Chapter 13
Nematodes as Biocontrol Agents

Tarique Hassan Askary

Abstract The high cost of chemical pesticides, their adverse effects on the environment and development of pest resistance demand an alternative approach for crop pests management, which should be ecofriendly and cost-effective. Entomopathogenic nematodes belonging to genera *Steinernema* and *Heterorhabditis* together with their symbiotic bacteria *Xenorhabdus* and *Photorhabdus*, respectively, and slug-parasitic nematodes *Phasmarhabditis* with its symbiotic bacteria *Moraxella* have been considered as promising biocontrol agents for the management of crop insect pests and slugs. These nematodes have short life cycle, wide host range, and can resist under unfavourable conditions and environmental extremes. Survival and pathogenicity of these nematodes vary from 5 to 35 0ºC. Nematodes can be mass produced under both *in vivo* and *in vitro* conditions. With the realization of these attributes among these bioagents there is a need to search out an ideal formulation and proper application technology to include them in pest management programme.

Keywords Bioagent • Entomopathogenic nematode • Heterorhabditis • In-vitro • In-vivo • Moraxella • Phasmarhabditis • Photorhabdus • Slug-parasitic • Steinernema • Xenorhabdus

13.1 Introduction

The use of nematodes as a biocontrol agent has been developed in the past 2 decades. Proper use of these bioagents on experimental scale has proved superbly successful in both short- and long-term pest suppressions. Crop insect pests are one

T.H. Askary (✉)
Plant Protection, KVK, Shuhama, Veterinary College Campus, SKUAST-K, Alastang, Srinagar, 190006, Jammu and Kashmir, India
e-mail: tariq_askary@rediffmail.com

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of the major limiting factors in sustaining the agriculture productivity and the indiscriminate use of chemical pesticides for its management has affected humans and their environment. Hence, the biological control of crop pests is an ideal alternative to reduce the overall use of chemical pesticides. Entomopathogenic nematodes belonging to the families Steinernematidae and Heterorhabditidae and slug-parasitic nematodes, *Phasmarhabditis* spp. are considered lethal parasites of crop insect pests and slugs, respectively, and have a high biocontrol potential, safe for humans, other non-target organisms and virtually posing no hazardous effect on the environment. These nematodes harbour symbiotic bacteria in their intestine, which are released after entering into the host. The bacteria produce a toxic substance that ultimately leads to killing of the host (Woodring and Kaya 1988). The focus of this chapter lies upon three nematodes, viz., *Steinernema, Heterorhabditis* and *Phasmarhabditis* important from biological control of view, limitations in their use and ideas to overcome the problem in the present context.

13.2 Historical Background

Nematodes from more than 30 families are known to be associated with insects and other invertebrates (Poinar 1979, 1990; Kaya and Stock 1997). However, only a few have established their potentialities as host enemies, while majority of them are more associated either for transport and dissemination or for sharing the same habitat (Sundarababu and Sankaranarayanan 1998). The nematodes from seven families, viz., Mermithidae, Allantonematidae, Sphaerularidae, Tetradonematidae, Phaenopsitylenchidae, Steinernematidae and Heterorhabditidae are important from biological control of view (Kaya and Stock 1997). Steinernematidae and Heterorhabditidae are of much interest and drew lot of attention on the part of research workers and practitioners (Lacey et al. 2001). These nematodes possess many attributes of parasitoids and pathogens. They are analogous to parasitoids because they have chemoreceptors and can actively search for their hosts (Kaya and Gaugler 1993; Gaugler et al. 1997). Their similarity to pathogens is due to their association with mutualistic bacteria, viz., *Xenorhabdus* and *Photorhabdus* for steinernematids and heterorhabditids, respectively. The nematode–bacterial complex is highly virulent, killing its host within 48 h through the action of mutualistic bacteria, can be cultured in vitro, have a high reproductive potential (Kaya and Gaugler 1993), have wide range of hosts, yet pose no threat to plants, vertebrates and many invertebrates (Akhurst 1990; Kaya and Gaugler 1993).

Steinernematidae comprises two genera: *Steinernema* and *Neosteinernema*. *Steinernema* has more than 50 species (Ganguly 2006), whereas *Neosteinernema* has only one species (Nguyen and Smart 1994). The family Heterorhabditidae has one genus *Heterorhabditis* with eight reported species (Adams and Nguyen 2002). However, these figures have increased as in the last few years a number of new species belonging to *Steinernema* and *Heterorhabditis* have been described from different parts of the world. Phan et al. (2005) described *Steinernema robustispiculum* from Vietnam. *S. seemae* and *S. masoodi* were described from India (Ali et al.

2005a), *S. khoisanae* from South Africa (Nguyen et al. 2006a), *S. leizhouense* from southern China (Nguyen et al. 2006b), *S. hebeiense* from northern China (Chen-ShuLong et al. 2006), *S. ashiuense* from Japan (Phan et al. 2006), *S. sichuanense* from east Tibetan mountains, China (Mracek et al. 2006), *S. cholashanensen* from Sichuan province of China (Nguyen et al. 2008a), *S. weiseri* from Turkey (Unlu et al. 2007), *S. costaricense* and *S. puntauvense* from Costa Rica (Uribe-Lorio et al. 2007), *Heterorhabditis safricana* from western cape province of South Africa (Malan et al. 2008) and *H. georgiana* from Georgia, USA (Nguyen et al., 2008b).

The first entomopathogenic nematode, *Aplectana kraussie* was reported by Steiner (1923), which was later named as *S. kraussie* by Travassos (1927). However, biocontrol potential of entomopathogenic nematode under field condition was recognized when Glaser (1932) reported the suppression of Japanese beetle with the application of *Neoaplectana glaseri*. Application of the nematode to 73 field plots in New Jersey resulted in 0.3–81% pest suppression and its persistence was noticed for 8.5 years after treatment (Glaser 1932; Glaser and Farrell 1935; Glaser et al. 1940). Schneider (1859) described the association of a nematode with the slug *Arion ater*. Maupas (1900) established culture of a nematode, *Phasmarhabditis hermaphrodita* (which he called *Rhabditis causenelli*) on rotting flesh and the dauer larvae used for this purpose was found in the intestine of *A. ater*. However, *Pp. hermaphrodita* was first described as a potential biocontrol agent by Wilson et al. (1993a). In 1994, the commercial product of this nematode was released for use by home gardeners under the trade name Nemaslug® (Glen et al. 1994, 1996). This nematode has now been on sale in several European countries (Ester and Wilson 2005).

