A Review of the Potential Use of Entomopathogenic Nematodes to Control Above-Ground Insect Pests in South Africa

T. Platt[1], N.F. Stokwe[1,2], A.P. Malan[1*]

[1]Department of Conservation Ecology and Entomology, Stellenbosch University, Private Bag X1, Matieland 7602, South Africa
[2]ARC Infruitec-Nietvoorbij, Private Bag X5026, Stellenbosch 7599, South Africa

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Entomopathogenic nematodes (EPNs), of the families Steinernema and Heterorhabditis, are insect parasites that have been successfully used as biological control agents of soil-based insect pests on the North American and European continents. The success of nematodes as biological control agents of the soil stages of pest insects has led to research into their use for control of above-ground insect pests. Laboratory-based studies have shown exceptionally good control, in most cases, against such pests as mealybugs, codling moth and leaf miners. As the life stages of the above-ground insect pests have not co-evolved together with those of EPNs, they are, generally, more susceptible than the soil-based life stages. However, EPNs are susceptible to desiccation and vulnerable to UV radiation, so that ensuring their survival beyond soil environments is problematic. The impetus to avoid environmental stressors can cause EPNs to seek sheltered, cryptic habitats on foliage, where their target insect pest (such as mealybugs) may be found. The current paper provides an overview of information on the application of EPNs as a biocontrol agent for the control of insect pests above ground and on foliage, with particular reference to research done in South Africa.

INTRODUCTION

Entomopathogenic nematodes (EPNs) from the order Rhabditida (Heterorhabditidae and Steinernematidae) are characterised by their exclusive pathogenicity to insects via their mutualism with symbiotic bacteria (Griffin *et al*., 2005). Various nematode families have been investigated as potential biocontrol agents, with over 30 having been linked to insects in some way (Kaya & Stock, 1997). However, current research focuses almost entirely on Steinernematidae and Heterorhabditidae (Grewal *et al*., 2005). The infective juvenile (IJ) stage, which is the free-living, non-feeding survival stage of the EPN life cycle, can easily be mass-cultured, formulated, and applied as a biological control agent for use against pest insects (Ferreira & Malan, 2014b; Campos-Herrera, 2015; Kagimu *et al.*, 2017).

Since the first implementation of EPNs as biological control agents of soil-based insect pests, investigations have been performed into their ability to control pest insect life stages found above ground. In particular, the success of EPN formulated products for soil application, as well as their above-ground application in the greenhouse production of crops (Shapiro-Ilan *et al*., 2006; Lacey & Georgis, 2012; Kutamanyane *et al*., 2018), has rekindled an interest in their commercial field application against above-ground insect pests (Arthurs *et al*., 2004; Le Vieux & Malan, 2013a; Platt *et al*., 2018; 2019a, b). However, as soil-adapted organisms, EPNs are poorly suited to above-ground environments, which often feature low relative humidity, extremes of temperature, and exposure to ultraviolet (UV) radiation. The above-mentioned factors result in rapid desiccation and death, negatively impacting on EPN's efficacy as biocontrol agents. The main factor appears to be humidity, with nematode survival being prolonged in humid environments (such as in rainforests or glasshouses) and curtailed in drier (i.e. Mediterranean or southern African) climates (Arthurs *et al.*, 2004).

Methods of improving EPN survival in above-ground environments are currently being investigated. Such methods include weather forecasting (De Luca *et al*., 2015), early morning or late afternoon application (De Waal *et al*., 2017), the addition of adjuvants (such as superabsorbent polymer formulations) (Shapiro-Ilan *et al*., 2010; De Waal *et al*., 2013), and pre- and post-application wetting (De Waal *et al*., 2010; Odendaal *et al*., 2016a), aimed at maximising the humidity levels experienced during and following application. De Waal *et al*. (2017) verified the positive effect of applying nematodes in the late evening and early morning

*Corresponding author: E-mail address: apm@sun.ac.za
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against codling moth in a pear orchard in South Africa. The addition of antidesiccants and surfactants to EPN foliar sprays has led to many examples of the enhancement of nematode efficacy (Glazer *et al*., 1992a; Head *et al*., 2004; De Waal *et al.,* 2013; Van Niekerk & Malan, 2014b).

In South Africa the grapevine mealybug, *Planococcus ficus* (Signoret), citrus mealybug, *Planococcus citri* (Risso), and the obscure mealybug, *Pseudococcus viburni* (Signoret), are important insect pests of grapevines, deciduous fruit and citrus, as their presence on export fruit results in rejection of consignments on phytosanitary grounds in many markets. EPNs are non-toxic and able to actively seek out hosts such as mealybugs in grape bunches (Lacey & Georgis, 2012), therefore they might be a promising alternative for mealybug control on table grapes destined for export, due to restrictions on the presence of chemical residues.

In the current review, available information on the above-ground application of nematodes is brought into context in terms of the control of key insect pests in South Africa, with special reference to the control of mealybugs on grapevines.

ENTOMOPATHOGENIC NEMATODES

Life cycle

EPNs belonging to the families Steinernematidae and Heterorhabditidae have been applied with great success as a biocide against a wide range of insect pests (Campos-Herrera, 2015). Both families have similar traits and life cycles, despite not being closely related (Blaxter *et al*., 1998), with the bacterial symbiont of *Steinernema* species belonging to the genus *Xenorhabdus*, whereas *Heterorhabditis* is associated with *Photorhabdus* (Griffin *et al*., 2005). Steinernematids and heterorhabditids have a free-living stage, called the infective juvenile (IJ), also known as the dauer juvenile. This stage occurs freely in the soil, where the IJs can actively seek out and find a suitable insect host.

Occurrence and distribution in South Africa

The first EPN recorded in South Africa was *Steinernema carpocapsae,* isolated from the black maize beetle, *Heteronychus arator* Fabricius (Coleoptera: Scarabaeoidea), collected from a maize field near Grahamstown in the Eastern Cape province (Harington, 1953). During the 1980's unidentified EPNs were applied to the above-ground larval stages of the sugarcane borer, *Eldana saccharina* Walker (Spaull, 1992).

