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# Enhancing the use of entomopathogenic nematodes for biological control of root pests: from field persistence to improved shelf-life

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## "Enhancing the use of entomopathogenic nematodes for biological control of root pests: from field persistence to improved shelf-life"

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## Table of content

Summary……………………………………………………………………………………………...4

Résumé………………………………………………………………………………………..................6

Acknowledgements……………………………………………………………………………….8

General introduction and thesis outline………………………………………………………..10

Chapitre I

Prevalence and activity of entomopathogenic nematodes and their antagonists in soils that are subject to different agricultural practices……………………………………………………………...33

Chapitre II

Highly potent extracts from pea (*Pisum sativum*) and maize (*Zea mays*) roots can be used to induce quiescence in entomopathogenic nematodes………..…………………………………………66

Chapitre III

Can cover crops enhance overwintering survival of entomopathogenic nematodes in annual crops? ………...........................................................................................................................................83

Annex……………………………………………………………………………………………110

Conclusion and outlook………………………………………………………………………..115

## Summary


Agriculture is evolving towards more sustainable strategies since the application of agrochemicals and physical management of the soil have severe environmental impact and deplete soil quality. The potential of using biocontrol agents to replace or in combination with pesticides has been investigated for several decades. Entomopathogenic nematodes (EPN) are very promising in this respect. EPN are obligatory parasites of insects and together with symbiotic bacteria kill their host very rapidly. EPN can be mass-produced and efforts to develop novel formulation and application methods are ongoing to reduce the cost and increase their biocontrol efficacy as compared to pesticides. Moreover, it is recognized that investigations into how soil characteristics affect naturally occurring and the released EPN are needed to develop management and application strategies that provide favorable conditions for EPN.

Understanding the conditions that are necessary for nematodes to survive and to remain active in the soil is a key aspect for the successful application of EPN. We used traditional insect baiting methods and quantitative polymerase chain reaction (qPCR) measurements to investigate the effect of different farming systems (conventional, bio-organic, and biodynamic) and crop type (winter wheat, maize, grass-clover ley) on EPN, as well as on other members of the associated food web. The abundance of EPN was very low in the numerous field sites that were investigated throughout Switzerland, and no difference was detected between the different farming management practices that were included in the studies. However, the crop type influenced the distribution of the investigated microorganisms that were found to be more abundant in winter wheat plots.

In this thesis I also investigated plant root exudates and extracts containing a compound or compounds that trigger a reversible state of quiescence in EPN. The eventual aim is to incorporate such “quiescence factors” (QF) in EPN formulations in order to increase the shelf-life of such formulations. We found that pea (*Pisum sativum*) root-cap exudate triggered quiescence in several species of EPN. We develop a method to extract the active compound(s) from deep-frozen roots to increase the concentration of the sought-after compound(s), and we showed that this extract was highly potent and could also be readily obtained from maize (*Zea maize*) roots. EPN were able to recover and infect insect larvae even after exposure to very high doses of QF. Efforts to identify the active compound(s) are still ongoing.

Finally, naturally occurring or applied EPN need to remain active in the soil for a sufficient time period for them to be able to play their biocontrol role. The fate and persistence of EPN depend greatly on the composition of the soil and its protection against biotic and abiotic stresses. We investigated the use of winter cover crops that may enhance the survival of EPN throughout the winter. Again using insect baiting and qPCR methods we showed that cover crops have minimal positive effects on the overwintering survival and activity of EPN. The abundance of naturally occurring EPN was confirmed to be very low, but applied EPN were able to survive the

winter. Their abundance decreased over time, but they were found to be as infectious after the winter (March) than just one month after application during the preceding fall (November).

Together, our data show that EPN in Swiss agricultural soil are scarce and their numbers do not seems to be affected by the different farming practices and they could be used in program involving heavily managed soils. Moreover, we bring strong support for the potential of a naturally plant-produced QF to be included in EPN formulation to increase their shelf-life. Finally, applied EPN persisted through the winter, but was moderately enhanced by the presence of a cover crop. We hypothesize that the low prevalence of EPN is due to a lack of suitable hosts, especially in agricultural fields. As yet, most Swiss crops have not suffered from extensive soil pest invasions and therefore EPN scarcity may not be relevant in these systems. This will change in case of pest outbreaks. The current EPN numbers in the soil may not be able to stop a spreading pest population and under those circumstances, an augmentation approach is advisable.

Keywords: Entomopathogenic nematodes · Annual crop · Agricultural practices · Cover crop · Quantitative real–time PCR · Quiescence · EPN formulation

**Résumé**

L'agriculture évolue vers des stratégies plus durables car l'application de produits agrochimiques et la lourde gestion des sols ont un impact sur l'environnement et réduisent la qualité des sols. La possibilité d'utiliser des agents biologiques, pour remplacer ou en combinaison avec les pesticides, est une solution envisagée depuis plusieurs dizaines d'années. Les nématodes entomopathogènes (NEP) sont très prometteurs à cet égard. Les NEP sont des parasites obligatoires d'insectes. Les NEP, combinés à leurs bactéries symbiontes, sont capables de tuer leurs hôtes très rapidement.

Les NEP peuvent, dès maintenant, être produits en masse, et de nombreux efforts sont mis en œuvre pour développer de nouvelles méthodes de formulations et d'applications afin de réduire leur coût et de les rendre compétitifs comparés aux pesticides. De plus, Il est important de connaître les caractéristiques et les effets des sols sur les micro-organismes pour développer des stratégies de gestions et d'applications qui permettent aux NEP, présents naturellement ou introduits, d'être dans de bonnes conditions pour jouer leurs rôles d'agents biologiques.

Comprendre les conditions nécessaires aux NEP pour survivre et rester infectieux dans un certain type de sol est un point clé pour le succès de leurs établissements. Nous avons utilisé une méthode commune qui consiste à utiliser une larve comme appât, ainsi que la technique de la réaction en chaine polymérase quantitative (RCPq) pour mesurer les effets de différents systèmes agricoles (conventionnel, organique, biodynamique) ainsi que du type de culture (blé d'hiver, maïs, fourrages graminée-trèfle) sur les NEP, mais aussi sur d'autres membres de leur chaîne alimentaire. Dans tous les champs que nous avons investigués à travers la Suisse, les NEP étaient peu abondant, et nous n'avons détecté aucune différence entre les différents systèmes agricoles inclus dans cette étude. Cependant, le type de culture a influencé la distribution des NEP et des membres de leurs chaînes alimentaires qui étaient plus abondants dans les parcelles de blés d'hiver.

Dans cette thèse, nous avons aussi investigué des exsudats et des extraits de plantes contenant un ou des composés qui déclenchent un état réversible de dormance (ou quiescence) chez les NEP. Le but éventuel étant d'intégrer ces « facteurs de quiescence » (FQ) dans les formulations de NEP pour augmenter leur durée de vie. Nous avons montré que les exsudats de l'apex racinaire de germinât de petit pois (*Pisum sativum*) déclenchent un état de quiescence chez plusieurs espèces de NEP. Puis nous avons développé une méthode d'extraction, à partir de racine gelée dans l'azote liquide, pour augmenter la concentration du ou des facteur(s) de quiescence, et montré que ces extraits sont très puissants. Ils peuvent également être obtenus à partir de l'intégralité de racines de maïs (*Zea maize*). Les NEP sont capables de se réveiller et d'infecter une larve malgré une exposition à une forte dose de FQ. Les efforts produis pour identifier le ou les composés actifs sont toujours en cours.

Finalement, les NEP, naturellement présents dans le sol ou introduits, ont besoin de rester infectieux pour une période de temps suffisante, leur permettant de jouer leurs rôles d'agents biologiques. Le sort et la persistance des NEP dans un milieu dépendent en grande partie de la composition du sol et des stress biotiques et abiotiques qu'ils peuvent y rencontrer. Nous avons testé la possibilité d'utiliser des parcelles sous couvert végétaux au cours de l'hiver pour augmenter la survie des NEP pendant la période froide. De nouveau nous avons utilisé la méthode d'appât et de qPCR et montré que le couvert végétal avait un effet marginal sur la survie et l'infectivité hivernale des NEP. L'abondance des NEP naturellement présents dans le sol était très basse comme dans nos précédents résultats, mais celle des NEP introduits étaient plus satisfaisante même après l'hiver. Leurs abondances ont décru au fil du temps, mais ils étaient plus infectieux après l'hiver (Mars) que seulement un mois après les avoir introduits (Novembre).

Globalement, nos données montrent que les NEP étaient peu abondants dans les sols agricoles Suisse et leurs nombres ne semblaient pas être affectés par les différents systèmes agricoles mis en place. Cela voudrait dire que les NEP pourraient être utilisés dans des programmes de lutte intégrés dans des sols dont la gestion est lourde. De plus, nous avons mis en évidence le grand potentiel d'un composé naturellement produit par les plantes pour augmenter la durée de vie des NEP et donc d'être inclus dans les formulations de NEP. Finalement la survie après l'hiver des NEP appliqués était satisfaisante mais n'était que marginalement améliorée par la présence d'un couvert végétal. Nous faisons l'hypothèse que le manque d'hôtes adaptés, surtout dans les cultures, peut expliquer la faible présence des NEP. En Suisse, le sol n'est pas sujet à une invasion massive d'une peste, et le rôle des NEP est pour le moment optionnel. Cependant, en cas d'invasion, la faible abondance que nous avons enregistrée pourrait ne pas être suffisante pour contrôler une population de peste qui se répandrait. C'est pour cela que nous suggérons de contrôler la population de NEP à nouveau, et si leur abondance est toujours aussi faible, nous conseillons d'introduire des NEP.

Mots-clés: Nématodes entomopathogènes · Cultures annuelles · Pratiques agricoles · Cultures de couverture · PCR quantitative en temps réel · Quiescence · Formulation de NEP

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Fouzy and get free

I started my thesis four years ago, not knowing whether it was the right thing for me to do. As I am finishing it, I still do not know if it was the right thing to do! But if my feelings did not change during those four years, did not deplete, and that I hold to the end it is because it has been such an amazing day to day experience to work in the FARCE laboratory and all the places we have been collaborating with. I have so many people to thanks; the list will be long…

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# Introduction
# and
# Thesis outline

## Introduction

*Preface*

To face unprecedented environmental pressures caused by climate change and increasing landscape modifications due to human activities, agriculture needs to adopt novel and sustainable cropping strategies. Farmers face two main challenges; to maintain a healthy and nutritious soil and to protect the crops from herbivores and diseases. Intensive agriculture that has been developed to satisfy a worldwide increasing demand for food and increased competitiveness of the market has resulted in a reduction of crop biodiversity, making them more vulnerable to pests (Root, 1973; Grez et al., 1995) and has degraded soil quality (Amundson et al., 2015; Virto et al., 2014). To address pest problems, chemical pesticides have been intensively used (Pimentel, 1997) leading to important environmental disturbances (van der Werf, 1996). As an answer to the degradation of soils, conventional farmers apply/applied mineral fertilizers, complementary nutrients that led to a modification of the soil chemistry, thereby modifying soil proprieties with a noteworthy decrease of organic matter and soil biological activity (White, 2009). It is increasingly recognized that a sustainable agriculture requires that the overall quality of the soil, a key element to optimal agricultural production, should be conserved or improved to enhance productivity in an eco-friendly manner. Prompt action is needed to tackle current soil-related problems, such as contamination by agrochemicals, depletion of resources and soil erosion (Komatsuzaki and Ohta, 2007; Lal, 2009; Abdollahi and Munkholm, 2014).

*Beneficial organisms*

Traditionally, farmers and scientist have focused their effort on suppressing detrimental organisms that are harmful for crops, such as herbivorous insects and plant pathogens. The conventional and intense use of chemicals to fight pests and diseases affects soil quality by decreasing biodiversity and affecting the populations of numerous non-target organisms (Köhler and Triebskorn, 2013). It is now widely recognized that sustainable solutions and strategies for the control of pests and diseases are needed (i.e. integrated pest management, biological control). For this purpose, a wide range of biological systems may be exploited as alternatives to chemicals (Nicot et al., 2011). Rather than solely focusing on the suppression of the pests, it is equally relevant to investigate solutions that help to enhance the abundance of beneficial organisms that can suppress pests (Higa and Parr, 1994). Naturally occurring beneficial organisms can also directly improve crop health, yield, and protect the soil against major disturbances (Cardinale et al., 2003; Lacey et al., 2015). Some strategies to use beneficial organisms for crop protection are already commonly practiced, notably involving the classical release of natural enemies that very specifically target invasive pest species (Mcevoy, 1996). An augmentative approach by regularly

releasing native natural enemies against persistent pests is largely limited to small-scale agriculture such as in greenhouse (Grewal et al., 2006). The use of beneficial organisms as biocontrol agents in large-scale agriculture needs to be optimized to increase their efficiency in a cost-effective manner (Lacey, 2015).

*Entomopathogenic nematodes, good candidates as biocontrol agent*

Entomopathogenic nematodes (EPN) have been studied intensively because of their tremendous potential as biological control agents (Lewis et al., 2006). EPN of the genera *Steinernema* and *Heterorhabditis* are obligate parasites of insects. They are associated with symbiotic bacteria of the genera *Xenorhabdus* and *Photorhabdus* respectively, which they carry with them and introduce into their insect host upon initial infection. Their combined effects are lethal to the insects (Dowds and Peters, 2002; Lewis 2002). The infective juveniles (IJs), the only stage of EPN that lives freely outside of the host, have the formidable task to locate and enter a host through the cuticle or the natural openings. Once inside, the IJs release the symbiotic bacteria, which will kill the host by septicemia within two to five days (Dillman et al., 2012). The IJs and the bacteria feed on the liquefying host, and IJs will mature to adults that reproduce and eventually will give a new generation of IJs. When the resources are depleted, the new IJs generation leave the dead host (Dillman et al., 2012) (Fig 1).

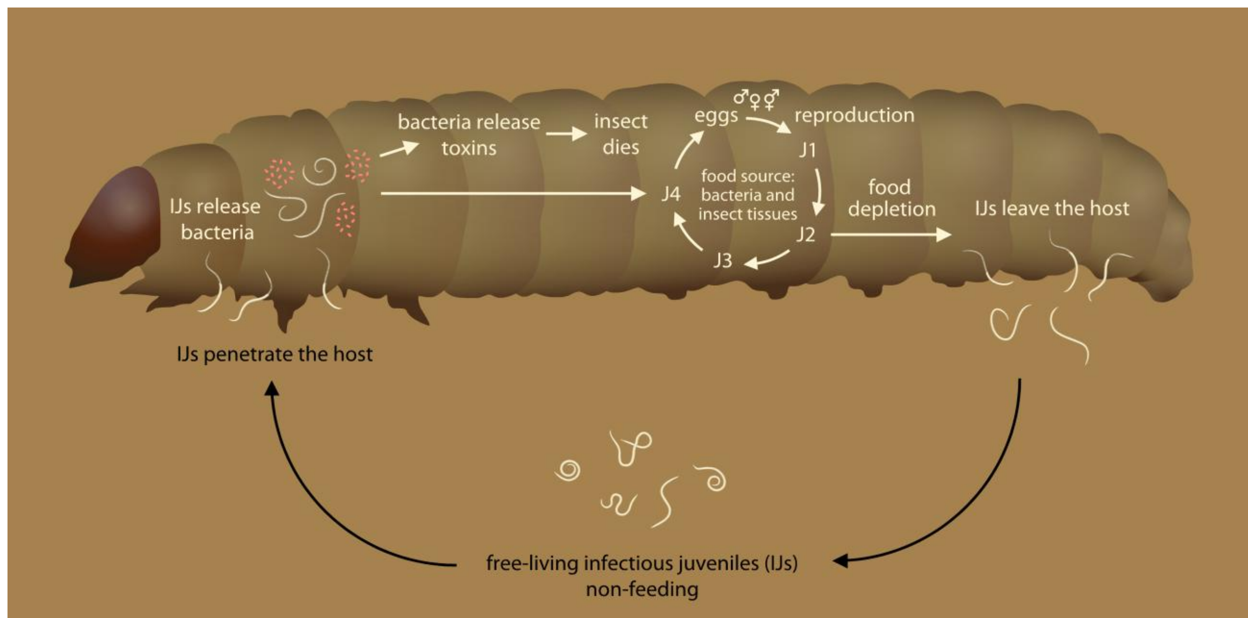


Fig 1. Entomopathogenic nematodes life cycle inside a larvae. Figure adapted from Stock (2015). Drawings by Geoffrey Jaffuel and Thomas Degen.

*Limitation of the use of entomopathogenic nematodes as biocontrol agent*

Unfortunately, despite their great potential as biocontrol agents, the application of EPN is currently rather costly compared to the use of pesticides and their commercial successes are

scarce and mainly limited to home gardening and small-scale farming (Georgis et al., 2006; Lacey et al., 2015). But some successful and encouraging cases of using EPN as biocontrol agent at large-scale application have been reported. There are several reasons for the limited success of EPN as biocontrol agents. For example, IJs do not feed until they infect a host and thus, without hosts, their survival and infectiousness decrease considerably over time (Shapiro-Ilan et al., 2006; Koppenhöfer, 2007). Another issue is the need to improve current methods of application. EPN are commonly sprayed or delivered through irrigation systems in water suspensions on plants or on the soil. Using those technics, EPN showed some successes in controlling pest populations, but also numerous failures (Shapiro-Ilan et al., 2006, Shapiro-Ilan and Dolinski, 2015). Most of the time, by using those methods, only a limited number of IJs will reach the target insect pests, and IJs suffer rapid desiccation and UV radiation exposure, which are both highly detrimental (Gaugler et al., 1997) (Fig 2). Therefore, various studies have focused on EPN formulation to increase the shelf-life and infectiousness of IJs over time and on the development of innovative application methods allowing a better release and efficiency of IJs in the field (Shapiro-Ilan et al., 2006, 2015; Hiltpold, 2015). One of the first factors to consider prior to release is the choice of the EPN species and the concentration threshold required to reach an efficient control of the pest (Shapiro-Ilan and Dolinski, 2015). EPN behavioral traits and tolerance to adverse abiotic factors vary from one species/population to another. Approaches such as those described in Grunder (2005) allow the selection of the appropriate species or strain against a specific targeted pest. Knowledge on the biotic and abiotic conditions present at the site of release is also necessary to choose the best adapted species or population of EPN to release.

After selecting the EPN species with the greatest control potential, developing an effective application method is critical. Most of the current application methods are derived from commonly used farming equipment and involve spraying or introduce EPN via irrigation systems (Shapiro-Ilan and Dolinski, 2015; Toepfer et al., 2010). These methods have their limitations due to factors such as pump type, pressure, nozzle shape that can reduce post-application EPN effectiveness (Baetman et al., 2007). Better adapted machineries with an increased compatibility with EPN are under investigation (Beck el al., 2013, 2014; Brusselman et al., 2012)

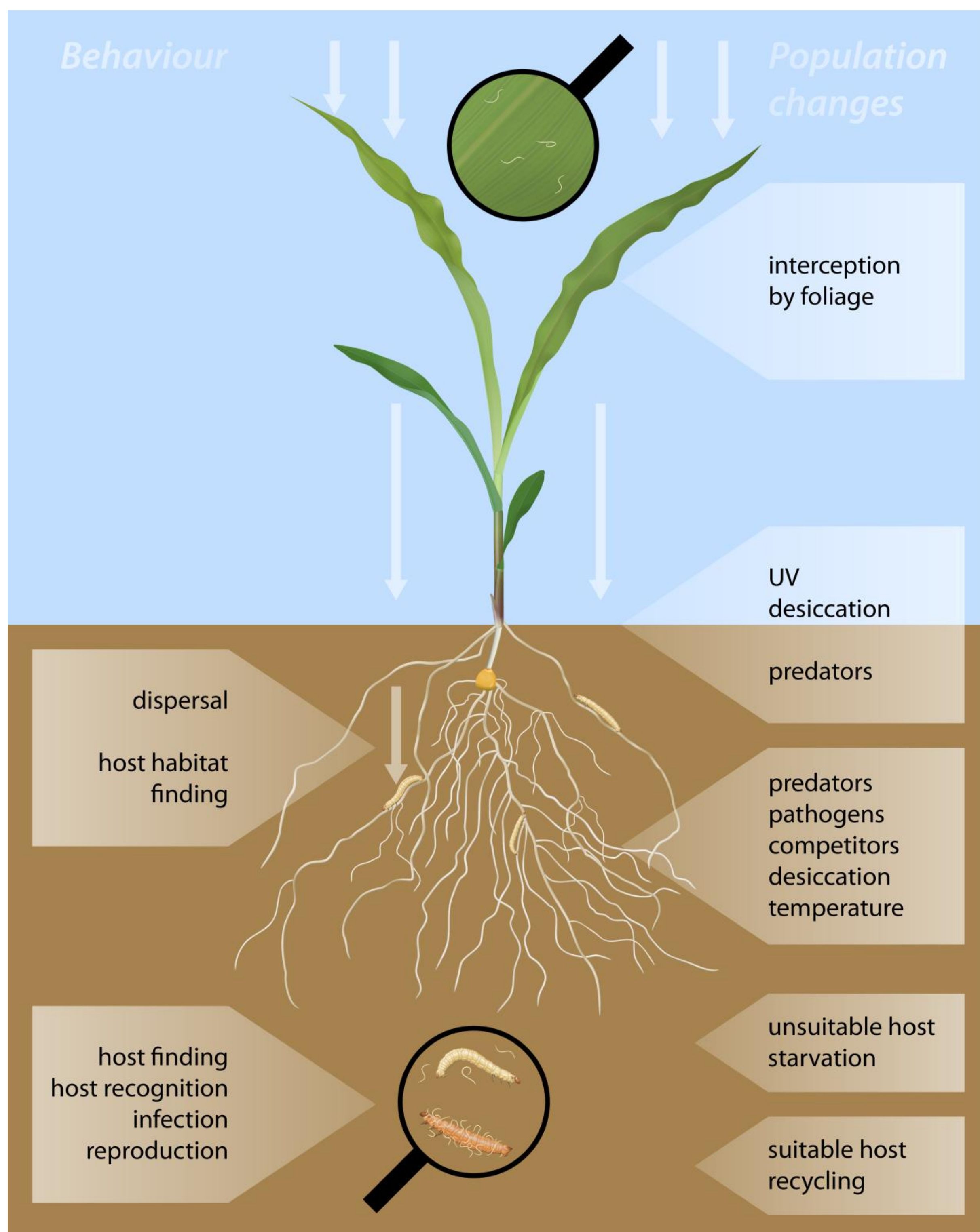


Fig 2. Behaviour and fate of applied entomopathogenic nematodes in the soil. Figure adapted from Griffin (2015). Drawings by Geoffrey Jaffuel and Thomas Degen.

*Entomopathogenic formulation and production*

The formulation in which EPN are shipped and applied is also key to their success as biological control agents. Efforts are made to develop formulations that are easy to handle, that are suitable for long-term storage and that are cost effective. The type of formulation also depends on where and against which target it is applied. For instance, aboveground formulations differ from formulations for belowground applications. Aboveground, the formulation should protect the EPN from desiccation and UV exposure. This can be achieved by adding surfactants and adjuvants, which has been shown to enhance the effectiveness of EPN (de Waal et al, 2013; Schroer and Ehlers, 2005). For belowground application, so far, the common commercialized formulation involves EPN in dry powder (i.e. vermiculite). This formulation needs to be diluted in water before application, with a shelf life of about one month. Attempts to formulate EPN in polysaccharide gel or capsule/beads are under intensive investigation. Such formulations may provide a practical medium for EPN release and their texture is reducing EPN mobility, prolonging their shelf-life (Kaya and Nelsen, 1995; Hiltpold et al., 2012; Kim et al., 2014; Hiltpold et al., 2015). Moreover, by adding specific compounds the shelf life of these formulations may be enhanced. Attempt to increase EPN shelf life focused on forcing them into a state of quiescence. Quiescence is naturally induced by unfavorable conditions such as a lack of oxygen, a lack of moisture, unsuitable temperature, and/or an osmotic stress (Barrett, 1991). The metabolism of quiescent EPN is significantly reduced, allowing energy conservation, prolonging their lifespan and infectiousness (Evans and Perry, 1976; Hiltpold et al., 2014). EPN quit being quiescent when the conditions turn more favorable. Quiescence may as well be chemically triggered, for example, by inducing an osmotic stress with a glycerol solution (Chen and Glazer, 2005) or with compound(s) found in plant roots (Hiltpold et al., 2014; Hubbard et al., 2005; Zhao et al., 2000). Several attempts to trigger quiescence in EPN formulation have been achieved. For instance, by triggering a partial state of anhydrobiosis (moisture stress) by slow desiccation, but none of the formulations obtained exceeded 4 months survival at room temperature (Grewal, 2002). A promising approach by Chen and Glazer (2005) involved an alginate bead formulation that included glycerol which significantly increased the shelf life of EPN up to 180 days. EPN recovered from the state of quiescence by simple dilution in water. Hiltpold et al., (2014) showed that over a period of 18 days, EPN preserved in quiescence triggered by exudates of pea root-cap were preserving better their lipid reserves, were more motile and infectious that EPN preserved only in water. The compound(s) present in plant exudates could potentially be used to increase shelf life of EPN in different types of formulations (Hiltpold et al., 2014). However, those compounds are not species-specific and thus may influence non targeted species of the soil fauna.

An alternative to artificial EPN formulations is the direct application of insect cadavers in the field site. A cadaver can potentially contain millions of EPN and directly applying cadavers provide several advantages. The cost of production is rather low as there is very few handling step (only to

infect insects) and IJs do not need to be stored. The insect cadaver also protects the EPN from harmful biotic and abiotic factors. EPN that come out of a cadaver have been shown to disperse better, survive better and to be more infectious than EPN stored in a water suspension. Insect cadavers can be enhanced with a protective coating that makes them easier to handle. Yet, the large-scale application of cadavers with adapted machinery remains challenging, and they are therefore mainly considered for small to medium sized growers, or for hard-to-reach cryptic habitat (Dolinski et al., 2015, Gumus et al., 2015).

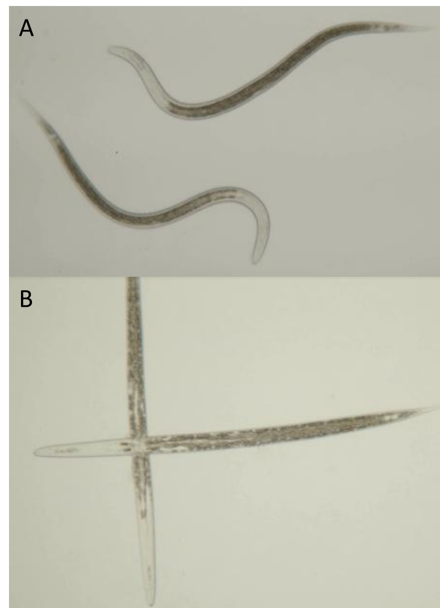


Fig 3. A: Active *Heterorhabditis megidis* in water solution. B: Quiescent *Heterorhabditis megidis* after 24h incubation in maize root extract (1 $mg.ml^{-1}$). Pictures by Neil Villard and Geoffrey Jaffuel (2015).

Formulation and application methods are not the only limiting factors for massive application; mass production in a cost effective manner is essential. Large-scale production of EPN can be achieved in liquid cultures inside bioreactors. This technology allows for the production of large number of IJs at comparably low costs, but further efforts to stabilize the process of production and to reduce the cost are needed (Ehlers, 2001, Ehlers and Shapiro-Ilan, 2005).

*Entomopathogenic nematodes commercialization*

Biocontrol agents production and commercialization is a flourishing market with around 225 microbial biopesticides produced over 30 countries (Kabaluk and Gazdik, 2007). Yet, the conventional pesticides market represents 90 % of the market compared with biopesticides (Bailey, et al., 2010), but a decline of 1.5% per year of conventional pesticide is registered from the 2000s (Piedra-Buena et al., 2015). In the case of EPN, they are nowadays widely used as biological control agent and several companies commercialize nematodes for a large variety of insect pest target. Commercially produced EPN are, for example, use to control the scarab larvae and the

mole crickets in lawn and turf, the fungus gnats in mushroom production, the black vine weevil in nursery plants, western flower thrips in greenhouses and foliar tunnels (e.g. for Chrysanthemums), in addition to other pest insects (Georgis et al., 2006, Lacey et al., 2006, Lacey and Georgis, 2012).

But the development and the use of biopesticides also depend on regulations that are still varying from a nation to another. It is urgent to advance on the clarification of their regulations as to define a legal framework for their release and use (Piedra Buena et al., 2015). This issue was revised in the frame of the REBECA project (Regulation of Biological Control Agents, UE) that provided a revision of the current situation on biological control agents producers and commercializers in Europe but as well as countries highly contributing such as US, Canada and Autralia. The REBECA project focused on providing insights about the production, the commercialization, the application and the risk assessment of beneficial organisms including EPN, providing new frames for their legislation (Loomans, 2007)

*Abiotic and biotic factors influence on entomopathogenic nematodes conservation and/or application*

To ensure that naturally occurring or applied EPN persist in agricultural soils it is also essential to provide a suitable soil medium (Shields, 2015). EPN survival and infectivity are greatly dependent on the soil's chemical and physical properties, as well as the management practices that may modify these and other proprieties. EPN survival can be strongly affected by framing practices such as tillage, farming systems and the application of agrochemicals (Rovesti et al., 1988; Susurluk and Ehlers, 2008; Duncan et al., 2007, 2013; Laznik et al., 2014; Lewis et al., 2015; Stuart et al., 2015; Shapiro-Ilan and Dolinski, 2015). Moreover, biotic factors, such as competitors and natural enemies, also affect the prevalence of EPN in the soil (Lewis et al., 2015; Kaya, 1990; Kaya and Koppenhöfer, 1996; Campos-Herrera et al., 2015a). A good understanding of the role of abiotic and biotic factors in determining the survival and effectiveness EPN as biocontrol agents in agroecosystems may help to provide new and relevant strategies to manage their abundance and activity.

*Abiotic factors influencing entomopathogenic nematode fitness*

The occurrence and persistence of EPNs can strongly be affected by both natural physical and chemical components such as soil pH, soil texture, soil structure and climate, together with physical and chemical disturbance resulting from human activity (Stuart et al., 2015). The abiotic factors influencing EPN have been extensively studied under laboratories conditions, but in the field the interactions are considerably more complex and information concerning the relative importance of abiotic factors is badly needed (Stuart et al., 2015). So far, studies have focused on

(i) moisture, (ii) soil texture and structure, (iii) temperature and (iv) soil chemistry as factors driving EPN survival, mobility and infectiousness (Stuart et al., 2015).

> *Moisture*. Soil nematodes are strongly influenced by moisture (Nickle, 1994). Moisture determines the water film thickness necessary for nematodes to move and survive, and affects the surface tension and the amount of oxygen present in the soil. These parameters influence EPN efficacy and survival, but their impact varies depending on their interaction with other environmental conditions. Moreover, different EPN species are differently adapted to local conditions (Stuart et al., 2015; Gaugler and Kaya, 1990; Shapiro-Ilan et al., 2002) i.e. in a study performed by Koppenhöfer and Fuzy (2007) involving 4 species of EPN (*S. scarabaei*, *S. glaseri*, *H. zealandica*, and *H. bacteriophora*), EPN infectivity against white grubs was found to be the highest at moderate soil moistures, and tended to be lower in wet and moderately dry soil. However in dry soil, only *S. scarabaei* showed some activity. In another recent study, El-Borai et al. (2016) showed that two very close species from the *Steinernema glaseri*-group, *Steinernema* sp. (Sx) and *Steinernema diaprepesi* were distinct in their moisture preference. In sand columns, *Steinernema diaprepesi* was migrating and settling towards dry soil (6%), whereas *Steinernema* sp. (Sx) preferred wetter soil (18%).

> *Soil texture and structure*. Both influence EPN survival and movement (Stuart el al., 2015). The effect of soil structure is rather clear, as poorly structured soils (small pore space) restricted the movement of EPN (Portillo-Aguilar et al., 1999). Also for soil texture and how it affects movement, survival and infectiousness of EPN is species-specific and condition dependent (Portillo-Aguilar et al., 1999; Stuart et al., 2015) i.e. the suppression of the citrus root weevil, *Diaprepes abbreviatus,* by *Steinernema riobrave* was found to be greater in coarse sandy soils than in fine textured soils (Shapiro et al., 2000; Duncan et al. 2001, 2003a) but the persistence of *Steinernema carpocapsae* and *H. bacteriophora* were not affected by soil texture in maize (Millar and Barbercheck, 2002).

> *Soil temperature*. It is an important parameter as it regulates both the survival of EPN and the parameters associated with reproduction such as the time to kill, the length of the life cycle, ability to move, ect. EPN temperature tolerance is associated with local conditions, but it can be modified through selection (Ehlers et al., 2005; Stuart et al., 2015; Glazer, 2015)

> *Soil chemistry.* Finally, EPN are sensitive to the chemistry of the soil and pH, but are rather tolerant to changes in soil chemistry. Most of the EPN tolerate pH varying from 4 to 8 without decreases in survival, but are sensitive to pH values outside this range (Kung et al., 1990). Concentrations of chemicals found in fertilizers such as KCL or $CaCl_2$, P, K and C:N ratio influence EPN performance and survival, but their effect varies among species (Stuart et al., 2015). The chemistry of the soil is also modified by the addition of pesticides and herbicides. Under certain conditions EPN have been shown to be compatible with several pesticides (eg. abamectin, acephate, aldicarb, dodine, fenamiphos, methomyl, parathion, and teflubenuron), but susceptible to

others (carbaryl, chlorpyrifos, dimethoate, endosulfan, fonofos, tefluthrin, imidicloprid) (Sahpio-Ilan and Dolinski, 2015). The compatibility of EPN with agrochemicals depends on a number of factors such as their concentration, the EPN species concerned, the timing of application, temperature, etc. Moreover, despite their survival compatibility with agrochemicals, sublethal effect affecting EPN reproduction potential and the time to kill insect larvae has been observed and should be taken into consideration (Gutiérrez et al., 2008).

*The natural enemies of entomopathogenic nematodes*

In an ecosystem, the members of the local fauna and flora interact in complex trophic webs (Fig 4). The soil biota undeniably influences the distribution, the survival and abundance of EPN. EPN will respond to stimuli from other organisms in various ways, and may approach, avoid or ignore the source. EPN are likely to interact with plants, host and non-host arthropods, competitors, predators, parasites and pathogens. The primary biotic factor affecting EPN populations is the presence of suitable host, which ensure reproduction. In the presence of suitable hosts, EPN populations are likely to grow and therefore their natural enemies and competitors will multiply as well and have a negative impact on these populations. Enemies and competitors of EPN are numerous. They include nematophagous fungi, microarthropods, ectoparasitic bacteria, and free-living nematodes. One of the most studied natural enemies of nematodes are nematophagous fungi, which may include fungi that develop inside the nematodes (i.e. *Hirsutella rhossiliensis*) and fungi that can trap and consume nematodes (i.e. *Arthrobotrys* spp.) (Kaya, 2002; Kaya and Koppenhöfer, 1996). Their abundances have been shown to increase as a response to EPN augmentation (Campos-Herrera et al., 2013). Other important enemies of EPN are bacteria, both endo- and ecto-parasite, protozoa and to a lesser extent virus (only one reported case) (Kaya and Koppenhöfer, 1996). Some of the bacteria that are harmful to EPN have been more extensively studied, such as the ecto-parasites form the *Paenibacillus* genus (El-Borai et al., 2005; Enright et al., 2005). Finally, micro-arthropods that are capable of nematophagy have been shown to increase in abundance following EPN application (Jabbour and Barbercheck, 2011).

EPN also face competition for the host cadaver with other organisms. The existence of deterrents emitted by EPN-infected cadavers can limit the competition with arthropods scavengers such as ants and flies (Gulcu et al., 2012). They may also contain antibiotics that prevent the growth of bacteria and fungi (Ehlers, 1996). Yet, a number of other nematode species or even other EPN are able to compete for the cadavers. It has long been assumed that only one species of EPN can develop per cadaver, but a number of studied have shown that several species can develop in the same cadaver, and produce mixed progeny (Kondo, 1989; Alatorre-Rosas and Kaya, 1990 reviewed in 1991; Koppenhöfer and Kaya, 1996; Koppenhöfer et al., 1995; Půza and Mrácek, 2009). The mixed progeny was never found to be balanced and in some case, one species displaces the other (e.g: Půza and Mrácek, 2009), or both species suffer from the competition (e.g:

Koppenhöfer and Kaya, 1996). Moreover, once the EPN reproduce inside the cadaver, free-living nematodes can enter and compete for the cadaver as a resource and may even displace EPN (Selvan et al., 1993, Koppenhöfer and Kaya, 1995; Duncan et al., 2003b; Stuart et al., 2006, 2015). The rapid progress in the development of advanced molecular technologies has opened the way to investigate more reliably and conveniently the inter- and intra-specific competition of EPN with other species. The competitive ability of free-living nematodes from the *Acrobeloides*-group reported by Campos-Herrera et al. (2012) was recently confirmed in a Swiss survey using *Galleria mellonella* soil baits; the free living nematodes were found to develop in the cadavers at the same time as EPN (Campos-Herrera et al., 2015a). However, they are not able to reproduce for a second generation (Campos-Herrera et al., 2012; Duncan et al., 2003b). But two species of free-living nematodes, *Oscheius tipulae* and *Oscheius onirici* were found to be able to reproduce over several generations in presence of EPN and to reduce the reproduction success of the latter (Campos-Herrera et al., 2015b).