13.3 Steinernematids and Heterorhabditids

13.3.1 Ecology and Distribution

After the baiting technique developed by Bedding and Akhurst (1975), random soil surveys were conducted globally in order to find entomopathogenic nematode in temperate, sub-tropical and tropical countries. These nematodes were common in both cultivated and uncultivated soils and their distribution was found to be worldwide (Hominick et al. 1996; Hominick 2002). Steinernematids were much more biologically diversified than Heterorhabditids. The most widely distributed species were *S. carpocapsae,* which has been isolated from Europe, Australia, New Zealand, India and America followed by *S. feltiae* from Europe, Australia and New Zealand (Poinar 1990). *S. carpocapsae* and *S. feltiae* were widely distributed in the temperate region, whereas *H. bacteriophora* in the continental Mediterranean climate and *H. indica* throughout the tropics and sub-tropics (Hominick 2002). Among the most thinly distributed species were *S. anomali*, which was recovered only from Russia, *S. rara* from Brazil, *S. kushidai* from Japan and *S. scapterisci* from Uruguay. The most prevalent species in the UK was *S. feltiae*, whereas in Northern Europe it was *S. affini* (Poinar 1990). The factors affecting the local distribution of

entomopathogenic nematodes are soil texture, vegetation and availability of suitable hosts (Griffin et al. 2005). *S. affini* was found largely in arable lands and grasslands but absent in forests, whereas *S. kraussie* was common in forests (Hominick 2002). *H. megidis* and *H. indica* were extensively found in sandy soils, resulting in a mainly coastal distribution (Griffin et al. 1994, 2000). The distribution of *H. indica* has also been reported from the soil samples collected from three sites in the date palm growing region in the eastern province of Saudi Arabia (Saleh et al. 2001). Uribe-Lorio et al. (2005) conducted a survey in north Pacific and southeast Caribbean regions of Costa Rica. Out of a total of 41 soil samples, five were positive for entomopathogenic nematodes, with three containing *Steinernema* and two containing *Heterorhabditis* isolates. Campos-Herrera et al. (2007) studied the distribution of entomopathogenic nematodes in natural areas and crop field edges in La Rioja, Northern Spain. Five hundred soil samples from 100 sites were assayed for the presence of entomopathogenic nematodes. There was no statistical difference in the abundance of entomopathogenic nematodes to environmental and physical-chemical variables, although, there were statistical differences in the altitude, annual mean air temperature and rainfall, potential vegetation series and moisture percentage recovery frequencies. Twenty isolates were identified upto species level and 15 strains were selected of which 11 were *S. feltiae*, two *S. carpocapsae* and two *S. kraussie*. *S. kraussie* was isolated from humid soils of cool and high altitude habitats and *S. carpocapsae* was found to occur in heavy soils of dry and temperate habitats. *S. feltiae* was the most common species with a wide range of altitude, temperature, rainfall, pH and soil moisture, although this species preferred sandy soils.

In course of evolution, entomopathogenic nematode like other terrestrial organisms have adopted unique survival mechanism to resist unfavourable condition and environmental extremes including absence of water, extreme temperature, lack of oxygen and osmotic stress. Survival and pathogenicity of *S. carpocapsae* has been found greater at lower temperature (5–25°C) than at higher temperature (35°C), whereas survival and pathogenicity of *S. glaseri* has been found greater at higher temperature (15–35°C) than at the lower temperature (5°C) (Kung and Gaugler 1991). The optimum temperature and moisture requirement for infectivity and survival vary with nematode species as has been reported in case of *S. abbasi*, *S. tami*, *S. carpocapsae*, *S. feltiae*, *S. glaseri* and *S. thermophilum* (Karunakar et al. 1999; Ganguly and Singh 2001; Ganguly and Gavas 2004). Cooler temperature has not been found detrimental to nematode survival (Kaya 1990) but exposure to nematode at 35°C or above have proved detrimental to infective juveniles (Schmiege 1963). Hazir et al. (2001) studied the effect of temperature on the infectivity, time of death, development and reproduction of *S. feltiae*. Five isolates of *S. feltiae* were used in the experiment: SN from southern France, Rafaela from Argentina, Monterey from California, MG-14 from Hawaii and Sinop from Turkey. The result indicated that all isolates caused 100% mortality of greater wax moth, *Galleria mellonella* larvae and developed and produced progenies between 8°C and 25°C. At 28°C none of the isolates produced progeny, and the nematodes developed to the first generation adults were unable to proceed to the next generation. In all isolates, penetration efficiency was highest at 15°C and 20°C and emergence time was

fastest at 20°C and 25°C. Bhatnagar and Bareth (2003) conducted an experiment to study the survival of *H. bacteriophora* in sandy loam soil at four moisture levels representing 25%, 50%, 75% and 100% of the field capacity. In saturated soils, 70% of the infective juveniles survived for 75 days. Nematode mortality reached 40% within 15 days in soil with 50% field capacity moisture level and within 5 days in soil with moisture level at 25% field capacity. Jothi and Mehta (2007) investigated the impact of different temperatures on the infectivity and productivity of four entomopathogenic nematodes, viz., *H. indica, H. bacteriophoa, H. zealandica* and *S. glaseri* on *G. mellonella*. All the species of entomopathogenic nematodes caused 100% mortality at a temperature ranging between 30°C and 40°C at 24 h after inoculation. At 48 h after inoculation *H. indica* and *H. bacteriophora* caused 100% mortality between 20°C and 27.5°C, whereas *H. zealandica* was effective at temperature between 22.5°C and 27.5°C. *S. glaseri* was found to be virulent even at 15°C and continued upto 27.5°C at 48 h after inoculation by causing 100% mortality.

13.3.2 Life Cycle

Life cycle of entomopathogenic nematode includes the egg, four juvenile stages and adult. The third stage is a free-living infective juvenile (dauer stage). The infective juveniles of both steinernematids and heterorhabditids carry in its gut bacteria of the genus *Xenorhabdus* and *Photorhabdus*, respectively (Boemare et al. 1993). The infective juvenile enters the host through mouth, anus or spiracles or penetrate through the intersegmental membranes of the insect cuticle as in case of *Heterorhabditis* sp. (Bedding and Molyneux 1982; Peters and Ehlers 1994) and reaches the haemocoel. In the haemocoel, infective juvenile releases cells of bacterial symbiont from its intestine. The nutrient-rich haemolymph of insect helps in the rapid multiplication of bacteria and ultimately results in killing the host within 48 h (Woodring and Kaya 1988). The infective juvenile then becomes feeding juvenile or functional third-stage juvenile and feed on the multiplying bacteria and degrading host tissues. The nematodes moult to fourth stage and finally develop into adult .The life cycle of steinernematids from infection to emergence of infective juveniles ranges from 7 to 10 days and for heterorhabditids ranges from 12 to 15 days (Sundarababu and Sankaranarayanan 1998). The number of generations may be more than one within the host cadaver depending upon the available resources.

Infective juveniles of Steinernematids develop into amphimictic females and males and never develop into hermaphrodites, whereas Heterorhabditids always develop into hermaphrodites in the first generation. Subsequent generation of heterorhabditids produces males, females and hermaphrodites (Dix et al. 1992). First-generation adults of steinernematids are termed as giant adults due to their larger size. This condition is believed to be due to the abundant available nutrition. The progeny of next generation, in most cases, find gradually depleting food supply due to regular progeny development. A full third-generation progeny may be observed when the food supply is in plenty (Adams and Nguyen 2002). Juveniles developing

with adequate food supply mature to adults, while those developing in crowded conditions with limited food resources results in infective juveniles. Under suitable condition infective juveniles exit the cadaver to seek new hosts.

The eggs are initially laid into the host medium but in older female or hermaphrodite, eggs hatch in the uterus, and the developing juveniles consume the parental tissues. This process is known as *endotokia matricida* (Johnigk and Ehlers 1999), i.e. intrauterine birth causing maternal death. The infective juveniles are provided with two layers of external membrane, the cuticle of the third and second stages, due to superimposed first and second moults. The sheath of *Heterorhabditis* spp. in particular helps in protection against desiccation, freezing and fungal pathogens (Timper and Kaya 1989; Campbell and Gaugler 1991a; Wharton and Surrey 1994). This tight-fitting sheath of heterorhabditids do not lose easily, whereas the loose-fitting sheath of steinernematids is soon lost, as the nematode moves through the soil (Campbell and Gaugler 1991b; Dempsey and Griffin 2003). The physiology of infective juveniles may also bestow resistance or hardiness. In addition, oral and anal openings of infective juveniles remain closed in soil, thus preventing entry by microbial antagonists and toxic chemicals.

