Entomopathogenic nematodes: can we use the current knowledge on belowground multitrophic interactions in future plant protection programmes? – Review

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Abstract: Plants under herbivore attack emit mixtures of volatiles that can attract the natural enemies of the herbivores. Entomopathogenic nematodes (EPNs) are organisms that can be used in the biological control of insect pests. Recent studies have shown that the movement of EPNs is associated with the detection of chemical stimuli from the environment. To date, several compounds that are responsible for the mediation in belowground multitrophic interactions have been identified. In the review, we discuss the use of EPNs in agriculture, the role of belowground volatiles and their use in plant protection programmes.

Keywords: insects; indirect plant defence; volatile organic compounds; chemical communication; insect parasitic nematodes

The rhizosphere represents the part of the soil that surrounds a plant’s root system. It contains a large number of organisms (Wenke et al. 2010). Several studies have shown that plants discharge numerous organic substances into the rhizosphere, which are categorised as amino acids, phenols, sugars, polysaccharides, amides and proteins, all of the higher molecular masses (Wenke et al. 2010; Voglar et al. 2019). Scientists have proven that plants can use their exudates to defend themselves against attacks by insects, bacteria and pathogenic fungi (Bais et al. 2006; Hiltpold et al. 2011).

An indirect defence is a mechanism whereby the plant releases certain volatile organic components that can repel plant pests or attract their natural enemies – predators, parasitoids, entomopathogenic nematodes (Turlings et al. 1990; Rasmann et al. 2005; Degenhardt 2009; Laznik & Trdan 2018). The benefits of an indirect plant defence include the reduction of herbivory and an increase in the reproductive fitness (Kessler & Baldwin 2001; Cheng et al. 2007). Volatile organic compounds (VOCs) are lipophilic components with a high vapour pressure that are released from leaves, blossoms, roots, and fruits into the atmosphere or soil (Dudareva et al. 2006). VOCs can be released by both damaged and undamaged plants and are classified as terpenes, benzenoids, derivatives of fatty acids, and phenylpropanoids (Bais et al. 2006). The release of VOCs depends on the type of damage (mechanical, herbivorous, etc.), as well as on the species of herbivorous insects (Gosset et al. 2009; Hiltpold et al. 2011; Laznik & Trdan 2013). Gosset et al. (2009) discovered that the plants damaged by the larvae of the Colorado potato beetle [Leptinotarsa decemlineata (Say, 1824)] release substances different from those released by the plants attacked by aphids. Exudates from plants have also other effects and roles. By root excretion, plants can encourage a beneficial symbiosis, regulate microbiological communities, inhibit the growth of neighbouring plants and regulate the physiochemical properties of the soil (Flores et al. 1999; Voglar et al. 2019).

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Furthermore, herbivores can accumulate plant toxins, which can be used for their defence against their natural enemies (Robert et al. 2017). For example, the larvae of the western corn rootworm (Diabrotica virgifera virgifera LeConte, 1868), which are among the most common pests of corn, are adapted to various plant defence mechanisms. Robert et al. (2017) showed that the larvae of the corn rootworm are able to accumulate benzoxazinoids, which are released during the herbivore attack by the corn (Zea mays Linnaeus) and they also act as a repellent to entomopathogenic nematodes. The results of their study show that herbivores can reactivate plant toxins, which can be used for their own defence against the enemies of the third and fourth trophic levels.

The multitrophic interactions pertinent to plant protection against herbivores describe the ecological influences of three or more trophic levels. These levels comprise the plants, the pests and their natural enemies (Turlings et al. 1990; Dicke 1999; Kessler & Baldwin 2002; Heil 2008; Degenhardt 2009; Ali et al. 2010; Hiltpold et al. 2013; Jagodič et al. 2017; Voglar et al. 2019) (Figure 1). Entomopathogenic nematodes (EPNs) are organisms that can be used in classical, conservational, and augmentative biological control programmes. Several studies showed that EPNs can use belowground volatiles as a cue to locate their hosts. In the review, we discuss the use of EPNs in agriculture, the role of belowground volatiles and their use in plant protection programmes.