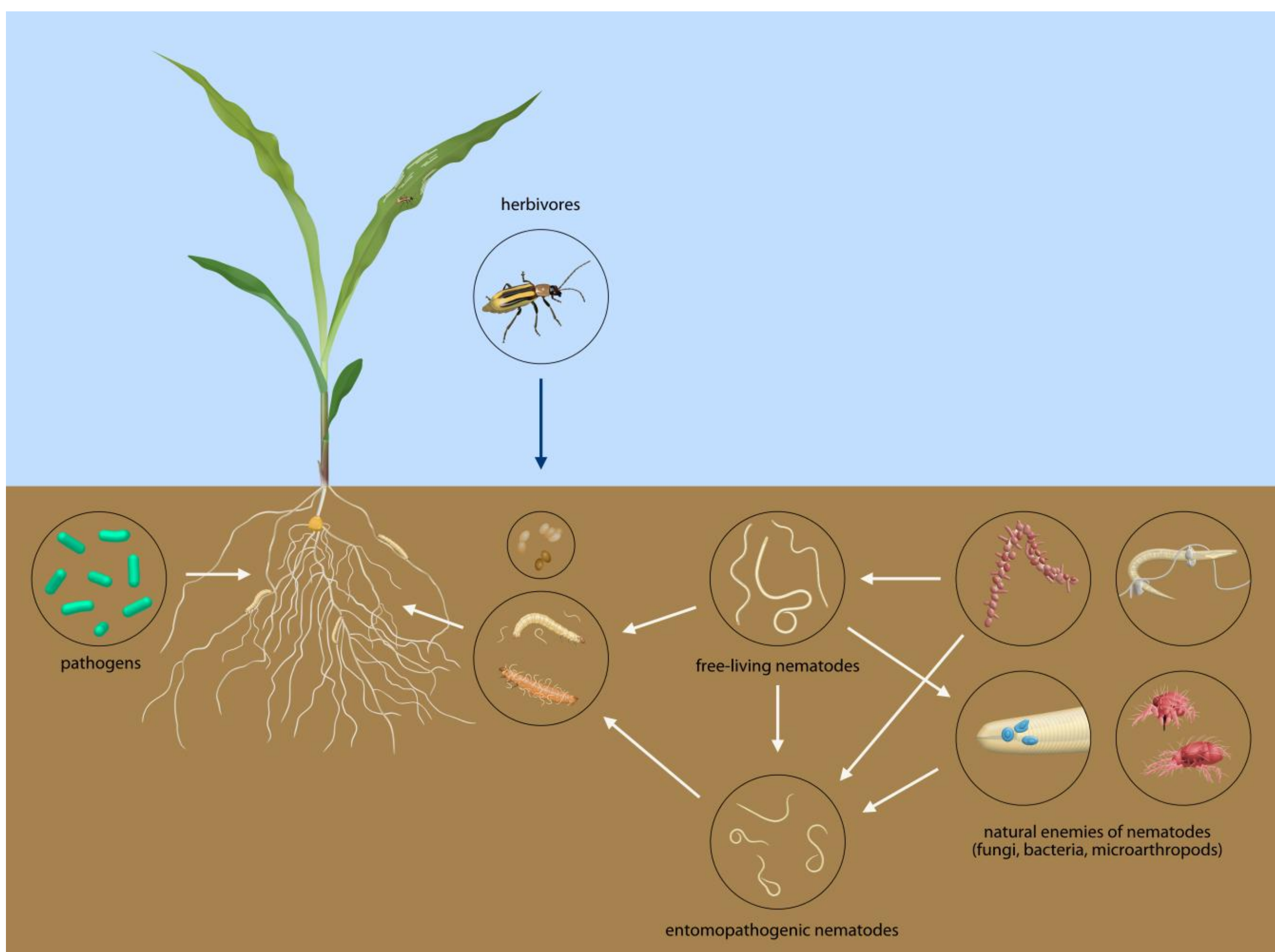


Fig 4. Trophic web interactions influencing the abundance of entomopathogenic nematodes.
Drawings by Raquel Campos-Herrera, Geoffrey Jaffuel and Thomas Degen.

*Using molecular techniques to investigate the entomopathogenic nematode food web*

Traditionally, the EPN communities have been investigated with the use of the insect baiting method, Baerman funnel extractions and sucrose centrifugation (Hominick, 2002). During the last decades, molecular methods such as PCR-based techniques evolved in such manner that we are able to explore the diversity of organisms, their interaction and function in ecosystems with great precision (Campos-Herrera et al., 2013). Among those techniques, quantitative real-time PCR (qPCR) proved to be very efficient for belowground systems. Simultaneous identification and quantification of target species is achievable because of species-specific primers and probes, and allows one to investigate species that previously went undetected due to incomplete isolation methods. Recently, EPN assemblages and food webs including ectoparasitic bacteria and fungal antagonists, as well as competitors such as free-living nematodes have been intensively studied using qPCR data (Atkins et al., 2005; Zhang et al., 2006; Torr et al., 2007; Pathak et al., 2012; Campos-Herrera et al., 2011a, 2011b, 2012, 2015a, 2015b). These studies have revealed the associations of the different species interacting with EPN in agricultural fields and natural areas (Campos-Herrera et al., 2012, 2013, 2016). The combination of molecular tools and traditional measurements of EPN activity with sentinel insects can provide insight into EPN abundance and activity and how these are affected by abiotic and biotic factors and how these factors may be altered by management practices in agroecosystems. This knowledge may help to develop novel conservation and augmentation strategies that can increase the effectiveness of EPN as biocontrol agents.

## Thesis outline

*EPN prevalence in different farming systems*

The Swiss National Research Program 68 (NRP68) focused on the use of soil as a natural resource that can serve to improve plant health and protection, and thereby can help to increase crop yield. In the context of this research program, we investigated the natural occurrence of beneficial organisms in Swiss soils and management practices that might help to enhance soil-provided ecological services in an agroecosystem. A screening of beneficial organisms and good knowledge of the factors that determine their abundance in a given agroecosystem is a first step towards the development of sustainable biological control of soil pests.

We first investigated the effect of abiotic and biotic factor affecting the prevalence and activity of EPN and the soil-borne organisms that are part of the same food web. Abiotic factors that are typically modified by different soil management, such as tillage and fertilization, but also soil proprieties such as soil humidity, clay content or pH have all been shown to have an impact on

EPN prevalence (Stuart et al., 2015, Lewis et al., 2015). Biotic factors such as the presence of competitors and natural enemies of EPN that can affect their distribution and persistence were also investigated. For this we focused on free-living nematodes, which can compete with EPN inside insect cadavers, as well nematophagous fungi and ectoparasitic bacteria, which are natural enemies that feed on EPN. We performed experiments at different sites throughout Switzerland to assess the impact of these biotic and abiotic factors on the EPN food web.

In this thesis report, I focus on a survey that was performed at a long-term field trial (since 1978), which aims at comparing biodynamic, bio-organic and conventional farming systems in a 7-year crop rotation system (Mäder et al., 2002). We investigated the prevalence and activity of EPN and their associated soil food web depending on (*i*) three crop types (wheat, maize and grass-clover pasture) and (*ii*) farming practices, i.e. conventional, organic and biodynamic, which differed in fertilization, tillage and pesticide use. The overall assumption was that soils with high levels of natural organic matter and minimal soil disturbance preserve higher abundance of soil microorganisms, including EPN and associate organisms. Soil from the trial field was sampled twice in April and October 2013. The sampled were sieved and nematodes and associated organisms were extracted by sucrose centrifugation (Jenkins 1964). DNA was then extracted and species-specific primers and probes were used to determine the identity and prevalence of EPN, free living nematodes, and nematophagous fungi and bacteria (Campos-Herrera et al., 2015a). We linked the information obtained on EPN prevalence with their activity, using a classical insect bating method.

*Increasing the shelf-life of entomopathogenic nematodes*

In commonly use formulations EPN move about and rapidly waste energy (mainly in the form of lipids) and over a short time will become less suitable to achieve good control of the target pest. Hubbard et al. (2005) showed that the root-cap exudates can induce a state of dormancy called quiescence in a wide range of nematodes genera and species including one species of EPN, *S. feltiae*. Subsequent studies showed that several EPN species experienced quiescence when exposed to exudate from pea germinates (Hiltpold et al., 2014). It is still unknown what compound(s) trigger(s) quiescence. Quiescence factors (QF) are produced by various families of plants, probably as a defense mechanism against plant pathogenic nematodes (Hubbard et al., 2005). EPN have been shown to recover from the induced state of quiescence by simply diluting the solution containing the exudate (Hiltpold et al., 2014). While under quiescence, EPN preserve their lipid reserves and after recovering they are more motile and infectious that EPN simply preserved in water (Hiltpold et al., 2014). Therefore, QF can potentially be used to increase the shelf life and lifespan of EPN in formulations and to better control the timing of delivery of the EPN. However, the use of exudate in practice is tedious, as it is difficult to collect and it contains only relatively low concentrations of QF. To overcome these problems, we developed a high throughput

method for the extraction of QF from deep frozen root. We tested the potency of such extracts from pea (*Pisum sativum*) and maize (*Zea maize*) to trigger quiescence in EPN (*H. megidis*), as well as the ability of EPN to recover from quiescence and to infect insect larvae. We also tested whether the QF was exclusively produced from the root-cap of the root or was also present in the rest of freshly germinated roots. Moreover, we showed variability in the induction of quiescence depending on the availability of oxygen and we explored the consequences of this limitation for the usefulness of QF. The resulting extract method is currently being used to perform bio-guided fractionation in order to isolate and identify the QF.

*Increasing EPN persistence*

Good persistence of EPN in the field is critical to their successful application against pests. To better understand how and why EPN persist in certain areas is important to develop management and application strategies for their optimal use in biocontrol. The persistence in the field is linked to the conditions of soil in the field. It might be possible to modify certain soil proprieties to render the soil more suitable for EPN populations. We investigated the potential of cover crops to enhance soil conditions in a manner that would allow better EPN persistence. Cover crops are known to conserve soil quality by increasing organic matter, water retention, nutrient levels and soil strength (Cooper et al., 2016). The presences of cover crops can also increase the abundance of the soil biota (Altieri et al., 1999). To investigate if cover crops are also beneficial for EPN persistence we investigated EPN presence in plots with and without winter cover crops. We also studied the impact on competitors and enemies of EPN. Two experiments were performed comparing persistence of natural occurring, as well as augmented populations of EPN using traditional insect baiting methods and qPCR data, and was correlated with the measured prevalence of their natural enemies and competitors.

The first experiment (CC27) investigated the persistence of applied EPN throughout winter, comparing soil covered with pea (*Pisum sativum*), mustard (*Brassica juncea*), and bare soil as control. We applied two species of EPN, one isolated in Switzerland, the native *S. feltiae* R5-S and a commercial strain *H. bacteriophora* (Andermatt Biocontrol, AG). We measure the abundance (qPCR) and activity (insect baiting) of EPN at three time points; 1) before application, 2) one months following application and 3) four months following application.

The second experiment (CC29) investigated the abundance of naturally occurring EPN under pea (*Pisum sativum*) as a winter cover crop. This experimental field was also subjected to different tillage regimes. This allowed us to compare the abundance of EPN in tilled versus not tilled plots (direct seeding) at three time points; 1) at the end of the cover crop period, 2) after the plots have been tilled or prepared for direct seeding and the subsequent crop, winter wheat (*Triticum aestivum* L var. Ariana), planted and 3) at the winter wheat growing stage. Finally, we evaluated the impact of different soil textures that are present at the field site.

For both experiments (CC27 and CC29), the prevalence of key EPN species and the other members of the soil food web (nematophagous fungi, bacteria and free-living nematodes) were investigated (Campos-Herrera et al., 2015a).

**References of the general introduction**

Abdollahi, L., Munkholm, L. J., 2014. Tillage system and cover crop effects on soil quality: I. Chemical, mechanical, and biological properties. *Soil Science Society of America Journal.* 78: 262–270.

Alatorre-Rosas, R., Kaya. H. K., 1990. Interspecific competition between entomopathogenic nematodes in the genera *Heterorhabditis* and *Steinernema* for an insect host in sand. *J. Invertebr. Pathol.* 55 : 179–188.

Altieri, M. A., 1999. The ecological role of biodiversity in agroecosystems. *Agric Ecosyst Environ.* 74: 19–31.

Amundson, R., Berhe, A. A., Hopmans, J. W., Olson, C., Sztein, A. E., and Sparks, D. L. 2015. Soil science. Soil and human security in the 21st century. *Science.* 348:1261071.

Atkins, S. D., Clark, I. M., Pande, S., Hirsch, P. R., Kerry, B. R., 2005. The use of real-time PCR and species-specific primers for the identification and monitoring of *Paecilomyces lilacinus Microbiol. Ecol.* 51: 257–264.

Bailey, K. L., Boyetchko, S. M., Längle, T., 2010. Social and economic drivers shaping the future of biological control: A Canadian perspective on the factors affecting the development and use of microbial biopesticides. *Biol. Control. 52*: 221–229.

Bateman, R. P., Matthew, G. A., Hall, F. R., 2007. Ground–based application equipment. In. Lacey L.A, Kaya H. K. (Eds.), Field manual of techniques in invertebrate pathology 2nd ed., pp. 73–98. Dordrecht, The Netherlands: Springer.

Barrett J. 1991. Anhydrobiotic nematodes. In Evans K (Ed.) *Agricultural zoological reviews.* Vol. 4. Intercept, Andover, pp 161–176

Beck, B., Brusselman, E., Nuyttens, D., Moens, M., Pollet, S., Temmerman, F., et al., 2013. Improving foliar applications of entomopathogenic nematodes by selecting adjuvants and spray nozzles. *Bio. Sci. and Tech.* 23: 507–520.

Beck, B., Brusselman, E., Nuyttens, D., Moens, M., Temmerman, F., Pollet, S., et al., 2014. Improving the biocontrol potential of entomopathogenic nematodes against *Mamestra brassicae*: effect of spray application technique, adjuvants and an attractant. *Pest Manag Sci* 70: 103–112.

Brusselman, E., Beck, B., Pollet, S., Temmerman, F., Spanoghe, P., Moens, M., et al., 2012. Effect of the spray application technique on the deposition of entomopathogenic nematodes in vegetables. *Pest. Manag. Sci.* 68(3): 444–453.

Campos-Herrera, R., Ali, J. G., Diaz, B. M., Duncan, L. W., 2013. Analyzing spatial patterns linked to the ecology of herbivores and their natural enemies in the soil. *Front. Plant Sci.* 4 : 1–18.

Campos-Herrera, R., El-Borai, F. E., Duncan, L. W., 2012. Wide interguild relationships among entomopathogenic and free-living nematodes in soil as measured by real time qPCR. *J. Invertebr. Pathol.* 111: 126–135.

Campos-Herrera, R., El-Borai, F. E., Stuart, R. J., Graham, J. H., Duncan, L. W., 2011b. Entomopathogenic nematodes, phoretic *Paenibacillus* spp., and the use of real time quantitative PCR to explore soil food webs in Florida citrus groves. *J. Invertebr. Pathol*. 108: 30–39.

Campos-Herrera, R., Jaffuel, G., Chiriboga, X., Blanco-Pérez, R., Fesselet, M., Půža, V., Mascher, F., Turlings, T. C. J., 2015a. Traditional and molecular detection methods reveal intense interguild competition and other multitrophic interactions associated with native entomopathogenic nematodes in Swiss tillage soils. *Plant Soil.* 389: 237-255.

Campos-Herrera, R., Johnson, E.G., Stuart, R. J., Graham, J. H., Duncan, L. W., 2011a. Long-term stability of entomopathogenic nematode spatial patterns in soil as measured by sentinel insects and Real-Time PCR assays. *Ann. Appl. Biol.* 158: 55–68.

Campos-Herrera, R., Puza, V., Jaffuel, G., Blanco-Pérez, R., Čepulytė Rakauskienė, R., Turlings, T. C. J., 2015b. Unraveling the intraguild competition between *Oscheius* spp. nematodes and entomopathogenic nematodes: implications for their natural distribution in Swiss agricultural soils. *J. Invertebr. Pathol*. 132: 216-227.

Cardinale, B. J., Harvey, C. T., Gross, K., Ives, A. R., 2003. Biodiversity and biocontrol: Emergent impacts of a multi-enemy assemblage on pest suppression and crop yield in an agroecosystem. *Ecol Lett*. 6: 857–865.

Chen, S. B., Glazer, I. 2005. A novel method for long–term storage of the entomopathogenic nematode *Steinernema feltiae* at room temperature. *Biol. Control.* 32(1): 104–110.

Cooper, J, Baranski, M, Stewart, G, Nobel-de Lange, M, Bàrberi, P, Fließbach, A, et al., 2016. Shallow non-inversion tillage in organic farming maintains crop yields and increases soil C stocks: a meta-analysis. *Agro. for Sustain. Devel*. 36: 22.

de Waal, J. Y., Malan, A. P., Addison, M. F., 2013. Effect of humidity and a superabsorbent polymer formulation on the efficacy of *Heterorhabditis zealandica* (Rhabditida: Heterorhabditidae) to control codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae). *Bio. Sci. and Tech.* 23: 62–78.

Dillman, A. R., Chaston, J. M., Adams, B. J., Ciche, T. A., Goodrich-blair, H., Patricia, S., Sternberg, P. W., 2012. An entomopathogenic nematode by any other name. *Plos Patho.* 8(3): 8–11.

Dolinski, C., Shapiro-Ilan, D, Lewis, E. E., 2015. Insect cadaver applications: Pros and Cons. In: Campos-Herrera, R. (Ed.). *Nematode pathogeneses of insects and other pests*, Springer International Publishing, Switzerland, pp. 207-230.

Dowds, B. C. A. and Peters A., 2002. Virulence Mechanisms. In Gaugler R (Ed) *Entomopathogenic Nematology*. CABI International, pp. 79-98.

Duncan, L. W., Dunn, D. C., Bague, G., Nguyen, K., 2003b. Competition between entomopathogenic and free-living bactivorous nematodes in larvae of the weevil *Diaprepes abbreviatus. J. Nematol*. 35: 187–193.

Duncan, L.W., Graham, J.H., Dunn, D.C., Zellers, J., McCoy, C.W., Nguyen, K., 2003a. Incidence of endemic entomopathogenic nematodes following application of *Steinernema riobrave* for control of *Diaprepes abbreviatus*. *J. Nematol.* 35: 178– 186.

Duncan, L. W., Graham, J. H., Zellers, J., Bright, D., Dunn, D. C., El-borai,F. E., Porazinska, D. L., 2007. Food web responses to augmenting the entomopathogenic nematodes in bare and animal manure–mulched soil. *J. Nematol.* 39:176–189.

Duncan, L.W., McCoy, C.W., 2001. Hydraulic lift increases herbivory by *Diaprepes abbreviatus* larvae and persistence of *Steinernema riobrave* in dry soil. *J. Nematol.* 33: 142–146.

Duncan, L. W., Stuart, R. J., El-Borai, F. E., Campos–Herrera, R., Pathak, E., Giurcanu, M., Graham, J. H. 2013. Modifying orchard planting sites conserves entomopathogenic nematodes, reduces weevil herbivory and increases citrus tree growth, survival and fruit yield. *Biol. Control.* 64: 26–36.

El-Borai, F. E., Duncan, L. W., Preston, J. F., 2005. Bionomics of a phoretic association between *Paenibacillus* sp. and the entomopathogenic nematode *Steinernema diaprepesi. J. Nematol.* 37: 18–25.

El-Borai F., Killiny N., Duncan L. W., 2016. Concilience in entomopathogenic nematode responses to water potential and their geospatial patterns in Florida. *Front Microbiol.* 7: 1–12.

Enright, M. R., Griffin, C. T., 2005. Effects of *Paenibacillus nematophilus* on the Entomopathogenic nematode *Heterorhabditis megidis*. J. *Invertebr. Pathol.* 88, 40–48.

Ehlers, R. U., 1996. Symbiotic relation within the nematode-bacterium complex. Symbiosis and pathogenicity of the nematode-bacterium complexes. N. Boemare, R. U. Ehlers, A. Fodor and A. Szentirmai, European Commission: 45-51.

Ehlers R. U., 2001. Mass production of entomopathogenic nematodes for plant protection. *Appl Microbiol Biotechnol.* 56: 623–633.

Ehlers, R. U., Shapiro-Ilan, D. I. 2005. Mass production. In Grewal P. S, Ehlers R. U, Shapiro-Ilan D. I. (Eds.), *Nematodes as biocontrol agents* pp. 65–78. Oxfordshire, UK: CABI Publishing.

Ehlers R. U., Oestergaard, J., Hollmer, S., Wingen, M., Strauch, O., 2005. Genetic selection for heat tolerance and low temperature activity of the entomopathogenic nematode-bacterium complex *Heterorhabditis bacteriophora- Photorhabdus luminescens. BioControl* 50: 699–716.

Evans, A. A. F., Perry, R. N., 1976. Survival strategies in nematodes. In Croll N .A. (Ed), *The Organization of nematodes*. London & Ney York: Academic Press, pp. 383-424

Gaugler, R., Kaya, H. K., 1990. *Entomopathogenic nematodes in biological control.* Boca Raton, FL: CRC.

Gaugler, R., Lewis, E., Stuart, R. J., 1997. Ecology in the service of biological control: the case of entomopathogenic nematodes. *Oecologia* 109:483–489

Georgis, R., Koppenhöfer, A. M., Lacey, L. A., Bélair, G., Duncan, L. W., Grewal, P. S., Samish, M., Tan, L., Torr, P., van Tol, R. W. H. M., 2006. Successes and failures in the use of parasitic nematodes for pest control. *Biol. Control.* 38:103–123

Glazer, I., 2015. Improvement of entomopathogenic nematodes: A genetic approach. In: Campos-Herrera, R. (Ed.). *Nematode pathogeneses of insects and other pests*, pp. 29-55, Springer International Publishing, Switzerland.

Grewal, P. S. 2002. Formulation and application technology. In R. Gaugler (Ed.), *Entomopathogenic nematodes*. New York: CABI, pp. 265–28

Grewal, P. S, Ehlers, R-U, Shapiro-Ilan, D. I, 2006. Nematodes as biocontrol agents, CABI Publishing, Oxon, UK

Grunder, J. M., 2005. *Quality control of entomopathogenic nematodes*. Wädenswil, CH: Agroscope FAW.

Gumus, A., Karagoz, M., Shapiro-Ilan, D., Hazir, S., 2015. A novel approach to biocontrol: Release of live insect hosts pre-infected with entomopathogenic nematodes. *J. Invertebr. Pathol*. 130: 56–60.

Gulcu, B., Hazir, S., Kaya, H. K., 2012. Scavenger deterrent factor (SDF) from symbiotic bacteria of entomopathogenic nematodes. *J. Invertebr. Pathol.* 110:326–333.

Gutiérrez, C., Campos-Herrera, R., Jiménez, J., 2009. Comparative study of the effect of selected agrochemical products on *Steinernema feltiae* (Rhabditida: Steinernematidae). *Biocontrol Sci. Technol*. 18: 305–309.

Higa, T., Parr, J. F., 1994. Beneficial and effective microorganisms for a sustainable agriculture and environment. Atami, Japan: International Nature Farming Research Center.

Hiltpold I., 2015. Prospects in the application technology and formulation of entomopathogenic nematodes for biological control of insect pests. In: Campos-Herrera, R. (Ed.). *Nematode pathogeneses of insects and other pests*, Springer International Publishing, Switzerland, pp. 187-207.

Hiltpold, I., Hibbard, B. E., French, B. W., Turlings, T. C. J., 2012. Capsules containing entomopathogenic nematodes as a Trojan horse approach to control the western corn rootworm. *Plant and Soil*. 358(1-2): 11–25.

Hominick, W.M., 2002. Biogeography. In: Gaugler, R. (Ed.), Entomopathogenic Nematology, CABI Publishing, Wallingford, UK, pp. 115-143.

Hubbard, J. E., Yolanda F. L., Schmitt M., M., McCLure, M. A., Stock, S. P., Hawes, M. C., 2005. Increased penetration of host roots by nematodes after recovery from quiescence induced by root cap exudate. *Nematology*. 7(3): 321–331.

Jabbour, R., Barbercheck, M. E., 2011. Soil microarthropod response to the application of entomopathogenic nematode-killed insects in maize and flower strip habitats. *Pedobiologia*. 54: 243–251.

Jaffuel, G., Hiltpold, I., Turlings, T. C. J., 2015. Highly potent extracts from pea (*Pisum sativum*) and maize (*Zea mays*) roots can be used to induce quiescence in entomopathogenic nematodes. *J. Chem.Ecol*. 41: 793–800.

Jenkins, W. R., 1964. A rapid centrifugal-flotation technique for separating nematodes from soil. *Plant Dis. Rep.* 48:492

Kabaluk, T.,Gazdik, K., 2007. Directory of microbial pesticides for agricultural crops in OECD countries. Agriculture and Agri-Food Canada.

Kaya, H. K., 2002. Natural enemies and other antagonists. In R. Gaugler (Ed.), *Entomopathogenic nematology.* New York: CABI Publishing, pp. 189–202.

Kaya, H. K., Koppenhöfer, A. M., 1996. Effects of microbial and other antagonistic organism and competition on entomopathogenic nematodes. *Biocontrol Sci. Technol.* 6: 357–372.

Kaya, H. K, Nelsen C. E., 1985. Encapsulation of Steinernematid and Heterorhabditid nematodes with calcium alginate: a new approach for insect control and other applications. *Environ. Entomol.* 14: 572–574.

Kim, J., Jaffuel, G., Turlings, T. C. J., 2014 Enhanced alginate capsule properties as a formulation of entomopathogenic nematodes. *BioControl. 60*(4): 527-535.

Koppenhöfer, A. M., Fuzy, E. M., 2007. Soil moisture effects on infectivity and persistence of the entomopathogenic nematodes *Steinernema scarabaei*, *S. glaseri*, *Heterorhabditis zealandica*, and *H. bacteriophora*. *Appl. Soil Ecol.* 35: 128–139.

Koppenhöfer A. M. 2007. Nematodes. In: Lacey, L. A., Kaya, H. K. (Eds.) *Field manual of techniques in invertebrate pathology*, 2nd edn. Springer, Berlin, Germany, pp. 249–264

Koppenhöfer, A. M., Kaya, H. K., 1995b. Density-dependent effects on *Steinernema glaseri* (Nematoda : Steinernematidae ) within an insect host. *J. Parasitol.* 81: 797–799.

Koppenhöfer, A. M., Kaya, H. K., Shanmugam, S., Wood, G. L., 1995a. Interspecific competition between steinernematid nematodes within an insect host. *J. Invertebr. Pathol.* 66 : 99–103.

Koppenhöfer, A. M., Kaya, H. K., 1996. Coexistence of two steinernematid nematode species (Rhabditida : Steinernematidae) in the presence of two host species. *Appl. Soil Ecol.* 4: 221–230.

Köhler, H. R. and Triebskorn R., 2013. Wildlife ecotoxicology of pesticides: can we track effects to the population level and beyond? *Science.* 341:759–65.

Komatsuzaki, M., Ohta, H, 2007. Soil management practices for sustainable agro-ecosystems. *Sustainability Science* 2: 103–120.

Kondo, E., 1989. Studies on the infectivity and propagation of entomogenous nematodes *Steinernema* spp. (Rhabditida: Steinernematidae) in the common cutworm, *Spodoptera litura* (Lepidoptera: Noctuidae). *Agrie. Bull.* 67: 1–87.

Kung, S., Gaugler, R., Kaya, H.K., 1990. Soil type and entomopathogenic nematode persistence. *J. Invertebr. Pathol.* 55:401–406.

Lacey, L. A., Georgis, R., 2012. Entomopathogenic nematodes for control of insect pests above and below ground with comments on commercial production. *J. Nematol.* 44: 218–225.

Lacey, L. A., Grzywacz, D., Shapiro-Ilan, D. I., Frutos, R., Brownbridge, M., Goettel, M. S., 2015. Insect pathogens as biological control agents: Back to the future. *J Invertebr Pathol.* 132: 1–41.

Lal, R., 2009. Soils and food sufficiency: A review. *Agro. for Sustain. Devel.* 29: 25–49. doi:10.1007/978-90-481-2666-8_4

Laznik, Z., Trdan, S., 2014. The influence of insecticides on the viability of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) under laboratory conditions. *Pest. Manag. Sci.* 70: 784–789.

Lewis, E. E., 2002. Behavioural Ecology. In Gaugler, R. *Entomopathogenic nematology* CABI International, pp. 205-223.

Lewis, E. E., Hazir, S., Hodson, A., Gulcu, B. 2015. Trophic relationships of entomopathogenic nematodes in agricultural habitats. In: Campos-Herrera, R. (Ed.). *Nematode pathogeneses of insects and other pests*, Springer International Publishing, Switzerland, pp. 139-163.

Lewis, E. E., Campbell, J., Griffin, C., Kaya, H., Peters, A., 2006. Behavioral ecology of entomopathogenic nematodes. *Biol. Control.* 38(1): 66–79.

Loomans, A. J. M., 2007. Regulation of invertebrate Biological Control Agents in Europe: Review and recommendations in its pursuit of a harmonised regulatory system. Report EU project REBECA, Specific Support Action, project no. SSPE–CT–2005–022709, pp. 1–26

Mäder, P., Flieβbach, A., Dubois, D., Gunst, L., Fried, P., Niggli, U., 2002. Soil fertility and biodiversity in organic farming. *Science.* 296: 1694–1697.

Mcevoy, P. B., 1996. Host specificity and biological pest control. *BioScience* 46(6): 401–405.

Millar, L. C., Barbercheck, M. E., 2002. Effects of tillage practices on entomopathogenic nematodes in a corn agroecosystem. *Biol. Control. 25*: 1–11.

Nicot, P C et al., 2011. Classical and augmentative biological control against diseases and pests: critical status analysis and review of factors influencing their success. Nicot, P. C. (Ed) International Organization for Biological and Integrated Control of Noxious Animals and Plants, West Palearctic Regional Section (IOBC/WPRS) pp 71-171

Nickle, W. R., 1984. *Plant and insect nematodes.* New York: Marcel Decker.

O'Callaghan, K. M., Zenner, A. N. R. L., Hartley, C. J., Griffin, C. T., 2014. Interference competition in entomopathogenic nematodes: male *Steinernema* kill members of their own and other species. *Int. J. Parasitol.* 44: 1009-1017.

Pathak, E., El–Borai, F. E., Campos–Herrera, R., Johnson, E. G., Stuart, R. J., Graham, J. H., Duncan, L. W., 2012. Use of real-time PCR to discriminate parasitic and saprophagous behaviour by nematophagous fungi. *Fun. Biol.* 116: 563–573.

Piedra-Buena, A., López-Cepero J., Campos-Herrera, R., 2015. Entomopathogenic nematode production and application: Regulation, ecological impact and non–target effects. In: Campos-Herrera, R. (Ed.). *Nematode pathogeneses of insects and other pests*, Springer International Publishing, Switzerland, pp. 255-282.

Pimentel, D., 1997. Techniques for reducing pesticide use: environmental and economic benefits. *Pest management in agriculture*. In Pimentel, D. (Ed). Chichester (UK): John Wiley & Sons, pp. 1-12

Portillo-Aguilar, C., Villani, M. G., Tauber, M. J., Tauber, C. A., Nyrop, J. P., 1999. Entomopathogenic nematode (Rhabditida: Heterorhabditidae and Steinernematidae) response to soil texture and bulk density. *Environ. Entomo*. 28: 1021–1035.

Půza, V., Mrácek, Z., 2009. Mixed infection of *Galleria mellonella* with two entomopathogenic nematode (Nematoda: Rhabditida) species: *Steinernema affine* benefits from the presence of *Steinernema kraussei*. *J. Invertebr. Pathol*. 102 : 40–43.

Root, R. B., 1973. Organization of a plant-arthropod association in simple and diverse habitats : the fauna of collards (*Brassica Oleracea*). *Ecol. Monogr.* 43:95–124.

Rovesti L., Deseö K. V., Heinzpeter E. W., Tagliente F., 1988. Compatibility of pesticides with the entomopathogenic nematode *Heterorhabditis bacteriophora* poinar (Nematoda: Heterorhabditidae). *Nematologica*. 34: 462–476.

Schroer, S., Ziermann, D., Ehlers, R. U., 2005. Mode of action of a surfactant–polymer formulation to support performance of the entomopathogenic nematode *Steinernema carpocapsae* for control of diamondback moth larvae (*Plutella xylostella*). *Bioc. Sci .and Tech.* 15(6): 601–613.

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

Shapiro, D.I., McCoy, C.W., Fares, A., Obreza, T., Dou, H., 2000. Effects of soil type on virulence and persistence of entomopathogenic nematodes. *Environ. Entomol.* 29: 1083–1087.

Shapiro-Ilan, D. I., Dolinski C., 2015. Entomopathogenic nematode application technology. *Nematode pathogeneses of insects and other pests*. Springer International Publishing, Switzerland, pp. 231-255

Shapiro-Ilan, D. I., Gouge, D. H., Koppenhöfer, A. M., 2002. Factors affecting commercial success: case studies in cotton, turf and citrus. In: Gaugler, R. (Ed.), Entomopathogenic Nematology. CABI, New York, pp. 333–356.

Shapiro-Ilan, D. I., Gouge, D. H., Piggott, S. J., Fife, J. P., 2006. Application technology and environmental considerations for use of entomopathogenic nematodes in biological control. *Biol. Control*. 38(1): 124–133.

Shields, E. J., 2015. Utlizing persistent entomopathogenic nematodes in a conservation or a more classical biological control approach. In: Campos-Herrera, R. (Ed.), *Nematode pathogeneses of insects and other pests*. Springer International Publishing, Switzerland, pp. 65-186.

Stock, S. P, 2015. Diversity, biology and evolutionary relationships. In: Campos-Herrera, R. (Ed.), *Nematode pathogeneses of insects and other pests*. Springer International Publishing, Switzerland, pp. 6.

Stuart, R. J., Barbercheck, M. E., Grewal, P. S., Taylor, R. A. J., Hoy, C. W., 2006. Population biology of entomopathogenic nematodes: concepts, issues, and models. *Biol. Control*. 38: 80–102.

Stuart, R. J., Barbercheck, M. E., Grewal, P. S., 2015. Entomopathogenic nematodes in the soil environement: distributions, interactions and the influence of biotic and abiotic factors. In: Campos-Herrera, R. (Ed.), *Nematode pathogeneses of insects and other pests*. Springer International Publishing, Switzerland, pp. 97-137.

Susurluk, A, Ehlers, R. U., 2008. Field persistence of the entomopathogenic nematode *Heterorhabditis bacteriophora* in different crops. *BioControl* 53: 627–641.

Toepfer, S., Hatala-Zseller, I., Ehlers, R. U., Peters, A., Kuhlmann, U., 2010. The effect of application techniques on field–scale efficacy: Can the use of entomopathogenic nematodes reduce damage by western corn rootworm larvae? *Agricand. Forest Entomo.* 12: 389–402.

Torr, P., Spiridonov, S. E., Heritage, S., Wilson, M. J., 2007. Habitat associations of two entomopathogenic nematodes: a quantitative study using real-time quantitative polymerase chain reactions. *J. Ani. Ecol*. 76: 238–245.

van der Werf, H. M. G., 1996. Assessing the impact of pesticides on the environment. *Agric. Ecosyst. Environ.* 60: 81–96.

Virto I., Imaz M. J., Fernández-Ugalde O, Gartzia-Bengoetxea N, Enrique A, Bescansa P., 2015. Soil degradation and soil quality in Western Europe: Current situation and future perspectives. *Sustain.* 7: 313–365.

Zhang, L. M., Liu, X. Z., Zhu, S. F., Chen, S. Y., 2006. Detection of the nematophagous fungus *Hirsutella rhossiliensis* in soil by real-time PCR and parasitism bioassay. *Biol. Control* 36: 316-332.

# Chapter I

# Prevalence and activity of entomopathogenic nematodes and their antagonists in soils that are subject to different agricultural practices

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## Abstract

Agricultural management practices can modify soil properties in ways that may disrupt the abundance and activity of beneficial organisms in the soil. We assessed the impact of different soil management practices on entomopathogenic nematodes (EPN), which have great potential as biological control agents against root-feeding insects. Soils were sampled during spring and autumn 2013 in all 96 plots of a long-term Swiss field trial (DOK experiment). By combining a traditional insect-baiting technique and real-time qPCR analyses, we identified and quantified over 20 soil-dwelling species (or genera). This allowed us to investigate how communities of natural EPN populations and their associated natural enemies and competitors are affected by (i) three crop types (wheat, maize and grass-clover ley) and (ii) farming systems, i.e. conventional, organic and biodynamic, which differed in fertilization, and pesticide use. We also determined the effects on soils' microbial biomass in terms of carbon (Cmic) and nitrogen (Nmic) and applied spatial distribution analysis (SADIE) to uncover patterns of aggregations and associations of the study organisms. Although manure based farming systems increased microbial biomass, the systems did not influence the presence of EPN or their antagonists. EPN was more abundant in winter-wheat plots than in maize and grass-clover ley plots. Overall, very low numbers of EPN were recorded, implying that their natural presence would not be sufficient to have a satisfactory suppressive effect on root-feeding pests and the application of EPN would therefore be an appropriate measure to protect yields in case of root pest outbreaks.

