Entomol 92:1086–1092

Shapiro-Ilan DI, Lewis EE, Campbell JF, Kim-Shapiro DB (2012) Directional movement of entomopathogenic nematodes in response to electrical field: effects of species, magnitude of voltage, and infective juvenile age. J Invertebr Pathol 109:34–40

Smits PH, Groenen JTM, De Raay G (1991) Characterization of *Heterorhabditis* isolates using DNA restriction fragment length polymorphisms. Rev Nematol 14:445–453

Steiner WA (1996) Dispersal and host-finding ability of entomopathogenic nematodes at low temperatures. Nematologica 42:243–261

Stock SP (1994) Isolation of entomopathogenic nematodes from the Pampean region of Argentina. In: Proceedings of the VIth international colloquium on invertebrate pathology and microbial control, abstracts. Montpellier, France, vol 2, p 13

Stock SP, Choo HY, Kaya HK (1997) An entomopathogenic nematodes, *Steinernema monticolum* sp. N. (Rhabditida: Steinernematidae) from Korea with a key to other species. Nematologica 43:15–29

Stuart RJ, Gaugler R (1994) Patchiness in population of entomopathogenic nematodes. J Invertebr Pathol 64:39–45

Stuart RJ, Gaugler R (1996) Genetic adaptation and founder effect in laboratory populations of the entomopathogenic nematode *Steinernema glaseri*. Can J Zool 74:164–170

Timper P, Kaya HK, Gaugler R (1988) Dispersal of the entomogenous nematode *Steinernema feltiae* (Rhabditida: Steinernematidae) by infected adult insects. Environ Entomol 17:546–550

Tomalak M (2005) Entomopathogenic nematodes isolated under urban trees and their potential natural hosts. Presented at Natural occurrence and evolution of EPN, Ceské Budejovice, 14–17 January 2005. http://www.cost850.ch/publications/20050114_ceske/COST850-16-Ceske-Tomalak.pdf

Travassos L (1927) Sobro genero Oxysomatium. Bol Biol (Sao Paolo) 5:20–21

Waturu CN, Hunt DJ, Reid AP (1997) *Steinernema karii* sp. n. (Nematoda: Steinernematidae), a new entomopathogenic nematode from Kenya. Int J Nematol 7(1):68–75

White CF (1927) A method for obtaining infective larvae from culture. Science 66:302–303

Womersley CZ (1990) Dehydration survival and anhydrobiotic potential. In: Gaugler R, Kaya HK (eds) Entomopathogenic nematodes in biological control. CRC, Boca Raton, pp 117–137

Woodring JL, Kaya HK (1988) Steinernematid and heterorhabditid nematodes: a handbook of techniques. Arkansas Agricultural Experiment Station Southern Cooperative bulletin 331, Fayetteville, Arkansas, p 30

Wouts WM (1981) Mass production of the entomogenous nematode *Heterorhabditis heliothidis (Nematode: Heterorhabditidae)* on artificial media. J Nematol 13:467–469

Wright PJ (1992) Cool temperature reproduction of steinernematid and heterorhabditid nematode. J Invertebr Pathol 60:148–151

Wright PJ, Noonan MJ, Jackson TA, Wouts WM (1988) Use of nematodes for control of pasture pests in New Zealand. In: Proceedings of the 5th Australasian conference on grassland invertebrate ecology, Melbourne, Australia, pp 82–87

Zervos S, Johnson SC, Webster JM (1991) Effects of temperature and inoculum size on reproduction and development of *Heterorhabditis heliohidis* and *Steinernema glaseri* (Nematoda: Rhabditoidea) in *Galleria mellonella*. Can J Zool 69:1261–1264