13.3.3 Nematode–Bacteria Symbiosis

The symbiotic association between entomopathogenic nematode and its bacteria have been reported by several workers (Kaya 1990; Kaya and Gaugler 1993; Tanada and Kaya 1993; Sicard et al. 2005; Somavanshi et al. 2006; Wang et al. 2007a). Infective juveniles of entomopathogenic nematode carry the bacteria *Xenorhabdus* (in case of steinernematids) or *Photorhabdus* (in case of heterorhabditids) belonging to Enterobacteriaceae (Forst et al. 1997; Nagesh et al. 2002). These bacteria are Gram-negative, anaerobes, nonspore former and do not have resistant stage. Infective juveniles of *Steinernema* sp. harbour *Xenorhabdus* sp. in a special intestinal vesicle, whereas those of *Heterorhabditis* sp. carry *Photorhabdus* sp. in the anterior two third part of the intestine (Forst and Clarke 2002).

Entomopathogenic nematodes, *Steinernema* and *Heterorhabditis*, belonging to different species harbour different species of bacteria (Table 13.1). The life cycle of nematode–bacteria association is composed of two stages: (i) a free stage in the soil, where the infective juveniles carry bacteria in their guts and search for new insect host, and (ii) a parasitic stage, where the infective juveniles infect insect, release their bacterial symbionts and reproduce in order to produce new infective juveniles (Emelianoff et al. 2007). Both partners benefit from the association. The bacteria provide a nutritive medium for the growth and reproduction of nematodes. These bacteria are also useful in other two ways: (i) largely responsible for the rapid death of the host, as well as (ii) suppressing other competing organisms by the production of antibiotics. On the other hand, nematode protects the bacteria from the external environment, carries them into the insect haemocoel and in some cases inhibits the insect immune response. Martens et al. (2003) suggested that

Table 13.1 Entomopathogenic nematodes and their symbiotically associated bacteria (Reproduced from Ganguly 2006)

Entomopathogenic nematode	Bacterium
Steinernema kraussei	*Xenorhabdus bovienii*
S. carpocapsae	*X. nematophila*
S. feltiae	*X. bovienii*
S. glaseri	*X. poinarii*
S. kushidai	*X. japonica*
S. intermedium	*X. bovienii*
S. affine	*X. bovienii*
S. cubanum	*X. poinarii*
S. bicornutum	*X. budapestensis*
S. longicaudatum	*X. beddingii*
S. rarum	*X. szentirmaii*
S. scapterisci	*X. innexi*
S. serratum	*X. ehlersii*
S. thermophilum	*X. indica*
Heterorhabditis bacteriophora subgroup Brecon	*Photorhabdus luminescens luminescens*
H. bacteriophora subgroup HP88	*Pp. luminescens laumondii*
H. bacteriophora subgroup NC	*Pp. temperata*
H. megidis Nearctic group (Ohio, Wisconsin)	*Pp. temperata*
H. megidis Palaearctic group	*Pp. temperata temperata*
H. indica	*Pp. luminescens akhurstii*
H. zealandica	*Pp. temperata*

Xenorhabdus nematophila initiates infective juvenile colonization of *S. carpocapsae* by competing for limited colonization sites or resources within the nematode intestine. Mahar et al. (2008) isolated the bacterial cells and metabolites of entomopathogenic bacterium *Pseudomonas luminescens* from *H. bacteriophora* and compared their effectiveness to the larvae of diamondback moth, *Plutella xylostella*. All different instars of diamondback moth were susceptible to lethal effect of bacterium and its metabolites. However, bacterial cells of *Pp. luminescens* suspended in broth were slightly more lethal to diamondback moth larvae. Jan et al. (2008) in an experiment found that cells of the bacterial symbiont *X. nematophila* isolated from *S. carpocapsae* are lethal to the pupae of greater wax moth, *G. mellonella,* beet armyworm, *Spodoptera exigua*, diamondback moth, *Pp. xylostella* and blackvine weevil, *Otiorhynchus sulcatus* in the absence of nematode vectors. The cells of *X. nematophila* were found to enter the haemocoel of the pupae.

13.3.4 Host Range and Effects

Steinernematid and Heterorhabditid nematodes attack a far wide spectrum of insects and are being exploited worldwide to manage crop insect pests. The host range of these nematodes varies with the species (Table 13.2) and it has been observed to

Table 13.2 Host suitability of some *Steinernema* sp. against various insect pests

Steinernema sp.	Host insect
S. seemae, S. masoodi, S. thermophilum, S. glaseri, S. carpocapsae	Greater wax moth (*Galleria mellonella*)
S. carpocapsae, S. seemae, S. thermophilum, S. glaseri, S. masoodi	Rice moth (*Corcyra cephalonica*)
S. carpocapsae	Black cutworm (*Agrotis ipsilon*)
S. carpocapsae, S. feltiae, S. abbasi, Heterorhabditis indica	Tobacco caterpillar (*Spodoptera litura*)
S. glaseri, S. carpocapsae	White grub (*Holotrichia consanguinea*)
S. carpocapsae	Leaf minor (*Liriomyza trifolii*)
S. masoodi, S. seemae, S. carpocapsae, S. thermophilum	Gram pod borer (*Helicoverpa armigera*)
S. carpocapsae	Diamondback moth (*Plutella xylostella*)
S. seemae, S. masoodi	Legume pod borer (*Maruca vitrata*)
S. masoodi, S. seemae, S. carpocapsae	Blue butterfly (*Lampides boeticus*)
S. seemae, S. masoodi, S. carpocapsae	Bruchid (*Callosobruchus* sp.)
S. seemae, S. masoodi	Wheat flour beetle (*Tribolium castaneum*)
S. masoodi, S. seemae, S. carpocapsae	Grey weevil (*Myllocerus* sp.)
S. masoodi, S. seemae, S. carpocapsae	Bihar hairy caterpillar (*Diacrisia obliqua*)
S. masoodi, S. carpocapsae	Mealybug (*Centrococcus* sp.)

infect over 200 species of insects belonging to different orders (Woodring and Kaya 1988). *S. carpocapsae* has been found to parasitize more than 250 insect species from over 75 families in 11 orders (Poinar 1975). The host range of nematodes largely depends on foraging strategy varying from cruising to ambusher (Campbell and Gaugler 1997). Cruisers have an active searching strategy, moves through the soil and are more effective against those insects, which are less mobile (Lewis et al. 1993; Campbell and Gaugler 1997). The cruise foraging species are *Heterorhabditis* sp. and *S. glaseri* (Lewis 2002). Ambushers nictate during foraging by raising nearly all of their bodies off the substrate. *S. carpocapsae* and *S. scapterisci* are the extreme ambushers and may nictate for hours at a time (Campbell and Gaugler 1993). Heterorhabditids have a better host-finding ability than the Steinernematids (Choo et al. 1989). Motility and attraction are also responsible for host-finding ability of nematodes. There is a third type having intermediate foraging strategy whereby nematodes raise themselves on substrate for a short while, and has been reported in some species like *S. riobrave* and *S. feltiae* (Griffin et al. 2005). Susurluk (2008) compared the vertical movement of Turkish isolates of *S. feltiae* (TUR-S3) and *H. bacteriophora* (TUR-H2) at different temperatures in the presence and absence of larvae of the host insects, *G. mellonella*. It was observed that nematodes of both species moved faster towards the bottom of the column when an insect was placed there. *S. feltiae* showed greater vertical dispersal ability than *H. bacteriophora*. The vertical movement of both species increased as the temperature increased and lower temperature depressed the movement of *H. bacteriophora* more than *S. feltiae*. The nematodes that had migrated different distances were compared for their infectivity to *G. mellonella* and the positive

correlation between the distance travelled and infectivity indicated that there was a link between host-searching behaviour and infection behaviour in *S. feltiae* and to a lesser extent, also in *H. bacteriophora*.