## 1. Introduction

Agricultural production relies on healthy soils that guarantee essential soil functions such as carbon, nutrient, and water cycling. Traditionally, soil quality has been characterized by the presence of nutrients and water and their availability to the crop (Patzel et al., 2000). The development of agricultural practices such as fertilization and irrigation have largely enhanced crop yield; yet, protecting the crops against herbivores and diseases is also a fundamental aspect of these practices, as pests can reduce yields by up to 30% (Oerke, 2005). Crop rotation, cover crops and organic amendments in cropping systems aim at indirectly controlling pests and diseases; however, due to economic pressure, these strategies are often neglected in highly specialized agricultural cropping systems. Instead, different types of pesticides are readily applied. Soil pests are hard to reach with pesticides and exceedingly large amounts need to be applied in order to be effective. This has led to major environmental concerns, and an increasing number of pesticides are being banned (Pimentel, 1995; van der Werf, 1996). Alternatives are badly needed. As a first step, the current study explores the presence of natural biological control agents in agricultural soils in order to estimate their potential to suppress soil pest populations.

A large diversity of microorganisms have the potential to protect plants against pests and diseases if applied in an appropriate manner (Lacey et al., 2015). Efforts to develop biocontrol methods by augmenting beneficial soil organisms or by promoting their natural occurrence have been successful, but are still unsuitable for most types of large-scale agriculture (Bale et al., 2008; Lacey et al., 2015). The Swiss National Research Program 68 (NPR68; http://www.nfp68.ch/) aims to enhance the use of soil natural resources that can improve plant health and, thus, favor plant protection and yield. As a first step towards the development of new biological control methods for sustainable agriculture, a comprehensive inventory of selected beneficial organisms of a given agroecosystem is required, together with knowledge about the factors that might determine their abundance.

Entomopathogenic nematodes (EPN) of the families Steinernematidae and Heterorhabditidae are obligate parasites of insects and are considered excellent biological control agents (Georgis et al., 2006; Kaya et al., 2006; Lewis et al., 2006; Dolinski et al., 2012; Campos-Herrera, 2015; Lacey et al., 2015). Indeed, their infective juveniles (IJs) are able to kill an insect host within 2–3 days. The modus operandi of IJs is to actively seek and penetrate a suitable host. Once inside, IJs release their mutualistic α-Proteobacteria in the insect hemocoel. Toxins produced by the bacteria result in the death of insect host by septicemia. Thereafter, EPN and bacteria reproduce for several generations until the resources inside the host are fully consumed. Thousands of newly produced IJs then exit the cadaver and start a new life cycle (Adams and Nguyen, 2002; Dillman et al., 2012).

In the agroecosystem, EPN are affected by various abiotic soil properties such as soil texture, moisture, temperature, and soil organic matter, which might be drastically altered by

agricultural management practices, as well as biotic factors such as com-petitors and natural enemies (Stuart et al., 2006, 2015; Lewis et al., 2015). Understanding these interactions is essential to reveal ways to enhance the potential of EPN as biocontrol agents in a particular soil type and agricultural scenario. Indeed, this knowledge should allow us to apply EPN more judiciously and to increase their efficacy against targeted insect pests. Several studies have demonstrated negative effects of intensive soil management on EPN (Hummel et al., 2002; Campos-Herrera et al., 2008, 2010, 2014). Few studies even found a positive effect of organic soil management on EPN populations (Briar et al., 2007; Campos-Herrera et al., 2008, 2010). However, we cannot state this as a general rule, since a lack of such effects or even contrary findings have also been reported (Ferris et al., 1996; Bell and Raczkowski, 2008). This calls for more research on the factors that determine EPN occurrence in agricultural fields, but also on other members of the associated food web that may compete, kill or otherwise interact with EPN. Indeed, the distribution of EPN is also affected by the presence of other important organisms in the soil. For example, predators such as microarthropods or nematophagous fungi (NF), ectoparasitic bacteria or natural competitors such as free living nematodes (FLN) have a significant impact on the population dynamics of EPN (El-Borai et al., 2005; Enright and Griffin, 2005; Jabbour and Barbercheck, 2011; Campos-Herrera et al., 2012, 2013a; Pathak et al., 2012). These organisms have been shown to be spatially associated with EPN in the field (Campos-Herrera et al., 2013a). Besides their association with EPN, NF and FLN are also sensitive to abiotic factors (Persmark et al.,1996; Jaffee et al., 1998; Neher, 1999, 2010; Campos-Herrera et al., 2015a). Learning more about the factors that determine the prevalence of the organisms that are directly associated with EPN, can provide additional information on how farming practices might contribute to this important feature of soil health. Hence, the aim of this study was to investigate the effects of farming practices and crop type on the prevalence of EPN and their antagonists. We also investigated the microbial biomass carbon ($C_{mic}$) and nitrogen ($N_{mic}$) in the soils of each farming system. The overall expectation was that soils with high levels of natural organic matter and minimal soil disturbance sustain larger numbers of soil microorganisms, including EPN and associate organisms. To test this hypothesis we combined traditional insect baits and new molecular methods to screen for the presence, abundance and activity of organisms belonging to the EPN soil food web as proposed by Campos-Herrera et al. (2015a). We analyzed samples coming from a long-term field experiment that started in 1978, which aims at studying the effect of biodynamic, bio-organic and conventional farming systems in a seven-year crop rotation system (Mäder et al., 2002).

## 2. Material and methods

### 2.1. Field experiment design and soil sampling

The “DOK” experiment (Dynamisch-Organisch-Konventionell, in German) is located in Therwil, Switzerland (7 33˚E, 47 30˚N). The field experiment is managed by Agroscope (Reckenholz) and the Research Institute of Organic Agriculture (FiBL). The DOK field experiment consists of 96 plots, assigned to eight different treatments (12 replicates per treatment) corresponding to four farming practices and two different levels of organic fertilizer use (Table 1, Fig. S1). The conventional systems are managed in accordance with Swiss standards for integrated farming, applying crop rotation, cover crops and catch crops, integrated nutrient management, and use of pesticides according with economic thresholds (integrated pest control).

In the DOK field, a seven-year crop rotation has been applied since 1978 (Mäder et al., 2002; Fließbach et al., 2007). It changed in the course of time: in the 5th rotation from 2006 to 2012 it consisted of maize (*Zea maize* L.), winter wheat 1st year (*Triticum aestivum* L.) followed by catch crop rye (*Secale cereale* L.), soybean (*Glycine max* L.) followed by catch crop rye, potato (*Solanum tuberosum* L.), winter wheat 2nd year and two years of grass clover ley, standard mixture STM 330: *Trifolium pratense* L. 6%; *T. repens* L. 12%; *Dactylis glomerata* L. 17%; *Festuca pratensis Huds*. 36%; *Phleum pratense* L. 8%; *Lolium perennne* L. 21%.). This crop rotation was applied on three parallel main plots in the DOK experiment, displaced in time. In 2013, the year that we did the soil sampling, the crops planted were: maize var. Colisée (pre-crop grass-clover 2nd year), winter wheat 2nd year, var. Runal (pre-crop potatoes) and a grass-clover ley in its 1st year (pre-crop winter wheat 2, grass- clover planted in August 2012, standard mixture STM 330) with 32 replicates (plots) per crop (Fig. S1). The different management applied to the plots in 2012 and 2013 are presented in Table S1 and S2. On April 23rd and October 16th 2013, we randomly collected 20 soil cores per plot (2.5 cm diam. 20 cm depth), which were pooled in a plastic bag to obtain a composite sample of 3 kg of soil per plot. The plastic bags were transported to the laboratory and kept at 4˚C until use.

**Table 1**: Treatments corresponding to the four farming practices, with the different levels of organic fertilizer use per hectare. Livestock Unit (LU) are average stocking density in Switzerland.

| Management | Code | Organic fertilizer use | Other fertilizer use |
|---|---|---|---|
| Organic | O2 | 1.4 LU/ha | - |
| | O1 | 0.7 LU/ha | - |
| Dynamic | D2 | 1.4 LU/ha | - |
| | D1 | 0.7 LU/ha | - |
| Conventional | K2 | 1.4 LU/ha | Mineral |
| | K1 | 0.7 LU/ha | Mineral |
| No fertilization, biodynamic | N | - | - |
| Only Mineral fertilizers (since 1985) | M | - | Mineral |

### 2.2. Nematode extraction, estimation of the potential of the soil to suppress pest insects, and assessment of soil microbial biomass-carbon C ($C_{mic}$) and biomass nitrogen-N ($N_{mic}$)

Soil was thoroughly mixed in the laboratory and stored at 4˚C in the dark before further processing. Nematodes were extracted no later than 2–4 days after collection, in such an order as to ensure that no temporal bias was introduced, following protocols described by Campos-Herrera et al. (2015a). Briefly, three fresh subsamples of 500 g were prepared from each of the 96 soil composite samples. One of these 500 g soil aliquots was used to extract nematodes and other associated organisms by sucrose centrifugation (Jenkins, 1964) for real-time qPCR analyses (Campos-Herrera et al., 2015a). A second 500 g fresh soil aliquot was baited with *Galleria mellonella* L. (Lepidoptera: Pyralidae) to test the suppressive potential of the soil. Following Bedding and Akhurst (1975) with few modifications, two portions of 250 g soil of the subsamples from each plots were baited with 5 final instar *G. mellonella* larvae (commercial stock, Au Pêcheur SARL Neuchâtel, Switzerland) in two independent rounds. Conditions and processing were carried out following Campos-Herrera et al. (2015a). After exposure for 4 days, the *G. mellonella* cadavers were recovered from the soil, thoroughly rinsed with tap water, and individually placed in White traps, which are routinely used to collect emerging nematodes (White, 1927). Every 2–3 days, we checked for the organisms responsible for the mortality of *G. mellonella* larvae under the stereoscope. In case of nematode infection, nematodes were recovered at emergence. After a month of incubation, cadavers for which no obvious cause of death could be discerned were discarded after dissection. We confirmed EPN infection by performing Koch's postulate (Lacey, 1997). Each nematode isolate that was obtained from the soil samples (Original isolated from *G. mellonella*, hereafter OG) was placed in a 5.5 cm Petri dish containing four *G. mellonella* larvae.

Larval mortality was assessed twice a week and emerging nematodes recovered (Multiplication of nematodes in *G. mellonella* larvae, hereafter, MG). A second round was performed to confirm the negative results (Campos-Herrera et al., 2015a).

The third aliquot of 500 g fresh soil was used to measure soil microbial biomass carbon ($C_{mic}$) and nitrogen ($N_{mic}$), which was determined by chloroform-fumigation-extraction (CFE) following Fließbach et al. (2007). Triplicates of 20 g (dry soil) per sample were fumigated with chloroform and subsequently extracted in 80 mL of 0.5 M K2SO4 solution. In order to be able to relate our findings to a given amount of dry soil, we dried 180 g aliquots of wet soil of all the samples at 40˚C for 3–4 days and estimated the water content.

2.3. Identification and quantification of entomopathogenic nematodes and members of their soil food web

The cultures of the organisms used as positive controls, the subsequent DNA extraction, and the standard curves establish-ment for the different targeted organisms were generated following Campos-Herrera et al. (2015a). We employed the Power Soil DNA Isolation Kit (MoBio laboratories, Inc.) for the DNA extraction of both soil samples and pure cultures when living material was available. In some cases (see Table S3), when no living material was available, we employed plasmids with the whole sequence of interest to establish our positive control (see Campos- Herrera et al., 2015a). Aliquots of DNA samples not used in the study were stored at 80˚C. The quality and quantity of each DNA sample was analyzed using 1 mL per duplicate in a Nanodrop 1000 (Thermo Scientific, Wilmington, DE, USA). The identification and quantification of a total of 24 organisms comprising EPN and members of the same soil food web (Table S3) was performed using real–time PCR protocols following the MIQE procedure (Bustin et al., 2009). The species were selected based on previous surveys of Swiss soils, plus we added some species with a high potential to be found in temperate climates (Steiner, 1996; Kramer et al., 2001; Hominick, 2002; Campos-Herrera et al., 2015a). Specifically, we screened for a total of 13 EPN species (*Heterorhabditis megidis*, *H. bacteriophora*, *H. zealandica*, *Steinernema feltiae*, *S. carpocapsae*, *S. glaseri*, *S. kraussei-silvaticum*, *S. affine*, *S. bicornotum*, *S. intermedium*, *S. weiseri*, *S. poinari*, *and S. intermedium group*), six nematophagous fungi (NF) (*Arthrobotrys oligospora*, A. dactyloides, *A musiformis*, *Purpureocillium lilacinus*, *Hirsutella rhossiliensis*, and *Catenaria* sp.), four free-living nematodes (*Acrobeloides*-group, *Oscheius tipulae*, *Oscheius onirici* and *Oscheius* sp. 3) and one ectoparasitic bacterium (*Paenibacillus nematophilus*) (Atkins et al., 2005; Zhang et al., 2006; Torr et al., 2007; Pathak et al., 2012; Campos-Herrera et al., 2011a, 2011b, 2012, 2015a, 2015b). In some cases, we described the maximum taxonomical resolution that our molecular tools can provide, which concerns the data for *S. kraussei-silvaticum* or *Acrobeloides*-group, as discussed in Campos-Herrera et al. (2012, 2015a).

We employed optical 100–well gene disc (Biolabo, scientific instruments, Switzerland) reaction plates on the Corbett Research real time PCR machine. All samples were run in duplicates (unknown, positive and negative controls) in all the reactions. Final reaction, concentrations, and protocols have been described by Campos-Herrera et al. (2015a). The soil DNA extractions were individually screened with the corresponding unique species-specific primers/probes combination for each species, which represent >80 full-plate reactions (including some re-runs for confirmation). Numbers of nematodes collected from the soil samples were assessed from a 10-fold dilution of the DNA, whereas total DNA without dilutions was used for the identification and quantification of NF and ectoparasitic bacteria using the adapted qPCR procedure as described for EPN (see details in Campos-Herrera et al., 2015a). A correction factor was derived from the dilution series to transform qPCR data to numbers of IJs. Similarly, aliquots of nematodes corresponding to all the MG isolates were used for DNA extraction, but using QIAamp DNA mini kit (Qiagen). DNA quality and quantity was verified (Nanodrop system) and samples were diluted to the range of 0.5–1 ng/mL.

Real time qPCR was also used to assess the abundance and identity of the progeny of each MG, screening for all the EPN and FLN species that were initially detected in the soil samples. In this case, aliquots of these samples were pooled four by four and adjusted to have a final 10-fold dilution volume. When a species was detected in the pooled sample, we analyzed the corresponding samples individually to identify the exact sample(s) that contained the species. In addition to this, the sequences corresponding to the ITS region of selected MG isolates were amplified and sequenced as described by Campos-Herrera et al. (2015a) to confirm species identity.

### 2.4. Statistical analysis

Data from the *G. mellonella* baits allowed us to estimate the suppressive capacity of each sampled plot (here called “activity” and defined as the percentage of larval mortality per plot, averaged by treatment). The activity was determined with respect to the total mortality, caused by any agent (i.e nematode, bacteria, fungi, virus, etc.) and caused by nematodes or EPN only. A General Linear Model (GLM) fitting a binomial distribution was used to analyze the data obtained by *G. mellonella* baits using management practices, crop and the period of sampling as factors.

All the organisms’ variables quantified in the soil by using qPCR were expressed per 100 g of dry soil. Thereafter, data were transformed for further analyses following Campos-Herrera et al. (2015a). The parasitism of nematodes by NF was determined by dividing the DNA quantity of each species by the total amount of DNA and expressed as “infection rate” (Campos-Herrera et al., 2012; Duncan et al., 2013). To estimate the total FLN and NF, we standardized the units of measurement among species to be on a scale of 0–1, by dividing all data within a species by the highest measurement for that species (de Rooij-van der Goes et al., 1995). EPN abundance was

expressed as number of IJs per 100 g of soil. We also calculated the EPN species richness, equivalent to the total number of species per plots.

$C_{mic}$ and $N_{mic}$ values obtained by CFE were analyzed without any transformation. A linear model with stepwise procedure was used to analyze qPCR and CFE data, using (i) farming system, (ii) crop and (iii) period of sampling as factors and every sample as a random factor to account for repeated measurements. Normality and equal variances of the data were assessed, but most of the data failed those assumptions, thus the reported F-values were obtained from the original model whereas P-values were obtained by 1000 permutations using the "pbkrtest" package of R 3.1.0 (CRAN). Data are presented as mean SEM of the untransformed values.

The competition among nematodes (FLN and EPN) for a cadaver (MG samples) was determined using relative proportion of Ct values obtained from the qPCR for each species. Because a low Ct value indicates a high concentration and vice versa, we subtracted the total number of cycles run during the qPCR process (n = 36) to the final Ct value to obtain a proxy of relative proportion per nematode species in each of the cadaver (Campos-Herrera et al., 2015b). In addition to this, we used Cts obtained from both nematodes isolated from the soil and nematodes that emerged from the subsequent Koch's postulate larvae. We compared the proportion of nematodes detected in these cadavers to determine species prevalence.

As suggested by Campos-Herrera et al. (2013a), we employed SADIEshell (v2.0) (Kelvin F. Conrad & IACR-Rothamsted 2001), to explore the spatial distribution of the different investigated trophic guilds. SADIEshell uses the measurements of abundance in relation to the coordinates. This provides the degree of clustering of the data (patches or gaps) that can be associated with the species distribution and the association or dissociation of two trophic guilds populations. The resulting cluster indices were used to draw contour maps that represent the distribution of the targeted species. We focused on the "index of aggregation" (Ia), explained as follows: value of Ia = 1 suggests a spatially random pattern, Ia > 1 suggests an aggregated pattern and Ia < 1 indicates a regular pattern (Perry, 1998). In addition to this, we obtained the index of association (X) among selected organisms. This index describes the degree of association or dissociation of two variables, with a positive X index suggesting a local association, and a negative value indicating a local disassociation. Coordinates (x,y) were assigned to the plots and used to perform the different analyses described above. The analyses were carried out using qPCR transformed data (for NF, EPN and FLN) and values obtain from CFE (for $C_{mic}$ and $N_{mic}$).

## 3. Results

*3.1. Measurements by qPCR of entomopathogenic nematodes and members of their soil food web*

EPN were detected in 63.5% of the plots. Overall, we detected 6 out of the 13 targeted EPN species: *S. affine*, *S. carpocapsae*, *S. feltiae*, *S. poinari*, *H. bacteriophora* and *H. megidis*. The most common species was *H. megidis*, which was found in 30.2% of the plots, followed by *H. bacteriophora*, detected in 29.1% of the plots, but only in the spring sampling. *S. carpocapsae*, *S. feltiae*, *S. poinari* and *S. affine* were less abundant and were detected in 13.5%, 7.3%, 5.2% and 4.2% of the plots, respectively. Because of the low quantities detected (on average below 1 IJ/100 g of dry soil), statistical analyses were only performed for the total number of IJs, irrespective of species. The EPN community showed significantly higher number of IJs in October than in April (Table 2). Also, significantly higher numbers of IJs were recorded in plots planted with winter wheat and maize than those in the recently sown artificial grass-clover plots (Table 2, Fig. 1a), whereas farming system did not significantly affect the EPN community (Table 2, Fig. 2a). The EPN richness was only affected by the sampling period ($P < 0.001$) (data not shown).

NF were detected in almost all the plots (98.9%) and we detected the presence of 4 of the 6 targeted NF species in the soil. The dominant species were *Catenaria* sp. and *H. rhossiliensis*, which were found in 89.6% and 90.6% of the plots, respectively. *P. lilacinus* and *A. oligospora* were less abundant with 52.1% and 72.9% of detection, respectively. *Arthrobotrys dactyloides* and *A. musiformis*, both trapping NF species, were not detected. The NF community was more abundant in October than in April (Table 2). Higher values, but not statistically significant, were recorded in plots planted with winter wheat than in those with grass-clover ley and maize (Table 2, Fig. 1b). Like with the EPN guild, the farming practices did not have a significant impact on the targeted NF community (Table 2, Fig. 2b).

We did not detect the ectoparasitic bacterium *Paenibacillus nematophilus* in any of our sample. By contrast, the free living nematodes (FLN) that compete with EPN for cadavers, namely *Oscheius* spp. and nematodes belonging to the *Acrobeloides*-group were detected in all of the 96 plots. Out of the 4 species targeted, the only nematode not detected was *Oscheius* sp 3. More precisely, the *Acrobeloides*-group was found in all of the plots, whereas *Oscheius* onirici and *Oscheius tipulae* occurred both in 95.8% of the plots. In agreement with the EPN, the FLN were significantly more abundant in October than in April (Table 2). Similarly to the other trophic guilds, significantly higher values were recorded in plots planted with winter wheat than in those with grass-clover ley and maize (Table 2, Fig.1c), and again no differences were found among different farming practices (Table 2, Fig. 2c)

*3.2. Soil supression and microbial biomass*

Considering all the plots and both periods of sampling, the mean total activity of the soil, defined as the mean percentage of *G. mellonella* larval mortality by various known causes, including nematode, bacteria and fungi, was 30.7%. The activity was spread over the entire DOK field with 87.3% of the plots showing some activity. Of all larvae, 12.6% were infected by nematodes (FLN + EPN), which is almost half of the total measured activity. Nematode activity was registered in 35.5% of the plots, but EPN were responsible for only half of the nematode activity (5.8%). Total numbers and activity of nematodes differed significantly between the two sampling periods, with higher percentage of cadavers with nematodes in October than in April (Table 2). The crop type significantly shaped the activity of nematodes, with higher activity observed in the wheat plots than in the plots with the two other crops (Table 2, Fig. 3a), but this was not the case for the total activity (Table 2, Fig. 3b). The farming practices applied to the different plots, however, did not have any influence on the activity of organisms (Table 2, Fig. 4). As far as the microbial biomass proxies $C_{mic}$ and $N_{mic}$ were concerned, the type of crop did not affected $C_{mic}$ or $N_{mic}$ (Table 2). As expected, $C_{mic}$ and $N_{mic}$ were significantly influenced by the farming practices (Table 2, Fig. S2), with the highest values observed in the biodynamic ($N_{mic}$ and $C_{mic}$) and organic (only $C_{mic}$) plots. Finally, the period of sampling influenced both $N_{mic}$ and $C_{mic}$, with higher values in October (Table 2).

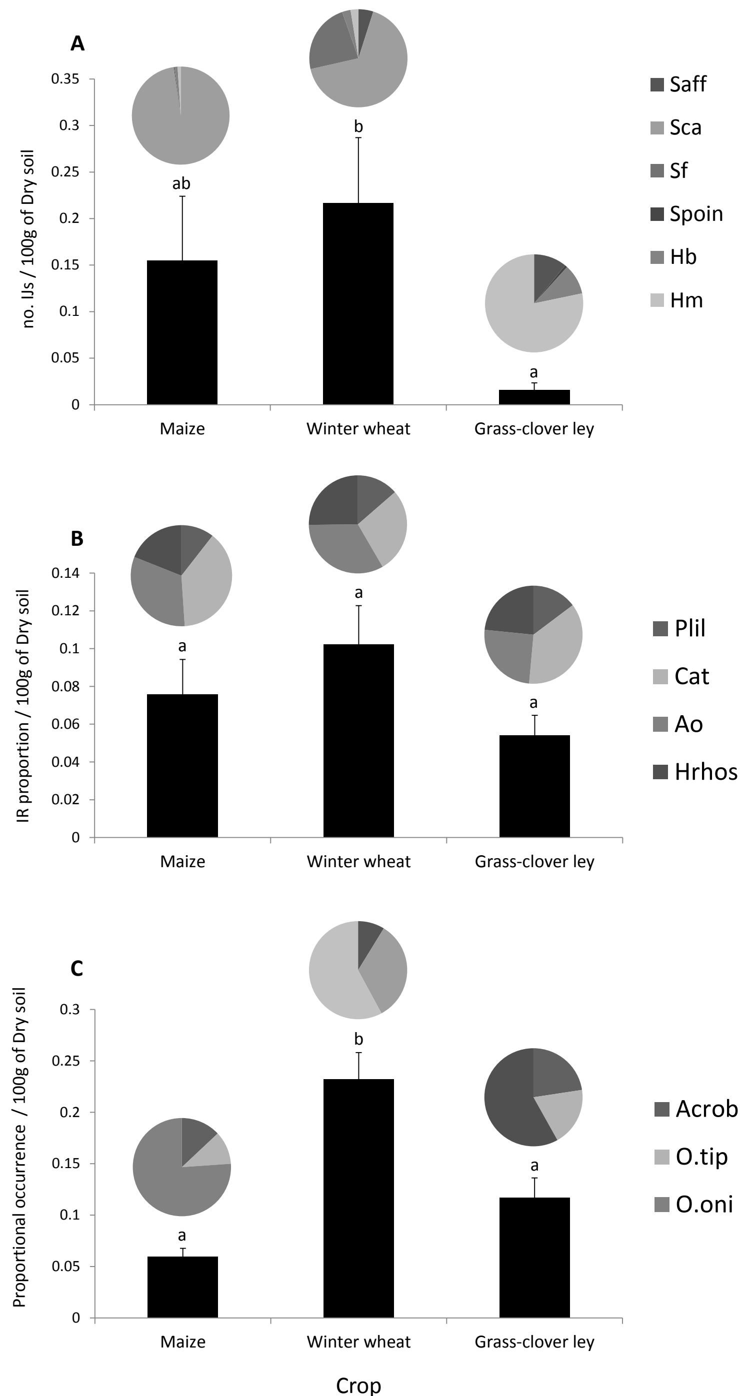


**Fig. 1.** Influence of crop type (maize, winter wheat, grass-clover ley) on natural occurrence of entomopathogenic nematodes (EPN) along with selected key members of their soil food web: nematophagous fungi (NF) and free-living nematodes (FLN). Data show the average value for the combined species-specific quantification of each of the organisms in the DOK field trial combining two different sampling periods in 2013. (a) Total number of EPN infective juveniles (IJs) of the species *Steinernema affine* (Saff), *S. carpocapsae* (Sca), *S. feltiae* (Sf), *S. poinari* (Spoin), *Heterorhabditis bacteriophora* (Hb), *H. megidis* (Hm). (b) Total NF infection rate (IR) proportion for the species *Purpureocillium lilacinus* (Plil), *Catenaria* sp. (Cat), *Hirsutella rhossiliensis* (Hrhos), and *Arthrobotrys oligospora* (Ao). (c) Total proportional occurrence of FLN for the *Acrobeloides*-group (Acrob), *Oscheius onirici* (O.oni) and *Oscheius tipulae* (O.tip). The proportional presence of each EPN, NF or FLNs species is shown with the corresponding proportion of the pie graph above each column. Data are shown as means ± SEM. Lowercase letters indicate statistical differences.

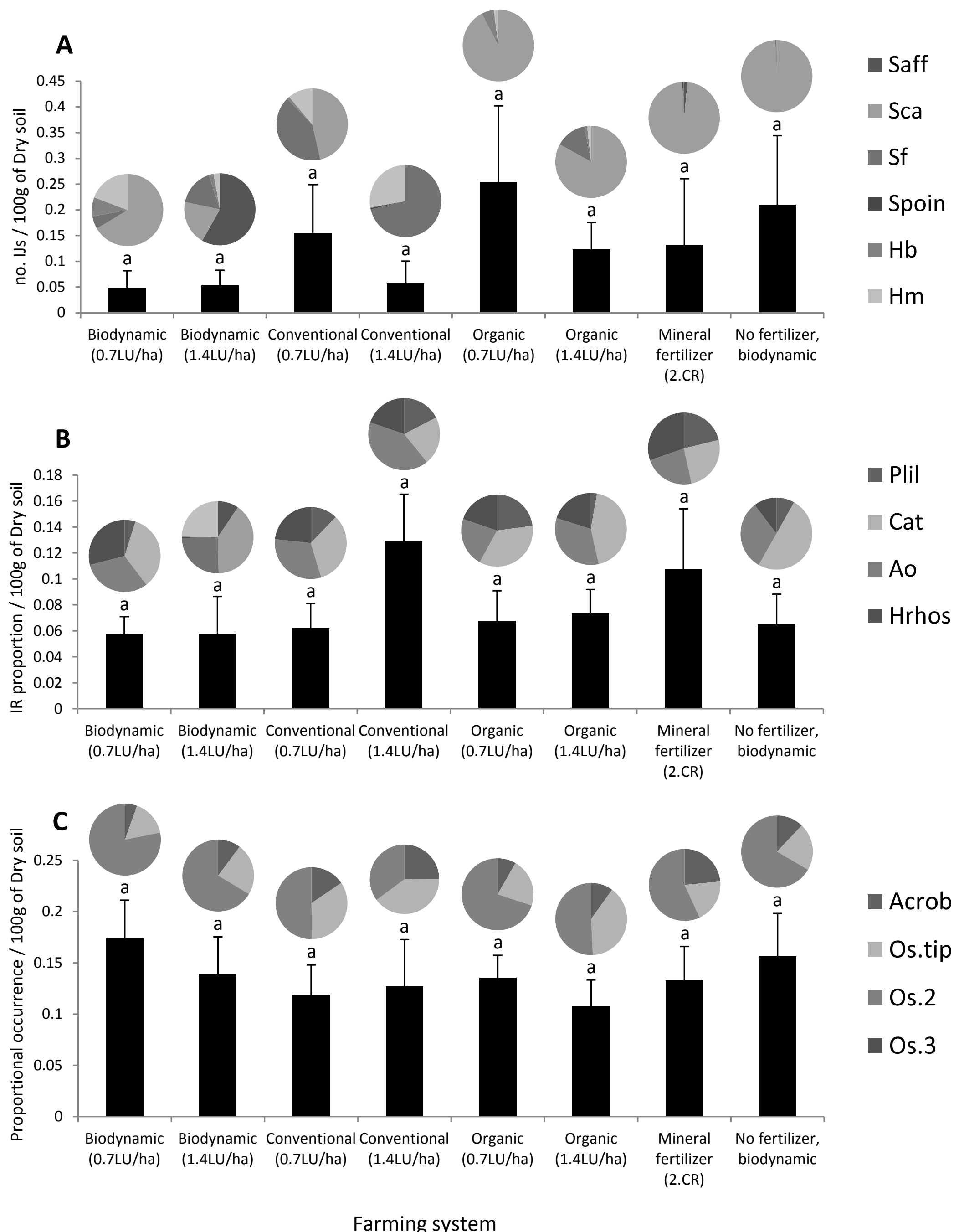


**Fig. 2.** Influence of management practices on the natural occurrence of entomopathogenic nematodes (EPN) along with selected key members of their soil food web: nematophagous fungi (NF) and free-living nematodes (FLN). Data present the average value of the combined species-specific quantification of each of the organisms in the DOK field trial combining two different sampling points in 2013. (a) Total number of EPN infective juveniles (IJs) of the species *Steinernema affine* (Saff), *S. carpocapsae* (Sca), *S. feltiae* (Sf), *S. poinari* (Spoin), *Heterorhabditis bacteriophora* (Hb), *H. megidis* (Hm). (b) Total NF infection rate (IR) proportion for the species *Purpureocillium lilacinus* (Plil), *Catenaria* sp. (Cat), *Hirsutella rhossiliensis* (Hrhos), and *Arthrobotrys oligospora* (Ao). (c) Total proportional occurrence of FLN for the *Acrobeloides*-group (Acrob), *Oscheius onirici* (O.oni) and *Oscheius tipulae* (O.tip). The proportional presence of each EPN, NF or FLNs species is shown with the corresponding proportion of the pie graph above each column. Data are shown as means ± SEM. Lowercase letters indicate statistical differences.

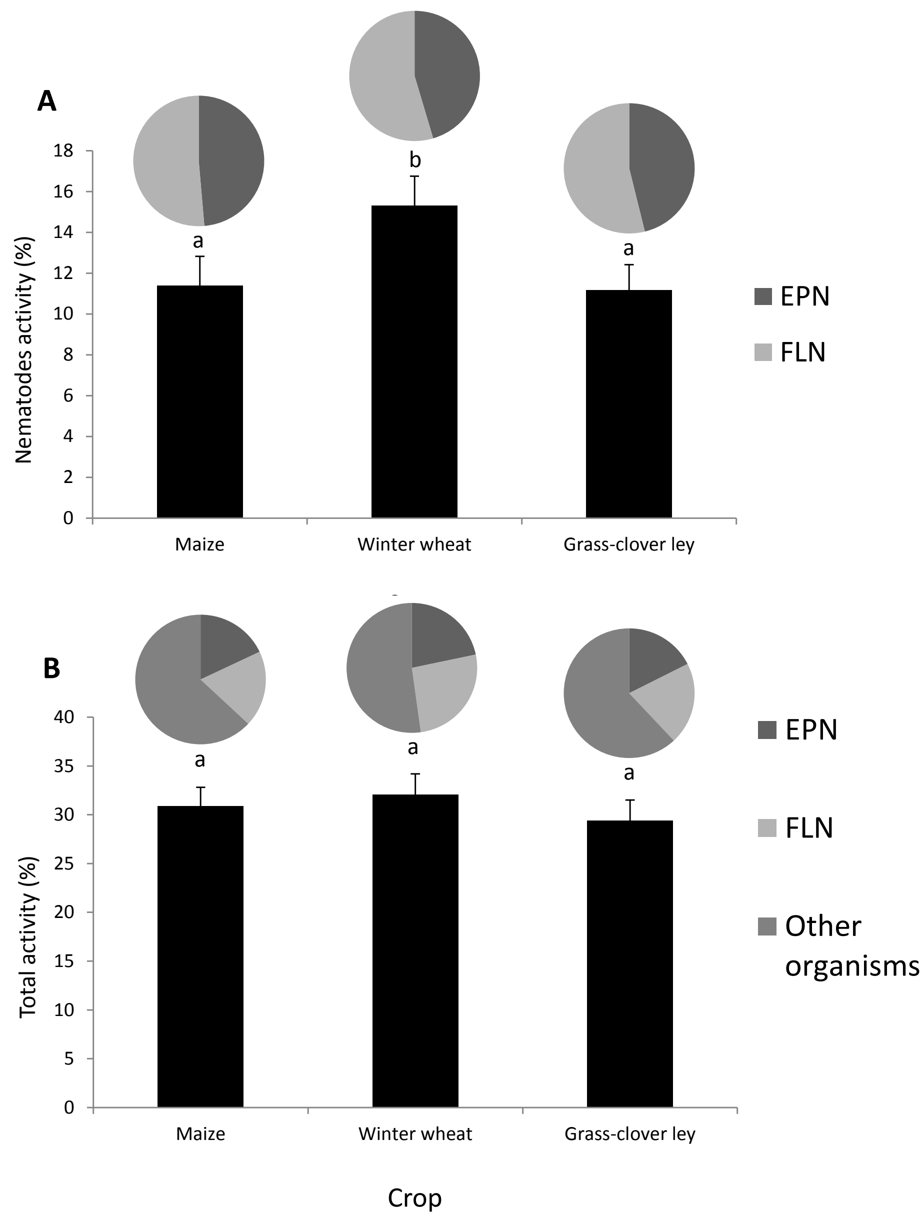


**Fig. 3.** Influence of crop type (maize, winter wheat, grass-clover ley) on activity recorded from *Galleria mellonella* baiting as expressed by the average percentage of larval mortality. (a) Mean activity of nematodes (EPN + FLN) and (b) mean activity of all organisms (EPN + FLN + Other organisms, mainly bacteria and fungus). Data are shown as means ± SEM. Lowercase letters indicate statistical differences.

