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Novelties in Pest Control by Entomopathogenic and Mollusc-Parasitic Nematodes

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Additional information is available at the end of the chapter

Abstract

Entomopathogenic and molluscoparasitic nematodes are important parasites of many insects and molluscs, respectively. Due to their infectivity, the possibility of mass production by industrial techniques and the relative safety to nontarget organisms and environment, these organisms represent an attractive agent for biological control of many pests. This chapter summarises the current knowledge of the diversity of these organisms. In this chapter, we review the recent advances in production, storage, application techniques genetic improvement and safety of these organisms.

Keywords: nematodes, entomopathogenic, molluscoparasitic, *Steinernema*, *Heterorhabditis*, *Phasmarhabditis*, diversity, occurrence, rearing, application, safety

1. Introduction

Entomopathogenic nematodes (EPNs) in the families of *Steinernematidae* and *Heterorhabditidae* are important parasites of many insect species. Due to their ability to infect various insects, the possibility of mass production by industrial techniques and the relative safety to nontarget organisms and environment, EPNs represent an attractive agent for biological control of many insect pests.

Over the past decade, a large number of new EPN species have been described from throughout the world. New lineages present a unique combination of characteristics and thus have a great potential for biological control of particular insect pests.

Mollusc-parasitic nematodes (MPNs) represent a taxonomically more diverse group, consisting of members of seven families (Agfidae, Alaninematidae, Alloionematidae, Angiostomati-

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dae, Cosmocercidae, Diplogasteridae, Mermithidae and Rhabditidae). However, to date, only *Phasmarhabditis hermaphrodita* (Rhabditidae) has been commercialised. It is likely that other mollusc-parasitic nematodes have a potential to provide new bio-agents for slug and snail control. MPN biology is mostly unknown, but recently published descriptions of several new species provided at least some notes about MPN biology.

This chapter provides thorough information about the diversity and biology of EPNs and MPNs. We also focus on the recent advances in production, storage and application techniques.

2. Overview of EPN and MPN biology and diversity

2.1. Diversity of entomopathogenic nematodes

EPNs are common in all types of soils and more frequently inhabit agricultural and secondary forest ecosystems, which represent suitable conditions for insect host populations. These organisms have a worldwide distribution [1]. Over the past few decades, numerous surveys were performed mainly in Europe [2] and North America [3]. However, recently a huge effort for the study of EPNs field occurrence was recorded from other continents of all zoogeo-graphical regions. Results of these surveys increased rapidly a number of new described species, especially from South Africa, Ethiopian region [4], Southeast Asia, Indo-Malaysian region [5] and tropical areas in Neotropical region [6]. Over the past decade, regularly used DNA analysis facilitated discrimination of the morphologically almost identical sibling species.
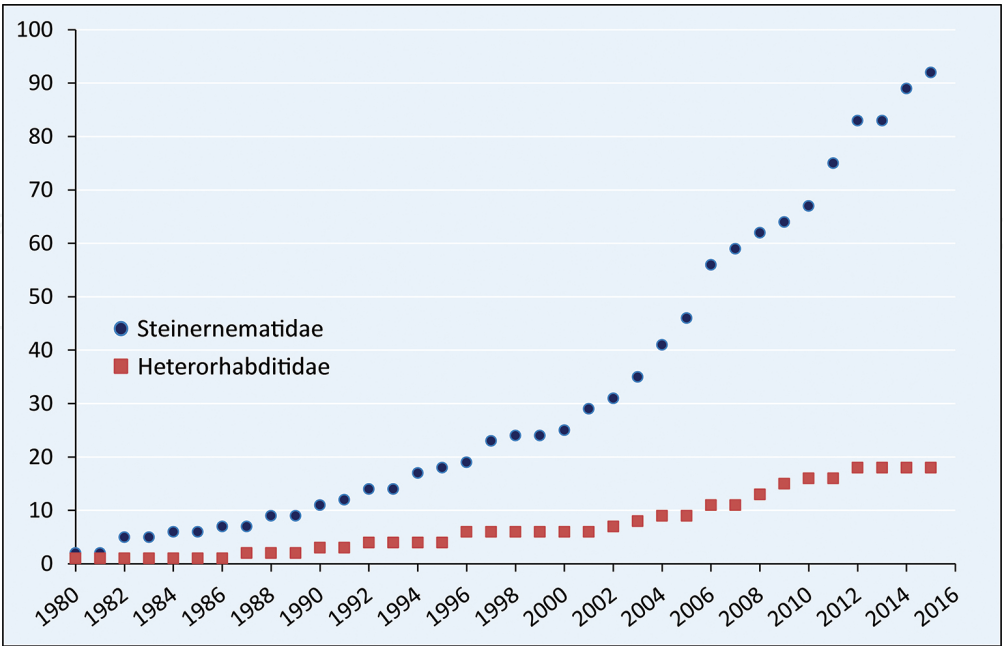

Figure 1. The increasing number of recognised steinernematid and heterorhabditid species based on published data.

This led to the tremendous increase in the known EPN diversity. From the year 2000, the number of the described steinernematids and heterorhabditids more than tripled from 25 to 92 and from 6 to 18, respectively (**Figure 1**). Understanding of EPNs diversity should be considered as a basic requirement for a successful field control of noxious insects.

It is generally accepted that steinernematids are more common in cooler, temperate zone whereas heterorhabditids prefer warmer, tropical and subtropical conditions (torrid zone) [7]. Geographically, the torrid zone lies between the Tropic of Capricorn and the Tropic of Cancer parched with heat. In this zone, many new species have been recently detected from Vietnam and southern China. Temperate zones contain the areas or regions between the tropic of Capricorn and the Antarctic circle or between the tropic of Cancer and the arctic circle, having a moderate climate. According to the number of described species, this zone seems to be the richest for the EPNs occurrence. Frigid zones represent the areas or regions between the Antarctic circle and the south pole or between the arctic circle and north pole, intensively cold, have probably a low EPNs occurrence represented only by several findings. *Steinernema kraussei* and recently *Steinernema affine* are the only species with a link to the frigid zone [8, 9].