The insects killed by nematodes are flaccid and do not undergo putrefaction because the mutualistic bacteria produce antibiotics, which prevent the growth of secondary micro-organisms. Also the cadaver differs in colour. Insects killed by steinernematids turn ochre, yellow brown or black, whereas those killed by heterorhabditids turn red, brick- red, purple, orange or sometimes green (Sundarababu and Sankaranarayanan 1998). The insect infected with heterorhabditids, luminesce in the dark and this is due to the symbiotic bacteria *Photorhabdus luminescens* present in the intestine of the nematodes. The internal tissues of the killed insects become gummy or sticky.

Cannayane et al. (2007) conducted a laboratory experiment to test the pathogenic potential of *H. indica* and *S. glaseri* on cardamom root grub, *Basilepta fulvicorne*. After mortality the cadaver of *B. fluvicorni* exhibited brick red to brown colour when infested with *H. indica* and also luminescent under ultraviolet, whereas, yellow and flaccid nature was due to *S. glaseri* infestation.

The efficacy of Steinernematids and Heterorhabditids in the management of crop insect pests has been worked out by several workers in the past. Kumar et al. (2003) studied the efficacy of Heterorhabditids against *S. litura* collected from castor bean. The insect mortality was significant within 48 h of exposure when infective juveniles of *Heterorhabditis* were released against the larva of *S. litura* at the rate of 50, 75, 100, 125 and 150 infective juveniles per 100 g of soil. Narayanan and Gopalakrishnan (2003) reported that mustard sawfly, *Athalia lugens proxima* was highly susceptible to *S. feltiae* on radish under field condition. Toledo et al. (2006) for the first time demonstrated the infectivity of *H. bacteriophora* on third instar of tropical fruit fly, *Anastrepha serpentina* under laboratory conditions. Adjei et al. (2006) reported that *S. scapterisci* applied in stripe to a 10 ha bahia grass pasture reduced populations of mole crickets, *Scapteriscus* spp. by 79.2% over a period of 3 years. Infection on *Tipula paludosa*, a turf grass pest on golf courses was studied under laboratory condition against *Heterorhabditis* and *Steinernema* and it was observed that these nematodes were virulent against *T. paludosa* (Simard et al. 2006). Shapiro-Ilan and Cottrell (2006) also reported the susceptibility of lesser peach tree borer, *Synanthedon pictipes* against *S. carpocapsae* and *S. feltiae*. Cuthbertson et al. (2007) tested the efficacy of *S. feltiae* under both laboratory and glass house condition against sweet potato white fly, *Bemisia tabaci*. They observed 90% mortality in second instar of *B. tabaci* under laboratory condition and 80% under glass house condition. Ramos-Rodriguez et al. (2007) reported that under laboratory bioassay *S. riobrave* significantly reduced survival of larva, pupae and adults of a store grain pest red flour beetle, *Tribolium castaneum*. In an experiment, *S. thermophilum* when applied at 3000 infective juveniles per millilitre caused 46% mortality of diamondback moth infesting cabbage, whereas, mortality at 2,000 infective juveniles per millilitre was 40.5% (Somavanshi et al. 2006). Elawad et al. (2007) assessed the pathogenicity of *H. indicus* a local isolate of UAE against red palm weevil, *Rhynchophorus ferrugineus*. The result indicated that nematode was

effective in declining the population of *R. ferrugineus* under both laboratory and field conditions. However, a higher concentration of *H. indicus* was required for field application. Khan et al. (2007) tested the pathogenicity of *S. masoodi* against final instars of six insect pests, i.e. *G. mellonella, Pp. xylostella, Pieres brassicae, Corcyra cephalonica, Helicoverpa armigera* and *A. proxima*. Six concentrations of the nematode were used, i.e. 25, 50, 75, 100, 125 and 150 infective juveniles per larvae. The nematode was found to be pathogenic to all the six insects with a considerable degree of variability in pathogenicity. Koppenhofer et al. (2008) conducted a series of laboratory and green house experiments to evaluate the comparative effectiveness of *S. scarabaei*, *H. bacteriophora* and *H. zealandica* for the control of second and third instar of cranberry white grub, *Phyllophaga georgiana* in cranberries. The result indicated that *S. scarabaei* was the most effective species causing 76–100% mortality of *Pp. georgiana* under green house condition. However, under laboratory condition *S. scarabaei* was more effective against third instar than second instar of *Pp. georgiana*. In an experiment under laboratory condition, Entonem and Larvanem, the two commercial products of *S. feltiae* and *H. bacteriophora*, respectively, were evaluated against *Parahypopta caestrum*, the major insect pest of *Asparagus officinalis* in Greece. *S. feltiae* caused insect mortality within 24 h, however, the highest level of mortality was observed at 48 h. In contrast, *H. bacteriophora* required 96 h to achieve the highest level of mortality. However, under field condition the two nematodes provided equal insect suppression (Salpiggidis et al. 2008).

13.3.5 Mass Production

The two different techniques for mass production of entomopathogenic nematodes are (i) in vivo, and (ii) in vitro. Production of entomopathogenic nematodes depend upon the area to be applied as well as the type of nematode species used. If a small plot is to be applied as for research purpose, the in vivo production technique would be appropriate, otherwise for fields in vitro methods are used.

13.3.5.1 In Vivo Production

White trap (White 1927) is one of the most common methods to produce entomopathogenic nematodes. Insects are inoculated with entomopathogenic nematodes on a petridish lined with filter paper. After 2–5 days, the infected insects are transferred to the White trap. The White trap consist of an inverted watch glass placed in a petridish on which Whatman paper of appropriate size is placed and moistened with sterilized distilled water. Adequate amount of distilled water is also maintained on and around the watch glass. As the infective juveniles emerge from the cadaver they migrate to the surrounding water and get trapped. The nematodes are harvested from the White trap and collected in a beaker. The concentration of nematodes can be accomplished by

Fig. 13.1 Entomopathogenic nematode *Steinernema masoodi* multiplying over the body of *Galleria mellonella* larva (Reproduced from Ali et al. 2005b)

gravity settling (Dutky et al. 1964) and/or vacuum filtration (Lindergen et al. 1993). Entomopathogenic nematodes produced in vivo are highly virulent and infective. The last instar of the greater wax moth, *G. mellonella*, is generally used for in vivo production of entomopathogenic nematodes as this insect is highly susceptible, easily available and produces high yields (Fig. 13.1) (Woodring and Kaya 1988).

Other Lepidopterans and Coleopterans have also been used for in vivo production of nematodes (Shapiro-Ilan and Gaugler 2002). Nematode yield depends upon the insect host size. In general yield of nematode is proportional to the size of the insect host (Blinova and Ivanova 1987; Flanders et al. 1996), however, yield per milligram insect (within host species) and susceptibility to infection is inversely proportional to size or age of host (Dutky et al. 1964; Shapiro-Ilan et al. 1999). The major drawback of in vivo technique is cost of production, which tilts towards the higher side, as two different organisms, host insect and entomopathogenic nematode are to be cultured simultaneously. But such limitation has not restricted the production technology to sustain itself as a cottage industry (Gaugler et al. 2000; Gaugler and Han 2002). In vivo production of entomopathogenic nematodes is likely to continue as small ventures for niche markets or in those countries where labour cost is low. The production and application of entomopathogenic nematodes in infected host cadaver is also an alternative to encourage this technology (Shapiro-Ilan et al. 2001, 2003).