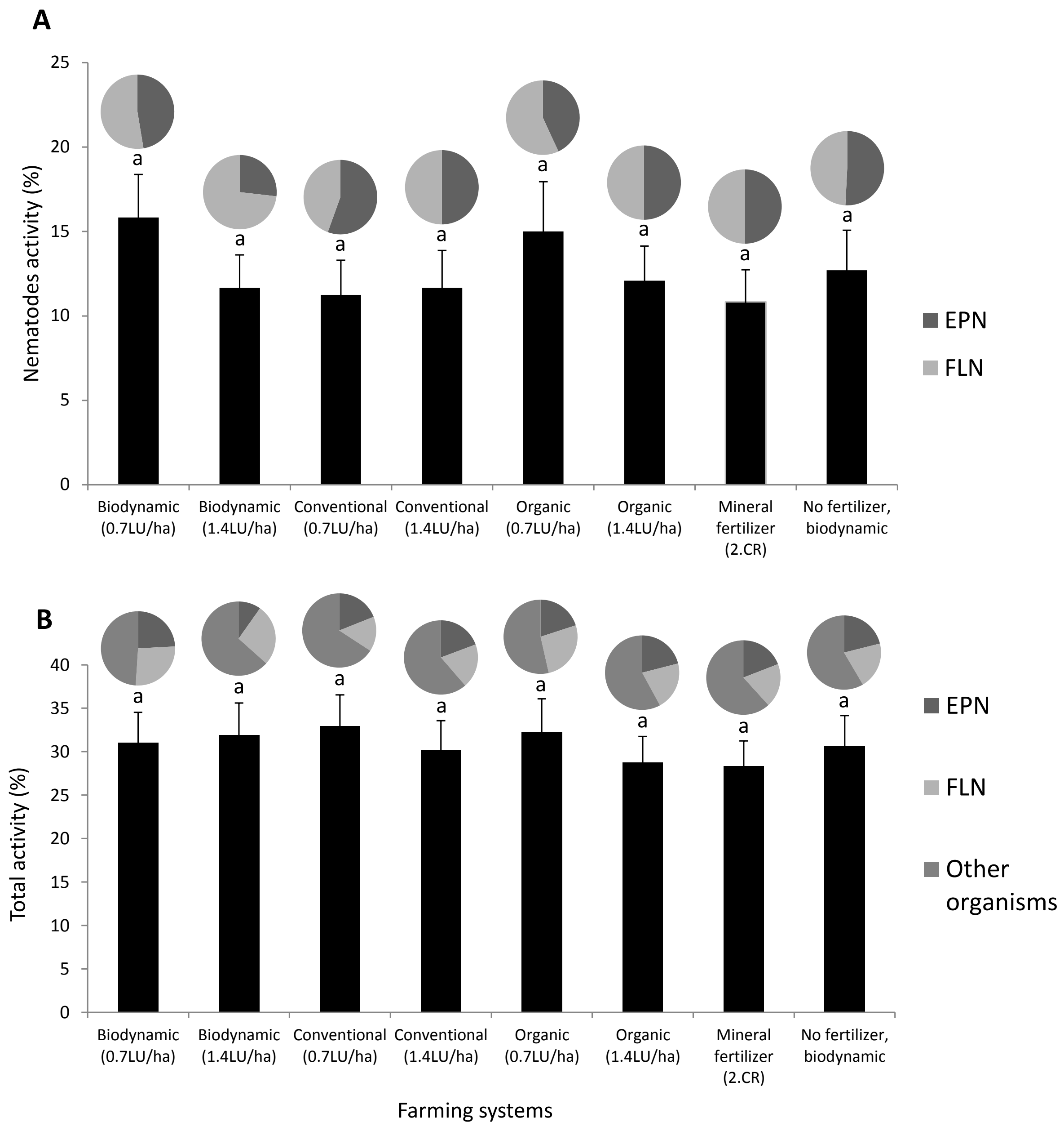


**Fig. 4.** Influence of management practices (biodynamic 0.7 LU/ha and 1.4LU/ha; organic 0.7 LU/ha and 1.4LU/ha; conventional 0.7 LU/ha and 1.4LU/ha; Mineral fertilizer; No fertilizer, biodynamic) on activity recorded from *Galleria mellonella* baiting expressed as the average percentage of larvae mortality. (A) Mean activity of nematodes (EPN + FLN) and (b) mean activity of all organisms (EPN + FLN + Other organisms, mainly bacteria and fungus). Data are shown as means ± SEM. Lowercase letters indicate statistical differences.

*3.3. Inter and intra-specific nematode guild competition for the cadavers*

The qPCR data collected from Koch's postulate cadavers (MG) revealed the same species as were detected in the soil sample, but with the addition of *S. kraussei-silvaticum*. Mixed nematode progenies (EPN + FLN) were found in 44% of all the MG records (n = 254). In total, EPN represented 11.5% of the nematodes reproducing from the Koch's postulate cadavers, whereas free-living *Oscheius* spp. represented 88.5%. In the soil samples, Steinernematidae and Heterorhabditae showed similar proportional occurrences, accounting for 2.6% and 4.3%, respectively, while Heterorhabditae were almost absent in MG cadavers (0.3%) in favor of the Steinernematidae (11.1%). The *Oscheius* spp. were even more dominant in the MG (88.5%) than in the soil samples (66.7%). The *Acrobeloides*-group, very abundant in the soil (26.2%), did not reproduce during any of the subsequent MG steps (Fig. 5).

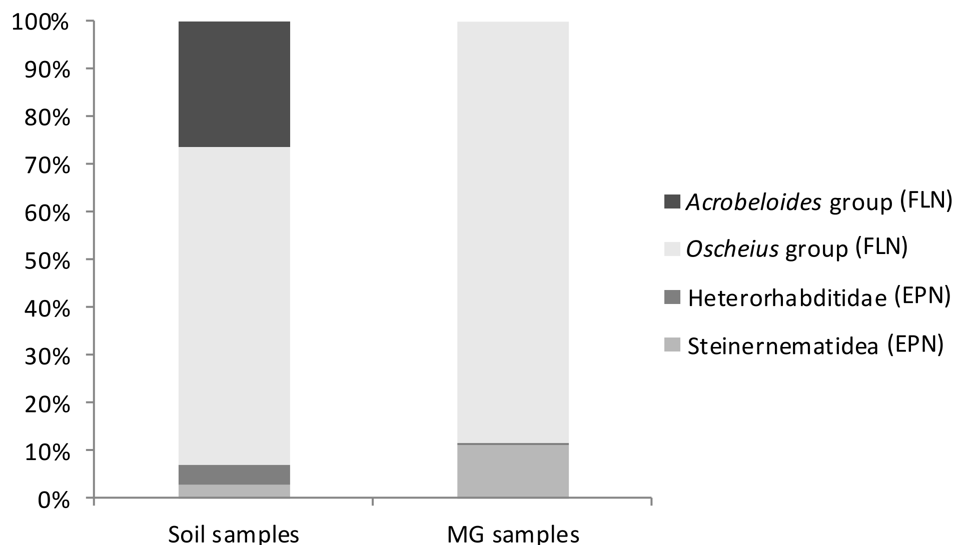


**Fig. 5.** Relative proportions of emerging EPN species in both soil samples and MG samples (Koch's postulate cadavers) after *Galleria mellonella* baiting. Proportions were obtained from Ct values of qPCR species-specific quantification of each of the organisms in the DOK field trial combining two different sampling points in 2013.

*3.4. Spatial distribution and association*

Spatial distribution and association Results from SADIE analyses suggest that EPN were randomly distributed in the DOK field. FLN, $C_{mic}$ and $N_{mic}$ were aggregated and NF followed a regular pattern (Table S4). EPN were associated in the field with all of the four tested parameters (NF, FLN, $C_{mic}$ and $N_{mic}$) (Table S5). These associations in the distributions of EPN, FLN and NF throughout the DOK field were transposed to a contour map in Surfer 12 (Trial version, Golden Software, 2015), (Fig. S3).

Table 2. Statistical analysis of activity measured by *Galleria mellonella* bait data, natural occurrence of the organisms (in 100 g of dry soil) detected by qPCR in the soils and microbial biomass detected by CFE. Data are presented as $Chisq_{df}$ (for binomial analyses), $F_{df}$ (for permutation analyses) and probability levels: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, n.s., no significant.

| | Farming system (F) | Crop type (C) | Period (P) | F*C | C*P | F*P | F*C*P |
|---|---|---|---|---|---|---|---|
| **GM bait data** | | | | | | | |
| All organisms | | | | | | | |
| Activity (%) | n.s. | n.s | $307.24_1$*** | n.s. | n.s. | n.s. | $38.2_{14}$*** |
| Entomopathogenic nematodes | | | | | | | |
| Activity (%) | n.s. | n.s. | $110.6_1$*** | n.s. | n.s. | n.s. | $29.6_{14}$** |
| Nematodes (all) | | | | | | | |
| Activity (%) | n.s. | $9.04_2$* | $165.4_1$*** | n.s. | n.s. | n.s. | $28.08_{14}$* |
| **qPCR data** | | | | | | | |
| Entomopathogenic nematodes | | | | | | | |
| Total IJs | n.s. | $4.3_2$* | $5.0_1$ * | n.s. | $4.0_2$* | n.s. | $1.6_{14}$*. |
| Free-living nematodes | | | | | | | |
| ng DNA, (0-1) scaled | n.s. | $20.9_2$*** | $6.1_1$* | n.s. | $21.4_2$*** | n.s. | n.s. |
| Nematophagous fungi | | | | | | | |
| Total IR[1] | n.s. | n.s. | $6.8_1$** | n.s. | n.s. | n.s. | n.s. |
| **CFE data** | | | | | | | |
| Cmic (mg/kg) | $23.2_7$*** | n.s. | 8.5** | n.s. | $4.7_2$*. | n.s. | n.s. |
| Nmic (mg/kg) | $11.4_7$*** | n.s. | $219.4_1$*** | n.s. | n.s. | n.s. | n.s. |

[1] IR, infection rate for the nematophagous fungi (NF), equivalent to pg NF DNA / total DNA

## 4. Discussion

Entomopathogenic nematodes, which can serve as biological control agents, were found to be very rare in the soil of the DOK field (less than 1 IJs per 100 g of dry soil). With the exception of *S. poinari*, all of the EPN species that we detected have previously been reported for Swiss soils (Steiner, 1996; Kramer et al., 2001; Hominick, 2002; Campos-Herrera et al., 2015a). Interestingly, and contrary to our hypothesis, differences in farming system did not affect the presence or abundance of EPN or any of the associated food web members included in this study, except for the microbial biomass measurements. On the other hand, the cropping sequence (pre-crop) strongly affected EPN density. For most of the species, only trace levels were detected. The low numbers were similar to those recorded in other long-term Swiss field experiments dealing with other agronomic treatments, such as arable land under various tillage practices (Campos-Herrera et al., 2015a). The current study, as well as the findings by Campos-Herrera et al. (2015a), support the idea that the disturbance caused in the soil as consequence of arable crop production negatively affects the natural occurrence of EPN to the extent that it might mask possible additional detrimental effects caused by other soil modifications. It is also possible that the low EPN numbers

are caused by an absence of suitable insect hosts due to effective crop rotation strategies applied in Switzerland. The DOK experiment started 38 years ago and involves a seven-year crop rotation, well balanced between cereals, root crops, leguminous crops and grass-clover ley. Our experiment was also preceded by crop rotations, including three years of grass-clover. There was no indication that any of these treatments affected EPN presence. It should be noted that the overall scarcity of the EPN might have been the main reason for the fact that we did not find any difference between the practices. At higher EPN densities these practices may have an effect and EPN scarcity could be simply explained by the general absence of insect hosts (Stuart et al., 2015). Several studies have correlated the abundance of hosts with the abundance of EPN, but found contrasting findings. Those correlations are difficult to interpret as EPN infect a wide variety of insects, but also have more or less suitable hosts (Puza and Mrácek, 2005; Mrácek et al., 1999, 2005). It is likely that in the DOK field management practices, as well as the lack of hosts, interferes with the proliferation of EPN. Moreover, the EPN were out-numbered by competing FLN species, in particular two species from the Oscheius genus, *O. tipulae* and *O. onirici*. Nematodes emergence from *G. mellonella* cadavers by EPN (5.8%) was similar to the one by FLN (6.8%). Cadavers that were obtained following Koch's postulate, i.e. killed by nematode offspring that originated from the cadavers collected from the original soil samples, showed a dominance of the two *Oscheius* spp. These FLN have been recently reported as important competitors of the EPN for the cadavers in a species-specific and density-dependent manner (Campos-Herrera et al., 2015b). This strong competition for the cadavers by the FLN limits the reproduction potential of EPN under those circumstances, as FLN may displace EPN from the cadaver. Nematodes from the *Acrobeloides*-group can interfere with the reproduction of EPN, but this potential interference is EPN species-dependent (Campos-Herrera et al., 2012). It seems that *Acrobeloides* nematodes can reproduce concomitantly with EPN-cadavers, but since they cannot kill the insect they will not contribute, but rather interfere with pest control (Campos-Herrera et al., 2012). For *Oscheius* spp. this is less clear (Dillman et al., 2012; Campos-Herrera et al., 2015b) and needs to be further investigated.

Despite the low numbers of IJs, the results showed a relatively high diversity of EPN species, with a total of seven species detected at the DOK site. But there was no difference in species richness among the different farming systems nor was there a difference among crop type. Under different circumstances (i.e. other crop included in rotations, type of soil, climate conditions) with more insect hosts and/or fewer competing FLN, the EPN diversity might be considerably higher.

The long-term conventional application of fertilizers had no impact on the abundance or the activity of nematodes, nor did it affect the associated organisms. In a comparable study in La Rioja (Spain) by Campos-Herrera et al. (2008, 2010), almost no EPN were detected in annual arable crops, independently of whether organic or conventional management was applied. Similarly, Jaffee et al. (1998) observed that the use of chemical fertilizers in the conventional systems do not have any effect on EPN. But, some other studies in different types of crops have shown that the

effect of conventional soil management depends on the period of sampling (Campos-Herrera et al., 2010), or did not detect any effect (Bell and Raczkowski, 2008). The only parameters that were influenced by the farming practices were those related to of the microbial biomass, with both $C_{mic}$ and $N_{mic}$ showing the highest values in the biodynamic plots, and increased values for $C_{mic}$ in organic plots. These findings are consistent with what has been found before in the same field site over a period of 21 years (Fließbach et al., 2007; Esperschütz et al., 2007), and with the recent evaluation of the soil microbial diversity under long-term organic and conventional farming (Hartmann et al., 2015). The microbial biomass has followed the same pattern over many years in the DOK field, highlighting the consistency of plot management. It should be noted that in addition to the agricultural practices and crops that were planted, plots differed in soil clay content due to a specific gradient of clay that is present in the DOK field (P. Mäder, personal communication). However, we did not find any correlation between clay content and the presence of any of the organisms (data not shown) and conclude that the clay content was not one of the main factors shaping the distribution of the studied species in the DOK field.

To our knowledge, this is the first large scale, replicated study to investigate whether crop type influences the distribution of nematodes and their associated food web members at the same field site. The EPN, as the other associated organisms investigated were found to be most numerous in wheat plots (after potatoes as preceding crop). In accordance, the highest activity of nematodes was recorded in the wheat plots. Interpretation should be done with caution since we were investigating a field subjected to crop rotation and it is well possible that the preceding crop (Fig. S1), at least in part, influenced the observed pattern. Records from the DOK field suggest a generally low prevalence of root-feeding insects in wheat plots, but an infestation by wireworms was noticed on the preceding potatoes (P. Mader, personal communication). It is, therefore, indeed possible that rotation and the soil fauna established during the earlier cultivation had an effect on EPN presence in the following winter wheat plots. Nevertheless, differences in rhizosphere structure and soil community among wheat, maize and grass-clover fields (Demarta et al., 2014), as well as differences in the complexity of these habitat (Jabbour and Barbercheck, 2008) may explain the pattern observed for EPN.

We found seasonal variability in the abundance of all the organisms that were investigated, densities and activities being higher in autumn (October sampling) than in spring (April sampling). While this inference is only based on two sampling events, the phenology we observed is consistent with data reported by other studies (Efron et al., 2001; Campos-Herrera et al., 2008, 2010, 2015a). The advantage of repeating our measurements two times over the year, one in spring and another in autumn is that we can assess if the differences due to the crop and the management were consistent over time. It is remarkable that even with the expected temporal differences, we obtain very consistent pattern in response to the management practices and the crop type, for both sampling periods.

Using SADIE analyses, we showed how EPN presence is associated in the field with the other trophic guilds (FLN, NF and microbial biomass). EPN are likely to suffer from predation and competition, which may significantly decrease their efficiency as biocontrol agents (Stuart et al., 2015). This is relevant for crop protection strategies that included EPN augmentation, as the response of FLN and NF populations will play a key role in the successful establishment of EPN populations. These results and those of studies in other crop systems start to provide evidence that the trophic cascades in the soil can impact crop health and even yield (Campos-Herrera et al., 2013b, 2014; Duncan et al., 2013). Our results further confirm that qPCR data and traditional baiting results can be combined to assess the spatial distribution of trophic groups from soil samples (Campos-Herrera et al., 2013a).

Overall, we found a high proportion of mixed progeny of nematodes emerging from Koch's postulate cadavers, with FLN species and different EPN species co-existing in the same cadaver. Studies on interspecific (Kondo, 1989; Alatorre-Rosas and Kaya, 1990 reviewed in 1991; Koppenhöfer and Kaya, 1996; Koppenhöfer et al., 1995; Duncan et al., 2003; Puza and Mrácek, 2009) and intraspecific competition (Selvan et al., 1993; Koppenhöfer and Kaya, 1995; O'Callaghan et al., 2014) among EPN show that cadavers can produce mixed progenies, but that in some cases one of the species is more suppressed than the other (e.g: Puza and Mrácek, 2009) or the numbers of both species are dramatically reduced (e.g: Koppenhöfer and Kaya, 1996). Competition of EPN with FLN within cadavers has also been reported, but has been less extensively studied than competition between EPN species (Campos-Herrera et al., 2012; Duncan et al., 2003). Recently, relying on molecular data, Campos-Herrera et al. (2015a) found that several EPN and FLN species can coexist in the same cadaver. In a subsequent study, by using qPCR assays Campos-Herrera et al. (2015b) showed that two FLN species, *Oscheius tipulae* and *Oscheius onirici*, were unable to kill a *G. mellonella* larvae by themselves, but were readily able to reproduce in larvae co-infected with EPN. The current data support these observations, i.e. FLN species belonging to *Oscheius* spp. were able to reproduce and could be maintained over several generations on *G. mellonella* larvae, as long as they were in the presence of EPN. More importantly from an agronomic perspective, our results suggest that in the DOK field, EPN may be prevented from reproducing to their full potential due to strong competition with FLN. Their main competitors (*Oscheius* spp.) were much more abundant than EPN in both the soil and the cadavers used to fulfill Koch's postulate.

## 5. Conclusions

The differences in fertilization methods and plant protection for the four farming systems evaluated in this study had no impact on the abundance or the activity of nematodes, nor did they affect the associated organisms. However, crop type and the crop history (crops in rotation) might have an important effect. EPN, as well as the associated organisms were found to be most

numerous in wheat plots (after potatoes), which is in agreement with the highest activity of nematodes recorded in the plots with wheat. We consider that under this ecological scenario, the biocontrol service offered by the native EPN might be very limited, as they occurred only in very low numbers, showed weak activity, and were in strong competition with other nematode guilds. It may therefore be advisable to use an augmentative approach in the case of a serious root pest outbreak, but further research is needed to confirm the effectiveness of such a strategy. It also remains to be determined whether the association of EPN species with *Oscheius* species and other FLN is detrimental or beneficial in a biological control approach, this is the subject of ongoing studies. EPN release as biocontrol appears to be most promising in intensive conventional crop rotation with monocultures, but there is also great potential in organic and integrated farming for specific crops such as potatoes, as they suffer from severe wireworm infestations.

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**References**

Adams, B.J., Nguyen, K.B., 2002. Taxonomy and systematics. In: Gaugler, R. (Ed.). Entomopathogenic Nematology CABI Publishing, Wallingford, UK, pp. 1-34.

Alatorre-Rosas, R., Kaya. H.K., 1990. Interspecific competition between entomopathogenic nematodes in the genera Heterorhabditis and Steinernema for an insect host in sand. J. Invertebr. Pathol. 55, 179–188.

Atkins, S.D., Clark, I.M., Pande, S., Hirsch, P.R., Kerry, B.R., 2005. The use of real-time PCR and species-specific primers for the identification and monitoring of *Paecilomyces lilacinus* FEMS Microbiol. Ecol. 51, 257–264.

Bale, J.S., van Lenteren, J.C., Bigler, F., 2008. Biological control and sustainable food production. Philos Trans. R. Soc. Lond. B Biol. Sci. 363, 761–776.

Bedding, R.A., Akhurst, R.J., 1975. A simple technique for the detection of insect parasitic rhabditid nemadodes in soil. Nematologica 21, 109–110.

Bell, M.C., Raczkowski, C.W., 2008. Soil property indices for assessing short-term changes in soil quality. Renew Agr. Food Syst. 23, 70–79.

Briar, S.S., Grewal, P.S., Somasekhar, N., Stinner, D., Miller, S.A., 2007. Soil nematode community, organic matter, microbial biomass and nitrogen dynamics in field plots transitioning from conventional to organic management. Appl. Soil Ecol. 37, 256–266.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Hugget, J., Kubista, M., et al., 2009. The MIQE guidelines: Minimun information for publication of quantitative real-time PCR experiments. *Clínica/ Chemistry 55,* 611-622.

Campos-Herrera, R. (ed.). 2015. Nematode Pathogenesis of Insects and Other Pests. Springer International Publishing Switzerland.

Campos-Herrera, R., Gómez-Ros, J.M., Escuer, M., Cuadra, L., Barrios, L., Gutiérrez, C., 2008. Diversity, occurrence, and life characteristics of natural entomopathogenic nematode populations from La Rioja (Northern Spain) under different agricultural management and their relationships with soil factors. Soil Biol. Biochem. 40, 1474–1484.

Campos-Herrera, R., Piedra-Buena, A., Escuer, M., Montalbán, B., Gutiérrez, C., 2010. Effect of seasonality and agricultural practices on occurrence of entomopathogenic nematodes and soil characteristics in La Rioja. Pedobiologia 53, 253–258.

Campos-Herrera, R., Johnson, E.G., Stuart, R.J., Graham, .J.H., Duncan, L.W., 2011a. Long-term stability of entomopathogenic nematode spatial patterns in soil as measured by sentinel insects and Real-Time PCR assays. Ann. Appl. Biol. 158, 55–68.

Campos-Herrera, R., El-Borai, F.E., Stuart, R.J., Graham, J.H., Duncan, L.W., 2011b. Entomopathogenic nematodes, phoretic *Paenibacillus* spp., and the use of real time quantitative PCR to explore soil food webs in Florida citrus groves. J. Invertebr. Pathol. 108, 30–39.

Campos-Herrera, R., El-Borai, F.E., Duncan, L.W., 2012. Wide interguild relationships among entomopathogenic and free-living nematodes in soil as measured by real time qPCR. J. Invertebr. Pathol. 111, 126–135.

Campos-Herrera, R., Ali, J.G., Diaz, B.M., Duncan, L.W., 2013a. Analyzing spatial patterns linked to the ecology of herbivores and their natural enemies in the soil. Front. Plant Sci. 4 (September), 1–18.

Campos-Herrera, R., Pathak, E., El-Borai, FE., Schumann, A., Abd–Elgawad, M.M.M., Duncan, L.W., 2013b. New citriculture systems suppresses native and augmented entomopathogenic nematodes Biol. Control 66, 183–194.

Campos-Herrera, R., El-Borai, F.E., Ebert, T.E., Schumann, A., Duncan, L.W., 2014. Management to control citrus greening alters the soil food web and severity of a pest-disease complex. Biol. Control 76, 41–51.

Campos-Herrera, R., Jaffuel, G., Chiriboga, X., Blanco-Pérez, R., Fesselet, M., Půža, V., Mascher, F., Turlings, T.C.J., 2015a. Traditional and molecular detection methods reveal intense interguild competition and other multitrophic interactions associated with native entomopathogenic nematodes in Swiss tillage soils. Plant Soil 389, 237-255.

Campos-Herrera, R., Puza, V., Jaffuel, G., Blanco-Pérez, R., Čepulytė Rakauskienė, R., Turlings, T.C.J., 2015b. Unraveling the intraguild competition between *Oscheius* spp. nematodes and entomopathogenic nematodes: implications for their natural distribution in Swiss agricultural soils. J. Invertebr. Pathol. 132, 216-227.

Demarta, L., Hibbard, B.E.O. Bohn, M., Hiltpold, I., 2014. The role of root architecture in foraging behavior of entomopathogenic nematodes. J. Invertebr. Pathol. 122, 32–39.

de Rooij-van der Goes, P.C.E.M., van der Putten, W.H., van Dijk, C., 1995. Analysis of nematodes and soil-borne fungi from *Ammophila arenaria* (Marram grass) in Dutch coastal foredunes by multivariate techniques. Eur. J. Plant Pathol. 101, 149–162.

Dillman, A.R., Chaston, J.M., Adams, B.J., Ciche, T.A., Goodrich-Blair, H., Stock, S.P., Sternberg, P.W., 2012. An entomopathogenic nematode by any other name. PLoS Pathogens 8, e1002527.

Dolinski, C., Choo, H.Y., Duncan, L.W., 2012. Grower acceptance of entomopathogenic mematodes: case studies on three continents. J. Nematol. 44, 226–235.

Duncan, L.W., Dunn, D.C., Bague, G., Nguyen, K., 2003a. Competition between entomopathogenic and free-living bactivorous nematodes in larvae of the weevil *Diaprepes abbreviatus.* J. Nematol. 35, 187–193.

Duncan, L.W., Stuart, R.J., El-Borai, F.E., Campos–Herrera, R., Pathak, E., Giurcanu, M., Graham, J.H., 2013. Modifying orchard planting sites conserves entomopathogenic nematodes, reduces weevil herbivory and increases citrus tree growth, survival and fruit yield. Biol. Control. 64, 26–36.

Efron, D., Nestel, D., Glazer, I., 2001. Spatial analysis of entomopathogenic nematodes and insect hosts in a citrus grove in a semi-arid region in Israel. Environ. Entomol. 30, 254–261.

El-Borai, F.E., Duncan, L.W., Preston, J.F., 2005. Bionomics of a phoretic association between *Paenibacillus* sp. and the entomopathogenic nematode *Steinernema diaprepesi.* J. Nematol. 37, 18–25.

Enright, M.R., Griffin, C.T., 2005. Effects of *Paenibacillus nematophilus* on the entomopathogenic nematode *Heterorhabditis megidis*. J. Invertebr. Pathol. 88, 40–48.

Ferris, H., Venette, R.C., Lau, S.S., 1996. Dynamics of nematode communities in tomatoes grown in conventional and organic farming systems, and their impact on soil fertility. Appl. Soil Ecol. 3, 161–175.

Fließbach, A., Oberholzer, H.-R., Gunst, L., Mäder, P., 2007. Soil organic matter and biological soil quality indicators after 21 years of organic and conventional farming. Agric. Ecosyst. Environ. 118, 273–284.

Georgis, R., Koppenhöfer, A.M., Lacey, L.A., Bélair, G., Duncan, L.W., Grewal, P.S., Samish, M., Tan, L., Torr, P., van Tol, R.W.H.M., 2006. Successes and failures in the use of parasitic nematodes for pest control. Biol. Control 38, 103–123.

Hartmann, M., Frey, B., Mayer, J., Mäder, P., Widmer, F., 2015. Distinct soil microbial diversity under long-term organic and conventional farming. ISME J. 9, 1177–1194.

Hominick, W.M., 2002. Biogeography. In: Gaugler, R. (Ed.), Entomopathogenic Nematology, CABI Publishing, Wallingford, UK, pp. 115-143.

Hummel, R.L., Walgenbach, J.F., Barbercheck, M.E., Kennedy, G.G., Hoyt, G.D., Arellano, C., 2002. Effects of production practices on soilborne entomopathogens in Western North California vegetable systems. Environ. Entomol. 31, 84–91.

Jabbour, R., Barbercheck, M.E., 2008. Soil and habitat complexity effects on movement of the entomopathogenic nematode *Steinernema carpocapsae* in maize. Biol. Control 47, 235–243.

Jabbour, R., Barbercheck, M.E., 2011. Soil microarthropod response to the application of entomopathogenic nematode-killed insects in maize and flower strip habitats. Pedobiologia 54, 243–251.

Jaffee, B.A., Ferris, H., Scow, K.M., 1998. Nematode-trapping fungi in organic and conventional cropping systems. Phytopathology 88, 344–350.

Jenkins, W.R., 1964. A rapid centrifual-flatation technique for separating nematodes from soil. Plant Dis. Rep. 48, 492.

Kaya, H.K., Aguillera, M.M., Alumai, A., Choo, H.Y., de la Torre, M., Fodor, A., Ganguly, S., Hazir, S., Lakatos, T., Pye, A., Wilson, M., Yamanaka, S., Yang, H., Ehlers, R.-U., 2006. Status of entomopathogenic nematodes and their symbiotic bacteria from selected countries or regions of the world. Biol. Control. 38, 134–155.

Koppenhöfer, A.M., Kaya, H.K., 1995. Density-dependent effects on *Steinernema glaseri* (Nematoda : Steinernematidae ) within an insect host. J. Parasitol. 81, 797–799.

Koppenhöfer, A.M., Kaya, H.K., 1996. Coexistence of two steinernematid nematode species (Rhabditida : Steinernematidae) in the presence of two host species. Appl. Soil Ecol. 4, 221–230.

Koppenhöfer, A.M., Kaya, H.K., Shanmugam, S., Wood, G.L., 1995. Interspecific competition between steinernematid nematodes within an insect host. J. Invertebr. Pathol. 66, 99–103.

Kondo, E., 1989. Studies on the infectivity and propagation of entomogenous nematodes *Steinernema* spp. (Rhabditida: Steinernematidae) in the common cutworm, *Spodoptera litura* (Lepidoptera: Noctuidae). Agrie. Bull. Saga Univ. 67, 1–87.

Kramer, I., Hirschy, O., Grunder, J.M., 2001. Survey of baited insect parasitic nematodes from the Swiss lowland. In: *Developments in Entomopathogenic Nematode/bacterial Research. Proceedings of the Workshop at National University of Ireland Maynooth. 13-15 April 2000.* EUR 19696-COST ACTION 819.

Lacey, L.A., (ed.). 1997. Manual of techniques in insect pathology. San Diego: Academic Press.

Lacey, L.A., Grzywacz, D., Shapiro-Ilan, D.I., Frutos, R., Brownbridge, M., Goettel, M.S., 2015. Insect pathogens as biological control agents: Back to the future. J. Invertebr. Pathol. 132, 1–41.

Lewis, E.E., Campbell, J., Griffin, C., Kaya, H.K., Peters, A., 2006. Behavioral ecology of entomopathogenic nematodes. Biol. Control 38, 66–79.

Lewis, E.E., Hazir, S., Hodson, A., Gulcu, B., 2015. Trophic relationships of entomopathogenic nematodes in agricultural habitats. In: Campos-Herrera, R. (Ed.). Nematode Pathogenesis of Insects and Other Pests, Springer International Publishing, Switzerland, pp. 139-163.

Mäder, P., Flieβbach, A., Dubois, D., Gunst, L., Fried, P., Niggli, U., 2002. Soil fertility and biodiversity in organic farming. Science 296, 1694–1697.

Mráček, Z., Becvár, S., Kindlmann, P., Jersakova, J., 2005. Habitat preference for entomopathogenic nematodes, their insect hosts and new faunistic records for the Czech Republic. Biol. Control 34, 27–37.

Mráček, Z., Becvár, S., Kindlmann, P., 1999. Survey of entomopathogenic nematodes from the families Steinernematidae and Heterorhabditidae (Nematoda: Rhabditida) in the Czech Republic. Folia Parasit. 46, 145–148.

Neher, D.A., 1999. Nematode communities in organically and conventionally managed agricultural soils. J. Nematol. 31, 142–154.

Neher, D.A., 2010. Ecology of plant and free-living nematodes in natural and agricultural soil. *Ann. Rev. Phytopathol.* 48, 371–394.

O’Callaghan, K.M., Zenner, A.N.R.L., Hartley, C.J., Griffin, C.T., 2014. Interference competition in entomopathogenic nematodes: male *Steinernema* kill members of their own and other species. Int. J. Parasitol. 44, 1009-1017.

Oerke, E.C., 2005. Crop losses to pests. J. Agric. Sci. 144, 31-43.

Pathak, E., El–Borai, F.E., Campos–Herrera, R., Johnson, E.G., Stuart, R.J., Graham, J.H., Duncan, L.W., 2012. Use of real-time PCR to discriminate parasitic and saprophagous behaviour by nematophagous fungi. Fun. Biol. 116, 563–573.

Patzel, N., Sticher, H., Karlen, D.L., 2000. Soil fertility - Phenomenon and concept. J. Plant Nutr. Soil Sci. 163, 129–142.

Perry, J.N., 1998. Spatial analysis by distance indices. J. Ani. Ecol. 64, 303-314.

Persmark, L., Banck, A., Jansson, H.-B., 1996. Population dynamics of nematophagous fungi and nematodes in an arable soil: vertical and seasonal fluctuations. Soil Biol. Biochem. 28, 1005–1014.

Pimentel, D., 1995. Amounts of pesticides reaching target pests: environmental impacts and ethics. J. Agric. Environ. Ethics 8, 17–29.

Půza, V., Mrácek Z., 2005. Seasonal dynamics of entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis* as a response to abiotic factors and abundance of insect hosts. J. Invertebr. Pathol. 89, 116–22.

Půza, V., Mrácek, Z., 2009. Mixed infection of *Galleria mellonella* with two entomopathogenic nematode (Nematoda: Rhabditida) species: *Steinernema affine* benefits from the presence of *Steinernema kraussei*. J. Invertebr. Pathol. 102, 40–43.

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

Esperschütz, J., Gattinger, A., Mäder3, P., Schloter, M., Fließbach, A. 2007. Response of soil microbial biomass and community structures to conventional and organic farming systems under identical crop rotations. FEMS Microbiol. Ecol. 61, 26–37

Steiner, W.A., 1996. Distribution of entomopathogenic nematodes in the Swiss Alps. Rev. Suis. Zool. 103, 439–452.

Stuart, R.J., Barbercheck, M.E., Grewal, P.S., Taylor, R.A.J., Hoy, C.W., 2006. Population biology of entomopathogenic nematodes: concepts, issues, and models. Biol. Control 38, 80–102.

Stuart, R.J., Barbercheck, M.E., Grewal, P.S., 2015. Entomopathogenic nematodes in the soil environement: distributions, interactions and the influence of biotic and abiotic factors. In: Campos-Herrera, R. (Ed.), Nematode Pathogenesis of Insects and Other Pests. Springer International Publishing, Switzerland, pp. 97-137.

Torr, P., Spiridonov, S.E., Heritage, S., Wilson, M.J., 2007. Habitat associations of two entomopathogenic nematodes: a quantitative study using real-time quantitative polymerase chain reactions. J. Ani. Ecol. 76, 238–245.

van der Werf, H.M.G., 1996. Assessing the impact of pesticides on the environment. Agric. Ecosyst. Environ. 60, 81–96.

White, G.F., 1927. A method for obtaining infective nematode larvae from cultures. Science 66, 302–303.

Zhang, L.M., Liu, X.Z., Zhu, S.F., Chen, S.Y., 2006. Detection of the nematophagous fungus *Hirsutella rhossiliensis* in soil by real-time PCR and parasitism bioassay. Biol. Control 36, 316-332.