Continent	*Steinernema/ Heterorhabditis*	From 2010	Species
Africa	16/6	11	*S. citrae, S. cameroonense, S. ethiopiense, S. nyetense, S. sacchari, S. innovationi, S. tophus, S. jeffreyense, S. fabii, S. pwaniensis, H. noenieputensis*
Asia	52/5	14	*S. minutum, S. nepalense, S. surkhetense, S. everestense, S. lamjungense, S. pui, S. changbaiense, S. tielingense, S. xinbinense, S. dharanaii, S. bifurcatum, S. huense, S. balochiense, H. beicherriana*
Australia	3/4	0	–
Europe	16/4	3	*S. schliemanni, S. vulcanicum, S. poinari*
North and Central America	15/9	1	*S. phyllophagae*
South America	15/4	5	*S. brazilense, S, unicronum, S. papillatum, S. goweni, H. atacamensis*

Table 1. Number of steinernematid and heterorhabditid species by continent and number and identity of the EPN species described from each continent since the year 2010 based on published data.

The highest species diversity of the genus *Steinernema* is found in the Asian continent, with 52 recorded species, whereas the area of North and Central America has the highest number of heterorhabditids with 9 recorded species (**Table 1**). The Asian and African continents are also the areas with the fastest growing numbers of the described EPNs with 11 and 14 described EPNs since the year 2010. Europe has the longest tradition of EPN research and is the most extensively and intensively sampled continent. Despite this fact, three new steinernematids have been recovered in the past 5 years. This fact suggests that we are likely to see much more new EPNs to be described from other continents in the future.

2.1.1. Geographic distribution of EPN species

Several EPNs are known to have a cosmopolitan occurrence, such as *S. kraussei, Steinernema glaseri, Steinernema feltiae, Steinernema carpocapsae, Heterorhabditis bacteriophora, Heterorhabditis indica, Heterorhabditis megidis* and *Heterorhabditis zealandica* (**Table 2**). Among them, we can distinguish those that prefer temperate or torrid zone, or occur in both these zones. Of these, *S. kraussei* is a Holoarctic species and its recovery from Neotropic in Colombia is a unique observation or doubtful result [10]. Similarly, *S. glaseri* and *S. carpocapsae* inhabit preferably Holoarctic temperate zone with links to torrid zone in Indo-Malaysian (India/Tamil Nadu) and Neoarctic (SE USA) regions [5, 11]. *S. feltiae* seems to be the best adapted species inhabiting all continents, warm and cool areas and wide spectrum of habitats. Surely, this is the most common steinernematid in Holoarctic and Neotropic and Australian regions, recently found also in Indo-Malaysian [12] and Afrotropical [13] regions. *H. bacteriophora*, originally described from Australian region, is the most widespread heterorhabditid. The nematode occurs in all zoogeographical regions including both torrid and temperate zones. *H. indica* is the nematode widespread over the torrid zone in tropical and subtropical areas of all zoogeographical regions, whereas *H. megidis* has been discovered only in temperate zone of Holoarctic. An interesting distribution is reported for *H. zealandica*, originally described from New Zealand, later found in the northeastern Europe. This species was recently reported also from northeastern China [14], Florida [15] and, surprisingly, also from South Africa [4].

Species	Distribution
S. carpocapsae	Worldwide, all continents
S. feltiae	Worldwide, all continents
H. bacteriophora	Worldwide, all continents
H. indica	Worldwide, all continents
H. zealandica	Worldwide, Australia, Africa, North America, Europe
S. glaseri	Holarctic—USA, Argentina, Azores, China, Korea and Spain
S. affine	Holarctic—Europe, Russia, Canada
S. kraussei	Holarctic—Europe, Russia, Canada
S. arenarium	Palearctic—Europe, Russia
S. intermedium	Palearctic—USA, Europe
S. poinari	Palearctic—Europe, Russia
H. megidis	Palearctic—North America, Asia, Europe
S. abassi	Northern Africa, India
S. weiseri	Central and Northern Europe, Turkey
S. yirgalemense	Central and Southern Africa
S. silvaticum	Central and Northern Europe, United Kingdom

Table 2. Entomopathogenic nematode species with a large geographic range and their distribution.

Bacterium	Nematode
X. bovienii	S. affine, S. jollieti, S. feltiae, S. cholashanense, S. ichnusae, S. intermedium, S. kraussei, S. sichuanense, S. weiseri, S. xueshanense
X. budapestensis	S. bicornutum, S. ceratophorum
X. beddingi	Unknown
X. cabanillasii	S. riobrave
X. doucetiae	S. diaprepesi
X. ehlersii	S. longicaudum
X. griffiniae	S. hermaphroditum
X. hominickii	S. karii, S. monticolum
X. indica	S. yirgalemense, S. abassi
X. innexi	S. scapterisci
X. ishibashi	S. aciari
X. japonica	S. kushidai
X. khoisanae	S. khoisanae, S. pwaniensis
X. koppenhoeferi	S. scarabei
X. kozodoii	S. apuliae, S. arenarium
X. magdalenensis	S. australe
X. mauleonii	Unknown
x. miraniensis	Unknown
X. nematophila	S. carpocapsae
X. poinarii	S. cubanum, S. glaseri
X. romanii	S. puertoricense
X. stockiae	S. huense, S. minutum, S. siamkayai
X. szentirmaii	S. rarum
X. vietnamensis	S. sangi
P. asymbiotica	H. gerrardi, H. indica
P. heterorhabditis	H. zealandica
P. luminescens	H. bacteriophora, H. georgiana, H. noenieputensis, H. sonorensis, H. indica
P. temperata	H. bacteriophora, H. downesi, H. georgiana, H. megidis

Table 3. Taxonomic correspondence of symbiotic bacteria of the genera *Xenorhabdus* and *Photorhabdus* to host entomopathogenic nematodes.

In contrast to ubiquitous species, the known geographic distribution of a majority of EPN species is much narrower, and some species are known just from a single country and even from a specific locality. This applies, for instance to *Steinernema vulcanicum*, to date found only

in Italian island of Sicily [16]. However, at least in some species, their known geographic range will probably expand with more data available in the future.

It is likely, that at least in some ubiquitous EPNs, their geographical distribution was recently enhanced by a human activity. This seems to be the case of *S. affine* that was known to occur throughout the Europe, and it was believed to have a Palearctic distribution. However, in 2005, it was recovered in British Columbia [17], North America. Such geographic distribution could be either due to its historically forming a disjunctive range of the species in a Holoarctic distribution or, and more likely, *S. affine* has been introduced into Greater Vancouver by immigrants and/or by imported commercial produce, such as potatoes (*Solanum tuberosum*), flower bulbs and other agriculture plants transported from Europe. Following its arrival, it then spread over the Greater Vancouver coastal area. In other species, it is often impossible to imply, whether they are indigenous to a given locality. It can be however assumed, that indigenous species should be considered only those isolated in natural, climax, ecosystems, for example, *Steinernema brazilense* [18].