13.3.5.2 In Vitro Production

Bedding (1984) developed a technique whereby huge number of infective juveniles may be economically produced using a chicken, duck or turkey offal medium on a

porous polyurethane foam substrate. The rearing container used in this method is a glass flask or autoclaved plastic bags aerated with aquarium pumps and inoculated with approximately 2,000 infective juveniles per gram medium. This method can be used to produce on an average one billion infective juveniles per bag of flask of 500 ml capacity (100 g medium). Currently, some companies, viz., Andermatt (Switzerland), Bionema (Sweden), Oviplant (Poland) and Biologic (USA) are using this technology of nematode production (Ehlers and Shapiro-Ilan 2005). This technique involves the following steps.

Preparation of Rearing Flasks/Bags

Small foam pieces are impregnated with chicken, duck or turkey offal homogenate at the rate of 12.5 parts medium to one part foam by weight. A wide mouthed Erlenmeyer flask of 500 ml capacity is filled with this foam homogenate mixture to the 250–300 ml mark (about 100 g). The mouth of the flask is wiped, plugged with cotton, wrapped with cheese muslin cloth and autoclaved at 121°C for 20 min.

Inoculation with Bacteria

Appropriate *Xenorhabdus* or *Photorhabdus* bacterial cells are aseptically transferred to 5 ml of nutrient broth in a test tube and kept overnight on a shaker. The flasks containing autoclaved material are inoculated with the bacterial culture by pouring the contents of one culture tube. The flask is shaken well and stored for 2–3 days at 25°C to allow multiplication of the bacteria.

Inoculation with Nematodes

Each flask colonized with the bacteria is inoculated with surface sterilized 500–1,000 infective juveniles of an appropriate species in 5 ml sterilize distilled water and are incubated at 25°C. The flask after inoculation should not be shaken vigorously to enable better feeding and reproduction of the nematode.

Harvesting

The nematodes can be harvested from the flask in about 15 days. A 20 mesh sieve is taken and foam pieces are piled 5 cm deep on it. The sieve is then placed in a pan and brought near water tap with water level adjusted so that the foam pieces are just submerged. It is left for 2 h. During this period infective juveniles will migrate into the water. The nematodes may be sedimented and rinsed to remove particulate matter and inactive or dead juveniles. The infective juveniles thus obtained should be rinsed with specialized distilled water for several times to make the suspension clear. Various other synthetic media tested to mass culture of entomopathogenic nematodes have been enlisted (Table 13.3).

Table 13.3 Different media recommended for production of entomopathogenic nematodes

Synthetic medium	Nematode species	Incubation period	Temperature	Nematode harvested	Reference
Beef extract, peptone, corn meal, water on sponge	*Steinernema feltiae*	–	–	–	Li (1984)
Dogfood agar medium	*S. feltiae*	–	–	10^5/g medium	Hara et al. (1981)
Kidney/fat homogenate	*S. feltiae*	2–3 weeks	25°C	3.8×10^7/30 flasks	Bedding (1981)
	S. bibionis			2.9×10^7/73 flasks	
	S. glaseri			8×10^6/11 flasks	
	Heterorhabditis bacteriophora			3.6×10^7/10 flasks	
	H. heliothidis			3.2×10^7/15 flasks	
3% Soyapeptone + 3% yeast extract + 10% chick embryo extract medium	*S. glaseri*	–	–	10^4/week for 93 days	Tarakanov (1980)
Nutrient broth yeast extract vegetable oil, flour coated on sponge	*H. heliothidis*	4 weeks	25°C	10^7/250 ml flask	Wouts (1981)
Wheat bran+ salad oil	*S. feltiae*	3 weeks	25°C	10^7/g medium	Abe (1987)
Wheat bran	*S. feltiae*	3 weeks	25°C	10^4/g medium	Abe (1987)

13.3.6 Formulation, Storage and Quality

The important aspects, which are to be kept in mind for commercialization of entomopathogenic nematodes as biocontrol agent are formulation, storage and quality control. Formulation refers to the preparation of a product from an ingredient by the addition of certain active (functional) and non-active (inert) substances. It provides means to improve the activity, delivery, ease to use, storage stability and field efficacy of the nematodes. Entomopathogenic nematode species have differential requirement for temperature, moisture and oxygen (Glazer 2002). These requirements may dictate the conditions for formulation and storage. As a result of varied nematode species, differential survival requirements and formulation types, an array of products can be developed for management of different insect pests. Entomopathogenic nematodes are live organisms and regardless of how they are formulated, their quality declines with time. Furthermore, all formulations are susceptible to temperature extremes, ultraviolet light, anoxic conditions and contamination (Lewis and Perez 2004). Infective juveniles of entomopathogenic nematode can be stored in water for several months in refrigerated bubbled tanks, however, high cost as well as quality maintenance are somewhat difficult through this method. Tolerance and activity of the nematodes at extreme environmental conditions can limit the shelf life, quality and field performance of the products (Ehlers et al. 2005). Till now no entomopathogenic nematode formulation has met the 2-year shelf life requirement of a standard chemical pesticide (Table 13.4).

The target in developing an ideal formulation is (i) maintenance of quality, (ii) increased storage stability, (iii) low transport cost, and (iv) enhancement of nematode survival during and after application. These can be achieved when absorbents, adsorbents, anticaking agents, antimicrobial agents, antioxidants, surfactants, carriers, preservatives, ultra violet protectants, etc. may be added to the formulation depending upon the need. Formulation of nematodes for storage and transport are generally done by two ways.

1. The nematodes are placed in inert carriers such as sponge and vermiculite that allow free gas exchange and movement of nematodes.
2. Addition of functional ingredients, which reduces nematode activity and metabolism.

It has been observed that sometimes nematodes escape from the inert carriers and dry out (Grewal and Peters 2005), therefore in formulations mobility/metabolism of nematodes is minimized through physical trapping, inclusion of metabolic inhibitors or through the induction of partial anhydrobiosis. Nematode metabolism is temperature driven and a warm temperature between 20°C and 30°C accelerates metabolic activities, thereby reducing nematode viability (Georgis 1990a). Formulations prepared in carriers such as alginate, clay, polyacrylamide gels, vermiculite, activated charcoal, etc. can be stored for at least 3 months under refrigeration or at room temperature. Temperature requirement during storage, however,

Table 13.4 Expected shelf life of different entomopathogenic nematode formulations

Formulation	Nematode species	Strain	Shelf life (months)	
			22–25°C	2–10°C
Sponge	*Steinernema carpocapsae*	All	0.03–0.01	2.0–3.0
	Heterorhabditis bacteriophora	HP88	0	1.0–2.0
		Hybrid	0	0.75–1.5
	H. indica	LN2	0.25	0
	H. marelata	Oregon	0	1.0–2.0
Vermiculite	*S. carpocapsae*	All	0.1–0.2	5.0–6.0
	S. feltiae	SN	0.03–0.1	4.0–5.0
Liquid concentrate	*S. carpocapsae*	All	0.16–0.2	0.4–0.5
	S. riobrave	RGV	0.1–0.13	0.23–0.3
Wettable powder	*S. carpocapsae*	All	2.0–3.5	6.0–8.0
	S. feltiae	UK	2.5–3.0	5.0–6.0
		ENO2	0.5–1.0	3.0–4.0
	S. glaseri	NJ43	0.03–0.06	1.0–1.5
	S. scapterisci	Uruguay	1.0–1.5	3.0–4.0
	H. bacteriophora	ENO1	0.5–1.0	2.0–3.0
	H. indica	LN2	0.25–0.50	0
	H. megidis	UK	2.0–3.0	4.0–5.0
	H. zealandica	X1	1.0–2.0	0
Water-dispersible granule	*S. carpocapsae*	All	4.0–5.0	9.0–12.0
	S. feltiae	SN	1.5–2.0	5.0–7.0
	S. riobrave	RGV	2.0–3.0	4.0–5.0
Alignate gel	*S. carpocapsae*	All	3.0–4.0	6.0–9.0
	S. feltiae	SN	0.5	4.0–5.0
Flowable gel	*S. carpocapsae*	All	1.0–1.5	3.0–4.0
	S. glaseri	NJ43	0.16–0.2	0.4–0.5
	S. scapterisci	Colon	0.1–0.13	0.23–0.3