## Supplementary material

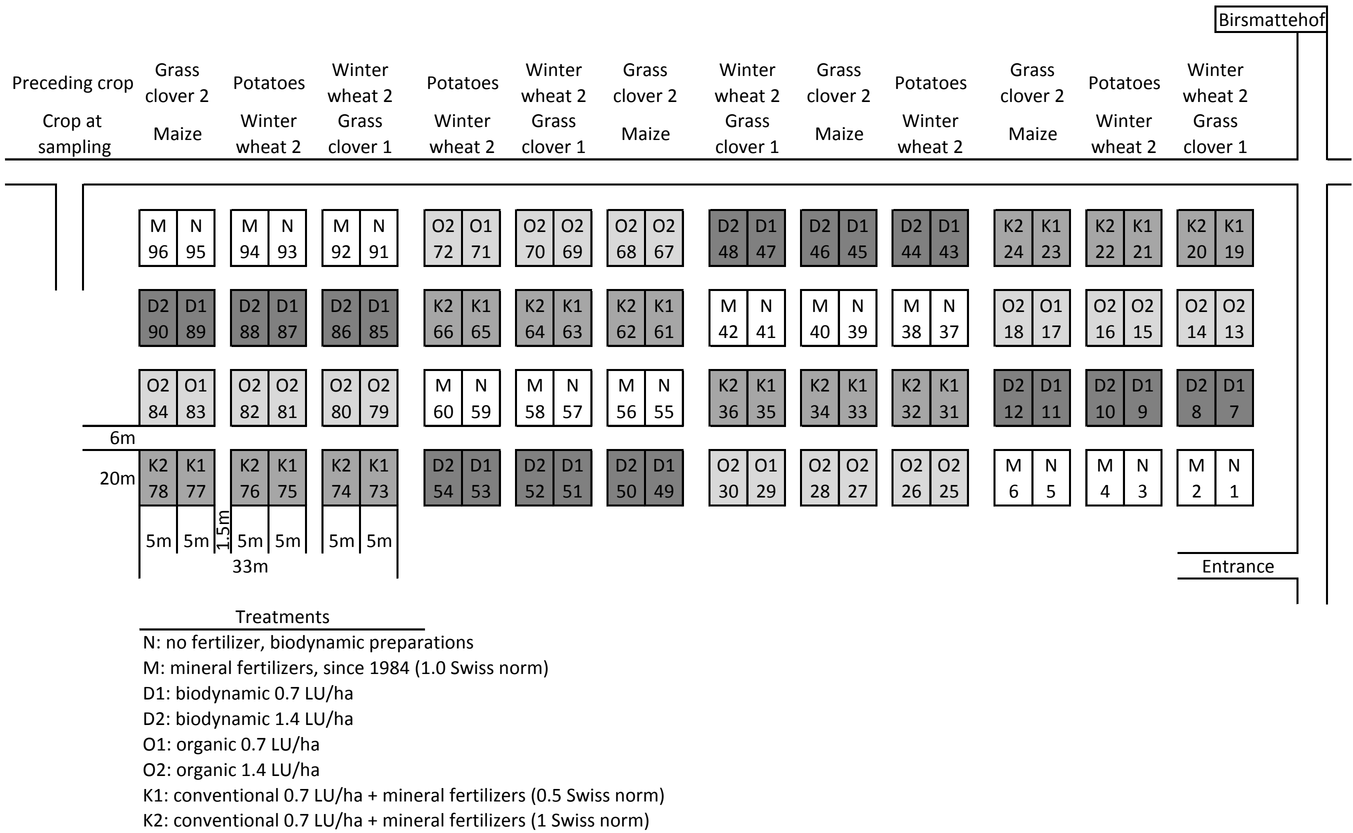



**Fig. S1.** Arrangement of the plots in the DOK field trial.

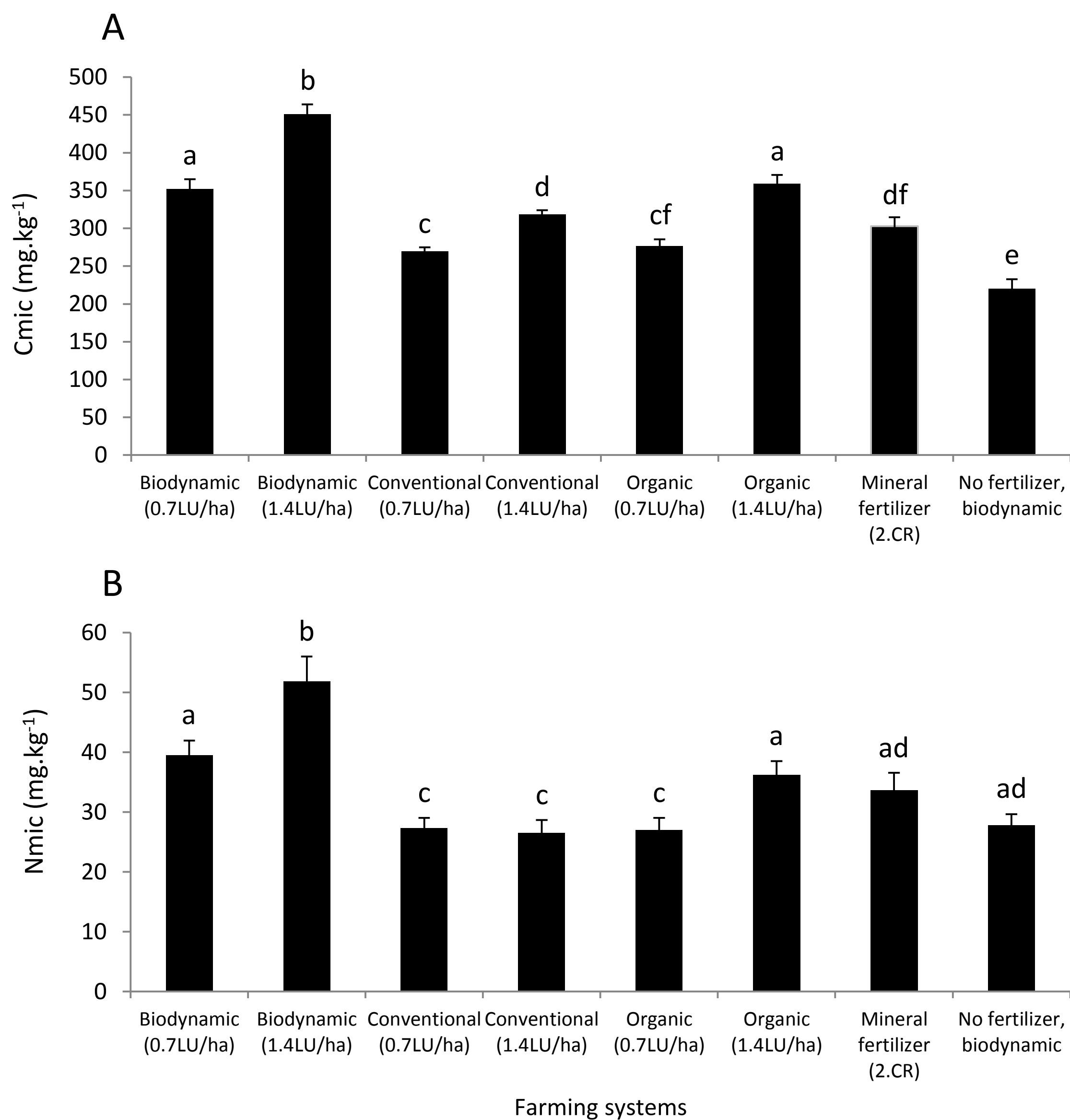
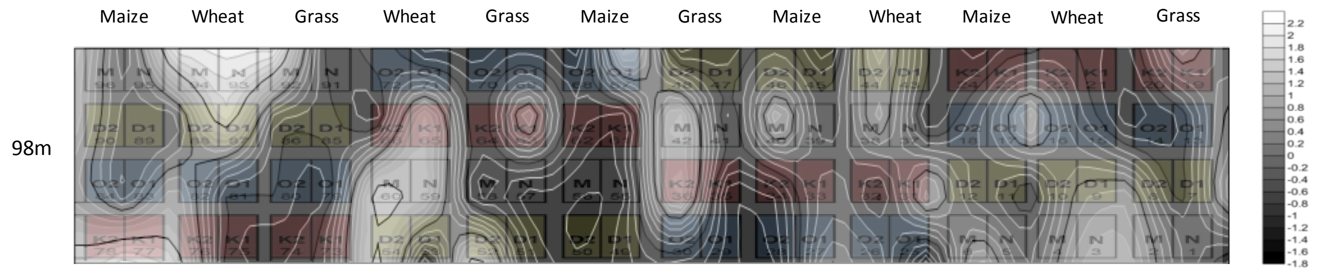



**Fig. S2.** Influence of management practices (biodynamic 0.4 LU/ha and 1.7LU/ha; organic 0.4 LU/ha and 1.7LU/ha; conventional 0.4 LU/ha and 1.7LU/ha; Mineral fertilizer (2. CR); No fertilizer, biodynamic) on $C_{mic}$ and $N_{mic}$ values recorded by chloroform-fumigation-extraction. (a) Mean $C_{mic}$ in $mg.kg^{-1}$ and (b) mean $N_{mic}$ in $mg.kg^{-1}$. Data are shown as means ± SEM. Lowercase letters indicate statistical differences.

NF




EPN

FLN

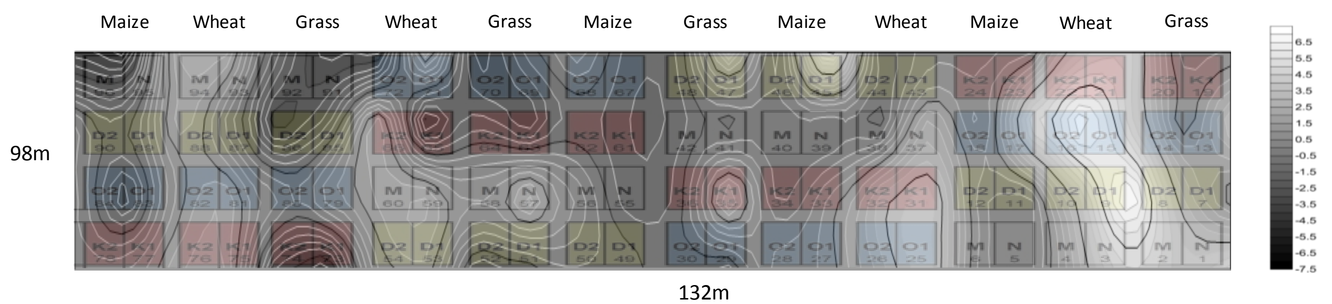



**Fig. S3.** Contour maps revealing the distribution of EPN, NF and FLN in the DOK field. Z values that were used to draw the maps were obtained with SADIEshell (v2.0).

**Table S2** Management applied at the DOK site in 2013

| Silage maize (pre-crop grass-clover 2) | | |
|---|---|---|
| Date | Farming system | Farm operation |
| 08.03.13 | D,N | spraying biodynamic preparations |
| 22.04.13 | D,N | spraying biodynamic preparations |
| 08.05.13 | D O K M | grass-clover cut |
| 13.05.13 | K | manure application |
| 14.05.13 | D O K M | Ploughing & rolling |
| 16.05.13 | D O | manure/ compost application |
| 27.05.13 | M | Fertiliser application |
| 27.05.13 | M | Fertiliser application |
| 27.05.13 | M | Fertiliser application |
| 27.05.13 | M | Fertiliser application |
| 27.05.13 | D O K M | harrowing |
| 28.05.13 | D O K M | maize seeding |
| 30.05.13 | D,N | spraying biodynamic preparations |
| 14.06.13 | D O | weeding |
| 17.06.13 | D O K | 1st slurry application |
| 20.06.13 | D O | weeding |
| 26.06.13 | K M | herbicide application |
| 26.06.13 | D O K M | taking Nmin-samples in D2, O2, K2, M |
| 02.07.13 | K M | banded N-application: 180kg N- NminN/ha |
| 02.07.13 | D O | 2nd slurry application |
| 02.07.13 | D,N | spraying biodynamic preparations |
| 09.07.13 | K M | banded N-application |
| 09.07.13 | D O | weeding |
| 09.07.13 | D O K M | control of maizeborer |
| 17.07.13 | D O | weeding |
| 24.09.13 | D O K M | crop plant density determination |
| 25.09.13 | D O K M | harvest of silage maize + yield determination |
| 08.10.13 | D O K M | stubble mulching |
| 09.10.13 | D O K M | green manure seeding |
| 14.11.13 | D O K M | soil sampling |
| 19.11.13 | D,N | spraying biodynamic preparations |

| Winter wheat 2 cultivar Runal (pre-crop potato) | | |
|---|---|---|
| Date | Farming system | Farm operation |
| 20.02.13 | D O K M | Nmin-sampling |
| 08.03.13 | D,N | spraying biodynamic preparations |
| 20.03.13 | D O | 1st slurry application |
| 21.03.13 | M | 1st N-application |
| 21.03.13 | K | 1st N-application |
| 18.04.13 | K M | herbicide application, growth regulator |
| 18.04.13 | K M | 2nd N-application |
| 22.04.13 | D,N | spraying biodynamic preparations |
| 24.04.13 | D O | 2nd slurry application |
| 26.04.13 | D O | weeding |
| 14.05.13 | K M | 1st fungicide application |
| 27.05.13 | K M | 3rd N-application |
| 05.06.13 | D,N | spraying biodynamic preparations |
| 07.06.13 | K M | 2nd fungicide application |
| 02.07.13 | D O K M | bird protection net |
| 23.07.13 | D O K M | removing bird protection net |
| 23.07.13 | D O K M | straw sampling |
| 25.07.13 | D O K M | harvest and yield determination |
| 26.07.13 | D O K M | soil sampling |
| 02.08.13 | D O K M | straw removal |
| 07.08.13 | D O K M | stubble mulching |
| 09.08.13 | D O K | manure/ compost application |
| 12.08.13 | D O K M | tilling |
| 14.08.13 | D O K M | harrowing |
| 15.08.13 | M | N-application |
| 15.08.13 | D O K M | grass-clover seeding, rolling |
| 30.10.13 | D O K M | cleaning cut |
| 19.11.13 | D,N | spraying biodynamic preparations |

| Grass-clover SM330 (pre-crop winter wheat) | | |
|---|---|---|
| Date | Farming system | Farm operation |
| 08.03.13 | D,N | spraying biodynamic preparations |
| 20.03.13 | K M | fertilizer application |
| 20.03.13 | M | fertilizer application |
| 20.03.13 | M | 1. N-application |
| 21.03.13 | D O K | 1. slurry application |
| 22.04.13 | D,N | spraying biodynamic preparations |
| 07.05.13 | D O K M | determination of grass-clover composition (1st cut) |
| 08.05.13 | D O K M | 1. cut |
| 10.05.13 | D O K | 2. slurry application |
| 14.05.13 | M | 2. N-application |
| 30.05.13 | D,N | spraying biodynamic preparations |
| 05.06.13 | D,N | spraying biodynamic preparations |
| 19.06.13 | D O K M | 2. cut |
| 21.06.13 | D K | 3. slurry application |
| 21.06.13 | M | 3. N-application |
| 02.07.13 | D,N | spraying biodynamic preparations |
| 02.07.13 | O | 3. slurry application |
| 24.07.13 | D O K M | 3. cut |
| 08.08.13 | D O K | 4. slurry application |
| 08.08.13 | M | 4. N-application |
| 27.08.13 | D O K M | determination of grass-clover composition (4th cut) |
| 28.08.13 | D O K M | 4. cut |
| 16.10.13 | D O K M | 5. cut |
| 14.11.13 | D O K M | soil sampling |
| 19.11.13 | D,N | spraying biodynamic preparations |

Farming system code: D = Biodynamic, O = Organic, K = Conventionnal, M = Mineral fertilizer, N = No fertilizer, biodynamic

**Table S3:** Species and sources of nematodes, fungi and bacteria used in this study

| Type of organism/ species | Population | Material used/Unit of measurements | GenBank accession number |
|---|---|---|---|
| Nematodes: Entomopathogenic nematodes | | | |
| *Heterorhabditis bacteriophora* | Commercial | Infective juvenile (IJs) / no. IJs | KJ93576 |
| *Heterorhabditis megidis* | Commercial | Infective juvenile (IJs) / no. IJs | KJ938577 |
| *Heterorhabditis zealandica* | Btw | Infective juvenile (IJs) / no. IJs | GU174009 |
| *Steinernema affine* | CH | Infective juvenile (IJs) / no. IJs | KJ938567 |
| *Steinernema bicornotum* | D60 Pl | Infective juvenile (IJs) / no. IJs | KJ938568 |
| *Steinernema carpocapsae* | DOK-83 | Infective juvenile (IJs) / no. IJs | KJ818295 |
| *Steinernema feltiae* | RS-5 | Infective juvenile (IJs) / no. IJs | KJ938569 |
| *Steinernema glaseri* | NC | Infective juvenile (IJs) / no. IJs | GU173998 |
| *Steinernema intermedium* | 82 from USA | ITS rDNA sequence + pUC57/ pg DNA | AF171290 |
| *Steinernema intermedium*-group | VAD-1067 | Infective juvenile (IJs) / no. IJs | KJ696684 |
| *Steinernema kraussei* | OS | Infective juvenile (IJs) / no. IJs | KJ696686 |
| *Steinernema poinari* | 1160 | ITS rDNA sequence + pUC57 / pg DNA | KF241754 |
| *Steinernema weiseri* | 1117 | Infective juvenile (IJs) / no. IJs | KJ696686 |
| Nematodes: Free-living and competitors nematodes | | | |
| *Acrobeloides*-group | RT1-R15C | 18S rDNA sequence + pUC57 / pg of DNA | JQ237849 |
| *Oscheius tipulae* | MG68 P29 | Nematodes/ ng DNA | KJ938579 |
| *Oscheius onirici* | MG67 P20 | Nematodes/ ng DNA | KJ938578 |
| *Oscheius* sp. 3 | JU75 | 18S rDNA sequence + pUC57 / pg of DNA | AJ297890 |
| Nematophagous fungi | | | |
| *Catenaria* sp. | 1D | ITS rDNA sequence + pUC57 / pg of DNA | JN585805 |
| *Arthrobotrys dactyloides* | H55 | Pure culture / pg of DNA | KJ938574 |
| *Arthrobotrys musiformis* | 11 | Pure culture / pg of DNA | KJ938572 |
| *Arthrobotrys oligospora* | 8 | Pure culture / pg of DNA | KJ938573 |
| *Hirsutella rhossiliensis* | 2931 | Pure culture / pg of DNA | - |
| *Purpureocillium lilacinus* | 9357 | Pure culture / pg of DNA | KJ938575B |
| Ectoparasitic bacteria | | | |
| *Paenibacillus nematophilus* | NEM2 | 16S rDNA sequence of 490 bp + pUC57 / copy numbers | AF480936 |

**Table S4**: Results of SADIE analyses for the index of aggregation

| | Index of aggregation (I) | Probability of aggregation (Pa) |
|---|---|---|
| EPN | 0.854 | 0.6652 |
| FLN | 2.69 | 0.0003 |
| NF | 0 | 0.9998 |
| $C_{mic}$ | 1.767 | 0.0714 |
| $N_{mic}$ | 2.449 | 0.0478 |
| FLN+EPN activity | 1.777 | 0.03 |

**Table S5**: Results of SADIE analyses for the index of association

| Group 1 | Group 2 | Index of association (X) | Dutilleul adjusted probability (P) |
|---|---|---|---|
| EPN | NF | 0.0953 | 0.1855 |
| EPN | FLN | 0.475 | 0.322 |
| EPN | $C_{mic}$ | 0.1484 | 0.0729 |
| NF | FLN | -0.023 | 0.586 |
| EPN | $N_{mic}$ | -0.1112 | 0.8522 |

# Chapter II

# Highly potent extracts from pea (*Pisum sativum*) and maize (*Zea mays*) roots can be used to induce quiescence in entomopathogenic nematodes

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**Abstract**

Root exudates can play an important role in plant-nematode interactions. Recent studies have shown that the root cap exudates obtained from several plant species trigger a state of dormancy or quiescence in various genera of nematodes. This phenomenon is not only of fundamental ecological interest, but also has application potential if the plant-produced compound(s) could be used to control harmful nematodes or help to prolong the shelf-life of beneficial entomopathogenic nematodes (EPNs). The identification of the compound(s) involved in quiescence induction has proven to be a major challenge and requires large amounts of active material. Here, we present a high-throughput method to obtain bioactive root extracts from flash-frozen root caps of green pea and maize. The root cap extract obtained via this method was considerably more potent in inducing quiescence than exudate obtained by a previously used method, and consistently induced quiescence in the EPN *Heterorhabditis megidis*, even after a 30-fold dilution. Extracts obtained from the rest of the root were equally effective in inducing quiescence. Infective juveniles (IJs) of *H. megidis* exposed to these extracts readily recovered from their quiescent state as soon as they were placed in moist soil, and they were at least as infectious as the IJs that had been stored in water. Excessive exposure of IJs to air interfered with the triggering of quiescence. The implications of these results and the next steps towards identification of the quiescence-inducing compound(s) are discussed from the perspective of applying EPN against soil-dwelling insect pests.

## 1. Introduction

Plants produce a wide range of chemical organic compounds. When released into the environment, these metabolites mediate interactions with surrounding organisms (Barber et al., 1976), and can play major roles in defense, communication, attraction, and repellency (Vining, 1990). Aboveground interactions that are mediated by plant compounds generally are well documented, but it is only recently that the role of root-produced compounds in belowground interactions is receiving equal attention (Hartmann, 2007; Hiltpold et al., 2011; Rasmann et al., 2012; Turlings et al., 2012; van Dam, 2009). This seems pertinent because at least 20 % of the photosynthetically assimilated carbon is released by the roots (Barber et al., 1976; Kumar et al., 2006). Several effects of root-released chemicals on nematodes, bacteria, and fungi have been described (Bais et al., 2006), and evidence is accumulating that they also serve an important function in belowground tritrophic interactions among plants, herbivores, and entomopathogenic nematodes (Ali et al., 2010, 2012; Hiltpold and Turlings, 2012; Rasmann et al., 2005).

Initial research on soil-dwelling nematodes and root exudates focused on plant-parasitic nematodes, due to their importance as pests of crops. Plant parasitic nematodes use constitutively released root exudates to locate their host plant (Curtis et al., 2009; Prot 1980; Reynolds et al., 2011; Rolfe et al., 2000). Root exudates also are known to trigger egg hatching in several plant-parasitic nematodes (Den Nijs and Lock, 1992; Gaur et al., 2000; Khokon et al., 2009). Zhao et al. (2000) were the first to observe the intriguing phenomenon that root cap exudates induce a state of dormancy (quiescence) in a plant-parasitic nematode (*Meloidogyne incognita*), and they proposed it to be a defense mechanism against root tip penetration. Following up, Hubbard et al. (2005) found that the root cap exudates of a wide variety of plants can trigger quiescence in several species of plant parasitic nematodes, animal parasitic nematodes, and free living nematodes. At the time, only one species of entomopathogenic nematode (EPN), *Steinernema glaseri*, was tested and found to be susceptible to the exudates.

It also is known that roots under attack by herbivores release compounds that attract EPN as a secondary defense mechanism. For instance, when maize roots are damaged by the western corn rootworm, *Diabrotica virgifera virgifera*, they release (E)-β-caryophyllene, a volatile sesquiterpene that attracts the EPN *Heterorhabditis megidis* (Rasmann et al., 2005). This apparent defense mechanism has been confirmed for other plants and EPN systems (Ali et al., 2010, 2012; van Tol et al., 2001). More recently, several species of EPN also have been shown to be susceptible to root cap exudates. The exudates were found to induce quiescence in all tested EPN species, and their activity could be restored by diluting the exudate with water (Hiltpold et al., 2014).

Control of soil-dwelling insect pests mainly relies on pesticides causing environmental concerns (Köhler and Triebskorn, 2013). Hence, there is a clear need for sustainable alternatives that are based on ecologically sound crop management solutions. Biological control with EPNs

could offer a sustainable alternative to chemical pesticides, and thus have been under intensive research in this context (Lewis et al., 2006). The nematode of the *Steinernema* and *Heterorhabditis* genera are particularly promising, as these obligate parasites of insects rapidly kill their host after initial infection (Lewis et al., 2006). The infective juveniles (IJs), the free-living stage of EPNs, have evolved various strategies to locate and enter an insect host. Once inside, they release their symbiotic bacteria in the homeocel, which produce toxins and cause a lethal septicemia within 2 to 3 days (Adams and Nguyen, 2002; Dillman et al., 2012). The EPNs feed on the bacteria and reproduce into the cadaver. When the resource is depleted, a new generation of IJs is produced, and they leave the carcass and find new hosts (Dillman et al., 2012; Kaya and Gaugler, 1993).

Despite their efficiency in killing insect hosts, the use of EPNs as biocontrol control agents for soil insect pests remains challenging. One of the primary constraints is their short shelf life. Infective juveniles survive about a month in refrigerated vermiculite formulations, which is the prevailing storage method of commercially available EPN (Shapiro-Ilan et al., 2006). Prolonged storage diminishes EPN quality and their ability to reach a good level of control of the target pest (Grewal, 2002). Thus, storage limitation has been a critical aspect in EPN formulations. In addition, EPNs usually are sprayed on top of the soil, exposing them to UV light and desiccation (Lello et al., 1996). These constraints make the use of EPNs costly and only marginally effective in large scale application (Georgis et al., 2006). We propose that triggering a state of quiescence, to prolong shelf life, in combination with novel application methods may render EPNs much more effective as biological control agents.

The state of quiescence is characterized by a straight shape and non-motile state of nematodes. Quiescence normally is triggered by unfavorable environmental conditions, such as extreme temperature, lack of oxygen, a lack of moisture, and/or an osmotic stress (Barrett 1991). During this state of dormancy, the metabolism of nematodes is strongly reduced, allowing them to conserve energy, which can significantly prolong their lifespan and infectiousness (Hiltpold et al., 2014). Quiescence is reversible when the conditions turn more favorable. Quiescence also can be chemically triggered, for example with glycerol (Chen and Glazer, 2005) or with compound(s) in exudates of root caps (Hiltpold et al., 2014; Hubbard et al., 2005; Zhao et al., 2000). The advantages of glycerol and the so-called quiescence factors (QFs) are that they provide full control of the nematodes quiescence by dose regulation, as well as of the recovery by simple dilution. Hiltpold et al. (2014) showed that exposure to pea root cap exudates conserves EPN motility, infectiousness, and lipid content, which implies that including QFs in EPN formulations has potential to enhance their efficacy. Therefore it is worthwhile to identify the QF. However, collecting exudate is a tedious and highly time-consuming process. Moreover, the QF concentration contained in the exudate is low and its activity is quickly lost after a few dilutions (Hiltpold et al., 2014). It is for this reason that we set out to develop a high-throughput collection method that allowed us to obtain root extracts from root cap of green pea and maize, which were flash-frozen in liquid nitrogen.

To demonstrate that the root cap extract contains high concentrations of the QF, we incubated the EPN *Heterorhabditis megidis* in different dilutions of root cap extract and measured quiescence levels. To further improve the collection of QF, we also tested the possibility of using an extract of the entire root germinate instead of only the root cap. We compared quiescence of *H. megidis* IJs induced by the two different extracts. Furthermore, we determined whether quiescent IJs can recover from their inactive state and still efficiently penetrate and kill a target host. Two hosts were tested, larvae of the highly susceptible wax moth, *Galleria mellonella*, and larvae of the more resistant mealworm, *Tenebrio molitor* (Grewal and Peters 2005). In these tests, we compared the infectiousness of *H. megidis* IJs that had recovered from quiescence after storage in different root cap extract concentrations for 24 hr with the infectiousness of IJs that had been stored in water. Finally, because we hypothesized that quiescence is a response to oxygen deprivation, we also evaluated the effect of aeration of the storage solutions on the induction of quiescence in *H. megidis*.

## 2. Methods and Materials

### *2.1. Plants, nematodes and insects resources*

Pea (*Pisum sativum* L.) seeds (variety Lancet, Wyss Samen und Planzen AG, Switzerland) were first sterilized in 95 % ethanol for 5 min, then rinsed and immerged in distilled water for 12 hr. Soaked seeds were placed in plastic boxes (15×13.5×5 $cm^3$) containing phytoagar 1.0 % (Duchefa Biochemie, Haarlem, The Netherlands) and incubated at 25 °C in the dark for 3 days.

Maize (*Zea mayz* L.) seeds (cultivar Delprim, DSP SA, Switzerland) were sterilized in distilled water with 10 % bleach for 12 hr. Soaked seeds were placed in plastic boxes (30×30×10 $cm^3$) kept moist on paper towels, and incubated at 25 °C in the dark for 3 days.

*Heterorhabditis megidis* was obtained from Andermatt Biocontrol SA, Switzerland. The EPNs were reared in the laboratory by infecting *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvae (Au pêcheur, Neuchâtel, Switzerland), and new generations of IJs were recovered in White traps (White 1927) and stored at 10 °C before use. All experiments were performed with new, fresh IJs that were not more than 2-week-old.

One to 2-week-old larvae of the wax moth *Galleria mellonella* were used to rear the EPNs and for the infectiousness bioassays. These insects were obtained from Au Pêcheur SARL Neuchâtel (Switzerland). For the infectiousness test, we also used mealworm larvae (*Tenebrio molitor*), which were obtained from the University of Lausanne (Switzerland).

### *2.2. Extraction of root caps*

We collected 19.8 g (for maize) and 18.6 g (for pea) of the terminal 10 mm of the roots. This material was ground under liquid nitrogen in a pre-cooled mortar. The obtained extract was split

equally into two 50 ml Falcon tubes with 45 ml of MilliQ water, and subjected to ultrasonication for 1.5 hr. The root cap extract then was centrifuged at 4500 rpm for 10 min (Universal 16, Hettich Zentrifugen). The supernatants were pooled per plant species and filtered over a filter paper (Whatman 90 mm Ø, 4–7 µm particle retention) in vacuo and lyophilized, which resulted in 1.49 g (maize) and 1.52 g (pea) of dry root cap extract. The dry extracts were stored at −80 °C until further use. For maize, we repeated the entire procedure with the remaining parts of the root (root extract), thus excluding the ten first millimeter of the root cap.

2.3. *Collection of root cap exudate*

Following Hubbard et al. (2005) and Hiltpold et al. (2014), the root cap exudate was collected from 15 pea germinates by placing the terminal 10 mm of the root caps in a 1 ml drop of MilliQ water on a Teflon plate for 2 min. Then, the water drop was collected and centrifuged at 14,000 rpm for 10 min at 18 °C (eppendorf AG, centrifuge 5424), and the supernatant was stored at −20 °C (root cap exudate).

*2.4. Quiescence induction tests with H. megidis*

The sensitivity of *H. megidis* to the QF in the root cap extract and the root cap exudate at different concentrations was assessed following a previously described method (Hubbard et al., 2005; Hiltpold et al., 2014). A suspension containing 30 IJs per 50 µl of distilled water was prepared. Additionally, pea root cap extract was adjusted to seven different dilutions in MilliQ water: 10 mg (not diluted), 2 mg (×5), 1 mg (×10), 0.66 mg (×15), 0.5 mg (×20), 0.4 mg (×25), 0.33 mg (×30). Root cap exudate already in solution was adjusted to obtain the same seven dilutions (not diluted, ×5, ×10, ×15, ×20, ×25, and ×30). The control contained only MilliQ water. Suspensions (50 µl) with *H. megidis* IJs were poured into each well of a 96-well tissue plate (Sigma-Aldrich). Then, 175 µl of the different solutions of root cap extract or exudate were added, resulting in a total volume of 225 µl per well. After 12 hr, we counted the number of quiescent IJs in the wells (N=10/dilutions) under a microscope. Immobile and straight-shaped IJs were considered to be quiescent. The experiment was repeated three times with different batches of IJs. The same bioassay was used to assess the induction of quiescence by maize germinate root caps (0– 10 mm, N=12) compared to the rest of the maize germinate root (>10 mm, N=12). For the maize extract, we tested a reduced number of dilutions (not diluted, ×5, ×10, ×20, and ×30). The experiment was replicated twice.

The percentages of quiescent EPN were compared using Wilcoxon Signed-Rank test performed in R version 2.15.2 ( http://www.r-project.org/). Bonferroni correction was applied on the P-values to overcome multiple comparisons.

*2.5. Recovery and infectiousness of H. megidis*

The ability of *H. megidis* to infect and kill a host depending on storage (for 24 hr) in different concentrations of root cap extract was compared to *H. megidis* stored in water only. We tested the infectiousness using highly susceptible *G. mellonella* larvae, as well as more resistant *T. molitor* larvae. Suspensions (1.5 ml) of water with *H. megidis* at a concentration of 50 IJs in 10 µl were centrifuged in a 1.5 ml Eppendorf tube at 8000 rpm for 5 min (Eppendorf AG, centrifuge 5424), and the supernatant was replaced by different concentrations of root extracts (pea and maize). The final solutions were transferred into 4 ml glass tubes (BGB analytik, AG) for 24 hr to trigger quiescence before inoculation. The different concentrations of extract were: 10 mg (not diluted), 1 mg (×10), 0.5 mg (×20), 0.33 mg (×30), and water as control (N=15/concentration). Following Hiltpold et al. (2012), 50 ml Falcon tubes were filled with 60 % moist potting soil. One *G. mellonella* or one *T. molitor* larva was placed in an individual plastic specimen tube (1.5 ml Eppendorf tube pierced with 12 holes). The tubes containing the larvae then were each placed inside a 50 ml Falcon tube, 3 cm below the soil surface. They were placed at the edge of the tube so that the larvae could be observed. To each Falcon tube we added 50 µl of one of the dilutions, or water as control, containing IJs (ca. 250). Each solution was poured onto the soil, and a 1 cm soil layer was added to cover the drop. The tubes were not fully closed to allow gas exchange, and they were stored in the dark at room temperature. Every treatment was replicated 15 times. Every day, the success of *H. megidis* in infecting the larvae was evaluated for each tube by visual inspection, looking for red larvae, which is indicative of infection (Forst and Clarke 2002). Immobile larvae also were checked for mortality. Overall larval survival was evaluated 7 days after the start of an experiment. Parametric survival analyses were performed to evaluate significant differences in larval survival using the survival package of R program version 2.15.2 (http:// www.r-project.org/). The Weibull distribution of error produced the minimum error deviance and was therefore selected for further analyses.

*2.6. Quiescence factor aeration*

To verify if an excess availability of oxygen interferes with the triggering of quiescence, a set of three experiments was performed. The general setup involved ca. 1000 IJ's in 50 ml of water that were poured into a 4 ml glass tube (BGB analytik, AG). After application of the treatments described below, tubes were covered with one layer of parafilm to avoid evaporation, but allowing gas exchange. The tubes were stored at room temperature, and after 24 hr of exposure, quiescence was assessed.

In the first experiment, two different volumes of a solution of 1 mg of maize root cap extract were added to the tubes, a large volume of 1 ml (N=20) or a smaller volume of 200 µl (N=20).

In a second experiment, 1 ml of a solution of maize root cap extract at a concentration of 1

mg was added, half of the tubes were placed on an agitator to oxygenate the solution at 400 tr/ min (Edmund Bühler Compact Mixer Shaker KL-2) for 24 hr (N=20) and the other tubes (N=20) were just placed on a shelf next to the agitator. All tubes were kept at room temperature.

In the third experiment, maize root cap extract was prepared in a 250 ml Falcon tube and oxygenized for 3 hr with an aquarium air pump, before IJs were placed in the solution.

In parallel, a similar solution was made without pump aeration. One ml of the oxygenized solution (N=20) and non-oxygenized solution (N=20) were added to the tubes with the IJs. Twenty vials containing only 1 ml of water served as controls. The purpose of this latter experiment was to specifically test if the QF would become inactive after exposure to excess oxygen.

Every experiment was replicated twice. Binomial GLM performed in R version 2.15.2 ( http://www.r-project.org/) was used to assess difference between treatments.

## 3. Results

### *3.1. Extract versus exudate*

Root cap exudate was efficient only in inducing quiescence in *H. megidis* when not diluted, resulting in 90.34 % of quiescent EPNs, whereas there was no quiescence in the water controls (Bonferroni corrected P-value<0.001, W=900, P<0.001). All other root cap exudate dilutions were not significantly different from the water control (α<0.001; ×5: W=309.5, P=0.03; ×10: W=240.5, P=0.001; ×15: W=337.5, P=0.09; ×20: W=374, P=0.2; ×25: W=278.5, P=0.01; ×30: W=408.5, P=0.54). In contrast, root cap extract induced 100 % quiescence even at a 15× dilution, and quiescence induction was not significantly different from the undiluted root cap extract up to the 25× dilution (α<0.001; ×1, ×5, ×10 and ×15 induced 100 % quiescence, ×20: W=525, P=0.02; ×25: W=585, P=0.001; ×30: W=780, P<0.001). The undiluted root cap exudate was less effective in inducing quiescence than the undiluted root cap extract (α <0.001; W = 765, P < 0.001), this difference persisted until the×20 dilution of the extract (α<0.001; 25×: W=609, P=0.012; Fig. 1).

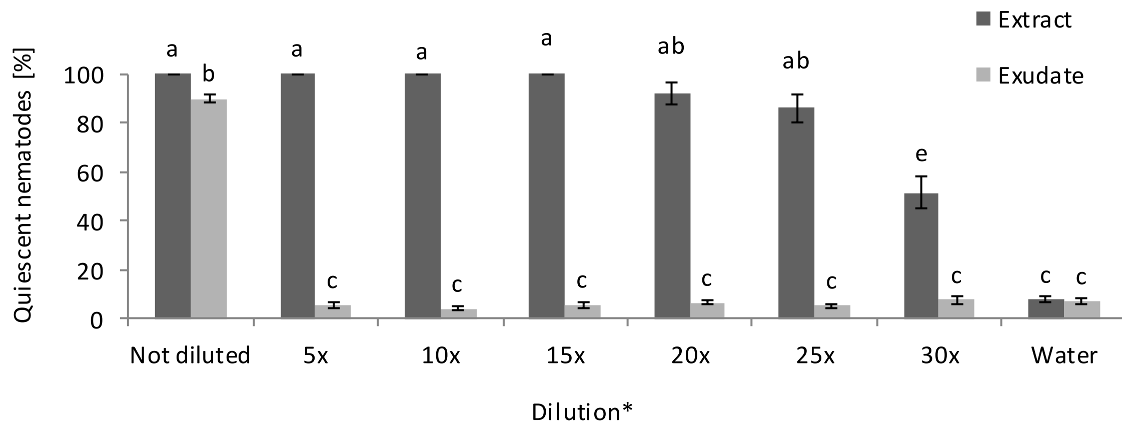


Fig. 1 Differences between pea root cap extract and root cap exudate in inducing quiescence in *Heterorhabditis megidis*. Overall, extract obtained by grinding the roots was more efficient in inducing quiescence than exudate. Only undiluted exudate induced more quiescence than water, whereas all of the tested extract dilutions induced more quiescence than water. Different small letters indicate statistical differences. Bars indicate standard error. *The values indicate dilutions of the root cap extract and root cap exudate. They do not include the additional 50 µl of infective juveniles (IJs) suspension that was added

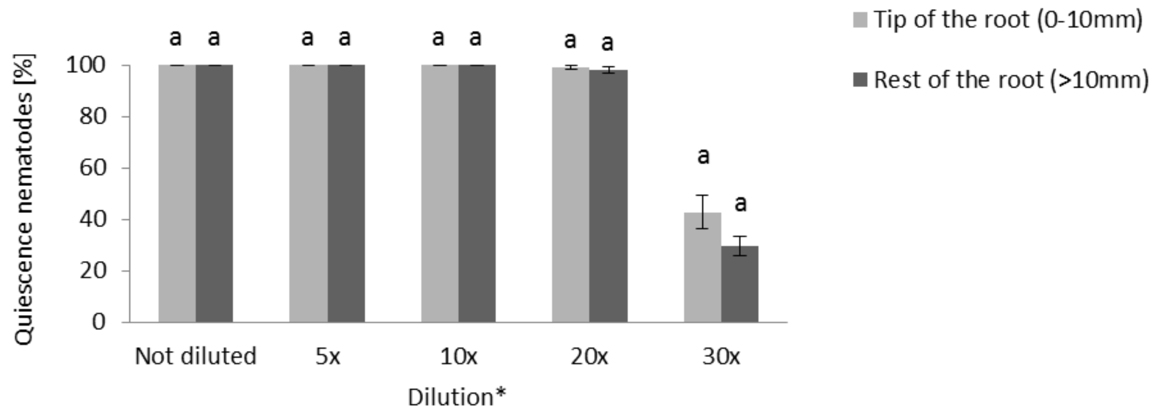


Fig. 2 Comparison of quiescence induction between extract from the first 10 mm of maize roots with extract from the rest of the root. The root extracts induced 100 % quiescence until the 1 mg dilution. Small letters indicate statistical differences. Bars indicate standard error. *The values indicate dilutions of the root cap extract and the rest of the root extract. They do not include the additional 50 µl of infective juveniles (IJs) suspension that was added.