**The use of entomopathogenic nematodes in agriculture**

Extensive research has demonstrated both their successes and failures in the control of insect pests of crops, trees, ornamental plants, lawn and turf (Ishibashi & Choi 1991; Hazir 2004; Head et al. 2004; Georgis et al. 2006; Laznik et al. 2010). EPNs naturally reside in the soil and are classified into the families of Steinernematidae and Heterorhabditidae (Ehlers 1998; Georgis et al. 2006; Poinar & Grewal 2012). EPNs form a dauer or stress-resistant stage known as the infective juvenile – IJ (Ehlers 1998). This developmentally arrested stage also plays a key role in the dispersal of the nematodes in the soil as they actively seek and infect suitable insect hosts (Campbell et al. 2003; Wilson et al. 2012). Additionally, the IJs are responsible for vectoring the entomopathogenic bacteria from one host to another. Upon locating and entering an insect host, IJs migrate to the haemolymph where they recover from their arrested state of development and release their bacterial symbionts. The bacteria reproduce, release toxins and kill the insect within 24–72 h (Shapiro-Ilan et al. 2003; Půža 2015). EPNs find their way through the soil by following chemical signals (chemotaxis). Through chemotaxis, they detect their hosts in the environment or detect the area in which the host is more likely to be present (Lewis 2002). Several studies have shown that the movement of EPNs in the soil is also affected by other factors – CO₂, plant exudates, pH, temperature, electric potential, VOCs (Burman & Pye 1980; Grewal et al. 1993; Rasmann et al. 2005; Shapiro-Ilan et al. 2012; Turlings et al. 2012).

The use of EPNs in a biological control has been traditionally associated with suppressing soil-inhabiting insect pests (Ishibashi & Choi 1991; Georgis et al. 2006). Research results from the last two decades indicate that they also have the potential to suppress above-ground insect pests, but only under certain circumstances (Hazir 2004; Head et al. 2004; Laznik et al. 2010). The reduced efficiency of EPNs in the suppression of above-ground insect pests is primarily due to their exposure to ultraviolet radiation (Gaugler et al. 1992), thermal extremes (Grewal et al. 1994), and insufficient moisture (Lello et al. 1996). These

![Figure 1. The possible influences of the different soil organisms on the root emissions of the volatile organic compounds](image-url)

1 – neighbouring plants; 2 – microbes, including bacteria and fungi; 3 – herbivores, including nematodes and insects; 4 – parasites and predators of the herbivores, such as entomopathogenic nematodes seeking an insect host.

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factors are of crucial importance for the survival of EPNs (Smits 1996). For this reason, EPNs are less efficient against above-ground insect pest outdoors, although previous laboratory tests showed a much higher efficiency (Laznik & Trdan 2011; Majić et al. 2019). The most common EPN formulation is an aqueous suspension (Cruz-Martínez et al. 2017). The equipment that is intended for spraying pesticides, manuring or irrigation can be used for EPN applications. For this purpose, a backpack, manual or tractor sprayers and sprinklers are suitable. IJs can pass through the spray tubes with diameters of at least 500 μm and are capable of withstanding pressures up to 2 000 kPa (Wright et al. 2005). Furthermore, IJs can tolerate short-term exposure (2–24 h) to many chemical and biological insecticides, fungicides, herbicides, fertilisers, and growth regulators and can be tank-mixed and applied together with such products (Head et al. 2000; Koppenhöfer et al. 2002; Krishnayya & Grewal 2002; De Nardo & Grewal 2003; Schroer et al. 2005; Laznik & Trdan 2014).

Nematode-pesticide combinations in tank mixes could offer a cost-effective alternative to foliar integrated pest management (IPM) systems.