varies with entomopathogenic nematode species. General range of storage temperature for steinernematids is 5–10°C, whereas for heterorhabditis it is 10–15°C (Georgis 1990b). In another approach functional ingredients such as alginate and flowable gel formulations are used to trap nematodes physically in order to reduce their movement. Also with the induction of partial anhydrobiosis, nematode activity and metabolism can be reduced. Grewal (2002) reported the storage of *S. carpocapsae* for 3–4 months at 25°C and *S. feltiae* for 2–4 weeks in alginate gel formulation. Bedding (1988) described a formulation whereby nematodes were mixed in clay for removing excess surface moisture and inducing partial anhydrobiosis. The formulation called 'sandwich' consisted a layer of nematode between two layers of clay.

Water-dispersible granule formulation is considered to be the first commercial formulation enabling storage of *S. carpocapsae* for 6 months at 25°C at a concentration of over 300,000 infective juveniles per gram (Grewal 2000). When stored at

Table 13.5 Formulations of *Steinernema* and *Heterorhabditis* developed by different countries

Nematode	Product	Country
Steinernema carpocapsae	Ortho biosafe	United States of America
	Bio vector	United States of America
	Exhibit	United States of America
	Sanoplant	Switzerland
	Boden nutzlinge	Germany
	Helix	Canada
S. feltiae	Manget	United States of America
	Nemasys	United Kingdom
	Stealth	United Kingdom
S. riobrave	Vector MG	United States of America
S. scapterisci	Proactant Sc	United States of America
S. kushidai	SDS biotech	Japan
Heterorhabditis megidis	Nemasys	United kingdom
H. bacteriophora	Otinem	United States of America
	E- Nema Gmbh	Germany

room temperature, water-dispersible granule formulations were found prone to microbial contamination. Therefore, antimicrobial and antifungal agents are often added to suppress the growth of these microbes.

Application of nematodes in infected insect cadavers have also been described by some workers (Shapiro-Ilan et al. 2001, 2003), which enables the slow release of nematode and therefore considered effective for small-scale application. Coating the cadavers with starch and clay mixture helps in preventing rupture during storage and shipping (Shapiro-Ilan et al. 2001).

Quality is measured in terms of degree of excellence of a product and quality control is a system of maintaining standards in manufactured products. According to Grewal and Peters (2005) quality of entomopathogenic nematode involves correct identity of species, total number of live nematodes, ratio of live and dead nematodes, matching of host finding behaviour to the target pest, pathogenicity and reproduction ability of nematodes in the target pest, age of the nematodes used, storability, heat tolerance and cold or warm temperature activity. Size and packaging, reliable instructions for the consumers, ease at transportation, absence of contaminants, product cost, availability and field efficacy are the other parameters required for the product quality (Grewal and Peters 2005). Some commercial products of entomopathogenic nematodes prepared in different countries are enlisted (Table 13.5).

13.3.7 Application Technology

Application technology aims at minimum loss during transfer of active ingredient, i.e. entomopathogenic nematodes from the mixing tank to the target insect. Several factors affect the ability to deliver infective juveniles in close proximity to the target

insect for achieving optimal results at the minimal possible cost. Since formulations of entomopathogenic nematodes have live, delicate and tiny organisms, a careful handling is required during its application so that the adverse effects of the surrounding are minimized in order to achieve the desired activity and efficiency. Survival of nematodes during and after application is also an important aspect to be considered. Application of nematodes is mostly targeted to the soil and cryptic habitats of insects (Hussaini 2001). The choice of application equipment, and manner in which the nematodes are applied, can have substantial impact on pest control efficacy (Shapiro-Ilan et al. 2006).While selecting an application system, some points, which need special attention are volume of the sprayer, agitation system, pressure, recycling time, environmental conditions and spray distribution pattern (Shetlar 1999). A high- or low-volume sprayer can be used to dispose the nematodes, but care should be taken that the pressure in the spray tank should not be too high (300 psi or 2,070 kPa); otherwise, it will prove detrimental to the nematodes. Repeated recirculation of the tank mix also decrease viability as the mechanical stress from the pump and nozzles may lead to the rise of temperature in the liquid (Nilsson and Gripwall 1999). Therefore, the best way is to maintain the temperature below 30°C within the pump, tank and nozzles (Grewal 2002) and this can be done by the use of lower-capacity pumps, such as diaphragm or roller pump. When applied in aqueous suspension the water should neither be too hot nor heavily chlorinated. At higher temperature, the solubility of oxygen decreases ultimately making the nematodes inactive. Another important issue is settling or sedimentation. When the density of infective juveniles to be used is 1.05 g/cm^2, it becomes heavier than the water and settles in spray tank (Wright et al. 2005). Infective juveniles larger in size settle faster than the smaller one. Sedimentation results in unequal distribution of nematodes particularly when used under irrigation system. Increasing the viscosity of water by adding carboxymethyl cellulose may reduce the sedimentation speed (Peters and Backes 2003). Above all, the right choice of nematode species or strain for a particular target insect pest is also very important (Shapiro-Ilan et al. 2008).

For soil application, larger capacity hydraulic nozzle is usually recommended. Nozzles with largest orifice create relatively the lowest shear stress on nematodes. Any obstacle such as smaller particles in the spray suspension may partly block the nozzle orifice, leading to a reduction in viability of the nematodes passing through the nozzle (Gwynn et al. 1999). When entomopathogenic nematode is to be applied in soil, pre- and post-application irrigation is usually recommended. This will help in going down the nematode deeper in soil and work efficiently against the target insect. Also the nematodes remain protected from the environmental extremities (Ali et al. 2005b).

Foliar application is also an interesting option, which requires careful handling of the nematodes as well as equipment to be used. Droplet size and spray distribution system are the other two important considerations for foliar application of entomopathogenic nematodes (Grewal 2002). Solid cone nozzle and flat fan nozzle deposit greater number of entomopathogenic nematode on leaves and give higher mortality of target insect (Lello et al. 1996). Addition of adjuvant to spray solution can also

help in increasing the deposition of entomopathogenic nematode on foliage. However, surface application on foliage faces hindrance as entomopathogenic nematodes cannot tolerate the effect of extreme temperature and ultraviolet radiation. Use of antidesiccant to retard evaporation of the nematode suspension on foliage and to prevent desiccation of nematodes has led to a great chance of success (Glazer and Novan 1990). Glycerine 10% has proved to be a more effective adjuvant for increasing survival and activity of nematodes on foliage (Nash and Fox 1969). But high cost of glycerine and risk of phytotoxicity at higher temperature limit its application. A better alternative for an effective protection against these external factors can be achieved by addition of fluorescent brightener and application during the period of moderate temperature and high humidity or late in the evening (Ali et al. 2005b). With some exceptions foliar applications have been less successful than soil applications due to nematode susceptibility to desiccation and ultraviolet rays, however, frequent low-rate applications of nematodes to foliage can result in substantial suppression of green house pests such as thrips (Shapiro-Ilan et al. 2006).