*3.2. Maize root cap extract versus the rest of root extract*

Extract obtained from maize roots also were efficient in inducing quiescence in *H. megidis*. Root cap extract and the rest of the root extract were equally efficient in inducing quiescence. The extracts induced 100 % quiescence until a dilution of 10× for both the root cap and the rest of maize roots. There also was no difference in QF activity between the extracts of the root cap and the extract of the rest of the root for the ×20 and ×30 dilutions (Bonferroni corrected P-value=0.002; not diluted and ×10 induced 100 % of quiescence; ×20: W=274, P=0.68; ×30: W=387, P=0.04; Fig. 2).

*3.3. Recovery and infectiousness of* H. megidis

Pea extract: *H. megidis* exposed to the different pea germinate root cap extract dilutions and water differed in their ability to kill *G. mellonella* larvae after recovery from quiescence (Weibull model: Chisq=13.21, P=0.001; Fig. 3). Overall, 81.3 % of *H. megidis*-exposed larvae were dead at the end of the experiment, and 96.7 % of the dead larvae displayed a red color. Except for the undiluted extract, IJs from all pea germinate extract dilutions were able to kill the host just as well as IJs that were kept in the control condition (water) (not diluted: =2.993, P=0.002; ×10: Z=0.114, P=0.9; ×20: Z=1.469, P=0.1; ×30: Z=−0.453, P=0.6, respectively; Fig. 3). Maize extract: *H. megidis* exposed for 24 hr to the different maize germinate root cap extract dilutions or to just water did not significantly differ in their ability to kill *G. mellonella* larvae after recovery from quiescence (Weibull model: Chisq=6.09, P=0.19). Overall, 92 % of the larvae were dead at the end of the experiment, and 89.70 % of the dead larvae displayed a red color. In all maize germinate extract dilutions, the IJs were able to kill *G. mellonella* larvae just as well as the ones that were kept in the control condition (water) (not diluted: Z=1.212, P=0.2; ×10: Z=−1.31, P=0.1; ×20: Z=−0.068, P=0.9; ×30: Z=−0.564, P=0.5; Fig. 4). Nevertheless, as for the IJs exposed to pea root extract, the undiluted treatment was the one with the lowest number of dead larvae (24 % of larvae remained alive at the end of the experiment compared to 0, 7.6, 6.66, and 6.66 % for the ×10, ×20, ×30 dilutions and water, respectively, Fig. 4). The results were similar when we used *T. molitor* larvae. Infective juveniles exposed for 24 hr to the different maize germinate root cap extract dilutions, or to just water did not significantly differ in their ability to kill *T. molitor* larvae after recovery from quiescence (Weibull model: Chisq =2.86, P=0.58). Overall, 85.3 % of the larvae were dead at the end of the experiment and 90.6 % of the dead larvae displayed a red color. Infective juveniles from all extract concentrations were able to kill *T. molitor* larvae just as well as the ones that were kept in the control condition (water) (not diluted: Z=0.794, P=0.4; ×10: Z=1.33, P=0.1; ×20: Z=1.005, P=0.3; ×30: Z= 0.021, P=0.9). Although at the end of the experiment the numbers of *G. mellonella* and *T. molitor* larvae killed were comparable, we found that the infection was slower for *T. molitor* (Weibull model: Chisq=15.27, P=0.019) (Fig. 5).

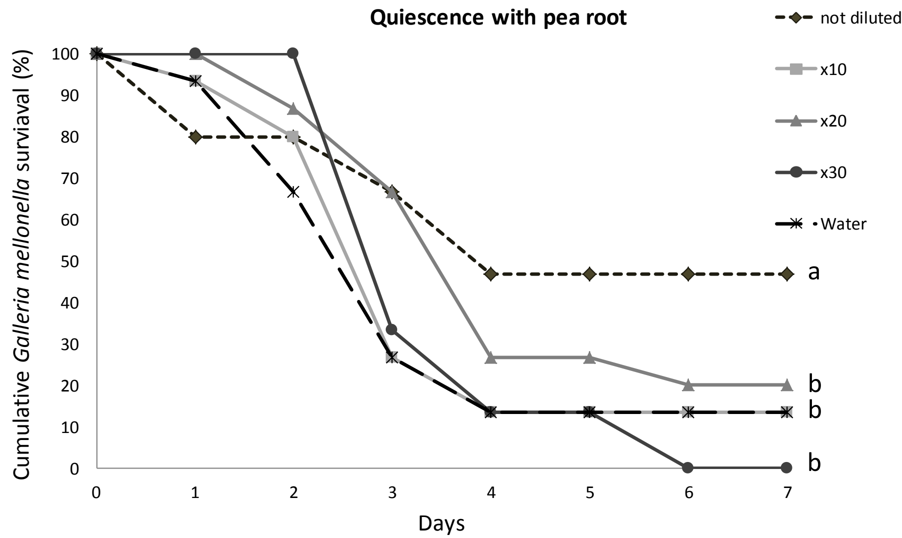


Fig. 3 Ability of *Heterorhabditis megidis* infective juveniles (IJs) to kill *Galleria mellonella* larvae after incubation in pea root extract (24 hr), as compared to incubation in just water. Nematodes incubated in all extract dilutions except the undiluted extract were as efficient as nematodes kept in water in killing host larvae. Different small letters indicate statistical differences.

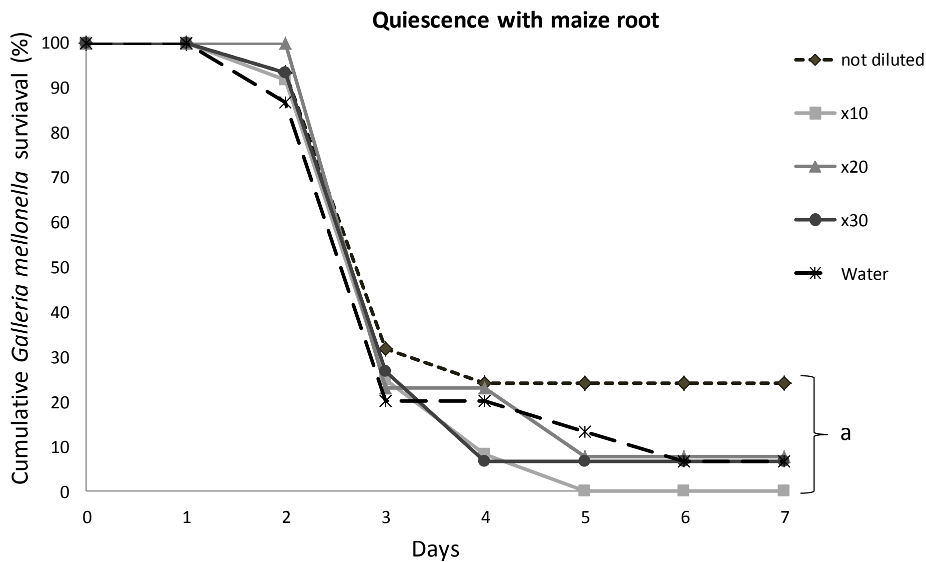


Fig. 4 Ability of *Heterorhabditis megidis* infective juveniles (IJs) to kill *Galleria mellonella* larvae after incubation in maize root extract (24 hr), as compared to incubation in just water. Nematodes incubated in all extract dilutions were as efficient as nematodes kept in water in killing host larvae. Different small letters indicate statistical differences.

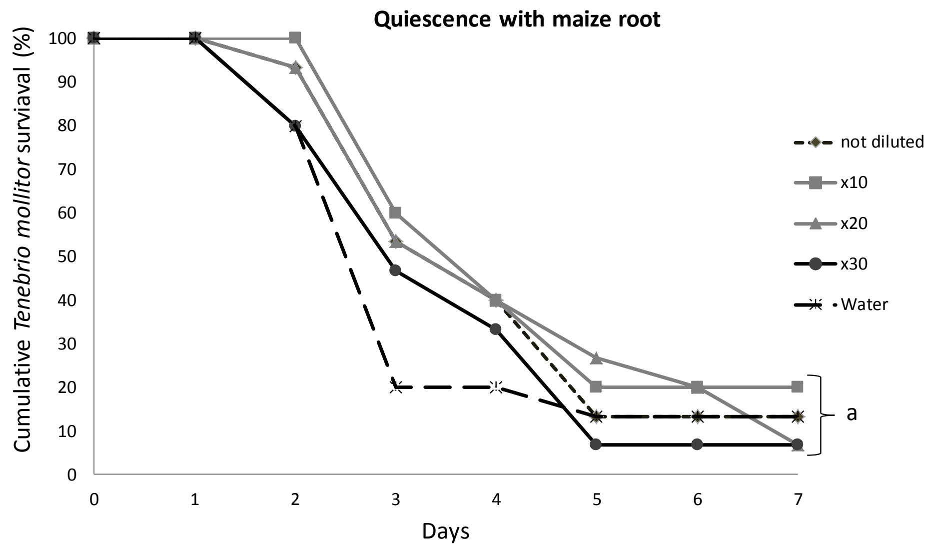


Fig. 5 Ability of *Heterorhabditis megidis* infective juveniles (IJs) to kill *Tenebrio molitor* larvae after incubation in maize root extract (24 hr), as compared to incubation in just water. Nematodes incubated in all extract dilutions were as efficient as nematodes kept in water in killing host larvae. Different small letters indicate statistical differences.

### *3.4. Aeration of the quiescence factor*

All the *H. megidis* IJs in the vials exposed to a large volume of solution of root cap extract were quiescent, contrary to all the vials filled with the smaller volume of extract, which only had a few quiescent IJs (DF=78, P<0.001). Moreover, none of the IJs exposed to root cap extract in vials that were shaken were quiescent, contrary to those of the unshaken vials (DF=78, P<0.001). This appeared not to be due to inactivation of the QF after exposure to oxygen, as IJs in oxygenated root cap extract showed the same rate of quiescence as IJs exposed in non-oxygenated root cap extract (DF=78, P=1). Finally, the IJs in the control vials with a larger volume (1 ml) of water showed no quiescence, unlike IJs in the same volume of root cap extract (DF=78, P<0.001) (Table 1).

Table1. Effect of volume, agitation and aeration of extract on *H. megidis* quiescence

| Parameter | Volume | | Agitation | | Aeration | | Control |
|---|---|---|---|---|---|---|---|
| Treatment | 1 ml extract | 200 µl extract | No agitation | Agitation | No aeration | Aeration | 1 ml water |
| *H.megidis* quiescence | + | - | + | - | + | + | - |

## 4. Discussion

For an optimal EPN formulation, especially in the context of commercialization, a prolonged infectiveness of EPNs is decisive (Grewal 2002). The possibility to induce quiescence in nematodes is a highly promising way to prolong the shelf life of EPNs and to maintain infectiveness for an extended period of time (Hiltpold et al., 2014). Quiescent factor is found in various genera of plants, and triggers a state of quiescence in all types of nematodes. Normally, the QF is collected from the exudate of root caps (Hiltpold et al., 2014; Hubbard et al., 2005). This collection method has several drawbacks, as it is time consuming and a tedious process, and only small quantities can be obtained. In this paper, we present a new and efficient way to collect QF from plants by using extraction of flash-frozen roots in liquid nitrogen. The root extracts that we obtained had high concentrations of QF and in all cases triggered 100 % quiescence at dilutions as low as 0.5 mg for pea root cap and 0.66 mg for maize root cap (0.5 mg, was not tested for maize). In contrast, root cap exudate had only low levels of QF and quickly lost its activity once diluted, which is consistent with the results obtained by Hiltpold et al., (2014).

Previous research on the QF focused on the root cap, and it was not known that the QF also is present in other parts of the root. Here we showed that the QF is constitutively produced in the entire root. The root cap extract and the extract of the rest of the root were equally efficient in triggering quiescence in H. megidis. This finding implies that considerably more plant material can be used to collect the QF. Whether the other root parts also release the QF into the rhizosphere remains to be determined, and we cannot exclude the possibility that the quiescence inducing compound(s) present in the rest of the root are different from those in the root cap.

It is important to note that a highly concentrated extract might contain too much QF for EPNs to recover from quiescence. This is important to consider when extracts are used in EPN formulation. We therefore tested the efficiency of the *H. megidis* IJs to recover from the state of quiescence, as well as their infectiousness after quiescence. For this, we used the relatively resistant host species *T. molitor*, as well as the very susceptible model species *G. mellonella*, and found that, after recovery, all the IJs exposed to the different dilutions of pea and maize extract for 24 hr were able to kill both hosts efficiently. The results imply that EPNs, even after exposure to high doses of QF, can be released in crop fields in a state of quiescence and still be able to kill a target pests.

In previous assays, we had noticed that root cap extract did not always trigger quiescence in *H. megidis* IJs. After further investigation, it was found that overexposure to air was possibly responsible for this absence of quiescence. We subsequently showed that quiescence induction by maize root cap extract is efficient only under relatively low oxygen conditions. Indeed, IJs kept in a small volume of root cap extract, representing a thin layer of solution and thus considerable exposure to air, did not turn into a state of quiescence, contrary to IJs that were kept in a larger volume (thick layer). Importantly, IJs kept in a larger volume of root cap extract that was shaken

also failed to become quiescent. Aeration of the root cap extract by means of an aquarium pump extract did not have any effect; IJs that were placed in extract that had first been aerated (thick layer) all became quiescent. As aeration did not change the proprieties of the QF in the root cap extract, it is clearly not that the QF is sensitive to exposure to air, but the exposure of EPNs to air interferes with the triggering of quiescence. A possible explanation is that a slightly weakened condition due to lack of oxygen may facilitate the triggering of quiescence. For the identification of the QF, we plan to use liquid chromatography to fractionate the exudate and bioassay guided isolation of the active compound(s) from these fractions. From the current results we learned that for these bioassays it is essential that we maintain the right conditions to ensure that a loss of activity is due only to a loss of active compound(s).

For now, we can only speculate on the ecological role of QFs in nature. It may well be a root defense against phytopathogenic nematodes (PPNs). Indeed, PPNs are considerable more sensitive than EPNs and may not recover from quiescence when exposed to high concentrations of QF (Hiltpold et al., 2014). Even if they recover, drugging PPNs may be an effective way for root tips to escape infection (Hiltpold et al., 2014). The eventual identification of QF should shed more light on its function and importance in shaping plant-nematode interactions.

In summary, we demonstrated that flash-frozen pea and maize roots extracts are highly effective in inducing quiescence in *H. megidis*, and are far more concentrated in QF than root exudates. At all concentrations, the state of quiescence was reversible after placing the IJs in soil with a high water content, and the IJs were still highly infectious after recovery. However, we did find that there is a limitation of the use of root cap extracts to induce quiescence: induction works only under conditions of slightly reduced oxygen availability. By using the presented method to obtain high concentrations of QF, we hope to be able to identify the key compound(s) that is (are) for quiescence induction.

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## References


Adams B, Nguyen K (2002) Taxonomy and systematics. In: Gaugler R (ed) Entomopathogenic nematology. CABI, New York, pp 1–34

Ali JG, Alborn HT, Stelinski LL (2010) Subterranean herbivore-induced volatiles released by citrus roots upon feeding by *Diaprepes abbreviatus* recruit entomopathogenic nematodes. J Chem Ecol 36:361–368. doi: 10.1007/s10886-010-9773-7

Ali JG, Alborn HT, Campos-Herrera R, Kaplan F, Duncan LW, Rodriguez-Saona C, Koppenhöfer M, Stellinski LL (2012) Subterranean, herbivore-induced plant volatile increases biological control activity of multiple beneficial nematode species in distinct habitats. PLoS ONE 7:1–8. doi: 10.1371/journal.pone.0038146

Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM (2006) The role of root exudates in rhizosphere interactions with plants and other organisms. Annu Rev Plant Biol 57:233–266. doi: 10.1146/annurev. arplant.57.032905.105159

Barber BYDA, Martin JK, June I (1976) The release of organic sub-stances by cereal roots into soil. New Phytol 76:69–80

Barrett J (1991) Anhydrobiotic nematodes. In: Evans K (ed) Agricultural zoological reviews. Vol. 4. Intercept, Andover, pp 161–176

Chen S, Glazer I (2005) A novel method for long-term storage of the entomopathogenic nematode *Steinernema feltiae* at room temperature. Biol Control 32:104–110. doi: 10.1016/j.biocontrol.2004.08.006 Curtis RHC, Robinson AF, Perry RN (2009) Hatch and host location. In: Perry R, Moen M, Starr J (eds) Root-knot nematodes. CABI Publ, Wallingford, pp 139–162

Den Nijs LJMF, Lock CAM (1992) Differential hatching of the potato cyst nematodes *Globodera rostochiensis* and *G. pallida* in root diffusates and water of differing ionic composition. Neth J Plant Pathol 98:117–128. doi: 10.1007/BF01996324

Dillman AR, Chaston JM, Adams BJ, Ciche TA, Goodrich-Blair H, Stock SP, Sternberg PW (2012) An entomopathogenic nematode by any other name. PLoS Pathog 8:8–11. doi: 10.1371/journal.ppat.1002527

Forst S, Clarke D (2002) Bacteria-nematode symbiosis. In: Gaugler R (ed) Entomopathogenic nematology. CABI Publishing, New York, pp 57–77

Gaur HS, Beane J, Perry RN (2000) The influence of root diffusate, host age and water regimes on hatching of the root-knot nematode, *Meloidogyne triticoryzae*. Nematology 2:191–199

Georgis R, Koppenhöfer M, Lacey LA, Bélair G, Duncam LW, Grewal PS, Samish M, Tan L, Torr P, van Tol RWHM (2006) Successes and failures in the use of parasitic nematodes for pest control. Biol Control 38:103–123. doi: 10.1016/j.biocontrol.2005.11.005

Grewal PS (2002) Formulation and application technology. In: Gaugler R (ed) Entomopathogenic nematology. CABI Publishing, New York, pp 265–287

Grewal PS, Peters A (2005) Formulation and quality control of entomopathogenic nematodes. In: Grewal PS, Ehlers RU, Shapiro-Ilan D (eds) Nematodes as biocontrol agents. CABI Publishing, Wallingford, pp 79–90

Hartmann T (2007) From waste products to ecochemicals: fifty years research of plant secondary metabolism. Phytochemistry 68:2831– 2846. doi: 10.1016/j.phytochem.09.017

Hiltpold I, Turlings TCJ (2012) Manipulation of chemically mediated interactions in agricultural soils to enhance the control of crop pests and to improve crop yield. J Chem Ecol 38:641–650. doi: 10.1007/ s10886-012-0131-9

Hiltpold I, Erb M, Robert CAM, Turlings TCJ (2011) Systemic root signal-ling in a belowground, volatile-mediated tritrophic interaction. Plant Cell Environ 34:1267–1275. doi: 10.1111/j.1365-3040.2011.02327.x

Hiltpold I, Hibbard BE, French BW, Turlings TCJ (2012) Capsules containing entomopathogenic nematodes as a Trojan horse approach to control the western corn rootworm. Plant Soil 358:11–25. doi: 10. 1007/s11104-012-1253-0

Hiltpold I, Jaffuel G, Turlings TCJ (2014) The dual effects of root-cap exudates on nematodes: from quiescence in plant-parasitic nematodes to frenzy in entomopathogenic nematodes. J Exp Bot. doi: 10.1093/jxb/eru345

Hubbard JE, Flores-Lara Y, Schmitt M, McCLure MA, Stock SP, Hawes MC (2005) Increased penetration of host roots by nematodes after recovery from quiescence induced by root cap exudate. Nematology 7:321–331

Kaya HK, Gaugler R (1993) Entomopathogenic nematodes. Annu Rev Entomol 38:181–206. doi: 10.1146/annurev.en.38.010193.001145

Khokon MAR, Okuma E, Rahman T, Wesemael WML, Murata Y, Moens M (2009) Quantitative analysis of the effects of diffusates from plant roots on the hatching of *Meloidogyne chitwoodi* from young and senescing host plants. Biosci Biotechnol Biochem 73:2345–2347. doi: 10.1271/bbb.90392

Köhler HR, Triebskorn R (2013) Wildlife ecotoxicology of pesticides: can we track effects to the population level and beyond? Science 341:759–765. doi: 10.1126/science.1237591

Kumar R, Pandey S, Pandey A (2006) Plant roots and carbon sequestration. Curr Sci 91:885–890

Lello ER, Patel MN, Matthews G, Wright DJ (1996) Application technology for entomopathogenic nematodes against foliar pests. Crop Prot 15:567–574. doi: 10.1016/0261-2194(96)00026-9

Lewis EE, Campbell J, Griffin C, Kaya H, Peters A (2006) Behavioral ecology of entomopathogenic nematodes. Biol Control 38:66–79. doi: 10.1016/j.biocontrol.2005.11.007

Prot J (1980) Migration of plant-parasitic nematodes towards plant roots. Rev Nemtology 3(2): 305–318

Rasmann S, Köllner TG, Degenhardt J, Hiltpold I, Toepfer S, Kuhlmann U, Gershenton J, Turlings TCJ (2005) Recruitment of entomopathogenic nematodes by insect-damaged maize roots. Nature 434:732– 737. doi: 10.1038/nature03451

Rasmann S, Hiltpold I, Ali J (2012) The role of root-produced volatile secondary metabolites in mediating soil interactions. In: Montanaro G, Cichio B (eds) Advances in selected plant physiology aspects. InTech Open Access Publisher, Croatia, pp 269–290

Reynolds AM, Dutta TK, Curtis RHC, Powers SJ, Gaur HS, Kerry BR (2011) Chemotaxis can take plant-parasitic nematodes to the source of a chemo-attractant via the shortest possible routes. J R Soc Interface 8:568–577. doi: 10.1098/rsif.2010.0417

Rolfe RN, Barrett J, Perry ARN (2000) Analysis of chemosensory re-sponses of second stage juveniles of Globodera rostochiensis using electrophysiological techniques. Nematology 2:523–533

Shapiro-Ilan DI, Gouge DH, Piggott SJ, Fife JP (2006) Application technology and environmental considerations for use of entomopathogenic nematodes in biological control. Biol Control 38:124–133. doi: 10.1016/j.biocontrol.2005.09.005

Turlings TCJ, Hiltpold I, Rasmann S (2012) The importance of root-produced volatiles as foraging cues for entomopathogenic nematodes. Plant Soil 358:51–60. doi: 10.1007/s11104-012-1295-3

Van Dam NM (2009) Belowground herbivory and plant defenses. Annu Rev Ecol Evol Syst 40:373–391. doi: 10.1146/annurev.ecolsys. 110308.120314

Van Tol RWHM, Van der Sommen ATC, Boff MIC, Van Bezooijen J, Sabelis MW, Smits PH (2001) Plants protect their roots by alerting the ennemies of grubs. Ecol Lett 4:292–294. doi: 10.1046/j.1461- 0248.2001.00227.x

Vining LC (1990) Functions of secondary metabolites. Annu Rev Microbiol 44:395–427. doi: 10.1146/annurev.mi.44.100190.002143 White GF (1927) A method for obtaining infective nematode larvae from cultures. Science 66:302–303

Zhao X, Schmitt M, Hawes MC (2000) Species-dependent effects of border cell and root tip exudates on nematode behavior. Phytopathology 90:1239–1245.

# Chapter III

# Can cover crops enhance overwintering survival of entomopathogenic nematodes in annual crops?

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**Abstract**

New approaches to crop production are necessary to confront contemporary challenges, such as climate change, the rapidly increasing human population, and environmental issues. Conservation agriculture, with limited disturbance to the environment, is arising as an alternative to conventional agriculture. In this context, cover crops show great promise as they may help to conserve soil quality by limiting soil erosion and by enhancing the content of nutrients, water, organic matter, and by regulating the populations of soil organisms. In this study, we investigated whether winter cover crops can enhance the persistence of entomopathogenic nematodes (EPN) over the winter season. EPN have great potential as biological control agents against root feeding insects and their abundance is an important measure of soil health. We compared the persistence of naturally occurring and augmented populations of EPN in plots with and without cover crops, pea (*Pisum sativum*) or mustard (*Brassica juncea*). The augmented plots were supplemented in autumn with infective juveniles (IJs) of either *Heterorhabditis bacteriophora* or *Steinernema feltiae*.

To identify and quantify the organisms in the associated soil food web we used the traditional baiting method with wax moth larvae, as well as quantitative real time PCR (qPCR) analyses to identify and quantify EPN in soil samples at the beginning and the end of the winter season. Numbers of naturally occurring EPN in the investigated fields were very low (< 1 EPN per 100g of soil) and were not affected by the presence or absence of cover crops. The persistence of augmented EPN was promising, but the cover crops only had a significant positive effect on the numbers of *S. feltiae* found in the middle of the winter. No such effect was found for *H. bacteriophora*. Yet, augmentation was found to enhance the suppressiveness of the soil, as the recorded EPN infectivity after the winter was higher than what was observed in autumn, one month after application. The qPCR analyses detected higher numbers of *H. bacteriophora* in the soil samples, but only *S. feltiae* successfully infected the baiting larvae in spring. In conclusion, the effect of cover crops on EPN persistence was only evident during mid-winter, and only in the plots augmented with *S. feltiae*. Moreover, we found that higher numbers of EPN in agricultural soils do not necessarily translate into high infectivity, which is the key factor determining their effectiveness in controlling soil pests.

## 1. Introduction

Improving soil quality and enhancing agricultural production in a sustainable manner is essential to face the challenge of ensuring food security for an increasing world population. Problems such as contamination of the environment with agrochemicals, depletion of natural resources and soil erosion call for novel strategies to optimize sustainable crop production (Komatsuzaki and Ohta, 2007; Lal, 2009, Abdollahi and Munkholm 2014). Concepts such as conservation agriculture, through adapted tillage, residue management and crop rotation, are key elements to improve modern agriculture (Verhulst et al. 2010).

One promising strategy is to use winter cover crops after summer productions, as these may help to diminish nutrient losses, reduce soil erosion and positively influence beneficial soil organisms (Dabney et al., 2001; Ewing et al., 1991; Fageria et al., 2005; Gómez et al., 2009; Hargrove, 1991, Snapp et al., 2005). Yet, the impact of cover crops on the soil biota, in general, and on soil beneficial organisms such as biological control agents, in particular, is poorly understood.

Entomopathogenic nematodes (EPN) are obligate parasites of insects and excellent biocontrol agents (Lacey et al., 2015). Infective juveniles (IJs) actively seek for a host that they invade and once inside they release their symbiotic α–Proteobacteria, which produce a toxin that generates a general septicemia in the host. Their high infectiousness and ability to kill an insect within 2-3 days and their relatively low impact on non-target species make EPN good candidates for sustainable pest management (Piedra Buena et al., 2015). However, the use of EPN in large-scale agriculture faces several constraints that make their application rarely cost-effective (Lacey et al., 2015).

As part of a research consortium that explores how soil health can be improved by applying ecological and rational approaches (NRP68: http://www.nrp68.ch), we studied how soil food webs, including EPN, can be better exploited to control soil-dwelling insect pests in annual crops. There is increasing interest in the factors that determine the presence and the dynamics of EPN populations, as well as their competitors and natural enemies, such as free-living nematodes, nematophagous fungi and bacteria (Campos-Herrera et al., 2013a, 2015a; Griffin 2015; Lewis et al., 2015). In agroecosystems, soil properties may be severely altered by agricultural management practices and EPN will be challenged with drastic changes of various abiotic and biotic factors (Stuart et al,. 2006, 2015; Lewis et al., 2015). Recent surveys of Swiss agricultural soils have shown that the natural occurrence of EPN is very low, independent of management practices (Jaffuel et al. 2016; Campos-Herrera et al. 2015b). Therefore EPN application to augment their numbers may be a promising strategy to improve the control of root pests. Overwintering ability of augmented EPN were assessed in field studies in Switzerland and were found to be poor (Imperiali, Chiriboga et al., in prep.), which is in agreement with the natural population dynamics observed for the native populations by Campos-Herrera et al. (2015b) and Jaffuel et al. (2016).

Yet, several other studies show that EPN can persist and remained infectious after overwintering (Duncan et al., 2013; Elmowitz et al. 2013; Cappaert et al. 2003), which implies that persistence might be climate-dependent. The aim of the current study was to explore ways to improve overwintering persistence of EPN. To this end, we compared field plots with winter cover crops and plots with bared soil to determine how cover crops may affect the population dynamic of EPN and other members of the associated soil food web.

Several previous studies used Baermann funnel extraction to survey the nematode community under cover crops. Overall, they found positive effects of cover crops on different genera of nematode populations, even in very different crop systems (Ito et al. 2015a, Ito et al. 2015b, DuPont et al. 2009, Ferris et al. 2012). The Baermann funnel technique only recovers nematodes that actively move, and therefore may miss certain nematodes. A study using insect baiting in different crops found a better persistence of *H. bacteriophora* in bean cultivation after red clover was used as a cover crop (Susurluk et al. 2008). The limitation with only using insect baits is that it only tests for infectivity and not overall EPN presence.

Here, we employed two distinct measurement techniques (traditional baiting with wax moth larvae and quantitative real time PCR analyses) to investigate whether certain cover crops can help to maintain higher levels of EPN densities in agricultural soils. We chose two cover crops: (i) pea (*Pisum sativum*) and (ii) mustard (*Brassica juncea*). The latter is known to produce bio-fumigants that may be detrimental to soil organisms, including nematodes (Ramirez et al. 2008; Henderson et al. 2009). We performed two independent field experiments. The first allowed us to evaluate how the naturally occurring EPN population persisted over winter under pea as cover crop *versus* bared soil. This was combined with comparison between tillage *versus* direct seeding management, as well as different soil textures. The second experiment was conducted to test how survival and activity of both native and augmented EPN population persist under cover crops (pea or mustard) as compared to barren soil. For both field experiments we also surveyed other key members of the soil food web (free-living nematodes, nematophagous fungi and bacteria). Thus, we also established how EPN application may affect the population dynamics of these associated organisms and how they in turn my affect EPN persistence. We also measured the microbial biomass carbon ($C_{mic}$) and nitrogen ($N_{mic}$) in the soils as an additional proxy for soil health.

## 2. Material and methods

### *2.1. Field experiments design*

We surveyed two ongoing field trials located in the Experimental Station Agroscope Changins, near Nyon (Switzerland, 46° 24’ N, 06° 14’ E, 430 m above sea level). In the first experiment CC27, we evaluated the impact of two types of cover crops. One was mustard (*Brassica juncea* var. Vitasso) with a possible deleterious effect on nematodes (Ramirez et al.,

2008) and the other pea (*Pisum sativum* var. Arkta), which we assumed to have a potentially neutral or positive impact on EPN. Bare soil plots were considered as control plots. Each plot was 8 m × 1.5 m, and the treatments were distributed following a randomized block design. The field is characterized by a texture of 24.6 % clay, 29.7 % sand and 45.7% silt, a pH of 7 and a soil organic matter content of 2.6 %. We also evaluated the effect of EPN augmentation before the winter season. We applied the following three treatments with nematodes to split-plots (within the cover crop plots) of 1 m × 1 m: 1) no augmentation (control), 2) application of *Steinernema feltiae* RS-5 (native) and 3) application of *Heterorhabditis bacteriophora* Andermatt (commercial). Both species of nematodes were previously detected in the same area (Campos-Herrera et al., 2015b). In each plot, we ensured a buffer between the mini-plots of 60-90 cm.

A native population of *Steinernema feltiae* was cultured in the laboratory using *Galleria mellonella* (Lepidoptera: Pyralidae) as host (au Pêcheur sàrl, Neuchatêl). Nematodes within two weeks after emergence or delivery (for the commercial species) were employed in the augmentation plots. For both nematode species, water suspensions were prepared the night before application to obtain the equivalent of 50 IJs/ $cm^2$ in the field. We verified activity in the laboratory for both species by infecting larvae of *G. mellonella.* In the field, the application was performed by using water cans (one per species), with the corresponding nematodes suspended in ca. 3 l of water per plot, ensuring the application of all of the material by rinsing the cans with an additional 1-1.5 l. In the control plots we just added the same amount of water. On November 18$^{th}$ 2014, before adding the nematodes, all the plots were first sampled to obtain information about the pre-application natural population (T0). The mini-plots were randomly arranged inside the plots and their respective location was taken into account to test for putative cross-contamination among mini-plots. (Supplementary data 1, SFig1)

In the second experiment, denominated CC29, we evaluated the effect of pea (*Pisum sativum* var. Arkta) as a cover crop, which was compared with bared soil as control. For this experiment we also considered the factor soil tillage, and of the plots were tilled at the end of October following a conventional procedure (20-25 cm depth), whereas the other plots were left untilled. Winter wheat (*Triticum aestivum* L var. Arina) was then sown in each plot. Moreover, the field shows a gradient in soil texture as described by Campos-Herrera et al. (2015b), which allowed us to compare between clay soils (CA, 17% sand, 32% silt, 51% clay; 4.3 % soil organic matter, pH 6.4) and clay loam soil (CL, 30% sand, 44% silt, 26% clay, 2.1 % soil organic matter; pH 7.1). Each plot was 18.5 m × 8 m, with 1m buffers between plots. The experimental design was a randomized split-block design including cover crop (cover *versus* bared soil) and tillage (till *versus* no-till), with three complete blocks for each of the soil types. (Supplementary data 2, SFig2).

*2.2. Soil sampling and biological characterization*

In each plot, we took a composite soil sample comprising 12 cores (3 cm diam. × 20 cm depth), with a total weight of ca. 1.2-1.5 kg. In CC27, soil cores were also taken at three time points: 1) pre-application (T0, November 18$^{th}$ 2014), 2) one month after application (pre-winter survival) (T1, December 9$^{th}$, 2014) and 3) four months after application to check for overwinter survival (T2, March 11$^{th}$, 2015). In CC29, soil cores were taken at three different time points as follow: at the end of the cover crop period (T1, October 9$^{th}$ 2014), after tillage (in the corresponding plots) and after sowing of winter wheat (*Triticum aestivum* L var. Ariana) (T2, November 18$^{th}$ 2014) and a third time in late winter, with soil covered with winter wheat (T3, March 11$^{th}$ 2015).In all the cases, the soil samples were brought to the laboratory, carefully mixed to ensure that all the cores were disaggregated, homogenized and stored at 4°C. They were processed within two to four days after sampling.

Following the protocols described by Jaffuel et al. (2016), aliquots of these soil samples were used for the following analysis: *i*) identification and quantification of EPN and several associated organisms, using real time qPCR (400 g of fresh soil); *ii*) measurement of EPN activity and potential suppressive effect of the soil (400 g of fresh soil), using *Galleria* mellonella baiting *iii*) measurement of water content (180 g of fresh soil), *iv*) measurement of soil microbial biomass carbon ($C_{mic}$) and nitrogen ($N_{mic}$) (120 g of fresh soil). The remaining soil samples were kept as backups at 4ºC until all the processing was finished, in case they were needed for additional measurements.

We followed the procedures and protocols described by Campos-Herrera et al. (2015b) for the molecular analysis of the EPN soil food web. Briefly, the nematode community was extracted from each sample by sucrose extraction (Jenkins, 1964), a method that allows the recovery of living and dead nematodes and of various microorganisms present in or on the nematodes (see review by Campos–Herrera et al., 2013a). Samples were transferred to a 1.5 ml Eppendorf tubes and DNA was extracted using PowerSoil$^{R}$ DNA Isolation Kit (MoBio, protocol for maximum yield). Following the procedures detailed in Campos-Herrera et al. (2015b, 2015c), we evaluated the presence and quantity of 18 members of the EPN soil food web (see Supplementary data 3, Stable 1). This included: a) seven species of EPN (previously reported for the area by Campos-Herrera et al., 2015b), b) six species of nematophagous fungi (NF) that prey on EPN and can strongly suppress their natural populations (Atkins et al., 2005; Zhang et al., 2006; Pathak et al., 2012), c) the ectoparasitic bacterium *Paenibacillus nematophilus* that can reduce the mobility of the EPN–infective juveniles (IJs) (Enright and Griffin, 2005; Campos–Herrera et al., 2011b), and d) the free-living nematodes (FLNs) that might compete for cadavers as a resource, including those in the *Acrobeloides*–group and three species of *Oscheius* (Campos-Herrera et al., 2012, 2015c).

Also, for the experiment in plot CC27 and post-application sampling times, procedures described in Campos-Herrera et al. (2015b) were followed for the quantification of the EPN activity

and potential suppressive effects of the soil using *Galleria mellonella* baits. In this case we used 2 aliquots of 200 g of fresh soil, to ensure using the same amount of soil for both nematode quantification methods. The DNA of the progeny leaving the *G. mellonella* cadavers was extracted using the QIAamp DNA mini kit (Qiagen), and species identity assessed by qPCR (Campos-Herrera et al., 2015b, Jaffuel et al., 2016).