Due to the sensibility of the nematodes to ultraviolet radiation, they have to be applied to plants in the evening, early in the morning or in cloudy weather, when the radiation is less intense (Lello et al. 1996; Laznik et al. 2010). Nematode survival and efficacy on the foliage are enhanced to varying degrees by the addition of various adjuvants (e.g., glycerol, various polymers) or UV-protective (brighteners) actions (Glazer et al. 1992; Grewal 2002), although more needs to be done to enhance the post-application survival. The greatest potential for using EPNs against foliar pests is almost certainly in IPM programmes, in conjunction with other biocontrol agents (Sher & Parella 1999) or selective chemicals (Rovesti & Deseö 1990; Head et al. 2000).

EPNs are considered exceptionally safe biological agents (Ehlers 2001). Because their activity is specific, they present no environmental risk other than being chemical agents for plant protection (Ehlers 1998). There have been no documented cases of environmental damage due to EPNs, since their first use for suppressing beetles of the species Popillia japonica Newman, 1841, in the USA (Glaser & Farrell 1935). The use of nematodes is safe for the users. EPNs and their bacterial symbionts are not harmful to mammals and plants (Boemare et al. 1982; Akhurst & Smith 2002).

The multitrifrophic interactions between the plants, soil-dwelling pests and entomopathogenic nematodes

The emission of belowground VOCs is a hereditary acquired characteristic similar to other plant characteristics. Studies in this field are scarce, so we have no direct proof that natural selection affects the emission of VOCs for the defence (direct and indirect) against herbivores (Hiltpold et al. 2013). Nevertheless, some authors report that the production of VOCs is hereditary (Ali et al. 2010; Hiltpold et al. 2013). Scientists have proven this concept in citrus and maize. In maize, the production of (E)-β-caryophyllene (E-β-C) reduces the attacks by harmful organisms and, subsequently, reduces the extent of the root destruction (Degenhardt et al. 2009). Several research teams have proven that the majority of maize cultivars known today have lost their ability to form such alarm signals due to the crossings and other plant-breeding procedures (Köllner et al. 2008; Laznik et al. 2011). Other authors consider that soil organisms exert a strong influence on the level of the variability of the emitted VOCs in plant populations (Maron & Kauffman 2006). Extensive attacks by soil organisms can cause increased emissions of more toxic VOCs.

Wang and Gaugler (1998) were among the first scientists who proved that VOCs released by roots of plants attract EPNs. They discovered that the IJs of Steinernema glaseri (Steiner, 1929) and Heterorhabditis bacteriophora (Poinar, Jackson, 1987) to the exudates emitted by conifer (Thuja occidentalis Linnaeus) roots damaged by the larvae of Otiorhynchus sulcatus (Fabricius, 1775). A similar study was also carried out by Boff et al. (2001). They discovered that the EPN H. megalidis was attracted to the exudates of strawberry roots damaged by O. sulcatus larvae.

Laznik and Trdan (2016a) investigated the effects of VOCs [released from carrots (Daucus carota Linnaeus) that were attacked by wireworms (Elateridae)] on EPNs. The key finding of their study was that undamaged carrot roots excreted substances (terpinolene) that had a repellent effect on the EPNs (Table 1). This means that plants emit chemical signals into the environment as a message that they are healthy and do not need additional protection (Laznik & Trdan
Table 1. The attraction behaviour of entomopathogenic nematodes (EPNs) towards the different compounds

<table>
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<tr>
<th>Compound</th>
<th>EPNs species</th>
<th>Behavioural response</th>
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2016a). These researchers continued their research with the inclusion of the potato (Solanum tuberosum Linnaeus) (Laznik & Trdan 2016b), whose tubers had been damaged by the larvae of the forest cockchafer (Melolontha hippocastani Fabricius, 1801). Their study included seven VOCs emitted by the damaged potato tubers: octanal, decanal, undecane, nonanal, 6-methyl-5-hepten-2-one, 1,2,4-trimethylbenzene and 2-ethyl-1-hexanol (Table 1). The research results showed that the EPNs were capable of distinguishing the individual VOCs that were released by the damaged or undamaged potato tubers, and the reaction of the EPNs to the VOCs was species-specific, prevailed over the host-seeking strategy and had been developed by the EPNs through evolution (Laznik & Trdan 2016b).