An investigation into biological control of the banded fruit weevil, *Phlyctinus callosus* (Schönerr) (Coleoptera: Curculionidae), from 1993 to 1994 yielded a heterorhabditid EPN species that was later confirmed to be *Heterorhabditis bacteriophora* Poinar (Grenier *et al*., 1996a, b). Since the first new EPN species from South Africa was described in 2006 as *Steinernema khoisanae* Nguyen, Malan & Gozel (Nguyen *et al*., 2006), several other new species have been described and their occurrence recorded. To date, 16 EPN species have been reported from South Africa, of which five are heterorhabditids, and 11 are steinernematids. Three of the five species of heterorhabditids and 10 of the 11 species of steinernematids were new species at the time of reporting (Malan *et al*., 2016; Hatting & Malan, 2017; Steyn *et al*., 2017a, b).

Use in above-ground biological control

EPNs have been successfully commercialised for use against insect pests in North America, Europe, Japan, China, and Australia (Ehlers, 1996; Kaya *et al*., 2006). Elsewhere research is still in relatively preliminary stages (Kaya *et al*., 2006). The most widely used commercial applications of EPNs have been aimed at the soil-based stages of insects (Wilson & Gaugler, 2004). Above-ground application against foliage-feeding insects has been rare, and has generally proved to be less successful than soil-based applications (Shapiro-Ilan *et al*., 2006).

Arthurs *et al*. (2004) conducted a meta-study of 136 trials on above-ground application of *Steinernema carpocapsae* (Weiser) Wouts, Mráček, Gerdin & Bedding, which has been the most commonly used species to control above-ground insect pests. The study showed that EPN efficacy tends to vary according to the targeted habitat. The most favourable habitat was boreholes (tunnels made by boring insects into leaves, stems, etc.), followed by cryptic habitats (micro-environments on the foliage of plants, sheltered from the environment by bark, leaves, or other structures), with exposed habitats (habitats open to the environment) being the least successful. EPN efficacy also varied according to trial location, with laboratory application (most controlled environment) generally being the most successful, followed by greenhouse application, with field application (the least-controlled application) the least successful.

Most studies that have been undertaken with above-ground application of EPNs to control insects have targeted the order Lepidoptera, whereas a smaller number of studies targeted Coleoptera, Diptera, Hemiptera, Hymenoptera, and Thysanoptera (Table 1). The above-ground stages of insects can be targeted with nematodes in different macro environments, such as covered areas like shade houses and glasshouses, or in large-scale field trials, whereas the microhabitat of the insect itself can be boring, cryptic, or exposed (Table 2).

Above-ground application of EPNs by insect order

The following sections discuss the prominent insect orders investigated for control with entomopathogenic nematodes, with a focus on the South African context.

Coleoptera

As major pests, coleopteran insects have been a significant focus for biological control using EPNs. *Steinernema feltiae* (Filipjev, 1934) Wouts, Mráček, Gerdin & Bedding was found to be ineffective for controlling the overwintering larval populations of large European elm bark beetle *Scolytus scolytus* (Fabricius) (Coleoptera: Curculionidae) at the doses applied (Finney & Walker, 1979). Testing a variety of EPN species against *Stethobaris nemesis* (Prena & O'Brien) (Coleoptera: Curculionidae) on leaf discs in the laboratory, Shapiro-Ilan & Mizell (2012) found that *S. feltiae* and *S. carpocapsae* both caused high levels of *S. nemesis* mortality.

Application of *S. carpocapsae* in an agar solution to potato foliage resulted in infection rates of 30% to 60% of adult Colorado potato beetles, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) (MacVean *et al*.,

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TABLE 1
Insect pests whose above-ground life stages have been targeted with entomopathogenic nematodes.

Order/Scientific name	Common name	Family	Target crop	Location	References
Coleoptera:					
Leptinotarsa decemlineata	Colorado potato beetle	Chrysomelidae	Potato	Colorado, USA; Havlíčkův Brod, Czech Republic	Welch & Briand (1961); MacVean *et al.* (1982); Adel & Hussein (2010); Hussein *et al.* (2012)
Phlyctinus callosus	Banded fruit weevil	Curculionidae	Deciduous fruit; grapevine	Western Cape, South Africa	Ferreira & Malan (2014a)
Scolytus scolytus	Larger European elm bark beetle	Curculionidae	Elm	Surrey, UK	Finney & Walker (1979)
Stethobaris nemesis	N/a	Curculionidae	Sycamore	Georgia, USA	Shapiro-Ilan & Mizell (2012)
Diptera:					
Ceratitis capitata	Mediterranean fruit fly	Tephritidae	Fruits	Western Cape, South Africa	Malan & Manrakan (2009); James *et al*. (2018)
C. rosa	Natal fly	Tephritidae	Fruits	Western Cape, South Africa	Malan & Manrakan (2009)
Liriomyza huidobrensis	Serpentine leaf miner	Agromyzidae	Leafy vegetables	York, UK; Harpenden, UK	Williams & Macdonald (1995); Williams & Walters (2000)
L. trifolii	American serpentine leaf miner	Agromyzidae	Chrysanthemum	Hawaii, USA; Ontario, Canada; California, USA	Harris *et al.* (1990); Hara *et al.* (1993); Broadbent & Olthof (1995)
Hemiptera:					
Bemisia tabaci	Silverleaf whitefly	Aleyrodidae	Cucumber; poinsettia; chrysanthemum; verbena	York, UK	Head *et al.* (2004); Cuthbertson *et al.* (2007, 2008)
Corythucha ciliata	Sycamore lace bug	Tingidae	Sycamore	Florida, USA	Shapiro-Ilan & Mizell (2012)
Planococcus citri	Citrus mealybug	Pseudococcidae	Citrus	Western Cape, South Africa	Van Niekerk & Malan (2013)
Planococcus ficus	Vine mealybug	Pseudococcidae	Grapevine	Western Cape, South Africa	Platt *et al.* (2018, 2019a,b)
Pseudococcus viburni	Obscure mealybug	Pseudococcidae	Apple	Western Cape, South Africa	Stokwe & Malan (2016)
Hymenoptera:					
Cephalcia lariciphila	Hymenopteran sawfly	Pamphilidae	Larch	Wales, UK	Georgis & Hague (1988)
Hoplocampa testudinea	Apple sawfly	Tenthredinidae	Apple	Quebec, Canada	Vincent & Bélair (1992)
Lepidoptera:					
Choristoneura occidentalis	Spruce budworm	Tortricidae	Fir	Canada	Kaya *et al.* (1981); Kaya & Reardon (1982)
C. rosaceana	Oblique banded leafroller	Tortricidae	Apple	Quebec, Canada	Bélair *et al.* (1999)