Unfortunately, the data on EPN diversity is partly influenced by the wrong identification of certain species. For instance, some EPNs originally described from South and North America, such as *Steinernema ritteri*, *Steinernema rarum*, *Steinernema scapterisci* and *Steinernema riobrave*, were later reported from Northeast China [14], which is at least doubtful. There are more species with reportedly very disjunctive distribution, overlapping different zoogeographical regions such as *Steinernema bicornutum* described from Serbia but later reported from Jamaica [19].

2.1.2. Habitat preference

EPNs inhabit most terrestrial habitats, but their occurrence has been evaluated mainly in relation to soil type and habitat [20]. Interestingly, heterorhabditids were equally abundant in turf and weedy habitats, but never found in closed-canopy forest [21]. In Germany, the rate of prevalence of steinernematids was highest in woodland (50.3%) where *S. affine*, *S. feltiae*, *Steinernema intermedium* and *Steinernema silvaticum* (=*Steinernema* sp. B) were the predominant species [22]. This fact can be explained by the higher insect host occurrence in woodland habitats in comparison with the usually poor field ecosystems. Many field studies solved an impact of various abiotic factors for EPNs recovery, survival etc. The EPN occurrence in Spain was evaluated through abundance, recovery frequency, larval mortality percentage and EPN population density. EPNs occurrence was also related to the selected soil physical and chemical variables as well as to some soil pollutants such as heavy metals and organochlorine pesticide residues. These factors help to understand how EPNs survive and disperse [23], but as usually no data were published about natural insect hosts. Recently, ten species of *Steinernematidae* including three undescribed and three species of Heterorhabditidae confirmed a rich EPNs fauna in northern China. Their occurrence was strongly associated to the prevailing climatic conditions, altitude, vegetation and soil types [24].

In general, the essential condition for the EPN occurrence and survival associates with biotic factors. Different species of EPNs occur in numerous habitats/ecosystems depending prefera-bly on their insect host. It was demonstrated that at least some steinernematids show a distinct

habitat preference that may reflect the distribution of suitable hosts, which are adapted for the habitat [7]. Even though, these nematodes are ubiquitous, their recovery from the field is influenced by a number of biotic factors, including nematode antagonists and host range that is dependent on the suitability for penetration of different insect hosts by nematodes, possibility of finding a suitable host in the habitats (e.g., leaf-feeding insects cannot be readily attacked in the natural habitat), and by the natural population density [25]. Till present, the impact of insect hosts has been, unfortunately, mostly overlooked. Insect aggregations and outbreaks of insect pests are a great opportunity to study EPN diversity and habitat preferences. Mráček and Bečvář [26] emphasised an essential impact of host aggregations on the incidence of EPNs. In their experiments, the high percentage, about 70%, of sampling sites with insect aggregations were nematode positive. Similarly, final mortality of the fly larvae and pupae from the bibionid (*Bibio marci*) nest aggregation caused by *S. intermedium* achieved 90% [27]. Even though, occurrence of suitable insect hosts in habitats seems to be elementary for the incidence of EPNs, at least some species are behaviourally adapted for different types of soil and habitat. In general, heterorhabditids prevail in light, sandy soils whereas soil type is less important for steinernematids and *S. kraussei*, *S. intermedium* and *S. silvaticum* are abundant species in forest habitats.

Competition between EPN species can also have an impact on their distribution. It was shown that even though *Heterorhabditis* and *Steinernema* can co-infect the host, they cannot coexist and one genus will prevail [28]. Two steinernematid species, on the other hand, can co-infect and reproduce within one host cadaver [29]; however, one or both species are often negatively affected by competition [30]. In British Columbia, Canada field sampling identified *S. affine* occurring together with *S. kraussei* at two sites [17]. In the field, the *Galleria* baiting and consequent laboratory experiments, *S. affine* appeared to be a more successful parasite than *S. kraussei*. When *Galleria mellonella* larvae were co-infected by *S. carpocapsae* and *S. glaseri*, the proportion of established females was reduced in cadavers and the progeny of *S. glaseri* was less affected by the mixed infection than that of *S. carpocapsae* [29]. Similarly, the interactions of two sympatric entomopathogenic nematodes, *S. affine* and *S. kraussei*, were studied in a series of laboratory experiments [30]. In the co-infections, *S. kraussei* was strongly negatively affected while *S. affine* was able to multiply in a higher number of hosts in comparison to single infection and it was also able to invade and multiply in hosts already infected and even killed by *S. kraussei* and it produced a normal amount of progeny. The field study in the original locality [31], however, found no spatial relationship between the two species, and no evidence suggesting any host differentiation between the two species was found. Authors assumed that both species share an ecological niche, and thus the avoidance of competition with the latter species seems to be a crucial factor for *S. kraussei*. Patchy distribution and implicit differences in horizontal distribution probably markedly contribute to the coexistence of both species.

2.1.3. Methods used for the study of EPN diversity

The outcome of the studies of EPN occurrence can be influenced by the method of isolation. A total of 40 soil samples from various habitats in Germany and the Czech Republic were studied for the presence of entomopathogenic nematodes using the *Galleria* baiting and a

sieving-decanting method for direct extraction of infective-stage juveniles [32]. All these species were recovered with both methods, but the baiting technique was generally less effective, and mixtures of several species in one soil sample were frequently undetected. The direct extraction method provided quantitative estimates of infective stage juvenile density, but no information on their infectivity or on morphological characters of adults and nematode cultures could be established. However, *Galleria* baiting could be negatively influenced by EPNs competition when one species' infective activity can be suppressed by another one [17, 31]. Besides these classical baiting methods, the quantitative real-time PCR (qPCR) techniques have been recently used to provide accurate and reliable methods to identify and quantify cryptic organisms in soil ecology [33]. By this method, six species of EPNs were recovered in Florida citrus (*Citrus* spp.) orchards (*S. glaseri, Steinernema diaprepesi, S. riobrave, H. indica, H. zealandica, Heterorhabditis floridensis* and an undescribed species in the *S. glaseri* group). The qPCR assay was more efficient than the *Galleria* baiting method for detecting the EPN species composition in species mixtures and represents a new challenge for the EPNs biodiversity studies. The classical *Galleria* baiting method uses larvae of the greater wax moth (*G. mellonella*) that are placed to the soil sample and invaded by EPN infective larvae. However, this method can miss inactive or competitively weak EPNs. In the qPCR method, the total DNA is extracted from the soil sample or the infected *Galleria* larva, and EPN species are detected and quantified by qPCR with species-specific probes.