Crespo et al. (2012) confirmed that black mustard (Brassica nigra Linnaeus) roots damaged by the larvae of the cabbage fly (Delia radicum Linnaeus) emit glucosinolates and numerous sulfur compounds. Additional studies were carried out by Jagodič et al. (2017). They studied the effects of six VOCs that were emitted by damaged roots of the black mustard on the EPN movement. They discovered that certain glucosinolates (allyl isothiocyanate, phenylethyl isothiocyanate, and benzonitrile) as well as sulfur compounds (dimethyl sulfide, dimethyl disulfide, dimethyl trisulfide) act as repellents to certain species of EPNs (Steinernema kraussei Steiner, 1923) (Table 1) (Jagodič et al. 2017).

EPNs are efficient biotic agents for suppressing western corn rootworm (WCR) larvae, which are among the most important pests in maize. The first studies with olfactometers showed that maize roots damaged by the larvae of the WCR attracted the EPN H. megidis to a greater extent than the undamaged or mechanically damaged maize roots (Rasmann et al. 2005). The difference between the undamaged maize roots and the roots damaged by the larval WCR was that the former released the terpene E-β-C, which acted as an attractant for H. megidis (Table 1) (Rasmann et al. 2005). The importance of this research is augmented by the fact that the results obtained in the laboratory study were also confirmed by the field experiments. It has been discovered that EPNs can locate their hosts from longer distances, as far as 50 cm away from the plants.

Research on the common milkweed (Asclepias syriaca Linnaeus) has shown that plants attacked by the soil pest Tetraopes tetraophthalmus (Forster, 1771) release VOCs that attract the EPN H. bacteriophora (Rasmann et al. 2011). Due to the complex composition of the VOCs released by the common milkweed under attack, it is difficult to determine which component attracts the H. bacteriophora. A follow-up field experiment established that the EPN affected the survival of the T. tetraophthalmus larvae, which consequently enabled the plants to regain their above-ground biomass (Rasmann et al. 2011). This experiment was among the first to prove that the natural enemies of herbivores contribute to the plants’ positive selection when producing signals in the case of herbivores.

Ali et al. (2010, 2011) discovered that certain species of EPNs were strongly attracted by exudates of citrus roots that had been damaged by the larvae of the diaprepes root weevil [Diaprepes abbreviatus (Linnaeus, 1758)]. They discovered that the citrus roots emitted a simple mixture of terpenoids. This type of defence mechanism in plants can be constitutive or established only in the case of an attack; in both cases, the exudates attract EPNs (Ali et al. 2011). The researchers also discovered that the VOCs that attracted the EPNs could also attract phytoparasitic nematodes, which can pose a great danger to the plants. This explains the fact that the majority of the studied plants released the VOCs only when attacked. The only citrus cultivar studied by Ali et al. (2011), which released the VOCs constitutively, was also resistant to the phytoparasitic nematodes.

Hallem et al. (2011) tested a spectrum of different plant VOCs (Table 1). They discovered that the VOCs attracted/repelled the IJs of H. bacteriophora and Steinernema carpocapsae (Weiser, 1955). EPNs have exceptionally well-developed chemosensory apparatuses, which are very sensitive to plant components, which they efficiently use to locate suitable hosts (Rasmann et al. 2012).

The application of VOCs in agriculture

EPNs have exceptional potential for suppressing different species of soil pests (Toepffer et al. 2009). Accurate knowledge of the VOCs emitted by plants could help improve the protection of agriculturally important plants from harmful organisms in the future, especially for protecting plants with EPNs (Degenhardt et al. 2003; Pickett et al. 2006; Turlings & Ton 2006; Hiltgold & Turlings 2008).