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TABLE 1 (CONTINUED)

Order/Scientific name	Common name	Family	Target crop	Location	References
Cydia pomonella	Codling moth	Tortricidae	Apple	Western Cape, South Africa	Kaya *et al.* (1984); Unruh & Lacey (2001); Odendaal *et al.* (2015); Lacey *et al.* (2005)
Diaphania hyalinata	Melonworm moth	Crambidae	Squash	Florida, USA	Shannag & Capinera (1995)
Earias insulana	Egyptian stemborer	Nolidae	Cotton	Bet Dagan, Israel	Glazer (1992)
Eldana saccharina	Sugarcane stalk borer	Pyralidae	Sugar cane	South Africa	Spaull (1992)
Euzophera semifuneralis	American plum borer	Pyralidae	Plum	New York State, USA	Kain & Agnello (1999)
Helicoverpa zea	Corn earworm	Noctuidae	Corn	Mississippi, USA	Bong & Sikorowski (1983)
Heliothis armigera	Cotton bollworm	Noctuidae	Bean	Bet Dagan, Israel	Glazer & Navon (1990)
H. viriscens	Tobacco budworm	Noctuidae	Tobacco	North Carolina, USA	Chamberlin & Dutkey (1958)
Herpetogramma phaeopteralis	Tropical sod webworm	Crambidae	Turfgrass	Florida, USA	Tofangsazi *et al.* (2014)
Holocacista capensis	Cape grapevine leafminer	Heliozelidae	Grapevine	Western Cape, South Africa	Steyn *et al.* (2019)
Hyphantria cunea	Fall webworm	Arctiidae	Cherry	Tokyo, Japan	Yamanaka *et al.* (1986)
Mamestra brassicae	Cabbage moth	Noctuidae	Cauliflower	Rumbeke-Beitem, Belgium	Beck *et al.* (2014)
Manduca sexta	Tobacco hornworm	Sphingidae	Tobacco	North Carolina, USA	Chamberlin & Dutkey (1958)
Operophtera brumata	Winter moth	Geometridae	Apple	N/a	Jacques (1967)
Ostrinia nubilalis	European corn borer	Crambidae	Cabbage	Bet Dagan, Israel	Ben-Yakir *et al.* (1998)
Phyllocnistis citrella	Citrus leaf miner	Gracillariidae	Citrus	Sydney, Australia	Beattie *et al.* (1995)
Platyptilia carduidactyla	Artichoke plume moth	Pterophoridae	Artichoke	N/a	Bari & Kaya (1984)
Plutella xylostella	Diamondback moth	Plutellidae	Kale; cabbage	Nairobi, Kenya, New Delhi, India	Nyasani *et al.* (2008); Baur *et al.* (1995, 1997, 1998); Schroer & Ehlers (2005); Mason *et al.* (1998); Somvanshi *et al.* (2006)
Prionoxystus robiniae	Carpenterworm	Cossidae	Oak	Kentucky, USA	Forschler & Nordin (1988)
Pryeria sinica	Euonymus leaf notcher	Zygaenidae	Japanese spindle	Korea	Lee *et al.* (2006)
Spodoptera exigua	Beet armyworm	Noctuidae	Nursery ornamentals	N/a	Begley (1990)
S. littoralis	African cotton leafworm	Noctuidae	Cotton	Bet Dagan, Israel	Glazer *et al.* (1992a,b)
Synanthedon culciformis	Large red-belted clearwing	Sesiidae	Alder, Sycamore	California, USA	Kaya & Brown (1986)

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TABLE 1 (CONTINUED)

Order/Scientific name	Common name	Family	Target crop	Location	References
S. exitiosa	Peachtree borer	Sesiidae	Apple	Italy	Deseo & Miller (1985)
S. myopaeformis	Red-belted clearwing	Sesiidae	Peach	British Columbia, Canada	Cossentine *et al*. (1990)
S. pictipes	Lesser peachtree borer	Sesiidae	Peach	Columbus, Ohio	Cottrell *et al*. (2011)
S. resplendens	Sycamore borer	Sesiidae	Alder, Sycamore	California, USA	Kaya & Brown (1986)
S. tipuliformis	Current clearwing	Sesiidae	Blackcurrant	Derwent Valley, Tasmania	Miller & Bedding (1982)
Tuta absoluta	Tomato leaf miner	Gelechiidae	Tomato	Barcelona, Spain	Batalla-Carrera *et al*. (2010); Van Damme *et al*. (2016)
Zeiraphera canadensis	Spruce bud moth	Tortricidae	Spruce	Canada	Eidt & Dumphy (1991)
Thysanoptera:					
Frankliniella occidentalis	Western flower thrips	Thripidae	Blueberries, Chrysanthemum, Saintpaulia	Canada, England, Ontario, UK, South Africa	Buitenhuis & Shipp (2005); Arthurs & Heinz (2006); Dlamini et al. (2019a)

1982). The addition of agar to the suspension increased the viability and infectivity of the nematodes, resulting in a significant reduction in the amount of leaf damage caused by *L. decemlineata* (Adel & Hussein, 2010; Hussein *et al*., 2012).

In South Africa, the indigenous banded fruit weevil (*Phlyctinus callosus* Schönerr) (Coleoptera: Curculionidae) tends to emerge above ground during late spring and early summer (Myburgh *et al*., 1973) in vineyards and orchards, where it is a serious pest. Ferreira & Malan (2014a) tested the pathogenicity of indigenous *Heterorhabditis zealandica* (Poinar) (Rhabditida: Heterorhabditidae) and *H. bacteriophora* to adults of the banded fruit weevil in the laboratory. Application of high EPN concentrations (400 IJs/insect) under optimum conditions and an exposure time of four days resulted in 41% to 73% mortality of banded fruit weevil larvae and 13% to 45% mortality of adult weevils.

Diptera

Harris *et al*. (1990) showed that applications of *S. carpocapsae* achieved mortality levels of 64% in larvae of the American serpentine leaf miner, *Liriomyza trifolii* (Burgess) (Diptera: Agromyzidae), on chrysanthemum, which was similar to control with the insecticide and anthelminthic abamectin. Further investigation by LeBeck *et al.* (1993) determined that all larval instars of *L. trifolii* were susceptible to *S. carpocapsae*, but that the second instar is the most susceptible. However, research conducted on control of *L. trifolii* on lima beans (Hara *et al*., 1993) and chrysanthemums (Broadbent & Olthof, 1995) primarily found that abamectin was more effective than *S. carpocapsae*.