2.1.4. Symbiotic bacteria

Entomopathogenic nematodes (EPNs) of the families *Steinernematidae* and *Heterorhabditidae* are mutualistically associated with specific symbiotic bacteria of the genus *Xenorhabdus* and *Photorhabdus*, respectively [34]. The relationship is obligate in natural environment [35]. Besides providing the food source to the nematodes, bacteria also protect cadaver against other microorganisms by production of bacteriocins, antibiotics and antimicrobials [36, 37] and against insect scavengers [38].

Single species of *Steinernema* may be associated with only one species of *Xenorhabdus*. The same applies to *Heterorhabditis* with the exception of *H. bacteriophora* that is associated either with *Photorhabdus luminescens* or with *Photorhabdus temperata*. On the other hand, species of *Photorhabdus* and certain species of *Xenorhabdus* are hosted by several species of *Heterorhabditis* and *Steinernema* (**Table 3**).

2.2. Mollusc-parasitic nematodes (MPNs)

Nematode parasites of molluscs (mollusc-parasitic nematodes) can be found in several families (e.g., Alloionematidae, Cosmocercidae, Mermithidae and Rhabditidae). Of these, only one species, *P. hermaphrodita* (Rhabditidae), has been commercialised. However, several other mollusc-parasitic nematodes might have a high potential to provide new bio-agents for harmful molluscs control. In the following section, we give an overview of the biology and diversity of MPNs.

Similarly to entomopathogenic nematodes, most of MPNs spend a part of their life cycle in the soil environment. Nematodes that infect host in the soil need some mechanism how to find an appropriate host. Soil dwelling invertebrates movement is usually slow, but still too fast for the nematodes and thus, during their evolution, parasitic nematodes developed useful adaptations. As known from EPNs, also *P. hermaphrodita* [39, 40] but very probably also many other nematodes, readily react to host-associated cues. This can be CO_2 or other volatile compounds produced by the living host, its faeces, mucus, etc. Parasitic nematodes react very strongly to all of them, not only to alive host. Of course it is not surprising statement, we know that *P. hermaphrodita* or other MPNs are able to complete their life cycles on slug faeces and other organic matter [41–43]. This type of behaviour provides the nematode also other advantage. Molluscs show a homing behaviour. They use the same shelters every day or night, and usually they cover this place by a big amount of mucus and faeces very soon. Therefore, the nematodes that readily react to these cues gain an advantage and increase their chance to meet the suitable host. Interesting finding is that *P. hermaphrodita* can strongly react not only to water soluble cues as most of other nematodes do but also to volatile cues [40], which can be related with its habitat, soil surface and organic matter, which is inhabited by its hosts.

Unlike EPNs, some MPNs are able to complete their life cycles in different organic matter in the soil. Naturally, the quality of the growing substrate affects nematode development, however, unlike in EPNs, the quality of the growing substrate is mostly expressed in the yield of dauer juveniles and not in the quality of progeny [42]. On the other hand, in EPNs, the substrate quality influences both yield and quality of IJs [44, 45]. The reason for this difference could be that while EPNs are the true parasites, MPNs retain both parasitic and free-living life cycles, and the ability to produce full quality dauer juveniles in a wide range of conditions is an essential advantage that helps them to survive in various changing environments.

2.2.1. Alloionematidae

Family Alloionematidae consists of three genera: *Alloionema* (with only one species: *A. appendiculatum*), *Neoalloionema* and *Rhabditophanes*. *Alloionema appendiculatum* is a common larval parasite of many terrestrial molluscs that was described from the body of slug *Arion ater* [46]. This nematode retains both parasitic and free-living life cycle [41]. Its dauer juveniles (third-stage larvae) invade foot muscle of snails and slugs and after moulting into the fourth-stage larvae, they stay encysted inside the host muscle. These fourth-stage larvae are able to leave slugs to mature and reproduce in the soil (parasitic generation). The progeny of the parasitic generation makes a free-living saprophytic generation that can live in a suitable organic material for very long time, at least 4 years in laboratory conditions [43]. Development of the saprophytic form is fast and the whole life cycle is completed within 72 or 96 h. When the source of food is depleted, new DJs are produced and spread in the soil to infect new hosts. All stages of both saprophytic and parasitic generations are bacteriophagous and freely associated with many bacteria, for example, *Acinetobacters* sp., *Pseudomonas* sp. and *Neisseria* sp. [43]. *A. appendiculatum* parasites in molluscs belonging to the families Agriolimacidae, Arionidae, Helicidae, Hygromiidae and Succineidae and its prevalence ranges from less than 0.01% in *Cantareus aspersus* up to 100% in some arionid slugs [43, 47].

2.2.2. Cosmocercidae

The nematodes in the family Cosmocercidae are usually parasites of reptiles and amphibians, but two genera are known as mollusc-parasites, namely *Nemhelix* and *Cosmocercoides*. *Cosmocercoides dukae* parasites in pallial cavity of many North American slugs and snails. *Nemhelix bakeri* and some other species of the genus parasite in reproductive organs of European helicid snails. Under natural conditions, *N. bakeri* is frequently associated with *Helix aspersa*. This nematode lives and reproduces in genital tract of its host. Infection of the new host by *N. bakeri* occurs only during mating, when the parasite is exchanged along with the spermatophores [48]. It means that juvenile molluscs are always free of infection. *N. bakeri* reduces the fecundity of their hosts [49] but their potential for mollusc biocontrol is still questionable.

2.2.3. Mermithidae

Mermithids are frequent parasites of many invertebrates in aquatic and terrestrial habitats, for example, *Romanomermis culicivorax* parasiting in mosquito larvae or *Mermis nigrescens* that is quite frequent parasite of grasshoppers and molluscs [50]. Mermithids are commonly found in mollusc hosts, but it seems that they use molluscs only as facultative hosts [47]. *M. nigrescens* has parasitic larvae and free-living adults that lay eggs on plants, especially on leaves, usually early in the morning or in the night, when there is high humidity. Eggs that are very resistant to dry conditions and UV radiation are able to persist on plants for the whole season. When the eggs are eaten by the suitable host, invasive larvae hatch and penetrate into the haemocoel through the gut wall and develop for several weeks. 'Grown up' larva leaves the host by penetrating its body wall. Host is usually infected with some pathogens through the opening and dies shortly afterward. Emerged larvae develop into post parasites in the soil and adults mate later. The whole development in the soil can take several months, and therefore the whole life cycle can take more than 1 year.