It has been discovered that the signals emitted by the above-ground parts of the plants can be improved or strengthened by the means of the genetic transformation of the plants. This has been proven both in laboratories and in greenhouses, where such manipulations improved the attractiveness of plants.
to predatory mites (Kappers et al. 2005) and parasitic wasps (Schnee et al. 2006; Fontana et al. 2011). The identification of the terpene E-β-C, which was emitted by maize roots damaged by herbivores, provides a great starting point opportunity to test this kind of approach to protect plants against insect pests. The scientists who carried out the experiment used American maize cultivars that had lost the ability to release E-β-C (Rasmann et al. 2005; Köllner et al. 2008); thus, they could confirm that the plants’ signals, emitted under the herbivore attack, could be used to further improve the protection of the plants against the herbivores. The signalisation in the maize was restored in a non-productive line by means of the genetic transformation of the gene for the E-β-C synthesis, which was identified in oregano (Origanum vulgare Linnaeus) (Crocoll et al. 2010). The successful transformation of the maize plants made them release sesquiterpene in amounts equivalent to those released by normal maize plants when attacked by the WCR larvae. In contrast with the untransformed maize plants, the transformed plants emitted E-β-C constitutively and systemically (releasing the VOCs from the roots to the leaves) (Degenhardt et al. 2009). An extensive field experiment revealed that the transformed plants received much more EPN protection against root damage by the WCR larvae than the plants (Degenhardt et al. 2009). However, we should bear in mind that the constitutive release of such signals can negatively affect the agricultural applications. The emission of VOCs can also attract harmful organisms into the immediate vicinity of economically important field crops, and it can also be very energy inefficient, which can influence the qualities and quantities of the yields. For this reason, the scientists improved the procedure by placing an inducible promoter in front of the gene for the synthesis of E-β-C.

In addition to improving the signal itself, the responsiveness of the EPNs to the signals can also be improved. A large number of IJs are produced by every generation of the EPNs, and the generations are short, which makes them ideal study subjects for the facilitation of host-finding and the infectiveness itself (Gaugler et al. 1989; Gaugler & Campbell 1991; Griffin & Downes 1994). The increased responsiveness of the EPNs to the plant VOCs was proven again with E-β-C (Hiltpold et al. 2010a). Using an olfactometer and sprayers, the researchers proved the increased E-β-C responsiveness (Hiltpold et al. 2010a) in H. bacteriophora IJs. Unlike H. megidis, H. bacteriophora does not usually react to E-β-C (Rasmann & Turlings 2008; Hiltpold et al. 2010c), yet it only took six generations of selection to obtain a strain with high-levels of responsiveness (Hiltpold et al. 2010a). In a field experiment in Hungary, this EPN strain was more efficient in suppressing WCR larvae near maize roots that emitted E-β-C than the original strain, but no difference was observed between the H. bacteriophora strains in the maize that did not emit E-β-C (Hiltpold et al. 2010a), and the durability of both EPN strains was the same (Hiltpold et al. 2010b). The experiment also proved that the responsiveness to E-β-C was not very specific, as the chosen strain also displayed responsiveness to other sesquiterpenes, such as E-β-farnesene, whereas it responded to the monoterpenene α-pinene to a lesser degree (Hiltpold et al. 2010a). These data, together with those from the study of diffusion (Hiltpold & Turlings 2008), show that the attractiveness of the evaporative plants is not specific, as it also depends on the way the VOC compounds are diffused into the soil.

Scientists have already found ways to improve plant signalisation and the responsiveness of the EPNs to these signals. However, the difficult and labour-intensive field application of EPNs undoubtedly remains one of the largest obstacles for their use in plant protection. Maintaining large numbers of EPNs in the soil is seldom successful (Shapiro-Ilan et al. 2006). Hiltpold et al. (2008) consequently introduced a new application method based on capsules that contained EPNs. The precise formulation of the capsule’s shell enables the controlled release of EPNs. In theory, it would be possible to adjust the sowing machines to enable the insertion of the EPN capsules into the soil during sowing, which would save time, reduce the labour intensity, and prevent large EPN losses. The applications of the capsules could be especially efficient if they also contained attractants and other stimulative substances that would attract harmful organisms to the capsules.