Williams & Walters (1994, 2000) showed that all larval instars of the leafminer, *Liriomyza huidobrensis* (Blanchard) (Diptera: Agromyzidae), were susceptible to *S. feltiae*, with the second larval instar the most susceptible at conditions of relatively low humidity (Williams & Macdonald, 1995). Williams & Walters (2000) consolidated the aforementioned research by applying *S. feltiae* to Chinese cabbage plants infested with *L. huidobrensis*. They achieved 82% mortality of *L. huidobrensis*, a significant increase over mortality previously achieved with the insecticide heptenophos (≈ 20%).

The global importance of fruit fly has prompted investigations into the use of EPNs for biocontrol (Langford *et al*., 2014; Nouh & Hussein, 2014; Abbas *et al*., 2016). Laboratory studies have shown the potential of EPNs as biological control agents of many species in the genera *Anastrepha, Dacus, Bactrocera, Rhagoletis* and *Ceratitis,* focusing on the susceptibility of the third larval instar. Research in South Africa has been limited, but Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), and Natal fruit fly, *Ceratitis rosa* (Karsch) (Diptera: Tephritidae), were tested for vulnerability to local EPNs. Although adult flies (i.e. the above-ground stage) of both species were found to be susceptible to EPNs, they were less susceptible than the soil-based third instar larvae, indicating that soil-based EPN applications are probably more feasible (Malan & Manrakan, 2009; James *et al*., 2018).

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TABLE 2
Above-ground life stages of insect pests targeted with entomopathogenic nematodes in different environments (table compiled from numerous references in reference list).

Family	Scientific name/family	Target crop	Insect stage	Pest habitat	Lab-oratory	Glass-house	Field	Nematode species
Agromyzidae	*Liriomyza huidobrensis*	Leafy vegetables	Larvae	Cryptic	x	x	-	Sf
	L. trifolii	Chrysanthemum	Larvae	Cryptic	x	x	-	Sc
Aleyrodidae	*Bemisia tabaci*	Cucumber; poinsettia, chrysanthemum, verbena	Nymphs; adults	Exposed	x	x	-	Sc; Sf
Arctiidae	*Hyphantria cunea*	Cherry	Larvae	Exposed	-	x	x	Sf
Chrysomelidae	*Leptinotarsa decemlineata*	Potato	Larvae	Exposed	x	x	x	Sc
Cossidae	*Prionoxystus robinae*	Oak	Larvae	Boring	-	x	-	Sf
Crambidae	*Ostrinia nubilalis*	Cabbage	Eggs; neonates	Boring	x	x	x	Hb; Sc
	Diaphania hyalinata	Squash	Larvae; prepupae pupae	Exposed	x	-	-	Hb; Sc; Sf; Sg
	Herpetogramma phaeopteralis	Turfgrass	Larvae	Exposed	x	x	-	Hb; Hi; Hm; Sc; Sf
Curculionidae	*Phlyctinus callosus*	Apples; pears; grapevine	Larvae; adults	Exposed	x	-	-	Hb; Hz
	Scolytus scolytus	Elm	Larvae	Boring	-	-	x	Sf
	Stethobaris nemesis	Sycamore	Nymphs	Exposed	x	-	-	Hb; Hi; Sc; Sf
Gelechiidae	*Tuta absoluta*	Tomato	Larvae	Boring	x	x	-	Hb; Sc; Sf
Geometridae	*Operophtera brumata*	Apple	Larvae	Exposed	x	-	-	Sc
Gracillariidae	*Phyllocnistis citrella*	Citrus	Larvae	Boring	-	-	x	Sc
Heliozelidae	*Holocacista capensis*	Grapevine	Larvae	Leaf mines	x	-	-	Hb; Hbau; Hi; Hn; Hz; Sjeff; Sy
Noctuidae	*Helicoverpa zea*	Corn	Larvae	Boring	-	-	x	Sc
	Heliothis armigera	Bean	Larvae	Exposed	x	x		Sf
	H. viriscens	Tobacco		Exposed	x	-	x	Sc
	Mamestra brassicae	Cauliflower	Larvae	Exposed	x	-	x	Sc
	Spodoptera exigua	Nursery ornamentals	Larvae	Exposed	-	-	x	Various
	S. littoralis	Cotton	Larvae	Exposed	x	-	-	Hb; Sc; Sg
	Earias insulana	Cotton	Larvae	Exposed	x	x	-	Sc
Pamphilidae	*Cephalcia lariciphila*	Larch	Larvae; prepupae	Exposed	-	-	x	Sf
Plutellidae	*Plutella xylostella*	Kale; cabbage	Larvae	Exposed	x	x	x	Various

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TABLE 2 (CONTINUED)

Family	Scientific name/family	Target crop	Insect stage	Pest habitat	Lab-oratory	Glass-house	Field	Nematode species
Pseudococcidae	*Planococcus citri*	Citrus	Nymphs; adults	Cryptic	x	x	x	Hz; Sy
	Planococcus ficus	Grapevine	Adults	Exposed	x	x	x	Sy
	Pseudococcus viburni	Apple	Nymphs; adults	Cryptic	x	-	x	Hb; Hz; Sc; Sy
Pterophoridae	*Platyptilia carduidactyla*	Artichoke	Larvae	Boring	-	-	x	Sc
Pyralidae	*Eldana saccharina*	Sugar cane	Larvae	Boring	-	-	x	*Heterorhabditis* spp.
	Euzophera semifuneralis	Plum	Larvae	Boring	-	-	x	Hb.; Sf
Sesiidae	*Synanthedon culciformis*	Alder; sycamore	Larvae	Boring	-	-	x	Sb; Sf
	Synanthedon exitiosa	Apple	Larvae	Boring	-	-	x	Sf
	Synanthedon myopaeformis	Peach	Larvae	Boring	-	-	x	Hh
	Synanthedon pictipes	Peach	Larvae	Boring	x	-	x	Hb; Hf; Hme; Hmeg; Hz; Sc; Sg; Sr
	S. resplendens	Alder; sycamore	Larvae	Boring	-	-	x	SB; Sf
	S. tipuliformis	Blackcurrant	Larvae	Boring	-	-	x	Sb
Sphingidae	*Manduca sexta*	Tobacco	Larvae	Exposed	x	-	x	Sc
Tenthredinidae	*Hoplocampa testudinea*	Apple	Larvae	Exposed	x	-	x	Hb; Sf
Thripidae	*Frankliniella occidentalis*	Chrysanthemum; Saintpaulia	Nymphs	Cryptic	x	x	-	Hb; Hbau; Hi; Hn; Hz; Sac; Sf; Sin; Sjeff; Sk; Sl; Sy; Tn
Tingidae	*Corythucha ciliata*	Sycamore	Adults	Exposed	x	-	-	Hb; Hg; Hi; Sr
Tortricidae	*Choristoneura occidentalis*	Fir	Larvae	Cryptic	-	-	x	Sc
	C. rosaceana	Apple	Larvae	Cryptic	x	-	x	Sc; Sf; Sg; Sr
	Cydia pomonella	Apple	Larvae	Cryptic	-	-	x	Sc; Sf
	Zeiraphera canadensis	Spruce	Larvae	Cryptic	-	-	x	Sc
Zygaenidae	*Pryeria sinica*	Japanese spindle	Larvae	Exposed	x	-	x	Sc