2.2.4. Rhabditidae

Rhabditidae is a large family consisting of many bacteriophagous free-living, phoretic and parasitic nematodes that are often associated with insects or terrestrial molluscs (e.g., *Rhabditis*, *Caenorhabditis* or *Phasmarhabditis*) and other invertebrates. The slug parasitic nematode *P. hermaphrodita* (Schneider) Andrassy is almost cosmopolitan species capable of infecting many slug and snail species, such as Arionidae, Agriolimacidae or Limacidae. The dauer juveniles (DJs) infect slugs in the area beneath the mantle surrounding the shell. They usually cause a disease with characteristic symptoms, particularly a swelling of the mantle. The infection often leads to the death of the slug, within 1–3 weeks. New DJs, which are released from the host cadaver, spread into the soil and look for new hosts [51]. Apart from the parasitic cycle, *P. hermaphrodita* also has a necromenic life cycle [41] and has been shown to reproduce on dead earthworms [52], leaf litter [53] and slugs or slug faeces [40]. *P. hermaphrodita* does not live in a strict association with only one species of bacteria as EPNs do, but is associated with many bacterial species [54, 55] that are common in its habitat. Bacterial species are responsible for the pathogenicity of nematode-bacteria complex towards their hosts [56].

3. Mass production

3.1. Entomopathogenic nematodes

An excellent review of the current situation regarding mass production of EPNs was published by Shapiro-Ilan et al. [57]. Therefore, in this chapter, we give only a short overview of the used methods.

The most simple method for EPN production is *in vivo* method, using living insects, mostly the greater wax moth (*Galleria mellonella*) larvae, or mealworms (*Tenebrio molitor*) that are both very susceptible to EPN infection, and their bodies contain enough nutrients for EPN reproduction. This method is simple and cheap, but is labour and cost-effective only at a small scale, and it is therefore appropriate for laboratory use or small-scale applications [58].

For large-scale production, solid or liquid fermentation *in vitro* technologies must be used. At first, EPNs were cultured axenically both in solid [59] and liquid media [60]. Nowadays, the nematodes are always cultured monoxenically to ensure quality consistency and predictability [61]. A symbiont is extracted from the nematodes, and subsequently sterile nematode eggs are applied to the medium pre-inoculated with bacterial symbiont.

EPN production in solid culture is usually performed in a three-dimensional rearing system with the liquid medium mixed with an inert carrier (e.g., pieces of polyurethane foam). Media were initially based on animal products (e.g., pig kidney) but were later improved by including various other ingredients (e.g., eggs, soy flour, peptone and yeast extract). The culture starts with the inoculation of the sterilised medium with bacteria followed by the nematodes. Nematodes are then harvested within 2–5 weeks by placing the foam onto sieves immersed in water. Only a few companies currently use this approach. A Chinese company Guangzhou Greenfine Biotechnology uses a solid culture method to produce several EPN species both for Chinese and international markets [57]. Other companies using this approach are Bionema (www.bionema.com), Andermatt Biocontrol AG (www.biocontrol.ch) and BioLogic USA (www.biologicco.com).

The *in vitro* liquid culture method is a complex process requiring medium development, understanding of the biology of the nematode-bacteria complex, the development of bioreactors and understanding and control of the process parameters. The process takes place in large bioreactors (up to 100.000 l). It is necessary to supply enough oxygen and prevent excessive shearing of the nematodes. Once the culture is completed, nematodes can be removed from the medium through centrifugation. This method is currently the most cost-effective [58], and thus the majority of EPN products result from liquid culture. Major producers using this method are BASF, Germany (www.agro.basf.com), E-Nema GmbH, Germany (www.e-nema.de), Koppert B.V., The Netherlands (www.koppert.com) etc.

3.2. Molluscoparasitic nematodes

In slug parasitic nematodes, there are two species that can be easily produced in a large scale, *P. hermaphrodita* and *A. appendiculatum*. The former is commercially produced as biocontrol

agents while the later only for scientific purpose. *A. appendiculatum* can be easily produced on homogenised pig kidneys placed agar plates [62], but this nematode can be also mass produced in a solid Bedding medium [63] with a slight modification.

In vitro methods for mass production of *P. hermaphrodita* were developed in 1990s by Wilson [51]. Wilson showed that *P. hermaphrodita* can grow in a xenic culture in solid foam chip according to Bedding [63] and also in liquid cultures. Actually this species is the only commercially produced MPN. Technology used for producing of *P. hermaphrodita* is a modified method used for mass production of EPNs. The nematodes are produced in air-lift fermenters, up to 20,000 l or more in the balanced medium that allows yielding about 100,000 dauer juveniles in 1 ml of the medium. When the maximum yield is obtained, nematodes are concentrated by centrifuged. *P. hermaphrodita* is currently produced by BASF company (www.agro.basf.com) under the trademark Nemaslug©.

4. Formulation and application

4.1. Formulation of entomopathogenic nematodes

Entomopathogenic nematodes are always applied as infective juveniles and are mainly used for controlling the larval or pupal stages of insect pests in the soil or cryptic habitats. Under specific conditions, EPNs can successfully suppress also foliar pests [64].

EPNs have been classically applied in the form of aqueous suspension using sprayers, mist blowers, or irrigation systems. This approach turned out to have several limitations, mainly due to the sensitivity of the nematodes to desiccation and UV radiation [65]. For this reason, several alternatives improving formulation and application have been proposed and established.

4.1.1. Cadaver application

Insect cadaver application [66] has been proposed as a method enhancing EPN persistence. In this method, EPNs are applied in the infected insect host cadaver directly to the target site, and pest control is achieved by the infective juveniles that emerge from the host cadavers.

Insect cadaver application proved to be superior in EPN infectivity, survival, dispersal and pest control efficacy in some instances [67, 68]. EPN delivery can be further improved by formulating the infected hosts in coatings [69]. The cadaver application method has so far only been used commercially on a small scale relative to conventional methods [70], and it is especially useful for small- and medium-sized growers due to easier application and reduced storage costs [71].