It seems that the application of VOCs represents a promising tool in the biological control of plant pests (Kaplan 2012). A number of scientists have tried to genetically transform plants to increase their potential for VOC emissions (Brillada et al. 2013). However, such a system in which the plants’ behaviour is deliberately and successfully used in the biological control is of a much more complex nature (Khan et al. 1997; Hassanali et al. 2008). A major restricting factor may be the fact that the emission of VOC’s as
indirect defences would not be only a matter of the breeding procedures, and this could frequently compromise the useful interactions between the plants and the third trophic level. For example, commercial varieties of cotton (*Gossypium hirsutum* Linnaeus) release seven times lower VOC quantities compared to natural cotton lines (Loughrin et al. 1995). Similarly, the roots of the North American maize varieties do not release the terpene E-β-C, which attracts EPNs (Rasmann et al. 2005). Therefore, it is not very probable that the VOCs were deliberately chosen in plant breeding programmes, though the amounts of released VOCs have not diminished in certain *Brassica* Linnaeus (Benrey et al. 1998) or maize cultivars (Gouinguené et al. 2001).

Agricultural ecosystems are complicated to manipulate (Kaplan 2012), and substantial research on the protection of plants against harmful substances is required to thoroughly understand how such systems work. What are the most frequent problems encountered by scientists during the integration of VOCs for agricultural purposes? We have already established that the increased attraction of parasitoids to VOCs did not increase the level of parasitism in the field (von Mérey et al. 2012; Vieira et al. 2013). Parasitised herbivores also grew larger and used more resources than unparasitised herbivores (Alleyne et al. 1997; Harvey et al. 2010). We should bear in mind that the VOCs represent information that is accessible to other organisms, and they can also be used by herbivores and hyperparasitoids to locate their hosts. For example, maize plants that were treated with evaporative components had more intense degrees of WCR and *Spodoptera frugiperda* (Smith, 1797) infestation, which resulted in more damage than in the control plants (von Mérey et al. 2011).

**CONCLUSION**

VOCs merely represent information, and parasitoids and predators can quickly learn or adjust to avoid plants that emit VOCs without herbivores. It is also important to bear in mind that agricultural ecosystems are not always able to maintain populations of the useful arthropods that are required for the stable biological control. Finally, the integration of such biologic strategies will require a large amount of effort, as these strategies do not enable the complete removal of the harmful organisms from the system.

The endeavours to develop an efficient biological strategy for the plant protection will have to adopt a broader perspective, paying more attention to the direct effects of the VOCs on the harmful organisms and pathogens. Repulsion was an important property when VOCs were successfully used in agricultural experiments (Hassanali et al. 2008; Braasch & Kaplan 2012; Xiao et al. 2012), but the antimicrobial properties of VOCs should be given more attention. In the defence against harmful organisms, wild plants do not rely solely on one defence strategy; consequently, the biological control strategies should also follow suit.

The quantity of the studies of multitrophic interactions with chemical mediation is multiplying rapidly. There is also a growing number of studies that show that root exudates are active players in the rhizosphere and that they can influence the formation of their growth environment, by protecting plants and optimising their efficiency. It has been proven that they chemically affect soil microorganisms and other useful soil organisms, such as EPNs (Voglär et al. 2019).

We primarily lack a solid understanding of the co-evolutionary perspectives in rhizosphere ecology and soil interactions. A solid understanding of these processes would facilitate approaches that preserve the well-established beneficial interactions between the domestication and cultivation of economically important cultivars. We should emphasise that the possible consequences of such manipulations should be evaluated prior to the actual practical applications of the plant protection techniques discussed herein. It is a fact that we can find hundreds of different species of microorganisms in a handful of soils and that a change in one parameter can cause unexpected consequences in an established ecosystem and disturb the stability of the soil's function. Since a soil is a complex and heterogeneous ecosystem, the application of different strategies cannot be generalised, and the strategies will require very precise assessments in the different cases. The knowledge acquired this way will further enable the improvement of sustainable practises.

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