Heterorhabditis bacteriophora = Hb; *H. baujardi* = Hbau; *H. floridensis* = Hf; *H. georgiana* = Hg; *H. heliothidus* = Hh; *H. indica* = Hi; *H. megidis* = Hmeg; *H. mexicana* = Hme; *H. noenieputensis* = Hn; *H. taysearae* = Ht; *H. zealandica* = Hz; *Steinernema anomaly* = Sa; *S. bibionis* = Sb; *S. carpocapsae* = Scap; *S. citrae* = Scit; *S. feltiae* = Sf; *S. glaseri* = Sg; *S. innovationi* = Sin; *S. jeffreyense* = Sjeff; *S. karii* = Sk; *S. khoisanae* = Sk; *S. litchii* = Sl; *S. rarum* = Srar; *S. riobravis* = Srio; *S. sacchari* = Sac; *S. weiseri* = Sw; *S. yirgalemense* = Sy; *Thripinema nicklewoodi* = Tn

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Hemiptera

Investigations into the use of *S. feltiae* to control the silverleaf whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae), found that *S. feltiae* was unable to achieve significant control of *B. tabaci* by itself (inducing pest mortality of between 10% and 32% on tomato, cucumber, verbena, poinsettia and chrysanthemum), but the efficacy of the EPN application could be enhanced by 15% to 31% with the use of adjuvants (Head *et al*., 2004). Combining applications of *S. feltiae* with imidacloprid provided significantly more comprehensive control than the use of either treatment alone (Cuthbertson *et al*., 2007). Shapiro-Ilan & Mizell (2012) showed that five species of EPNs, but particularly *Heterorhabditis indica* Poinar, Karunakar & David, had potential as biocontrol agents for the sycamore lace bug, *Corythucha ciliata* (Say) (Hemiptera: Tingidae), a hemipteran pest of ornamental plants.

Mealybugs (Pseudococcidae) are among the most important pests in South African agriculture, and research to develop methods of foliar application of EPNs against them is ongoing. *Planococcus citri* (Risso) is a major pest of citrus (Hattingh & Moore, 2003), *Planococcus ficus* (Signoret) is a major pest of grapevines (Walton, 2003) and the obscure mealybug, *Pseudococcus viburni* (Signoret), is regarded as the main mealybug pest of deciduous fruit (Prinsloo & Uys, 2015).

Van Niekerk & Malan (2012) screened potential EPN candidates for the foliar control of *P. citri*, finding *Steinernema yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler & Adams and *H. zealandica* to be the most effective nematode species. They then tested both species in combination with various agrochemicals and natural enemies, in response to which neither species was shown to decrease in infectivity. Both EPN species were, however, highly infective to the larvae of the ladybird *Cryptolaemus montrouzieri* (Coleoptera: Coccinellidae) (Mulsant), which is a biocontrol predator of *P. citri,* indicating that these organisms should not be used together in an IPM system (Van Niekerk & Malan, 2014a).

Van Niekerk & Malan (2015) investigated the use of adjuvants to overcome a key obstacle to the application of EPNs to foliage, namely the need to maintain suitable levels of relative humidity (RH) to allow for EPN infection of the citrus mealybug. Application of the adjuvant Zeba® (3 g/L) increased the effectiveness of *H. zealandica* against *P. citri* by 22% at 80% RH, with the combination of both Zeba® and Nu-Film-P® significantly increasing the amount of nematodes deposited on the leaves. In a semi-field trial in a citrus orchard, significantly higher levels of control (53%) were achieved by adding Zeba®. The study showed that the addition of an adjuvant improved the ability of *S. yirgalemense* to infect *P. citri* by retarding desiccation and by buffering the nematodes from the harsh environmental conditions (Van Niekerk & Malan, 2014b).

In South Africa, Le Vieux & Malan (2013a, b; 2015) demonstrated high susceptibility of adult *P. ficus* to six different indigenous EPN species (with *S. yirgalemense* the most promising) in laboratory studies, and olfactometer studies indicated that *S. yirgalemense* actively moves towards the vine mealybug, which could prove advantageous for finding mealybugs in their cryptic habitats prior to desiccation. Platt *et al*. (2018) recently also showed that *P. ficus* females are highly susceptible to four South African EPN species, with the highest mortality of 90% caused by *Heterorhabditis noenieputensis* Malan, Knoetze & Tiedt in laboratory bioassays. They also showed that a combination of Nu-Film® and Zeba® increased the deposition of *S. yirgalemense* on grapevine leaves. Results from a growth chamber bioassay, using grapevine leaves, showed 84% mortality of *P. ficus* females when combined with the two adjuvants, while in a glasshouse trial 88% mortality of *P. ficus* females was obtained (Platt *et al*., 2019a). *Steinernema yirgalemense* was also tested in a semi field trial in a vineyard, combined with adjuvants, causing 66% female mortality, which varied with the nematode concentration and the time of application during the day (Platt *et al*., 2019b). The high susceptibility of *P. ficus* to EPNs and the tendency of mealybugs to form colonies in cryptic habitats above ground make *P. ficus* an ideal candidate for control using nematodes. EPNs could be applied to target mealybugs on leaves and bunches during the growing season, and after leaf drop to target overwintering mealybugs under the bark on vine cordons and stems.