Recently, the use of live insect hosts pre-infected with entomopathogenic nematodes against insect pests living in cryptic habitats was tested [72]. In this study, the release of the pre-infected lawn caterpillar, *Spodoptera cilium* (Lepidoptera: Noctuidae) against *S. cilium* in Bermudagrass arenas was as equally successful as standard aqueous application. The use of pre-infected *G.*

mellonella against the goat moth *Cossus cossus* (Lepidoptera: Cossidae) in chestnut (*Castanea sativa*) logs was much more efficient in comparison to the standard aqueous application. This novel approach thus showed an immense potential to control insect pests living in hard-to-reach cryptic habitats.

4.1.2. Capsules

Formulation of EPNs in polymer-based capsules can protect EPNs from desiccation and UV radiation and from biotic stressors such as their natural enemies. This approach was first used with *S. feltiae* and *H. bacteriophora* that were encapsulated in calcium alginate and fed to larvae of *Spodoptera exigua* [73]. In the following study, [74] tomato seeds were placed into the alginate matrix containing nematodes. When the seed germinated, the nematodes escaped from the capsule and could infect the host.

The higher efficiency can be further achieved by addition of another compatible pesticide [58]. Also, the recently proposed 'lure and kill' approach based on the application of the nematodes in capsules with insect attractant may reduce the number of nematodes necessary to control the insect pest as has been shown [75]. These authors developed alginate capsules containing EPNs and buried them in the rhizosphere of maize (*Zea mays*). The addition of attractants and feeding stimulants to the shell attracted the pest larvae as much as maize roots and in field trials, encapsulated *H. bacteriophora* nematodes were more effective in comparison to the nematodes applied in the aqueous suspension on the soil surface. Further studies improve capsule properties in order to increase EPN retainment within the capsules [76].

4.1.3. Shelf life

Besides aforementioned cadaver and gel formulations, EPNs are formulated in water-dispersible granules, nematode wool, gels, vermiculite, clay, peat, sponge, etc. The formulation, together with nematode species, strongly affects the shelf life of the EPN-based products. Actively moving nematodes are metabolically very active and use energy reserves soon [77]. Thus, they can remain alive and infective for 1–6 months under refrigeration ranges. EPNs with reduced mobility (formulations in gels) are still infective after up to 9 months of storage, whereas EPNs formulated in partial anhydrobiosis (formulations in water soluble powders) remain so for up to 1 year.

The root exudates were revealed to induce quiescence in EPNs that is reversible after placing the IJs in soil with high water content [78]. This approach could be used to prolong the shelf life of beneficial entomopathogenic nematodes (EPNs).

4.2. Formulation of molluscoparasitic nematodes

As was mentioned in the previous subchapter, the only commercial product based on MPNs (*P. hermaphrodita*) is Nemaslug© (BASF). Experiments with other nematodes species as bio-agents, for example, *A. appendiculatum* [42, 79] and some other rhabditids [80] were already done, but the results and the potential of these nematodes for the use in bio-control are still questionable and too far from practical impact.

General recommendation is to apply *P. hermaphrodita* on wet soil in the dose of 300,000 DJs/m^2 and water the soil immediately after application. The optimal application time is early evening when the soil temperature is about 15°C. Nematode efficacy can be increased by cultivation of soil just after application [81]. Nematodes are protected against UV radiation and drying. Nozzles and filters should have holes at least 1 mm wide, and the pressure should not exceed 5 bar. It is good to avoid application of *P. hermaphrodita* in the areas that were treated with some toxic chemicals, for example, pellets based on methiocarb used against noxious slugs. Combination with metaldehyde is safe for nematodes, because this compound does not affect them in concentration recommended for field application [82].

There are various strategies for the application. Common strategy is to apply the nematodes over the whole soil surface, and the alternative strategies are based on local applications. Slugs, *Deroceras reticulatum* and others, tend to avoid places treated with *P. hermaphrodita* [83]. Therefore, there were some ideas to apply the nematodes only around individual plants or in bands centred on plant rows. Unfortunately, the assumption of protecting plants using this approach with a lower amount of nematodes was not confirmed. There is no significant benefit associated with band or local application as opposed to uniform application [84]. The number of DJs decrease in time and the repellent effect to slugs subsides. The method of the reduction of the dose of nematodes but without lowering of the efficacy against slugs was published by Grewal et al. [85]. The principle is to apply nematodes in dose 0.6×10^6/m^2 only under artificial shelters that are used by slugs during day. This method provides almost the same effect as uniform application of 0.3×10^6/m^2. Highly effective can be repeated application of lower than recommended dose. In Brussels sprouts (*Brassica oleracea*) application of 50,000 DJs/m^2 is three times repeated in 1-month interval. It represents 50% reduction of the previously recommended single application, while the efficacy is almost the same as in case of using metaldehyde pellets [86].

P. hermaphrodita is applied in many plants in greenhouses, vegetables, ornamentals and in arable crops, for example, *Cymbidium* sp., lettuce, cabbage, Brussels sprouts, *Asparagus* sp., oilseed rape, wheat or sugarbeet and many other crops. The most common target pest are *Deroceras* sp. and *Arion* sp. Repeated uniform application on the soil surface is usual, but *P. hermaphrodita* can also be applied in the plastic tunnels or pots used in greenhouses. In arable crops, the nematodes have future especially in organic farms.

P. hermaphrodita is formulated in, for example, vermiculite [87] that slightly dehydrate and immobilise nematodes that can save energy more effectively in this state. Formulated nematodes are packed into polyethylene bags that allow exchange of air but retain water. The final product can be stored in a refrigerator for up to six months [47].

4.3. Genetic improvement

Genetic improvement has been an important contributor to the enormous advances in productivity that have been achieved over the past 50 years in plant and animal species that are of agricultural importance [88]. For entomopathogenic nematodes, main target characteristics are virulence, host range, heat and desiccation tolerance and shelf life. Glazer [89] summarised the four potential genetic-manipulation strategies: artificial selection, hybridisa-

tion, mutation and recombinant DNA techniques. Because it is unlikely that a transgenic EPN strain would meet public acceptance as a control agent [90], hybridisation and selective breeding are the most promising approaches to enhance EPN characteristics.

In a pioneer selection study performed with EPNs, the host-finding ability of *S. feltiae* was enhanced 20-fold to 27-fold after 13 selection rounds [91]. However, relaxation of the selection pressure produced a gradual decrease in host-finding. Similarly, Salame et al. [92] increased downward migration and infectivity of *S. feltiae*.