Water content was measured for 180 g of fresh soil of each sample, incubated at 40 ºC for a week. Finally, triplicates of 20 g (dry soil) per sample were used to measure soil microbial biomass carbon ($C_{mic}$) and nitrogen ($N_{mic}$) by chloroform-fumigation-extraction (CFE) following Fließbach et al. (2007). Each sample of 20 g of dry soil was extracted in 80 mL of 0.5 M $K_2SO_4$ solution.

*2.3. Data transformation and statistical analysis*

Values obtained from qPCR for each organism (see units in Supplementary data 3, Stable 1) were adjusted with the appropriate correction factor used for the qPCR runs, and transformed to express these quantities for 100 g of dry soil.

The infection rate (IR), a proxy for the parasitism of nematodes by NF was determined by dividing the DNA quantity found in each plot by the total amount of DNA (Duncan et al., 2013; Campos–Herrera et al., 2012). We standardized the units of measurement among species (0–1) by dividing all data within a species by the highest measurement for that species (de Rooij van der Goes et al., 1995). The FLN data were expressed in pg DNA, whereas EPN were expressed as number of IJs, being all standardized (0-1) as described above for NF to allow comparison among species (Jaffuel et al., 2016)  (Supplementary data 3, STable 1).

Data from the *G. mellonella* larvae baits were analyzed following Jaffuel et al. (2016). The "suppressiveness" or "activity" of each plot is defined as the percentage of larval mortality observed in the soil samples per plot. We distinguished between the mortality caused by any agent (i.e nematode, bacteria, fungi, virus, etc.) and mortality caused only by nematodes. We fitted a binomial (or quasibinomial) distribution to a General Linear Model (GLM) to analyze the data obtained with *G. mellonella* larvae baits using the EPN augmentation, the cover crops and the period of sampling as factors. $C_{mic}$ and $N_{mic}$ values obtained by CFE were analyzed without any transformation.

The CC27 experimental field presented a split-plot configuration with blocks randomly distributed across the field. The qPCR and CFE data were analyzed with a linear mixed model (lmer, package "lme4") using as fixed factors i) cover crop (pea *versus* mustard *versus* bare soil), ii) EPN application (*S. feltiae versus H. bacteriophora versus* no EPN), iii) period of sampling and as nested factor; every individual samples depending on the period of sampling to account for the repeated measurement over time and the split-plot configuration with blocks randomly distributed across the field. For the CC29 experimental field, which also presents a split-plot configuration, the same statistical procedure was used with the following fixed factors: *i*) cover crop, *ii*) soil texture, *iii*)

tillage status and *iv*) period of sampling. The data were log or log+1 transformed. Most of the data failed the assumptions of normality and equal variances, thus the reported F-values were obtained from the original model, whereas *P*-values were obtained by 1000 permutations using the package "pbkrtest". The multiple comparisons among factors were obtained by performing a Tukey test using the package "lsmeans" (CRAN, v1.2-3). The statistical analyses were carried using R 3.2.4 (CRAN) and data are presented as mean ± SEM of the untransformed values.

## 3. Results

### *3.1. Impact of cover crops on population dynamics of entomopathogenic nematodes: fate after augmentation*

The cover crop treatment (pea, mustard and bare soil) did not directly influence the total EPN densities of the inoculated plots in field CC27 ($P = 0.5$) (Table 1). But the interaction of the cover crop treatment with the application treatment and the sampling period was significant ($P = 0.02$) (Fig 1A). In the *S. feltiae* augmented plots, the cover crop treatment (both pea and mustard) significantly enhanced the *S. feltiae* presence at the mid-winter measurement (December); with 2 times more *S.fetiae* IJs in pea plots ($P = 0.02$) and 2.5 fold more in mustard plots ($P = 0.02$), as compared to the bare soil plots. No significant differences were observed at any time point for plots augmented with *H. bacteriophora* (Fig 2). Overall, the application of EPN was effective as we found a highly significant increase in the numbers of EPN detected following application ($P < 0.001$). As a consequence, EPN detection was significantly different for the different sampling dates ($P < 0.001$), as was the interaction with the application treatment ($P < 0.001$) (Table 1, Fig 3).

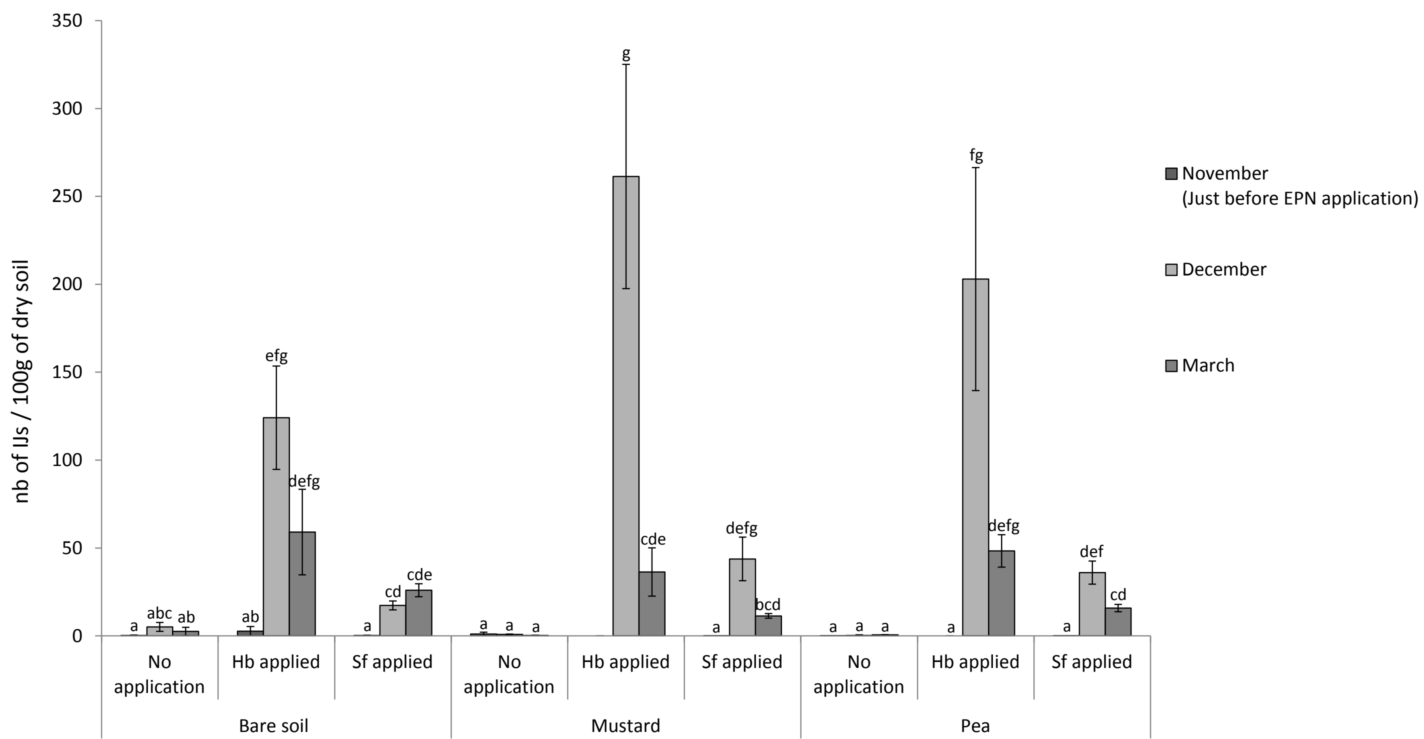


**Fig 1**. Mean occurrence of entomopathogenic nematodes (EPN) depending on the cover crop cultivated (mustard, pea or bare soil), the species of EPN applied (*H. bacteriophora* and *S. feltiae*) along the sampling period. Data are shown as means of IJs in 100g of soil ±SEM. Different letters indicate statistically significant differences of the interaction of the cover crop treatment, the EPN application treatment and the sampling period.

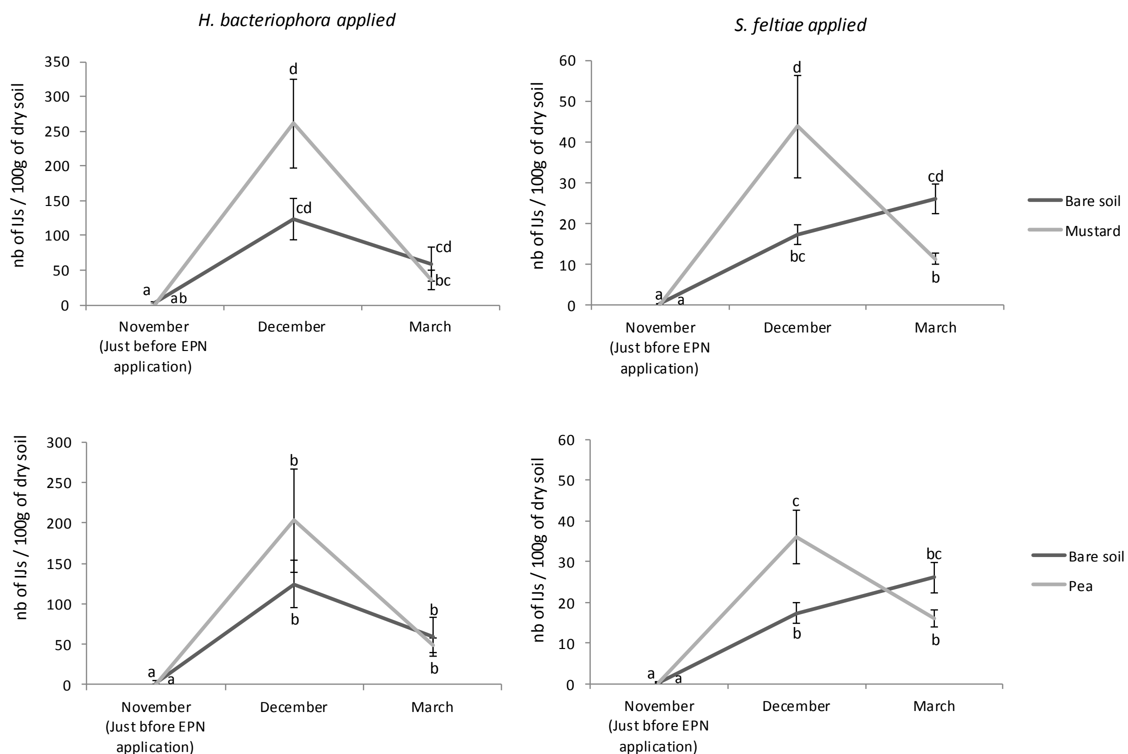


Fig 2. Mean occurrence of entomopathogenic nematodes (EPN) depending on the cover crop cultivated (mustard versus bare soil, pea versus bare soil) and the species of EPN applied (*H. bacteriophora and S. feltiae*) along the sampling period. Data are shown as means of IJs in 100g of soil ±SEM. Different letters indicate statistically significant differences of the interaction of the cover crop treatment and the sampling period

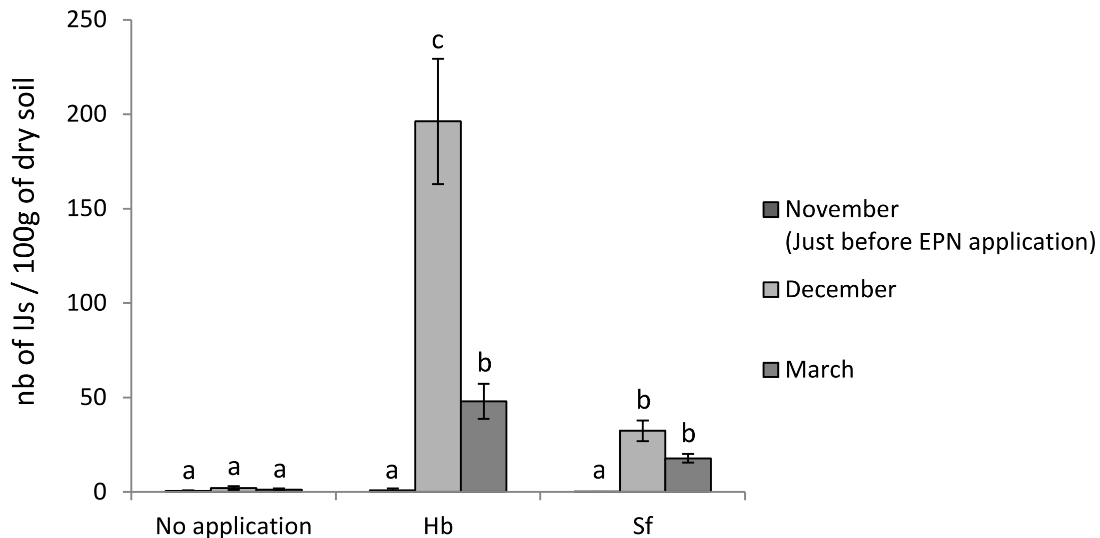


**Fig 3**. Mean occurrence of entomopathogenic nematodes (EPN) in the CC27 parcel depending on the application treatment (no application, Hb = application with *H. bacteriophora* and Sf = application with *S. feltiae*) for the three sampling dates. Data are shown as the mean number of IJs in 100g of soil ±SEM. Different letters indicate statistically significant differences in the interaction between the application treatment and the sampling period.

At T0, before the application of EPN, we only detected the presence of 3 naturally occurring EPN species: *Steinernema affine*, *Steinernema poinari*, *Heterorhabditis bacteriophora* (Table 2). The mean number of naturally occurring IJs was very low, with, on average, fewer than 1 IJs/ 100g of dry soil (0.47±0.3), and they were found in only 27.7% of the plots.

At T1, one month after the application of *S. feltiae* and *H. bacteriophora* in selected plots, the mean number of IJs per 100g of dry soil in augmented plots was 114.2±23.7 compared to 2.1±1.0 in the control plots ($P < 0.001$). In their augmented plots the average *H. bacteriophora* number was 65.8±18.9 IJs per 100g of dry soil. *S. feltiae* IJs were detected in much lower numbers in their specific plots, 10.9±3.1 IJs per 100g of dry soil. There was overall an increase in EPN species richness: at T1 we detected all of the seven targeted species, whereas we detected only three at T0 (Table 2).

At T2, after the winter, the mean number of IJs per 100g of dry soil in augmented plots was 32.8±5.6 compared to 1.1±0.8 in the control plots ($P < 0.001$). The most frequently detected species was the naturally occurring endemic species *H. megidis*, found in almost half of the plots (47%) but in very low numbers (mean: 0.6±0.3 IJs per 100g of dry soil). The applied species *S. feltiae* was the second most frequently detected species (in 41% of the plots). As at T1, with a mean of 5.4±1.5 IJs per 100g of dry soil its density was lower compared to that of *H. bacteriophora* (15.9±4.8), but *H. bacteriophora* was detected in fewer plots (33.3%). Fewer EPN species were detected at T2 than at T1, with only four of the seven targeted species (Table 2).

**Table 1** (CC27): Effect of cover crop and EPN application, as well as sampling period on occurrence and activity of various soil organisms and on microbial biomass. Statistics are presented as Chisq (for binomial analyses) and F (for permutation analyses); probability levels: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, n.s., not significant. Activity was assessed with the *Galleria mellonella* baiting method, natural occurrence of the organisms was determined by qPCR and microbial biomass was detected by CFE.

| Measured parameter | Cover (C) | Application (A) | Samling period (P) | C*A | C*P | A*P | C*A*P |
|---|---|---|---|---|---|---|---|
| | Df = 2 | Df = 2 | Df = 2 | Df = 4 | Df = 4 | Df = 4 | Df = 8 |
| **qPCR data** | | | | | | | |
| Entomopathogenic nematodes | | | | | | | |
| Total IJs | n.s. | 105.7*** | 189.5*** | n.s. | ns | 38.6*** | 1.7* |
| Free-living nematodes | | | | | | | |
| ng DNA, (0-1) interval-scaled | n.s. | n.s. | 54.6*** | n.s. | 4.5** | n.s. | n.s. |
| Nematophagous fungi | | | | | | | |
| Total IR[1] | n.s. | n.s. | n.s. | n.s. | 2.0* | n.s. | n.s. |
| **GM bait data** | | | | | | | |
| | Df = 2 | Df = 2 | Df = 1 | Df = 4 | Df = 2 | Df = 2 | Df = 4 |
| All organisms | | | | | | | |
| Activity (%) | n.s. | 54.09*** | n.s. | n.s. | n.s. | n.s. | n.s. |
| Entomopathogenic nematodes | | | | | | | |
| Activity (%) | n.s | 40.10*** | n.s. | n.s. | n.s. | 10.57** | n.s. |
| Nematodes (all) | | | | | | | |
| Activity (%) | n.s. | 39.52*** | 3.93* | n.s. | n.s. | 7.68* | n.s. |
| **CFE data** | | | | | | | |
| Cmic (mg/kg) | n.s. | n.s. | 12.0*** | n.s. | 3.1* | n.s. | n.s. |
| Nmic (mg/kg) | n.s. | n.s. | 6.4** | n.s. | n.s. | n.s. | n.s. |

[1] IR, infection rate for the nematophagous fungi (NF), equivalent to pg NF DNA / total DNA

### *3.2. Impact of cover crops on the natural enemies of entomopathogenic nematodes*

#### *3.2.1. Nematophagous fungi and ectoparasitic bacteria*

The presence of cover crops (pea, mustard *versus* bare soil) did not directly favor the presence of the NF in CC27 ($P = 0.5$), nor did the EPN application treatment ($P > 0.9$). The period of sampling also did not impact the abundance of NF ($P > 0.09$) but was significantly interacting with the cover crop treatment ($P = 0.03$) (Table 1).

At T0, four out of the six tested NF were detected (Table 2), and the infection rate (IR) was 0.4±0.07. At T1, one month after the EPN augmentation, the NF community structure only showed minor changes, with a very slight change in species prevalence (Table 2). The EPN augmentation did not change the presence of NF in the field with an overall IR of 0.5±0.05 ($P = 0.9$). At T2, five months after EPN augmentation the NF community structure had changed and they were considerably less prevalent (Table 2), but the overall IR was slightly higher (0.6±0.07) ($P = 0.9$).

The ectoparasitic bacterium *P. nematophilus* was not detected in any of these samples.

*3.2.2. Free-living nematodes*

The cover crop treatment and the application treatment had no impact on the numbers of the selected free-living nematode (FLN) species in CC27 ($P = 0.7$ and $P = 0.2$ respectively) (Table 1). There was a significant difference in FLN presence among the sampling dates ($P < 0.001$) and there was a significantly interaction between sampling date and cover crop treatment ($P < 0.001$) (Table 1).

At T0, before EPN augmentation, FLN (*Acrobeloides*-group + *Oscheius* spp.), which compete with EPN inside insect cadavers, were detected in all of the 36 plots. Their prevalence was very high (Table 2). The 0-1 standardized values used as proxy for FLN quantity were also high with an average of 0.8±0.08. At T1, 1 month after EPN application, the FLN community was qualitatively similar to the baseline community (T0). However, the EPN augmentation influenced the FLN proportional occurrence, with a much lower numbers expressed in the corrected value of 0.3±0.05 after augmentation. After the winter (T2), the proportional occurrence of FLN had returned to almost its original state (0.7±0.06), with similar population prevalence as at the two earlier sampling times (Table 2). *Oscheius* sp. 3 was not detected in any of the samples at any sampling time.

3.3*. Assessment of the suppressive activity of augmented soils*

The general assessment did not reveal an effect of the cover crops on the activity of all organisms together (total activity) nor on nematodes alone ($P = 0.7$ and $P = 0.2$ respectively). Yet, as expected, the application treatment significantly shaped the activity of EPN ($P < 0.001$), with a higher activity in the plots where *S. feltiae* were released ($P < 0.001$). Plots inoculated with *H. bacteriophora* did not show more activity than control plots ($P = 0.1$, Table 1, Fig 4). The mean total activity of the soil, which represents the mean proportion of dead *G. mellonella* larvae per plot killed by any organisms (i.e. bacteria, fungi, nematodes and other agents), was 20.3% and 24.1% for T1 and T2, respectively. Out of the 36 plots, 33 showed nematode activity at T1 and 35 at T2. The part of the soil activity due to nematodes only (FLN+EPN) was of 8% at T1 and 10.9% at T2. More than half of the plots showed nematode-related activity (58.3% and 75% for T1 and T2, respectively).

The nematode progeny from infected *Galleria mellonella* cadavers was identified. In all case, the progeny was mixed, with up to five different species detected in the same cadaver. At T1, on average over all the plots, the augmented nematodes *S. feltiae* represent 21.8 % of emerging nematodes, followed by *H. bacteriophora*, also augmented, with 19.9 %. At T2, after the winter, we observed a slight decrease of emerging *S. feltiae* (19.6 %) and a drastic decrease of *H. bacteriophora*, which were not detected in baits anymore. For the two last sampling periods, the species that was applied turned out to be the main species infecting the *Galleria mellonella* baits. In plots augmented with *H. bacteriophora*, *H. bacteriophora* was the dominant EPN species

(emerging from 20 % of the baits, only at T1), but they were still underrepresented with respect to FLN (Fig 5). In *S. feltiae* augmented plot, *S. feltiae* emerged from 51 % of the bait larvae and was by far the most abundant emerging nematode. In the non-augmented control treatments, FLN made up the majority of the nematodes emerging from the baits (Fig 5).

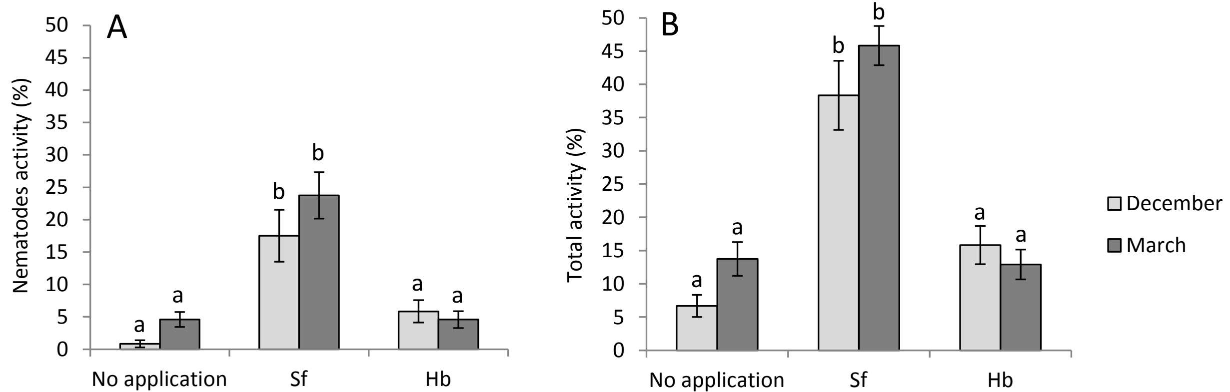


**Fig 4**. Activity recorded from *Galleria mellonella* baiting. The bars represent the average percentage of larval mortality. **A.** Mean activity of nematodes (EPN + FLN) and **B.** Mean activity of all organisms (EPN + FLN + Other organisms) depending on the application treatment (No EPN = no application, Sf = application of *S. feltiae* and Hb = application of *H. bacteriophora*) for both December and March sampling. Data are shown as mean activity ± SEM. Different letters indicate statistically significant differences.

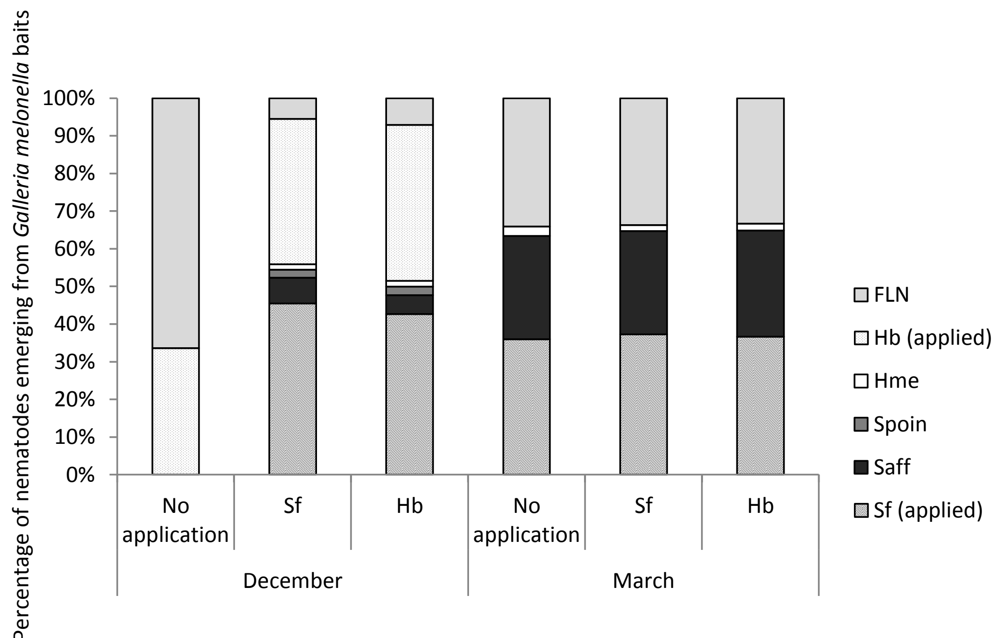


**Fig 5**. Species composition of EPN and FLN emerging from baited *Galleria mellonella* larvae with respect to the application treatment. Proportions were obtained from Ct values of qPCR species-specific quantification of each of the organisms in the CC27 field trial. *Steinernema affine* (Saff), *S. carpocapsae* (Sca), *S. feltiae* (Sf), *S. poinari*, (Spoin), *S. kraussei-silvaticum* (Skr), *Heterorhabditis bacteriophora* (Hb), *H. megidis* (Hm), Free-living nematodes (FLN).

**Table 2:** Species survey of entomopathogenic nematodes, nematophagous fungi and free-living nematodes (or group) in the CC27 and CC29 field. In parenthesis are the percentages of detection over all plots; the mean number of IJs for EPN, mean IR for NF, mean corrected data for FLN

| Field | Sampling period | Date | EPN qPCR (N of detection; mean IJs) | NF qPCR (N of detection; mean IR) | FLN qPCR (N of detection; mean corrected data) |
|---|---|---|---|---|---|
| CC27 | T0 | 18/11/2014 | *Heterorhabditis bacteriophora* (19; 0.46)<br>*Steinernema poinari* (8; $9^{10-3}$)<br>*Steinernema affine* (3; $8^{10-3}$) | *Arthrobotrys oligospora* (92; 0.47)<br>*Hirsutella rhossiliensis* (78; 0.27)<br>*Paecilomyces lilacinus* (47; 0.47)<br>*Catenaria sp* (3; 5.310-6) | *Acrobeloides group* (100; 0.38)<br>*Oscheius tipulae* (89; 0.18)<br>*Oscheius onirici* (86; 0.26) |
| | T1 | 09/12/2014 | *Heterorhabditis bacteriophora* (50; 65.8)<br>*Steinernema feltiae* (42; 10.8)<br>*Steinernema poinari* (8; $1^{10-3}$)<br>*Steinernema caporcapsae* (6; 0.15)<br>*Heterorhabditis megidis* (3; $4^{10-3}$)<br>*Steinernema kraussei-silvaticum* (6; $3^{10-3}$)<br>*Steinernema affine* (3; 0.03) | *Arthrobotrys oligospora* (86; 0.34)<br>*Hirsutella rhossiliensis* (86; 0.30)<br>*Paecilomyces lilacinus* (72; 0.19) | *Acrobeloides group* (100; 0.02)<br>*Oscheius onirici* (92; 0.18)<br>*Oscheius tipulae* (86; 0.11) |
| | T2 | 11/03/2015 | *Heterorhabditis megidis* (47; 0.62)<br>*Steinernema feltiae* (42; 5.47)<br>*Heterorhabditis bacteriophora* (33; 15.97)<br>*Steinernema affine* (3; 0.19) | *Paecilomyces lilacinus* (78. 0.29)<br>*Arthrobotrys oligospora* (64; 0.33)<br>*Hirsutella rhossiliensis* (53. 0.31) | *Acrobeloides group* (100; 0.43)<br>*Oscheius onirici* (92; 0.14)<br>*Oscheius tipulae* (75; 0.10) |
| CC29 | T1 | 09/10/2014 | *Steinernema feltiae* (8; 0.11)<br>*Heterorhabditis bacteriophora* (4; 0.01)<br>*Steinernema poinari* (4; $3.4^{10-4}$) | *Hirsutella rhossiliensis* (67; 0.18)<br>*Arthrobotrys oligospora* (58; 0.26)<br>*Paecilomyces lilacinus* (42; 0.15) | *Acrobeloides group* (100; 0.11)<br>*Oscheius tipulae* (63; 0.08)<br>*Oscheius onirici* (54; 0.10) |
| | T2 | 18/11/2014 | *Heterorhabditis bacteriophora* (8; 1.06)<br>*Steinernema poinari* (4; $1.1^{10-4}$) | *Arthrobotrys oligospora* (63; 0.27)<br>*Hirsutella rhossiliensis* (50; 0.29)<br>*Paecilomyces lilacinus* (29; 0.14) | *Acrobeloides group* (100; 0.17)<br>*Oscheius tipulae* (36; 0.24)<br>*Oscheius onirici* (9; 0.11) |
| | T3 | 11/03/2015 | *Heterorhabditis megidis* (70; 0.62)<br>*Steinernema poinari* (4; $2.1^{10-3}$) | *Paecilomyces lilacinus* (86; 0.32)<br>*Arthrobotrys oligospora* (63; 0.32)<br>*Hirsutella rhossiliensis* (88; 0.15) | *Acrobeloides group* (100; 0.31)<br>*Oscheius tipulae* (83; 0.15)<br>*Oscheius onirici* (33; 0.13) |

N = number, IJs = Infective juvenile, IR = Infection rate

### *3.4. Impact of cover crop, tillage and soil type on the native populations of entomopathogenic nematodes*

None of the three treatments, i.e. pea cover crop *versus* bare soil, tillage *versus* direct seeding and soil type (clay loam soil *versus* clay soil) had an influence on the natural population densities of EPN observed in the CC29 field (Table 3), ($P = 0.5$, $P = 1$ and $P = 0.4$ respectively). The very low abundance of native EPN made it difficult to statistically detect such effects.

At T1 (before tillage), we detected 3 naturally occurring EPN species: *S. poinari*, *H. bacteriophora* and *S. feltiae* (Table 2). The number of IJ's was even lower than in the CC27 field at the baseline sampling period T0 (0.12 ± 0.1 per 100g of dry soil).

At T2, two months after half of the plots were prepared for direct seeding and half of the plots were tilled, we detected fewer EPN species than at T1; *S. feltiae* was absent (Table 2). The most abundant species was *H. bacteriophora*, which was found in only two plots, with a mean number of IJs of 1.0±0.9 per 100g of dry soil. Only a few individuals were detected for *S. poinari* (only in one plot).

At T3, after winter, we detected *S. feltiae* in only one plot and *H. bacteriophora* was no longer detected (Table 2). However, as in CC27, *H. megidis* appeared for the first time in the field with a mean number of IJs of 0.6±0.2 per 100g of soil.

### *3.5. Impact of cover crop, tillage and soil type on the natural enemies and competitors of entomopathogenic nematodes*

#### *3.5.1. Nematophagous fungi and ectoparasitic bacteria*

In field CC29, the cover crop treatment had a significant effect on the community of nematophagous fungi ($P < 0.01$), with more NF in bared soil compared to soil covered with pea (Fig 6A). Apart from the cover treatment, only the soil texture affected the occurrence of NF, with more NF found in clay soil than in clay loam soil ($P < 0.001$) (Table 3, Fig 6B). At T1, we detected the presence of the same three species of NF that were also found in CC27 field (Table 2). The calculated IR was 0.6±0.1. At T2, we found the same species as at T1 (Table 2). The calculated IR was slightly higher than at T1 with a value of 0.7±0.1. At T3, we observed a change in NF community structure with some differences in species prevalence (Table 2), and with an IR value as high as 0.8±0. The ectoparasitic bacteria *P. nematophilus* was not detected in any of the samples.

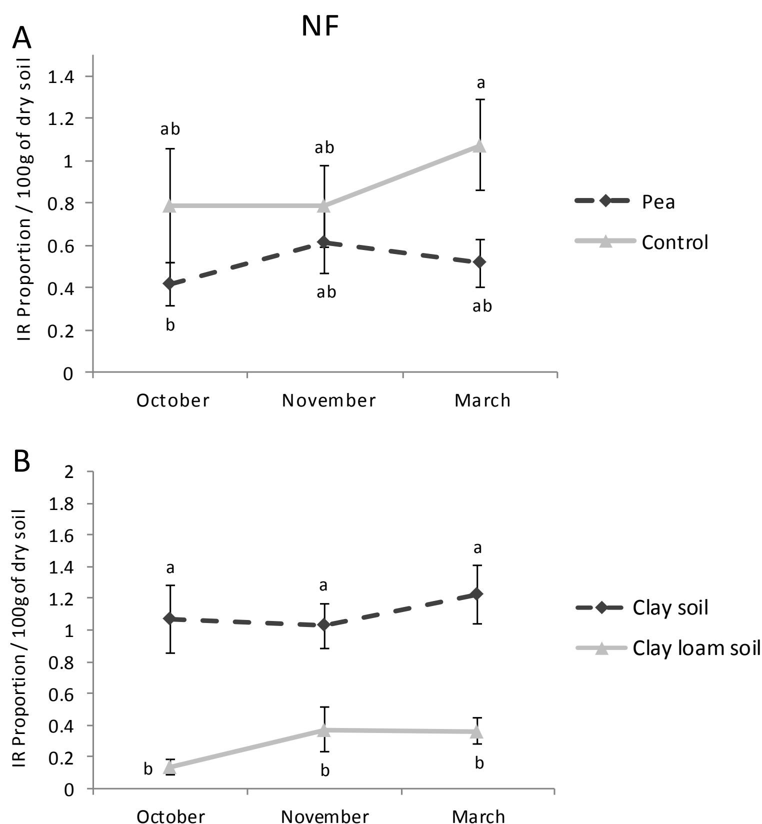


Fig 6. Natural occurrence of nematophagous fungi (NF) along the period of sampling depending on: **A.** cover crop (control = bare soil, pea = plot covered with pea *Pisum sativum*) and **B**. the soil types. Data are shown as means of IR proportion ±SEM. Different letters indicate statistically significant differences.

*3.5.2. Free-living nematodes*

The cover crop treatments did not have any impact on the species community of free living nematodes (*P* = 1) (Table 3), but there presence was significantly affected by tillage treatments, with more FLN in plots that were not tilled (direct seeding) (P < 0.01) (Table 3, Fig 7A). The FLN were also more numerous in clay soil than in clay loam soil (P < 0.01), and their number was increased during the sampling period (P < 0.01) (Table 3, Fig 7B).

At T1, T2 and T3, the FLN community structure was similar, with at least one of the FLN species or group found in all the plots (Table 2). However, FLN numbers as judged by the corrected values, was quite different for the three sampling time points with a value of 0.29±0.1 at T1 that increased at T2 and T3 to 0.52±0.1 and 0.58±0.1, respectively (Table 1, Fig. 7). *Oscheius* sp. 3 was not detected in any sample at any sampling time.