Steinernema yirgalemense Stokwe & Malan (2016) investigated the ability of EPNs to control *P. viburni*, one of three species of pseudococcids that are commonly found on pome fruit in the Western Cape province of South Africa (Wakgari & Giliomee, 2004). The researchers found that *H. zealandica* and *S. yirgalemense* were both able to reproduce in *P. viburni*, with the former displaying greater mealybug penetration, and also possessing the ability to infect *P. viburni* at the centre of infested apple cores, making it a potential candidate for the above-ground control of *P. viburni* in both apple and pear orchards.

Hymenoptera

To date, most research into the application of EPNs for the control of hymenopteran pests of foliage has focused on sawflies, outside the South African context. On evaluating *S. feltiae* for use against the web-spinning larch sawfly, *Cephalcia lariciphila* (Wachtl) (Hymenoptera: Pamphiliidae), in Welsh larch, Georgis & Hague (1988) found the infection of the larval stages to be prohibitively low when compared to application, at equivalent rates, to prepupae in the soil (3% to 39% versus 61% infection, respectively).

Vincent & Bélair (1992) took a similar approach, applying *S. carpocapsae* to dwarf apple trees in efforts to control the apple sawfly, *Holocampa testudinea* (Klug) (Hymenoptera: Tenthredinidae). The application of EPNs in this case was not found to significantly reduce the amount of primary damage, i.e. scarring of fruit as a result of sawfly burrowing into fruit. However, it did significantly reduce the amount of secondary damage incurred, in terms of the number of frass pellets deposited at the entry point of burrowing. Further research by Vincent & Bélair (1992) assessed the application of *S. carpocapsae* against *H. testudinea* over a period of three years. The amount of primary damage inflicted on the apple fruit by *H. testudinea* was reduced by 98% and 100% in the first two years, respectively, while the percentage of fruits exhibiting secondary damage was significantly reduced after

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a single application of *S. carpocapsae*. The effectiveness of the treatment was attributed to the cages used, which increased the RH, and, therefore, the extent of nematode longevity and mobility.

Lepidoptera

The research conducted by Bélair *et al*. (1999) into the application of *S. carpocapsae* against the oblique banded leafroller, *Choristoneura rosaceana* (Harris) (family Tortricidae), a pest of apples, concluded that the low efficacy of the nematode and the inability of the selected adjuvants to improve nematode efficacy indicated that the use of *S. carpocapsae* as a sole agent against the leafroller could not be recommended. On assessing the efficacy of *S. carpocapsae* in controlling the Western spruce budworm, *Choristoneura occidentalis* (Walsingham) (Tortricidae), in fir, Kaya & Reardon (1982) concluded that significant infectivity of the insect larvae and pupae could not be obtained, despite the use of adjuvants and the bagging of treated branches to enhance the extent of nematode survivability.

Cydia pomonella, the codling moth, has been a major target of research in terms of the foliar application of EPNs, due to its status as a serious pest of apples worldwide. The application of *S. feltiae* to codling moth diapausing larvae in corrugated cardboard on apple tree trunks resulted in 80% codling moth mortality in mid-autumn, with 32% mortality resulting therefrom in midsummer (Kaya *et al.*, 1981). Unruh & Lacey (2001) assessed the effect of applying a variety of methods to increase the infectivity of *S. carpocapsae* to codling moth larvae trapped in cardboard traps in apple orchards in Washington, USA. Their findings revealed that the application of EPNs to traps containing codling moth larvae was most effective under the relatively cool and humid conditions prevailing in the morning and evening, as well as in the case of both the pre- and post-wetting of the treatments. Odendaal *et al*. (2015) performed an investigation into the ability of South African EPNs to control codling moth in South African environments, by assessing the effectivity of the local species *Steinernema jeffreyense* Malan, Knoetze & Tiedt and *S. yirgalemense* in relation to the commercially available nematodes *S. feltiae* and two strains of *H. bacteriophora*. The researchers found that *S. jeffreyense* showed the highest efficacy (67%) when it was applied to codling moth larvae kept in small mesh cages. No adjuvants were added in the above-mentioned trials, with the cages merely being sprayed with water every 2 h for the first 6 h of the trial. This study indicates the potential for South African nematodes to be effective, if conditions of high humidity can be maintained.

Codling moth infestations have been shown to be persistent due to the contamination of fruit bins in orchards, even when other control methods were in place. On examining the ability of *S. carpocapsae* and *S. feltiae* to control the infestation of orchard fruit bins, Lacey *et al*. (2005) found that both species provided high mortality of cocooned codling moth larvae when they were applied together with wetting agents.

Two studies have been conducted in South Africa to determine the potential of EPNs to control codling moth infestations of wooden fruit bins. Using 25 IJs/ml as a discriminating dosage in laboratory trials, De Waal *et al*. (2010) determined the LD_{90} of codling moth to be 100 IJs/ml, using miniature bins under optimum conditions. The study also indicated that conditions of high humidity are crucial for obtaining the desired control, and that covering the bins with a tarpaulin, together with the use of adjuvants, improved the level of control significantly. Further studies by Odendaal *et al*. (2016a, b) evaluated the efficacy of *S. yirgalemense*, a local isolate, and two commercial isolates, *S. feltiae* and *H. bacteriophora*, for their potential to control codling moth in miniature bins at a concentration of 25 IJs/ml. The best control (75%) was obtained with *S. feltiae*, and the degree of control was significantly increased to >95% by the addition of adjuvants.

The diamond back moth, *Plutella xylostella* L. (Lepidoptera: Plutellidae) is a serious pest of cabbage and other crucifers. In laboratory trials, the LD_{50} for *P. xylostella* was found to be 12 *S. carpocapsae* larvae per insect (Zolfagharian *et al*., 2014). Field trials in Cuba, Marrero (2006) obtained 72% control with a spray application of *H. indica*, while Rodríguez *et al.* (2013) also showed a reduction in the number of *P. xylostella* on cabbage with *H. bacteriophora* in another trial.