Many studies also attempted to enhance EPN tolerance to environmental stresses. Ehlers et al. [93] enhanced the low-temperature activity of *H. bacteriophora* by reducing the mean temperature at which the dauer juveniles (DJs) were active from 7.3 to 6.1°C during five selective breeding steps. Nimkingrat et al. [94] enhanced cold tolerance in *S. feltiae* by selecting and hybridizing the most cold-active strains. The cold tolerance was lost after few reproductive cycles under standard conditions, but was recovered after seven selection cycles with exposure to low temperatures.

Ehlers et al. [93] increased the mean tolerated temperature from 38.5 to 39.2°C. (The heritability for heat tolerance was 0.68 and for activity at low temperature 0.38). Salame et al. [92] bred a heterogeneous population of the EPN *Steinernema feltiae* for desiccation tolerance. A high survival rate (>85%) at 85% relative humidity for 72 h was obtained after 20 selection cycles. Mukuka et al. [95] searched for the most desiccation and heat tolerant strains of *H. bacteriophora*. In the following study [96], the authors crossed the most tolerant strains, and by subsequent selection they further increased desiccation and heat tolerance. Mean tolerated temperature of the most thermotolerant strain was 44°C after adaptation (vs. 38.2°C recorded for the commercial strain). The most desiccation tolerant strain had a mean tolerated water activity (aw-value) of 0.65 (vs. 0.951 in commercial strain).

Perry et al. [90] concluded that screening among natural populations for high tolerance to desiccation is a feasible approach and cross-breeding and genetic selection can further improve tolerance. However, there is a crucial question of the stability of selected traits. In *Heterorhabditis* nematodes, the trait stabilisation can be achieved by creation of inbred lines in liquid culture [97, 98].

According to Glazer [89] for EPNs, we lack markers to follow transfer or enhancement/ degradation of traits and to identify 'beneficial genes' that can be transferred between populations. Further fundamental research in the field of the genetic architecture of key traits, such as infectivity, stress tolerance and reproduction, is needed.

Thanks to recent advances in EPN and bacteria genomics [99] it will be possible to determine genes from the whole genome that are being expressed, in order to detect those that are involved in a particular process and target them through genetic engineering methods.

4.4. Safety

Entomopathogenic nematode-bacteria complexes are pathogens capable of invading and killing a large number of insects and even other arthropods, for example, spiders, ticks and

millipedes [100]. It is thus necessary to establish the risk that these organisms applied for pest control pose to the environment and nontarget organisms.

Numerous studies have assessed the effect of these complexes on nontarget invertebrates, animals and humans and environment, and several conclusions can be drawn. The available data show that entomopathogenic nematode-bacteria complexes are generally safe to humans and animals, and their impact on nontarget insects and other invertebrates seems to be limited. An excellent review on this topic was given by Akhurst and Smith [101]. In this chapter, we shortly review the current knowledge and stress some recent findings.

4.4.1. Safety to the environment

Negative effect to the environment is likely to be much stronger if the introduced nematode establish in the target locality. Therefore, the establishment potential of the introduced beneficial nematodes represents a very important part of the risk assessment. The available information, however, is quite scarce and inconsistent. It has been shown that *H. bacteriophora* experimentally introduced to several fields in Germany persisted for a maximum of 2 years [102]. Exotic nematode *S. riobrave*, on the other hand, successfully established in the treated corn fields in USA [103]. Dillon et al. [104] reported the establishment of *S. feltiae* after application to forest clearcuts in Ireland, whereas *S. carpocapsae* and *H. bacteriophora* disappeared. Another example of the successful establishment is *S. scapterisci*, from Uruguay, was introduced in Florida, established in the target grassland areas, and even extended to other nonselected crops [105].

4.4.2. Safety to nontarget invertebrates

According to Bathon [106], the mortality of nontarget animals in the field may occur, but will be temporal, spatially restricted, affecting a part of the population, and its impact can be considered negligible. Piedra-Buena et al. [107] stated that the impact of EPNs in general on organisms considered 'non-target' is limited, with infections only occurring when these organisms are exposed to very high concentrations and under laboratory conditions.

Laboratory experiments have shown that EPNs can negatively affect a large number of invertebrates, including predatory insects [108], parasitoids [109, 110], Symphyla, Collembola, Arachnida, Crustacea, Diplopoda [111], terrestrial isopods, millipedes and Gastropods [112]. However, the field data generally show none or only a small reduction in field populations of nontarget species after applications of entomopathogenic nematodes [113, 114].

In a recent study, Dutka et al. [115] reported that *Bombus terrestris* is remarkably susceptible to two commercially available entomopathogenic nematode pest control products applied at the recommended field concentration. The authors imply that the fossorial habits of *B. terrestris*, and the overwintering of queens underground, may make this species uniquely vulnerable to biological pest control agents applied directly to the soil. However, it can be speculated that higher temperatures up to 30°C and a low relative humidity around 60% [116] within the bumblebee nest would not favour nematode infection and propagation.

4.4.3. Safety to humans and animals

Entomopathogenic nematode-bacteria complexes are generally considered safe to humans and animals. Many studies assessed the effect of EPNs on vertebrates. EPNs were applied orally, subcutaneously, peritoneally and intracerebrally to various vertebrates. In poikilotherms, the nematode application had usually no negative effect, with the exception of tadpoles, where nematode application caused mortality [117, 118]. However, the mortality was associated not with *Xenorhabdus* but with foreign bacteria entering the penetration holes made by the invading nematodes [101]. In homoioterms, no adverse effects have been recorded, with the exception of mice injected subcutaneously, where the nematodes caused the development of skin ulcers [119]. One case of possible human allergic response to EPNs was recorded in the person handling the concentrated nematode solutions during the harvesting, cleaning and storage stages of production [101].

The safety of bacterial symbionts has been tested by oral, intradermal, subcutaneous and intraperitoneal applications of the bacterial cells to various model vertebrates generally producing no adverse effect [120, 121]. There is, however, one exception, being *Photorhabdus asymbiotica*. Since 1989, some *Photorhabdus* strains have been identified as facultative human pathogens causing severe ulcerated skin lesions [122]. Ten years later, these clinical strains have been described as *P. asymbiotica* [123]. Mulley et al. [124] demonstrated that during a human infection, *P. asymbiotica* aggressively acquires amino acids, peptides and other nutrients from the human host, employing a so-called 'nutritional virulence' strategy. The authors further revealed that, interestingly, an insect Phenol-oxidase inhibitor Rhabduscin protects *P. asymbiotica* against the human complement pathway.