13.3.8 Compatibility with Pesticides

Entomopathogenic nematodes are compatible with many agrochemicals including herbicides, fungicides, acaricides, insecticides and fertilizers, as well as soil amendments (Rovesti and Deseo 1990; Gupta 2003). Infective Juveniles are tolerant to short-term exposures and therefore, can be tank mixed for applying together. Thus, entomopathogenic nematodes can also be included in the integrated pest mvanagement programme. But in several cases, nematode activity and its survival is reduced due to addition of some pesticides (Grewal et al. 1998) and sometimes chemicals used as inert ingredients or adjuvants used in formulation can prove toxic to nematodes (Krishnayya and Grewal 2002). Therefore, compatibility of each formulation with the specific nematode species should be evaluated before final application. There are various pesticides, which act synergistically with entomopathogenic nematodes and improve their efficacy in inundative applications. Easwaramoorthy and Sankaranarayanan (2003) have found that *S. glaseri* is compatible with carbofuran, phorate, quinalphos and aldrin. Compatibility of *S. carpocapsae* with dimethoate, endosulfan, malathion, mancozeb and zineb at recommended dosages have also been reported (Das and Divakar 1987). Gitanjalidevi (2007) conducted an experiment to test the effect on the viability and infectivity of freshly emerged infective juveniles of *Steinernema* sp. and *H. indica* on different formulations of formaldehyde, charcoal and alginate capsule. The result indicated that there was no significant difference in viability in the two nematode species in water + 0.1% formaldehyde + charcoal and water + 0.1% formaldehyde + alginate capsule treatment. The survival of the infective juveniles was highest in the formulation containing 0.1% formaldehyde + alginate capsule, followed by 0.1% formaldehyde + charcoal, for *H. indica and Steinernema* sp. Wang et al. (2007b) evaluated the combined efficacy of chemical pesticides, chlorpyriphos, imidacloprid and entomopathogenic nematode, *S. carpocapsae* against

Rhabdoscelus lineaticollis, a pest of palm and sugarcane. It was found that the mortality of *R. lineaticollis* was highest (88.89%) in the combined treatment of chlorpyriphos, imidacloprid and *S. carpocapsae* as compared to individual application of chlorpyriphos (72%), imidacloprid (25%) and *S. carpocapsae* (27.7–52.6%). Composted manure and urea do not influence *S. carpocapsae* but fresh manure may affect virulence (Shapiro-Ilan et al. 1997). Mahmoud (2007) conducted a laboratory bioassay to determine the potential of combination between *S. feltiae* and botanical insecticides, neem seed kernel extract, NeemAzal T (5%) and Neemix (4.5%) against the third-instar larvae of peach fruit fly, *Bactrocera zonata*. Of 25 treatment combinations between neem seed kernel extract and *S. feltiae,* 18 gave synergistic response, four were additive, none antagonistic and three without any response. Shapiro-Ilan et al. (2004) has reported antagonistic relationship between the fungi *Paecilomyces fumosoroseus* and *H. indica* or *S.carpocapsae*. Rumbos et al. (2007) investigated the effect of PL251, a strain of nematophagous fungi, *Pp. lilacinus* on the survival and virulence of *S. feltiae, H. bacteriophora* and *H. megidis* under controlled conditions. The survival and pathogenicity of all the three nematode species were not affected by PL251 application. In an experiment, *S. carpocapsae* when combined with nucleopolyhedrovirus against the beet armyworm *S. exigua*, caused additive mortality of sp. *exigua* larvae without causing any affect on reproduction of *S. carpocapsae* (Gothama et al. 1995, 1996). *Pasteuria penetrans*, a bacterial pathogen of plant parasitic nematodes did not infect *Steinernema* sp. under laboratory condition (Mohotti et al. 1998; Somasekhar and Mehta 2000). *Heterorhabditis* spp. and *S. glaseri* were also found not causing any infection on earthworm *Eudrilus eugeniae* (Prabhuraj et al. 2000).

13.4 Phasmarhabditis Hermaphrodita

Among the several slug-parasitic nematode species, *Pp. hermaphrodita* is considered to be the most successful capable of killing several slug species, the widespread pest of many agricultural and horticultural crops. In the recent years *Pp. hermaphrodita* has also been exploited as biocontrol agent. Schneider (1859) was the first to describe this nematode associated with the slug *A. ater*. Maupas (1900) established culture of *Pp. hermaphrodita* and maintained it on rotting flesh. Wilson et al. (1993c) patented the use of *Phasmarhabditis* as biological mulluscides on the basis that this nematode is capable of parasitizing and killing a wide range of agricultural and horticultural pest slug species.

13.4.1 Life Cycle

Till now not much extensive studies on *Phasmarhabditis* has been done, however, whatever the little information available indicates that life cycle of this nematode is dependent upon the slug species it encounters. Researchers have described three distinct life cycles of *Phasmarhaditis* sp.

1. Saprobolic – Where the nematodes have been reared on rotting flesh (Maupas 1900), on slug faeces (Tan and Grewal 2001) or on a wide range of bacteria (Wilson et al. 1995). Tan and Grewal (2001a) have the opinion that this nematode can be exploited for long-term inoculative slug control as it can persist in the environment without the living hosts. Recently, Rae et al. (2006) in an experiment found that *Pp. hermaphrodita* strongly attracted to dead slug *Deroceras reticulatum* than the live one, which adds weight to the hypothesis that this nematode is a facultative parasite capable of growing and reproducing on decaying plant and animal materials present in soil.
2. Necromenic – The infective juveniles of *Phasmarhabditis* get entrance into a slug, remain there without further development till the slug dies (Mengert 1953). After this infective juveniles feed on the slug cadaver, develop and reproduce. When the food starts depleting the formation of new infective juveniles takes place. These infective juveniles can be found in the mantle cavity, the general body cavity or the digestive tract of slugs. However, the entrance of nematode into slug and completion of life cycle there is parasitic or necromenic is still not fully understood (Wilson and Grewal 2005).
3. Parasitic life cycle: The infective juveniles enter into slug through the dorsal integumental pouch, through a short canal and reaches into the slug's shell cavity below the mantle (Wilson et al. 1993b; Tan and Grewal 2001). The development and reproduction of nematode takes place inside the slug. The infection in slug causes swelling of the rear half of the mantle where the nematodes reproduce. On an average 250–300 offspring of nematode is produced and once the second generation is produced these offspring spread throughout the slug's body and develop. The slug dies and third-generation nematodes are produced, which feeds on slug cadaver. When the food supply begins to deplete formation of infective juveniles takes place. Although the death of host generally occurs between 4 and 21 days, from the very time after infection the slug feeding is stopped (Glen et al. 2000; Grewal et al. 2001, Grewal et al. 2003).