Stem-boring lepidopteran larvae are attractive candidates for EPN application, as they obtain protection from harsh environmental conditions by means of boring tunnels or galleries into stems and leaves. Chief among these are the sesiids (Lepidoptera: Sesiidae), which are mostly obligate borers of plant stems. Kaya & Brown (1986) investigated the ability of *S. feltiae* to control the large red-belted clearwing, *Synanthedon culciformis* (Linnaeus) (Lepidoptera: Sesiidae), on alder, and the sycamore borer, *S. resplendens* (Edwards) (Lepidoptera: Sesiidae), on sycamore. The researchers found *S. feltiae* to be more effective against *S. culciformis* when it was applied directly to the borer galleries, due to the *S. culciformis* residing in the alder heartwood, which was moister than the sycamore heartwood. Deseo & Miller (1985) performed similar experiments, applying *S. feltiae* to apple trees in Italy to control two strains of red-belted clearwing, *Synanthedon myopaeformis (syn. S. typhiaeformis)* (Borkhausen) (Lepidoptera: Sesiidae). They concluded that the two specific strains of *S. feltiae* were capable of actively seeking out, and of migrating towards, *S. myopaeformis*.

The effects of EPNs against sesiids on peach have also been assessed. Cossentine *et al.* (1990) applied *H. bacteriophora* (heliothidus strain) to control the peach tree borer, *Synanthedon exitiosa* (Say) (Lepidoptera: Sesiidae), finding that a suspension of EPNs in and around the boreholes failed to reduce the number of adults emerging from the holes significantly. Cottrell *et al*. (2011), in testing several EPN species for efficacy against the lesser peach tree borer, *Synanthedon pictipes* (Grote & Robinson) (Lepidoptera: Sesiidae), compared two adjuvants (polyacrylamide gel and moistened baby diapers) with the aim of improving moisture retention and UV protection. Both adjuvants were found to improve the control of *S. pictipes* compared to the control.

Shannag & Capinera (1995) assessed five EPN species for the control of melonworm, *Diaphania hyalinata* (Linnaeus) (Lepidoptera: Crambidae), applying *S. carpocapsae* against *D. hyalinata* on squash foliage. Survival of EPNs on foliage

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was limited to only 0.25% after 18 hours under moderately humid conditions, however, this limited survival on foliage did not appear to impair infectivity, with field applications of 5 billion nematodes per hectare resulting in infection rates of between 52% and 55%.

Shapiro-Ilan *et al*. (2010) applied *S. carpocapsae* for the control of the late instars of the lesser peach tree borer, *S. pictipes* (Grote & Robinson) (Lepidoptera: Sesiidae), with applications of latex paint, moistened diaper, or gel spray post-application of EPNs to enhance nematode survival on the peach tree foliage. The application of Barricade® gel, after nematode application, enhanced the efficacy of *S. carpocapsae* against the peach tree borers on the foliage. Further research established that Barricade® could be used in a single-spray together with *S. carpocapsae*, and that the combination was at least as successful as chlorpyrifos against the lesser peach tree borer (Shapiro-Ilan *et al*., 2016). The susceptibility of different life stages of the South American tomato leafminer, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) to various EPN species has been tested with a view to foliar application. Van Damme *et al*. (2016) showed in laboratory studies that all the insect instars were susceptible to infection by *S. feltiae*, *H. bacteriophora* and *S. carpocapsae,* with *S. feltiae* causing 100% mortality under optimum laboratory conditions. The researchers found that improvements to spraying conditions and the addition of adjuvants allowed IJ concentrations as low as 6.8 IJs/cm^2 to achieve levels of control equivalent to the recommended IJ concentration of 27.3 IJs/cm^2 under standard conditions.
Recently it was found that different local EPN species were able to penetrate and infect larvae of the Cape grapevine leafminer, *Holocacista capensis* Van Nieukerken & Geertsema (Lepidoptera: Heliozelidae), in their galleries in grapevine leaves. High mortality of *H. capensis* larvae was recorded for *Heterorhabditis baujardi* Phan, Subbotin, Nguyen & Moens (92%), *H. noenieputensis* (85%) and *H. indica* (83%) under laboratory conditions (Steyn *et al*., 2019).

Thysanoptera

The major thysanopteran pest targeted with EPNs is the western flower thrips, *Frankliniella occidentalis* (Pergande) (family Thripidae), because it is difficult to control, due to its preference for cryptic habitats on plants. Buitenhuis & Shipp (2005) assessed the efficacy of *S. feltiae*, applied in conjunction with a wetting agent, against *F. occidentalis* on chrysanthemums in the flowering versus the vegetative (i.e. exposed) stage. They found no significant differences in mortality of larvae and pupae between the two plant stages, and, in addition, observed no significant mortality of adult thrips. Arthurs & Heinz (2006), in assessing the applications of *S. feltiae* against thrips on chrysanthemums, failed to reduce the amount of damage caused to the host plant.

In South Africa, 11 local EPN species and the exotic *S. feltiae* were tested under laboratory conditions for pathogenicity against western flower thrips. Generally, *Heterorhabditis* spp. were found to be more virulent than *Steinernema* spp. The study showed that *S. yirgalemense* (66 %)*, H. baujardi* (67 %) and *H. bacteriophora* (60 %) had potential for the control of *F. occidentalis* in terms of targeting its soil-dwelling stages. Results from a temporal development study showed that both *S. yirgalemense* and *H. baujardi* were able to complete their life cycles in the second stage larvae of *F. occidentalis* and to produce a new cohort of IJ (Dlamini *et al*., 2019a, b).

OVERCOMING CHALLENGES TO ABOVE-GROUND APPLICATION

The success of EPNs as biocontrol agents depends on their survival and their survival above ground is limited by several environmental factors, including temperature, ultraviolet (UV) radiation, and prevailing moisture/relative humidity (% RH) levels. This makes above ground applications of EPNs challenging.

Temperature

As nematodes are highly sensitive to changes in temperature, they must be kept in aqueous solutions ranging in temperature from 4°C to 30°C, with most species being intolerant to temperatures that are higher than 35°C for longer than 30 min at any one time (Grewal *et al.*, 1994). Relatively high temperatures also reduce the solubility of oxygen in solution. Depriving EPNs of oxygen for prolonged periods of time results in their deactivation and in their ultimate death (Wright *et al*., 2005). Different EPN species also have different thermal niches within which they can infect and establish themselves within their respective hosts. Grewal *et al.* (1994) listed the temperature niches for various species of nematodes in their interactions with last-instar *Galleria mellonella* Linnaeus (Tortricidae: Pyralidae) larvae. To minimise the negative effects of temperature, glasshouse and field applications of nematodes should take place either early in the morning or late in the afternoon. Nematodes which are tolerant to low temperatures, like *S. feltiae*, can be selected for use in relatively cool environments.