However, later studies identified also symbiotic strains of *P. asymbiotica* in association with *Heterorhabditis gerrardi* [125, 126] and *H. indica* [127], raising serious concerns about the safety of EPNs to humans.

European environmental risk assessment (ERA) excludes *Heterorhabditis indica* from the normal ERA exemption for EPNs, because of the rare association with this nematode of the symbiotic bacterium *Photorhabdus asymbiotica.* For this reason, there should be a precise identification of the symbiotic bacterium when *H. indica* is used for biocontrol [128].

Other commercially produced heterorhabditids, *H. bacteriophora* and *H. megidis*, have never been found in association with this bacterium and thus do not pose such a risk. Nevertheless, any contact between EPN-associated bacteria and human wounds should be avoided [129].

Very recently, Gengler et al. [130] have revealed the capacity of EPNs to act as an efficient reservoir ensuring exponential multiplication, maintenance and dissemination of the human pathogenic bacterium *Yersinia pseudotuberculosis*. The authors argue that if the similar relationship is between EPNs and *Y. pestis,* etiologic agent of plague, then it would enhance the understanding of long-term persistence of *Y. pestis* in plague endemic areas worldwide. Further research of this topic is necessary to determine any possible risk.

4.4.4. Phasmarhabditis hermaphrodita

The effect of commercial strain of *P. hermaphrodita* against many invertebrates has been tested in many studies. This organism is able to infect many slug and snail species, nontarget molluscs included. *Cepaea hortensis* and aquatic snail *Lymnaea stagnalis* are found susceptible to very high doses that several times exceed the recommended dose, whereas other aquatics mollusc, for example, *Physa fontinalis* are not [131–133]. Some other snails (*Succinaea putris*, *Pomatias elegans*, *Cepaea nemoralis* and others) can be infected with *P. hermaphrodita* but its effect on them is very low, if any. Negative effect on the earthworms *Lumbricus terrestris* and *Eisenia foetida* and others has never been found [134, 135], and no effect was found also against insects *Pterostichus melanarius*, *Zophobas morio* or *Galleria mellonella* [136, 137]. *P. hermaphrodita* is freely associated with many soil-dwelling bacteria [55, 138], and some of them, for example, *Stenotrophomonas maltophilia* [42] can be occasionally dangerous for human, especially those with a weakened immunity system.

4.5. Synergy with other biocontrol agents

Entomopathogenic and mollusc-parasitic nematodes are widely used in integrated and biological pest control systems. Entomopathogenic nematodes are relatively resistant to many pesticides in recommended dosage, except for some, for example, carbamates [82], and some authors reported synergy between EPNs and chemicals [139, 140]. But they are also influenced by many, especially soil dwelling, micro- and macro-organisms that can hardly suppress [141–143] or synergistically support them [144].

Synergy between entomopathogenic nematodes and other bio-agents are in great demand because this strategy can significantly reduce application rates and increase efficacy [145] that leads to higher economic profit. The great example of synergy between EPN *S. kraussei* (Nemasys L.) and insect-parasitic fungus *Metarhizium anisopliae* strain V275 was described [144]. Combination of a rates 1×10^{10} conidia and 250 000 IJs applied against overwintering larvae of black vine weevil *Otiorhynchus sulcatus* (Coleoptera: Curculionidae) resulted in 100% control, while the results in single applications were not so impressive. Similar results were obtained by Anbesse et al. [146] who tested synergistic effect of *H. bacteriophora* and *M. anisopliae* against barley chafer grub *Coptognathus curtipennis* (Coleoptera: Dynastidae) and Choo et al. [147] who reported synergy between *S. carpocapsae* and *Beauveria brongniartii* in control of oriental beetle *Exomala orientalis* (Coleoptera: Scarabeidae) grubs.

Synergistic effects were also found between EPNs and entomopathogenic bacteria *Bacillus thuringiensis* (Bt). Koppenhöfer and Kaya [148] reported additive and synergistic interaction between Bt and *S. glaseri* and *H. bacteriophora* that were applied against scarab grubs but also noted that these effects were not observed in case of *S. kushidai*. Similar reports about very low or absolutely no synergy between EPNs and other bio-agents, especially fungus and bacteria were published by many other authors [149, 150]. This inconsistence in results was explained by antagonism of nematodes symbiotic bacteria and other entomopathogens [151]. As stated in this study, bacteria *Photorhabdus luminiscens* is able to strongly suppress the growth and conidia production of *Beauveria bassiana*, *B. brongniarti* and *Paecilomyces fumosoroseus*, whereas

other bacterial symbiont *Xenorhabdus poinari* does not. Shapiro-Illan et al. [152] provide that neutral or negative interactions among EPNs and other bio-agents are also dependent on the specific pathogens, hosts, application parameters and environmental conditions.

Interestingly, the use of combination of several EPN species has been shown to increase the efficacy against insect pests. There was a very strong synergy of *Steinernema weiseri* with *H. bacteriophora* or *S. glaseri* applied on *Curculio elephas* (Coleoptera: Curculionidae) a major pest of chestnut [153].

Reports of synergy of EPNs and arthropod bio-agents are slightly less frequent, maybe because of the ability of EPNs to infect many of these organisms, but despite this there are some successful combined applications that clearly show synergistic effect [154]. These authors reported positive effect of combined application of predatory mite *Hypoaspis aculeifer* and *H. bacteriophora* or *S. feltiae* against soil-dwelling stages of western flower thrips *Frankliniella occidentalis*. Positive effects of the combined applications of EPNs and arthropod bio-agents can be mostly expected when EPNs are used against soil-dwelling stages and arthropods against leaf-living stages of insect pests.

Expectably there was also synergism of EPNs in combination with GM plants [155]. Entomo-pathogenic nematodes are not negatively influenced by the GM plant and can infect all soil-dwelling stages of insect pest that survive or avoid the effect of GM plant (e.g., Bt-corn) that results in higher efficacy of biocontrol.

Even though there are some reports of antagonism among nematodes and other bio-agents, we can say that, in general, higher diversity of predators and similarly also pathogens leads to better control of many pests [156], thanks to the synergy of their effects on pest populations. Conservation of natural enemies may carry additional benefits for biological control.

Acknowledgements

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