13.4.2 Nematode–Bacteria Association

The research on the association of slug-parasitic nematode, *Pp. hermaphrodita* with bacteria has not been carried out extensively as like entomopathogenic nematodes; therefore, a meagre information is available on this aspect. Tan and Grewal (2001b) on the basis of an experiment reported that *Pp. hermaphrodita* acts as a vector to transport the bacteria *Moraxella osloensis* into the shell cavity of the grey garden slug, *Derocerus reticulatum*. The infective juveniles of the nematode move through the soil, locate and infect the slug by penetrating through a natural opening at the backside of the mantle. Once inside the body of the host the infective juveniles release bacterial cells, start feeding on multiplying bacteria and develop into self-fertilizing hermaphrodites. This nematode–bacterial complex can cause the death of slug within 7–21 days after infection. Wilson (2002) reported association of

Pp. hermaphrodita with several bacterial isolates. In an experiment highest yield of *Pp. hermaphrodita* was obtained when cultured with the bacteria, *Providencia rettgeri, M. osloensis* (Wilson et al. 1995a) and two isolates of *Pp. fluorescens*. When a bioassay was conducted with these nematode–bacterial isolates against the slug *D. reticulatum* only, *M. osloensis* and *Pp. flourescens* were found to be pathogenic (Wilson et al. 1995b). However, no highly specific mutualistic association of *Pp. hermaphrodita* with bacteria has been found. Wilson and Grewal (2005) is of the opinion that lack of bacterial specificity as a food source as well as lack of a rigid cuticle in slugs indicate that more or less there is a general association of bacteria with *Pp. hermaphrodita*. It has been observed by researchers that the bacteria *M. osloensis* kill slugs only when they are carried by infective juveniles of nematodes (Tan and Grewal 2001b). New infective juveniles carry more viable cells of *M. osloensis* than the older one (Tan and Grewal 2001b). Tan and Grewal (2002) reported that *M. osloensis* produces a heat-stable endotoxin, which consists of a lipopolysaccharide lethal to slugs.

13.4.3 Host Range and Effects

The parasitic behaviour of *Pp. hermaphrodita* against different slug species have been studied by several workers (Wilson et al. 2000; Grewal et al. 2003). A single high dose of nematode, applied to slugs under soil condition caused significant mortality to three different pest families of slugs, i.e. *D. reticulatum*, *D. panormitanum, A. silvaticus, A. distinctus, A. intermedius, A. ater, Tandonia budapestensis* and *T. sowerbyi* (Wilson et al. 1993a). Coupland (1995) reported rapid killing of snails belonging to four species (*Theba pisana, Cernuella virgata, Cochlicella acuta* and *C. barbara*), when exposed to 300 infective juveniles per snail. Wilson et al. (2004) prepared a model to optimize biological control of slug *D. reticulatum* by using the nematode *Pp. hermaphrodita*. In this method the application rate of *Pp. hermaphrodita* was based on slug number per unit area. The accurate estimate of slug population density together with predictive modelling of slug population dynamics exploit the full potential of the model for optimizing the use of *Pp. hermaphrodita* for slug control. Hapca et al. (2007) investigated the response of *Pp. hermaphrodita* to the presence of slug mucus and finally concluded that nematodes exhibit both chemotactic and chemokinetic responses to a signal emanating from slug mucus.

13.4.4 Production and Formulation

Pp. hermaprodita has been grown successfully in xenic culture using solid foam culture and also in deep liquid culture on a flask shaker (Wilson et al. 1993b). An yield of 1 lakh infective juveniles per millilitre has also been achieved as reported by Wilson et al. (1995a). Once maximum yield of infective juveniles are obtained they are concentrated by centrifugation before formulation (Young et al. 2002).

Since 1994, the nematodes are being sold as commercial product under the trade name Nemaslug® (Glen et al. 1994, 1996) prepared by MicroBio Ltd. (now Becker Underwood) and now the sale of this biological molluscicide has increased to many other European countries like France, Germany, Switzerland, the Netherlands, Italy and Ireland. However, the shelf life of this product is very less when compared to other entomopathogenic nematodes such as *Steinernema* sp. or *Heterorhabditis* sp. (Ester and Wilson 2005).

13.4.5 Application Technology

The protocol used for applying slug-parasitic nematodes is more or less the same as for entomopathogenic nematodes such as application of nematodes in the early evening to avoid the ill effects of ultraviolet rays, a light irrigation in the soil immediately after application to save the nematodes from desiccation or application of nematodes in moist or damp soil (if condition prevails) or cultivating the soil immediately after application (Wilson et al. 1996; Hass et al. 1999) in order to remove the nematodes from surface, thus preventing the nematodes from desiccation and ultraviolet rays. The equipments used for application are watering can, knapsack sprayer and tractor-mounted sprayer (Ester and Wilson 2005). Uniform application of nematodes in soil as well as in narrow bands centred on the crop rows in row crops has also been reported (Hass et al. 1999). *Pp. hermaphrodita* can also be applied in combination with metaldehyde bait pallets, even at a very high concentration, thus showing its compatibility with chemical mulluscicide (Wilson et al. 2000).

13.4.6 Effects on Other Organisms

Pp. hermophrodita is considered as a lethal parasite for slugs, however, its affect on non-target organisms has not been extensively studied. Whatever, the information available makes the evidence clear that this nematode is safe for non-target snails, beneficial predators and earthworms. Under laboratory condition, the exposure of two snails, *Cepaea hortensis* and *Monancha cantiana*, to *Pp. hermaphrodita* showed susceptibility in snails, but no effect was found under field condition (Wilson et al. 2000). Morley and Morritt (2006) studied the effect of *Pp. hermaphrodita* upon the two fresh water snails *Lymnaea stagnalis* and *Physa fontialis* at 'spray tank' concentration and a 50% diluted 'spray tank' concentration over a period of 14 days. A significant mortality in *L. stagnalis* was found at both application levels, however, *Pp. fontialis* was unaffected. When bioassay of *Pp. hermaphrodita* was conducted against tenebrionid beetles *Zophoba morio* and *Tenebrio molitor* it was found that the nematodes do not infect either of the two organisms (Wilson et al. 1994). In another experiment under laboratory condition, adults of *Pterostichus melanarius*, the beneficial predatory carabid beetle was not killed

when exposed at a high dose of *Pp. hermaphrodita* (Wilson et al. 1993d). The effect of a commercial formulation of *Pp. hermaphrodita* on the earthworm *Eisenia fetida* was tested. Adults of *E. fetida* were exposed in 1-l glass beakers to *Pp. hermaphrodita* at three different concentrations (1×, 10× and 50× of the field-recommended rate of 3×10^9 billion nematodes/hectare) during a 14-day period in an artificial soil substrate. Also in this experiment injured earthworms with posterior ends removed were exposed to the 10× field-recommended rate of the nematode formulation. The results showed that neither intact nor injured *E. fetida* was susceptible to the nematodes during the 14 days of exposure even at a higher concentration, i.e. 10 and 50 times greater than the label dose (De-Nardo et al. 2004).

13.5 Constraints

The entomopathogenic nematodes, *Steinernema* and *Heterorhabditis*, as well as slug-parasitic nematodes, *Phasmarhabditis* offer the most promise for its commercial development as biocontrol agent. During the past 20 years a significant progress has been made in the development of nematode formulations, however, post-application survival is still a debatable issue. High product cost, limited product availability, lack of suitable production technology for different nematode species, low shelf life in comparison to traditional chemical pesticides and lack of proper technique (how to use) among the users are some hindrance coming in the way, which have still kept nematodes under-utilized in pest management programmes. Also, these beneficial nematodes always need a low temperature (whether formulated or not), which adds an additional expense for producers ultimately making the final cost high. Limited production capacity, poor shelf life and seasonal nature of demand further aggravate the problem.

13.6 Conclusions

In the present context the two basic elements necessary for entomopathogenic nematodes to be successful are (i) a suitable nematode for the target pest, and (ii) favourable economics for its commercialization. For sustainable agriculture an integrated approach of all the methods are required to obtain maximum effect without interfering with the effectiveness of other practices. Since entomopathogenic nematodes can interact synergistically with several chemicals and bioagents a combination of multiple tactics should be prepared to achieve a satisfactory result. In the recent years some progress has been made in developing application technologies, however, further improvements are still needed to make entomopathogenic nematodes compete with other insecticides. Increase in shelf life of nematodes, improvement in transport logistic and marketing will substitute insecticides and contribute to stabilize agriculture environments and crop yields.

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