Ultraviolet (UV) radiation

Exposure to UV light should be taken into consideration when applying EPNs above ground. UV light and sunlight have both been shown to affect the behaviour and pathogenicity of both plant- (Godfrey & Hoshino, 1933) and animal-parasitic (Stowens, 1942) nematodes significantly. Gaugler & Boush (1978) observed the effects of short UV radiation and natural sunlight on *S. carpocapsae*, in terms of their interaction with *G. mellonella* larvae. They found that exposure of IJs to short-term UV radiation for 7 min caused reduced pathogenicity and increased larval survival time post-infection. Exposure to direct sunlight also reduced their pathogenicity by as much as 95% after 60 min. Gaugler *et al*. (1992) found that *S. carpocapsae* IJs were rendered completely inactive after 10 min of moderate UV exposure, whereas *H. bacteriophora* was significantly affected after only 4 min, indicating that the susceptibility to UV light varies across species. In general, nematodes are known to move away from direct sunlight towards cryptic microhabitats. The challenge posed by this vulnerability to UV light could also be avoided with the application of nematodes either early in the morning or in the late afternoon, which would give them enough time to move towards the cryptic microhabitat in which the target host is also most likely to reside.

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Humidity

Temperature and UV radiation are contributing factors to the desiccation of IJs when they are applied above ground. However, nematode survival and viability on foliage appears to be directly related to the prevailing RH. Glazer (1992), comparing the survivability of *S. carpocapsae* on bean foliage at 45%, 60% and 80% RH, showed that nematode survival and pathogenicity both improved at 60% RH, and with the addition of antidesiccants. Glazer *et al*. (1992a, b) assessed the survival of *S. carpocapsae* IJs used to control the cotton pests, *Earias insulana* (Boisduval) (Lepidoptera: Nolidae), *Heliothis armigera* (Hübner) (Lepidoptera: Noctuidae), and *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) at low RH. The addition of antidesiccants to the nematode solutions applied to cotton plants resulted in between 85% and 95% insect mortality, compared to 22% in the case of the control, as well as a significant decrease in damage to the foliage compared to the control.

From research to date, it can be concluded that one of the possible means of overcoming environmental limitations, particularly humidity, when applying EPNs above ground is the addition of adjuvants to modify the characteristics of the nematode suspension. Adjuvants are broadly defined as additives to pesticide solutions that are intended to increase, or to modify, their effects (Krogh *et al*., 2003). The United States Environmental Protection Agency also includes safeners and synergists in its definition of adjuvants. In South Africa, guidelines regarding adjuvants are still being developed, while EU regulations refer to both the co-formulant and the adjuvant collectively as ‘’adjuvants’’. The additives are defined more by function than by form, with adjuvant formulations ranging from carbon-chain polymers (e.g. Anti-Stress 2000®), bicyclic oxazolidine (Moisturin®), di-1-p-menthene (Nu-Film-17®), acrylic resin (Shatter-Proof®), and polymeric terpene (Transfilm®) (Shapiro-Ilan *et al*., 2010).

Determining the toxicity of any adjuvant to the nematodes themselves is very important. Shapiro-Ilan *et al*. (2010) tested five adjuvants (Anti-Stress 2000®, Moisturin®, Nu-Film-17®, Shatter-Proof® and Transfilm®) at concentrations of 2%, 20% or 40% for their toxicity to *S. carpocapsae* and showed that the rate of nematode survival only decreased significantly at a concentration of 40%, compared to the control. This concentration far exceeded the recommended concentration of Shatter-Proof® (12.5%), the adjuvant selected for field trials, because it resulted in the lowest numerical mortality of nematodes in suspension.

Adjuvant efficacy varies on a case-by-case basis. In testing several adjuvants in combination with EPNs for the control of the diamondback moth, *Plutella xylostella* Linnaeus, Baur *et al*. (1997) found that, whereas the adjuvants tested served to increase the pathogenicity of the nematodes, the overall benefit attained was probably insufficient to warrant the use of EPNs against this pest. The researchers also observed that several of the adjuvants tested were phytotoxic to radish leaves, highlighting the importance of screening adjuvants not only for efficacy and for nematode mortality, but also for host plant toxicity.

The availability of a variety of adjuvants in the form of surfactants, gels and polymers is an area that remains to be actively explored (Shapiro-Ilan & Mizell, 2012; Malan & Hatting, 2015). Mixing nematode suspensions with such adjuvants, or with a combination of such adjuvants, should facilitate the use of the biocontrol agents in above-ground areas that were previously considered inaccessible for nematode application.

CONCLUSIONS

EPNs have potential value as a non-toxic alternative to manufactured chemical pesticides, thus allowing producers an additional biological tool with which to control pests in an environmentally sustainable way. Above-ground insects, like mealybugs, are expected, in general, to be relatively susceptible to EPNs, because the latter present a novel predator threat to mealybug against which they could not have evolved defences. EPNs are intensively used under cover in greenhouses and shade houses, in which the conditions tend to be relatively optimal.

Foliage-based pests residing in cryptic habitats above ground, such as beneath bark, in boreholes, or under leaves that are out of the reach of the sun, would appear to be ideal targets for EPNs that require conditions of shade, moderate temperature, and high humidity to survive and be infective. The application of EPNs to insect pests in controlled environments (such as the laboratory and the glasshouse) is evidence of their potential as the biocontrol agents of pests in environments in which the levels of humidity remain high, in which desiccation is relatively slow, and in which nematodes are able to find, and infect, their insect hosts. In contrast, EPNs tend to perform poorly against pests of foliage in the field. The main barrier to the successful application of EPNs in the control of foliar pests has been concluded to be the environment, mainly due to desiccation of EPNs in environments where the humidity cannot be directly controlled.

To counter this, novel application methods have been developed to retard desiccation of foliar-applied EPNs, ranging from the post-application spraying of a gel that was originally used in firefighting, to the envelopment of treated areas with moistened diapers. Simple management practices, such as altering the time of application to either late in the evening or in the early morning, can play an important role in attaining nematode efficacy, as nematodes need only a few hours of optimum conditions to be able to infect the host. In South Africa, an additional challenge is the development of methods to culture local EPN isolates on an industrial scale, which is a pre-requisite for commercialisation. The successful use of EPNs on foliage requires cultural and chemical methodology to be put in place to maximise the persistence and infectivity of EPNs on foliage, be it through time-sensitive application, spray methods, adjuvant formulation or any combination of the three.

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