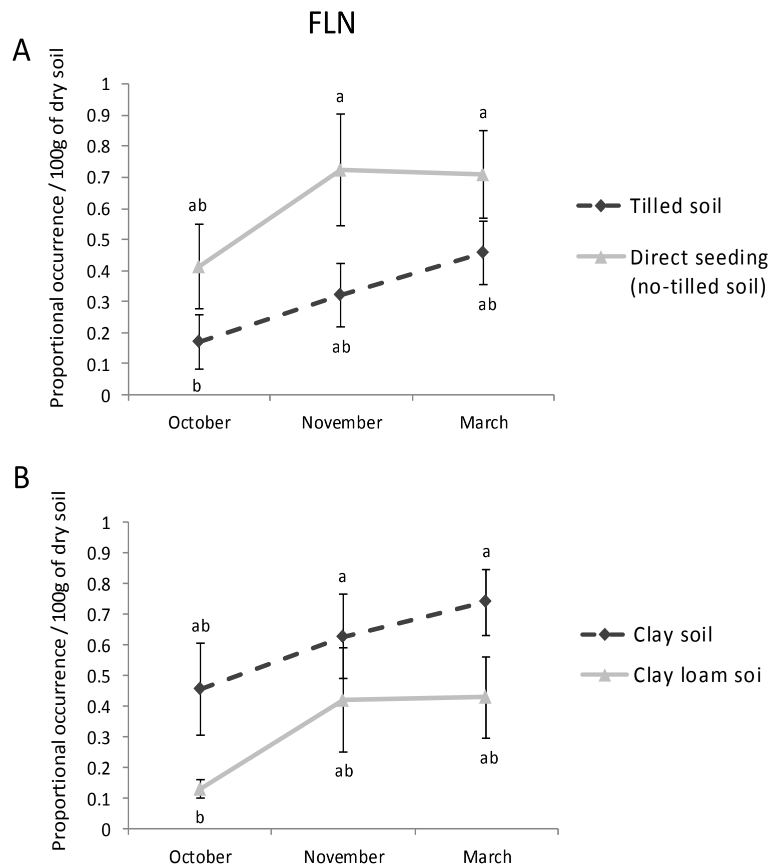


Fig 7. Natural occurrence of free-living nematodes (FLN) along the period of sampling depending on: **A**. tillage practice and **B**. soil type. Data are shown as means of proportional occurrence ±SEM. Different letters indicate statistically significant differences

**Table 3 (CC29):** Effect of cover crop, EPN application, soil type, tillage, as well as sampling period on activity and occurrence of various soil organisms and on microbial biomass. Statistics are presented as $F_{df}$ (for permutation analyses); probability levels: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, n.s., not significant. Natural occurrence of the organisms was determined by qPCR and microbial biomass was detected by CFE.

| Soil organism | Soil type (S) | Cover (C) | Sampling period (P) | Till (T) | S*C | S*P | S*T | C*P | C*T | P*T | C*T*S | S*C*P | S*P*T | C*P*T | S*C*P*T |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Df = 1 | Df = 1 | Df = 1 | Df = 2 | Df = 1 | Df = 2 | Df = 1 | Df = 2 | Df = 1 | Df = 2 | Df = 2 | Df = 2 | Df = 2 | Df = 2 | Df = 32 |
| Entomopathogenic nematodes | | | | | | | | | | | | | | | |
| Total IJs | n.s. | n.s. | n.s | n.s. | ns | n.s. | n.s. | n.s. | 2.56* | 3.68* | n.s. | n.s. | n.s. | n.s. | n.s. |
| Free-living nematodes | | | | | | | | | | | | | | | |
| IJs or ng DNA, (0-1) interval-scaled | 7.12** | n.s. | 4.31** | 6.77** | n.s. | n.s. | n.s. | n.s. | n.s. | n.s | n.s. | n.s. | n.s. | n.s. | n.s. |
| Nematophagous fungi | | | | | | | | | | | | | | | |
| Total IR[1] | 66.77*** | 8.73** | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s | n.s. | n.s. | n.s. | n.s. | n.s. |

[1] IR, infection rate for the nematophagous fungi (NF), equivalent to pg NF DNA / total DNA

### *3.6. Microbial biomass carbon ($C_{mic}$) and nitrogen ($N_{mic}$) assessment*

In field CC27, in which we measured microbial biomass carbon ($C_{mic}$) and nitrogen ($N_{mic}$), the cover crops affected $C_{mic}$ in interaction with the sampling period, with significant higher values recorded in covered plots (both pea and mustard) ($P < 0.01$) (Table 1, Fig. 8). The $C_{mic}$ and $N_{mic}$ values were significantly different for the different sampling periods and increased over the season ($P < 0.001$ and $P < 0.01$ respectively) (Table 1). Finally, $C_{mic}$ and $N_{mic}$ values were not affected by the EPN application ($P = 0.8$, and $P = 1$ respectively) (Table 1).

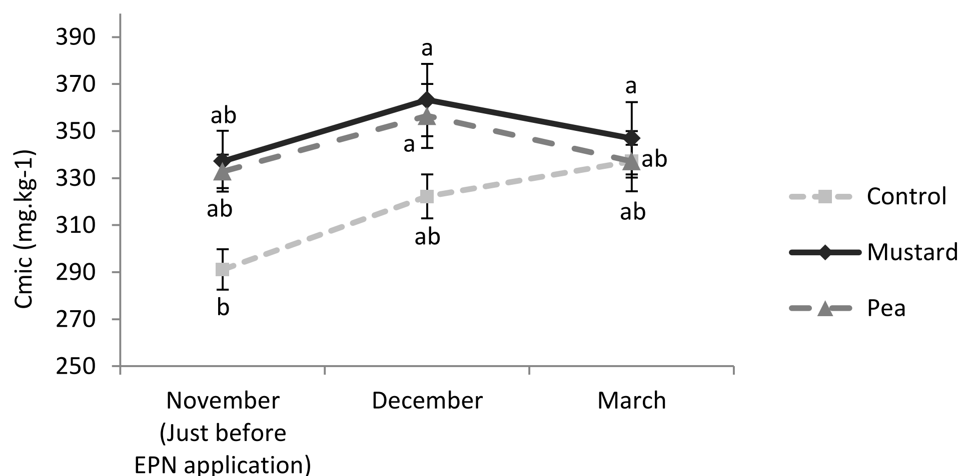


Fig 8. $C_{mic}$ measurement along the sampling period depending on the crop cover type (pea, mustard *versus* bare soil). Data are shown as mean $C_{mic}$ per mg $kg^{-1}$. Different letters indicate statistically significant differences.

## 4. Discussion

The effect of cover crops on EPN persistence was only evident at the middle of the winter season, and only in the plots augmented with *S. feltiae* (Fig. 2). At the end of the winter we no longer found a direct significant effect of cover crop on EPN presence in any of the two investigated fields, neither in the control (natural presence only) nor in the augmented EPN plots. However, the data showed a significant interaction among cover crop treatment, EPN application, and sampling date. These results are encouraging and indicate that cover crops may play a more important role at a specific period of the season and that they affect different species of EPN differently. Overall, the persistence of the applied EPN in the CC27 field was relatively good and actually better than persistence of the same species applied in 2013 by Chiriboga, Imperiali (in prep). We observed a substantial drop in EPN numbers from December to March. However, the recorded activity of nematodes (EPN+FLN) in CC27 was higher in March, four months after augmentation, than in December, one month after augmentation, which suggests that the EPN that survived through winter were considerably more infectious. Alternatively or simultaneously, the highly infectious EPN at the end of the winter may be the result of nematodes that were recycled in the field and thus represent a new generation of IJs in the soil. This indicates that, if suitable hosts are available, EPN have great potential to persist at this field site and that applying nematodes would only be needed once per year. Such applications would be necessary in case of pest problems, as natural population densities of EPN were found to be very low. The observed low numbers are in agreement with a previous study at the same field site and in four other fields at Agroscope Changins (Switzerland) (Campos-Herrera et al. 2015b Chiriboga, Imperiali, in prep).

Cover crops did not enhance the natural occurrence of EPN in CC29, and their numbers remained very low at all sampling points. Interestingly, in CC27 the number of EPN found in March was higher than in a previous experiment at very close field sites (Chirigoba, Imperiali in prep). Different soils, agricultural practices and microclimate conditions may explain these results, but we may also hypothesize that the field plots selected for the cover crop experiments was more suitable to EPN.

As mustard produces volatiles that are detrimental to EPN (Ramirez et al., 2008; Henderson et al. 2009), we could have expected a negative effect of mustard plants on EPN. Yet, we observed no difference in EPN persistence between pea and mustard cover plots. The harmful chemicals (glucosinolates) naturally produced by mustard plants have been found to suppress plant-parasitic nematodes (Snapp et al., 2005). Our results suggest that they have no such effect on EPN, this can be explained by the fact that glucosinolates are harmless in undamaged plants, where they are stored in the vacuole. They become toxic when the vacuoles are damaged and the glucosinolates come in contact with myronase, an enzyme that cleaves off the thio-linked glucose, resulting in the release of toxic isothiocyanates, thiocyanates, and nitriles (Chen & Andreasson,

2001). Therefore, the toxicity of glucosinolates is only relevant when the plants are damaged, as is the case of an attack by plant-parasitic nematodes.

In this study we also considered the infectivity potential of the EPN species employed. In our samples, even if applied at the same concentrations, the numbers of *H. bacteriophora* IJs that were detected by qPCR in field CC27 were on average five times higher than *S. feltiae*. However, the plots that showed the highest suppressive activity (infection rates of sentinel *Galleria mellonella* larvae) were the plots augmented with *S. feltiae*. *H. bacteriophora* was not even detected in any larvae baited in March. We can be confident that the activity was due to the EPN that were applied, because the qPCR analyses revealed that the augmented species did not move from the plots where they were applied. We found a few IJs from *H. bacteriophora* and *S. feltiae* in control plots or in plots where they were not applied, but their numbers were equivalent to what is known for their natural occurrence in the area (Campos-Herrera et al., 2015b). This implies that released EPN do not invade non-targeted sites. We can conclude that under the studied field conditions, *S. feltiae* persisted less well in the field, but at the end of season they were far more infective than *H. bacterophora*. *S. feltiae* therefore seems to be a better species to use for augmentation in Southwest Switzerland.

Previous surveys of Swiss soils suggest strong pressures by natural enemies and competitors (Campos-Herrera et al., 2015b, 2015c; Jaffuel et al., 2016), which could also explain the low numbers of EPN at the end of the season in the current study. We investigated these biotic factors and indeed found relatively high abundance of FLN and NF, which possibly had a negative impact on the EPN numbers. Similar to what was found by Campos-Herrera et al. (2015b) there was a strong effect of soil texture on the EPN antagonists, with more FLN and NF in clay soil than in clay loam soil (Fig. 6 and 7). Also, the FLN were more abundant in soil with direct seeding than in tilled soil confirming that FLN are sensitive to tillage (Campos-Herrera et al., 2015b).

The CC29 field has been subjected to different tillage treatments since 1969. Tillage practices impact the soil characteristics, and notably the soil organic matter content, which is known to be higher in no till plots (in clay soil) (Büchi et al., 2015). FLN depend greatly on the soil organic matter content, and therefore their higher abundance in direct seeding (no-till) plots may be explained by better soil conditions and fertility. In CC27, following the same pattern as EPN, FLN and NF were neither affected by the cover crop nor by the augmentation of EPN treatment. This implies that, under alike conditions, the EPN augmentation did not trigger a trophic cascade. This is different from previous studies (Campos-Herrera et al. 2013; Griffin, 2015; Lewis et al., 2015), which reported an increase of NF and FLN in plots augmented with EPN. The fact that we did not detect a trophic cascade following application of EPN in the CC27 field may be due to cold temperature that prevented FLN and NF to develop quick enough to react. It also means that the relatively minor effect of the cover crops on EPN persistence is probably not due to these antagonists.

We also measured microbial biomass carbon ($C_{mic}$) and nitrogen ($N_{mic}$) in the CC27 field as a proxy for soil health to be correlated with EPN augmentation. The microbial biomass proxies $C_{mic}$ and $N_{mic}$ were not influenced by the augmentation of EPN. The recorded $C_{mic}$ and $N_{mic}$ values were higher in covered plots, but there was not a clear significant effect. Like for EPN, the interaction of the cover crop treatment and the sampling period was significant for $C_{mic}$. Again, the effect of the cover crop on microbial biomass may depend on the period of the year. This fits the results of a meta-analysis, which revealed a mean increase of 20.7% of $C_{mic}$ and 21.6% of $N_{mic}$ in long-term rotation culture with a cover crop system as compared to a monoculture (McDaniel et al. 2014).

Overall, we did not find a strong positive effect of cover crops on EPN persistence, neither in control nor in the augmented plots. The only significant effect was observed in the middle of the winter for *S. feltiae*. Independent of cover crop, the overall persistence of the augmented EPN was very promising; the released EPN were detected in all of the augmented plots at the end of the season. Ongoing studies focus on alternative application methods that can enhance survival, prevalence and activity of the nematodes for prolonged periods of time.

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### References

Abdollahi L, Munkholm LJ. 2014. “Tillage system and cover crop effects on soil quality: I. Chemical, mechanical, and biological properties.” *Soil Science Society of America Journal,* 78: 262–270. doi:10.2136/sssaj 2013.07.0301

Atkins SD, Clark IM, Pande S, Hirsch PR and Kerry BR. 2005. “The use of real-time PCR and species-specific primers for the identification and monitoring of *Paecilomyces Lilacinus.*” *FEMS Microbiology Ecology,* 51 (2): 257–264. doi:10.1016/j.femsec.2004.09.002.

Büchi L, Amossé C, Wendling M, Sinaj S, Charles R. 2015. “Introduction of no till in a long term experiment on soil tillage in Switzerland.” *Aspect of Applied Biology,* 128: 49–56.

Campos-Herrera R, Ali JG, Diaz BM, Duncan LW. 2013a. “Analyzing spatial patterns linked to the ecology of herbivores and their natural enemies in the soil.” *Frontiers in Plant Science,* 4: 1–18.

Campos-Herrera R, El-Borai FE, and Duncan LW. 2012. “Wide interguild relationships among entomopathogenic and free-living nematodes in soil as measured by real time qPCR.” *Journal of Invertebrate Pathology,* 111: 126–135. doi:doi: 10.1016/j.jip.2012.07.006.

Campos-Herrera R, El-Borai FE, Duncan LW. 2015a. “Modifying soil to enhance biological control of belowground dwelling insects in citrus groves under organic agriculture in Florida.” *Biological Control*, 84: 53-63 doi:10.1016/j.biocontrol.2015.02.002

Campos-Herrera R, Jaffuel G, Chiriboga X, Fesselet M, Půža V, Blanco-Pérez R, Mascher F and Turlings T.C.J. 2015b. “Traditional and molecular detection methods reveal intense interguild competition and other multitrophic interactions associated with native entomopathogenic nematodes in Swiss tillage soils.” *Plant and Soil*, 389(1): 237-255. doi:10.1007/s11104-014-2358-4.

Campos-Herrera R, Půža V, Jaffuel G, Blanco-Pérez R, Čepulytė-Rakauskienė R, Turlings TCJ. 2015c. “Unraveling the intraguild competition between *Oscheius* spp. nematodes and entomopathogenic nematodes: Implications for their natural distribution in Swiss agricultural soils.” *Journal of Invertebrate Pathology,* 132: 216–227. doi:10.1016/j.jip.2015.10.007

Campos-Herrera R, Pathak E, El-borai FE, Schumann A, Abd-Elgawad MMM and Duncan LW. 2013b. “New citriculture systems suppresses native and augmented entomopathogenic nematodes.” *Biological Control,* 66 (3): 183–194. doi:10.1016/j.biocontrol.2013.05.009. doi: 10.1016/j.biocontrol.2013.05.009.

Campos-Herrera, R. 2015. Ed., "Nematode Pathogenesis of Insects and Other Pests." *Springer International Publishing Switzerland.*, 97-137 doi:10.1007/978-1-4419-7931-5.

Campos-Herrera R, El-Borai FE, Stuart RJ, Graham JH and Duncan LW. 2011. “Entomopathogenic nematodes , phoretic *Paenibacillus* spp., and the use of real time quantitative PCR to explore soil food webs in Florida citrus groves.” *Journal of Invertebrate Pathology*, 108(1): 30–39. doi:10.1016/j.jip.2011.06.005.

Cappaert DL, Koppenh AM. 2003. “*Steinernema scarabaei*, an entomopathogenic nematode for control of the European chafer.” *Biological Control,* 28: 379–386. doi:10.1016/S1049-9644(03)00118-X

Chen S, Andreasson E. 2001. “Update on glucosinolate metabolism and transport”. *Plant Physiology and Biochemistry*, 39: 743–758. doi:10.1016/S0981-9428(01)01301-8

Dabney SM, Reeves DW, Delgado JA. 2001. “Using winter cover crops to improve soil and water quality. *Communication in Soil Science and Plant Analysis,* 32: 1221–1250. doi:http://hdl.handle.net/10113/30335

de Rooij-van der Goes PCEM, van der Putten WH and van Dijk C. 1995. "Analysis of nematodes and soil-borne fungi from *Ammophila arenaria* (Marram grass) in Dutch coastal foredunes by multivariate techniques. *European Journal of Plant Pathology,* 101: 149–162.

Duncan LW, Stuart RJ, El-Borai FE, Campos-Herrera R, Pathak E, Giurcanu M, and Graham JH. 2013. "Modifying orchard planting sites conserves entomopathogenic nematodes, reduces weevil herbivory and increases citrus tree growth, survival and fruit yield." *Biological Control,* 64: 26–36. doi:10.1016/j.biocontrol.2012.09.006

DuPont ST, Ferris H, Van Horn M. 2009. “Effects of cover crop quality and quantity on nematode-based soil food webs and nutrient cycling.” *Applied Soil Ecology,* 41: 157–167. doi:10.1016/j.apsoil.2008.10.004

Elmowitz DE, Ebssa L, Koppenh AM. 2013. “Overwintering behavior of the entomopathogenic nematodes *Steinernema scarabaei* and *Heterorhabditis bacteriophora* and their white grub hosts. *Entomologia Eperimentalis et Applicata*, 246–258. doi:10.1111/eea.12092

Enright MR, Griffin CT. 2005. "Effects of *Paenibacillus nematophilus* on the entomopathogenic nematode *Heterorhabditis megidis*.” *Journal of Invertebrate Pathology,* 88: 40–48. doi:10.1016/j.jip.2004.10.002

Ewing RP, Wagger MG, Denton HP. 1991. “Tillage and cover crop management effects on soil water and corn yield.” *Soil Science Society of America,* 55: 1081. doi:10.2136/sssaj1991.03615995005500040031

Fageria NK, Baligar VC, Bailey BA. 2005. “Role of cover crops in improving soil and row crop productivity.” *Communication of Soil Science and Plant Analysis,* 36: 2733–2757. doi:10.1080/00103620500303939

Ferris H, Sánchez-Moreno S, Brennan EB. 2012. “Structure, functions and interguild relationships of the soil nematode assemblage in organic vegetable production.” *Applied Soil Ecology,* 61: 16–25. doi:10.1016/j.apsoil.2012.04.006

Gómez JA, Guzmán MG, Giráldez JV, Fereres E. 2009. “The influence of cover crops and tillage on water and sediment yield, and on nutrient, and organic matter losses in an olive orchard on a sandy loam soil.” *Soil Tillage Research,* 106: 137–144. doi:10.1016/j.still.2009.04.008

Griffin CT. 2015. “Behaviour and population dynamics of Entomopathogenic nematodes following application.” in Campos-Herrera, R. Ed., "Nematode Pathogenesis of Insects and Other Pests." *Springer International Publishing Switzerland,* 57-97 doi:10.1007/978-1-4419-7931-5.

Henderson DR, Riga E, Ramirez RA, Wilson J, Snyder WE. 2009. Mustard biofumigation disrupts biological control by *Steinernema* spp. nematodes in the soil. *Biological Control,* 48: 316–322. doi:10.1016/j.biocontrol.2008.12.004

Ito T, Araki M, Higashi T, Komatsuzaki M, Kaneko N, Ohta H. 2015. “Responses of soil nematode community structure to soil carbon changes due to different tillage and cover crop management practices over a nine-year period in Kanto, Japan.” *Applied Soil Ecology,* 89: 50–58. doi:10.1016/j.apsoil.2014.12.010

Ito T, Araki M, Komatsuzaki M, Kaneko N, Ohta H. 2015. “Soil nematode community structure affected by tillage systems and cover crop managements in organic soybean production.” *Applied Soil Ecology* 86: 137–147. doi:10.1016/j.apsoil.2014.10.003

Jaffuel, G, Mäder, P, Blanco-perez, R, Chiriboga, X, Fliessbach, A, Turlings, T. C. J, campos-herrera, R. 2016. Prevalence and activity of entomopathogenic nematodes and their antagonists in soils that are subject to different agricultural practices. *Agriculture Ecosystems Environment,* 230:329–340.

Hargrove WL. Cover crops for clean water. 1991. *Ankeny, IA Soil Water Conservation Society.*

Komatsuzaki M, Ohta H. 2007. “Soil management practices for sustainable agro-ecosystems.” *Sustainability Science,* 2: 103–120. doi:10.1007/s11625-006-0014-5

Lacey LA, Grzywacz D, Shapiro-Ilan DI, Frutos R, Brownbridge M, Goettel MS. 2015. “Insect pathogens as biological control agents: Back to the future.” *Journal of Invertebrate Pathology,* 132: 1–41. doi:10.1016/j.jip.2015.07.009

Lal R. 2009. “Soils and food sufficiency: A review." *Agronomy for Sustainable Development,* 29: 25–49. doi:10.1007/978-90-481-2666-8_4

Lewis EE, Hazir S, Hodson A, Gulcu B. 2015. “Trophic relationships of entomopathogenic nematodes in agricultural habitats.” In: *Nematode Pathogenesis of Insects and Other Pests* (ed. Campos-Herrera R), pp. 139-163. Springer International Publishing, Switzerland.

McDaniel MD, Tiemann LK, Grandy AS. 2014. “Does agricultural crop diversity enhance soil microbial biomass and organic matter dynamics ? A meta-analysis.” *Ecological Applications,* 24: 560–570.

Pathak E, El-Borai F, Campos-Herrera R, Johnson EG, Stuart RJ, Graham JH, Duncan LW and Pell J. 2012. “Use of real-time PCR to discriminate parasitic and saprophagous behaviour by nematophagous fungi.” *Fungal Biology,* 116 (5): 563–573. doi:10.1016/j.funbio.2012.02.005.

Piedra-Buena A, López-Cepero J, Campos-Herrera R. 2015. "Entomopahtogenic nematode production and application: Regulation, ecological impact and non-target effects." in Campos-Herrera, R. Ed., "Nematode Pathogenesis of Insects and Other Pests." *Springer International Publishing Switzerland* 255-282 doi:10.1007/978-1-4419-7931-5.

Ramirez RA, Henderson DR, Riga E, Lacey LA, Snyder WE. 2009. “Harmful effects of mustard bio-fumigants on entomopathogenic nematodes.” *Biological Control,* 48: 147–154. doi:10.1016/j.biocontrol.2008.10.010

Stuart RJ, Barbercheck ME, Grewal PS, Taylor RAJ and Hoy CW. 2006. “Population biology of entomopathogenic nematodes: concepts, issues, and models.” *Biological Control,* 38(1): 80–102. doi:10.1016/j.biocontrol.2005.09.019.

Stuart RJ, Barbercheck ME and Grewal PS. 2015. "Entomopathogenic nematodes in the soil environement: distributions, interactions and the influence of biotic and abiotic factors." in Jenkins W.R. 1964. "A rapid centrifual-flatation technique for separating nematodes from soil." *Plant disease Reporter,* 48, 492

Snapp, SS, Swinton SM, Labarta R, Mutch D, Black JR, Leep R, Nyiraneza J, and O'Neil K. 2005. “Evaluating cover crops for benefits, costs and performance within cropping system niches.” *Agronomy Journal,* 97:1-11.

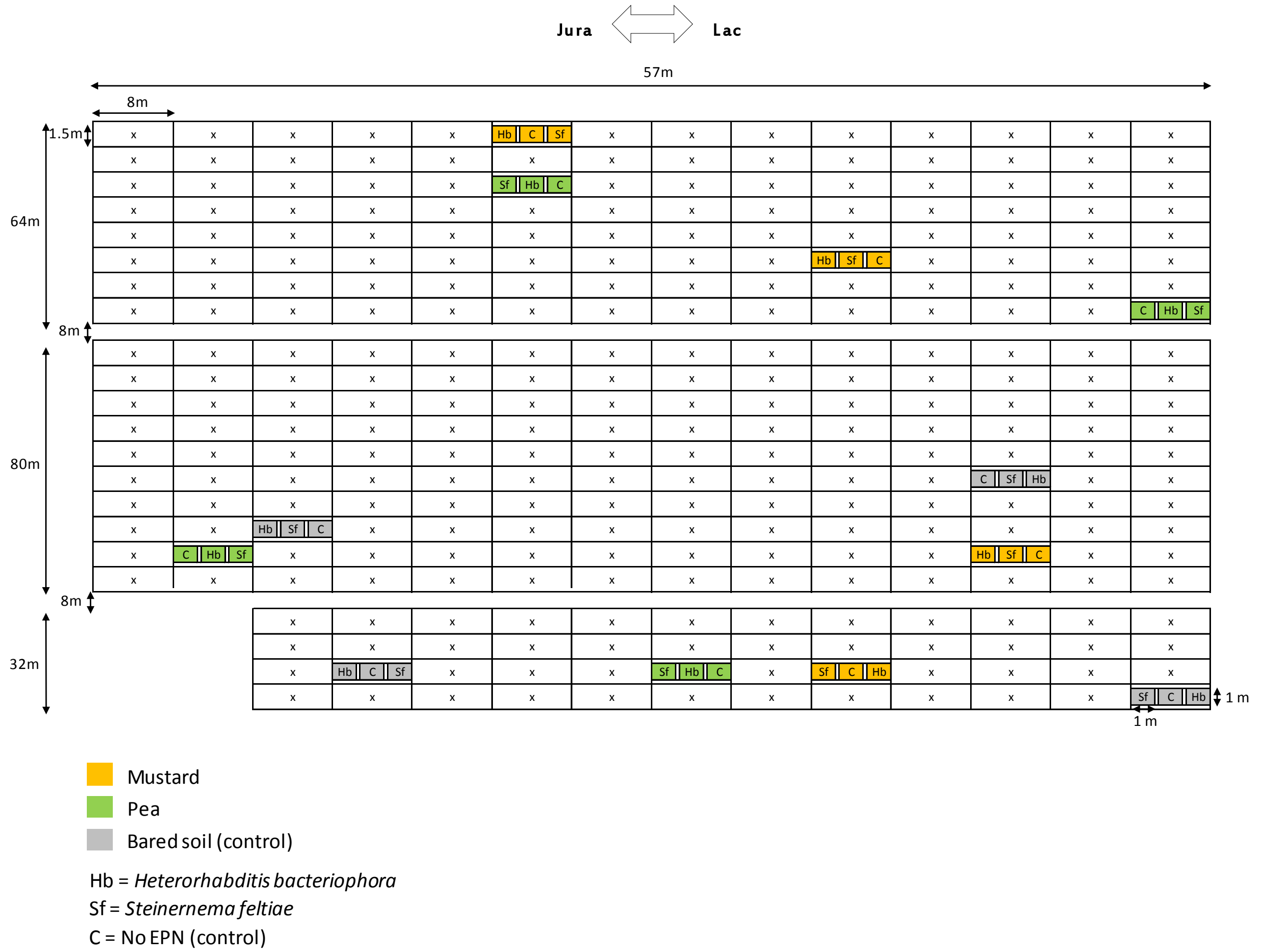
Susurluk A, Ehlers RU. 2008. “Field persistence of the entomopathogenic nematode *Heterorhabditis bacteriophora* in different crops.” *BioControl,* 53: 627–641. doi:10.1007/s10526-007-9104-2

Verhulst N, Govaerts B, Verachtert E, Castellanos-Navarrete A, Mezzalama M, Wall P, Chocobar A, Deckers J, Sayre KD. 2010. “Conservation agriculture, improving soil quality for sustainable production systems?” In: R. Lal and B.A.E. Stewart, editors, Food security and soil quality. *Advance in Soil Science*. CRC Press, Boca Raton FL. pp. 137–208.

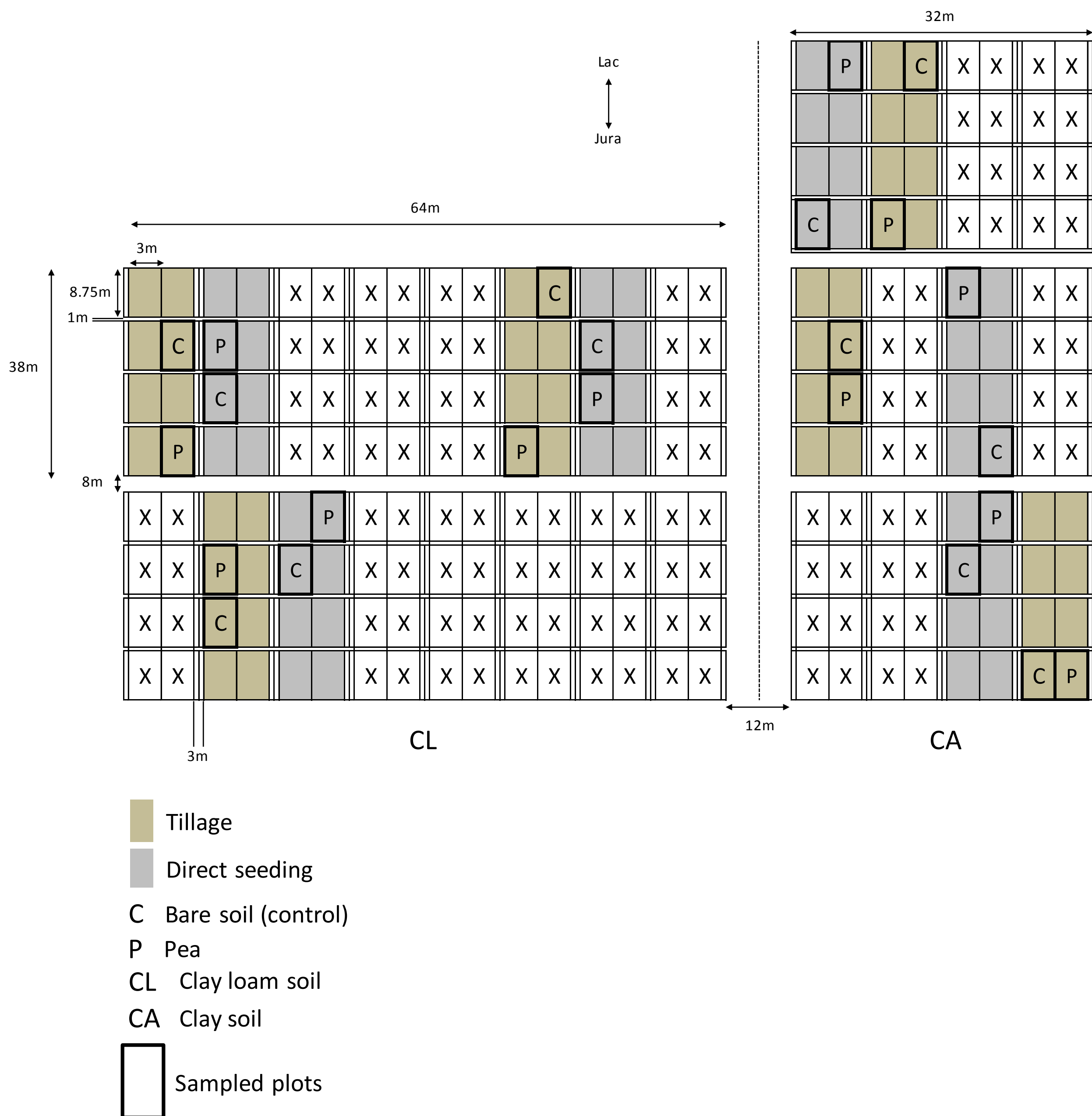
Zhang L, Liu X, Zhu S, Chen S. 2006. “Detection of the nematophagous fungus *Hirsutella rhossiliensis* in soil by real-time PCR and parasitism bioassay.” *Biological Control,* 36: 316–323. doi:10.1016/j.biocontrol.2005.08.002

## Supplementary material

Supplementary material 1, SFig1. CC27 plot distribution



Supplementary material 2. SFig 2. CC29 plots distribution



Supplementary data 3. Stable 1: Species and sources of nematodes, fungi and bacterium

| Type of organism/ species | Population | Material used/Unit of measurements | GenBank accession number |
|---|---|---|---|
| Nematodes: Entomopathogenic nematodes | | | |
| *Heterorhabditis bacteriophora* | Commercial | Infective juvenile (IJs) / no. IJs | KJ93576* |
| *Heterorhabditis megidis* | Commercial | Infective juvenile (IJs) / no. IJs | KJ938577* |
| *Steinernema affine* | CH | Infective juvenile (IJs) / no. IJs | KJ938567* |
| *Steinernema carpocapsae* | DOK-83 | Infective juvenile (IJs) / no. IJs | KJ818295* |
| *Steinernema feltiae* | RS-5 | Infective juvenile (IJs) / no. IJs | KJ938569* |
| *Steinernema kraussei* | OS | Infective juvenile (IJs) / no. IJs | KJ696686 |
| *Steinernema poinari* | 1160 | ITS rDNA sequence + pUC57 / pg DNA | KF241754 |
| Nematodes: Free-living and competitors nematodes | | | |
| *Acrobeloides*-group | RT1-R15C | 18S rDNA sequence + pUC57 / pg of DNA | JQ237849 |
| Nematophagous fungi | | | |
| *Catenaria* sp. | 1D | ITS rDNA sequence + pUC57 / pg of DNA | JN585805 |
| *Arthrobotrys dactyloides* | H55 | Pure culture / pg of DNA | KJ938574 |
| *Arthrobotrys musiformis* | 11 | Pure culture / pg of DNA | KJ938572 |
| *Arthrobotrys oligospora* | 8 | Pure culture / pg of DNA | KJ938573 |
| *Hirsutella rhossiliensis* | 2931 | Pure culture / pg of DNA | - |
| *Paecilomyces lilacinus* | 9357 | Pure culture / pg of DNA | KJ938575B* |
| Ectoparasitic bacteria | | | |
| *Paenibacillus nematophilus* | NEM2 | 16S rDNA sequence of 490 bp + pUC57 / copy numbers | AF480936 |

[a] Sequences generated for this study are followed by *.

# Annex

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RESEARCH PAPER

# The dual effects of root-cap exudates on nematodes: from quiescence in plant-parasitic nematodes to frenzy in entomopathogenic nematodes

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# Enhanced alginate capsule properties as a formulation of entomopathogenic nematodes

**Jinwon Kim · Geoffrey Jaffuel ·
Ted C. J. Turlings**

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Plant Soil
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REGULAR ARTICLE

# Traditional and molecular detection methods reveal intense interguild competition and other multitrophic interactions associated with native entomopathogenic nematodes in Swiss tillage soils

Raquel Campos-Herrera · Geoffrey Jaffuel · Xavier Chiriboga · Rubén Blanco-Pérez · Marie Fesselet · Vladimír Půža · Fabio Mascher · Ted C. J. Turlings

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# Unraveling the intraguild competition between *Oscheius* spp. nematodes and entomopathogenic nematodes: Implications for their natural distribution in Swiss agricultural soils

Raquel Campos-Herrera [a,*], Vladimir Půža [b], Geoffrey Jaffuel [a], Rubén Blanco-Pérez [a], Rasa Čepulytė-Rakauskienė [c], Ted C.J. Turlings [a]

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## Publications in preparation

Campos-Herrera, R., **Jaffuel, G.,** Chiriboga, X., Blanco-Pérez, R., Hug, A.-S., Meuli, R.G., Mascher, F., Turlings, T.C.J. Evaluation of entomopathogenic nematode soil food webs in Swiss soils reveals major differences between agricultural and natural habitats (Tentative title)*(In prep. To be submitted to Soil Biology and Biochemistry, November 2016)*

Imperiali, N*, Chiriboga, X*, **Jaffuel, G.,** Dennert, F., Schlaeppi, K., Fesselet, M., Blanco-Pérez, R., Villacrés, D., van der Heijden, M., Keel, C., Mauerhofer, M., Mascher, F., Turlings, T.C.J., Campos-Herrera, R. Can the combined use of beneficial Pseudomonas bacteria, arbuscular mycorrhizal fungi and entomopathogenic nematodes enhances the protection and performance of wheat? (Tentative title) (In prep. To be submitted in Journal of Agronomy and Crop Science October 2016) * equally contributing authors

Brütsch T*, **Jaffuel G***, Vallat-Michel A, Turlings T. C. J., Chapuisat M Wood ants enhance the antibiotic activity of tree-collected resin by applying glandular acids (Tentative title)*(In prep. To be submitted in September 2016)** equally contributing authors

Chiriboga, X.M., Campos-Herrera, R., **Jaffuel, G.,** Roeder, G., Turlings, T.C.J. Diffusion of the maize root signal (*E*)-β-caryophyllene in soils of different textures and the effects on the migration of the entomopathogenic nematode *Heterorhabditis megidis* (Tentative title) *(Submitted to Applied Soil Ecology, July 2016)*

## Attended meetings

*Conferences oral / poster presentation*

**NCCR final conference**, Neuchâtel, Switzerland (poster). January 2013. Encapsulation of entomopathogenic nematodes as biological control.

**25th IWGO Conference (IOBC-Global)**, April 2014, Chicago, U.S.A. (oral presentation). A root-produced quiescence factor may help to improve the shelf-life of entomopathogenic nematodes used in biological control.

**15th international symposium on insect-plant relationships** (SIP15), August 2014. Neuchâtel, Switzerland (poster). Low prevalence and strong competition of entomopathogenic nematodes in Swiss agricultural soils imply a need for an augmentation biological control approach for effective crop protection.

**15th meeting of the IOBC-WPRS working group microbial and nematode control of invertebrate pests**, June 2015, Riga, Latvia (oral presentation). Low prevalence and strong

competition of entomopathogenic nematodes in Swiss agricultural soils imply a need for an augmentation biological control approach for effective crop protection.

*Extension / vulgarisation*

**Cours AGRIDEA n° 1821.1** - PNR 68 - Biologie du sol - 23.01.2014

**Cours AGRIDEA n° 15.334** - PNR 68 - Biologie du sol II : Microfaune et microflore, booster de la fertilité des sols - 26.11.2015

# Conclusion
# and
# Outlook

## Conclusion

*EPN prevalence*

The survey performed in the frame of the NRP68 revealed very low numbers of naturally occurring EPN in all the sampled areas. Throughout this project we sampled natural areas such as forest, grasslands and different agricultural lands that were subjected to various management practices. In most of the agricultural fields that were sampled, the number of IJs was in average lower than 1 IJ per 100g of soil. The number of IJs in natural areas was higher than in “agricultural” lands (~ 2.5 fold on average; Campos-Herrera et al., in prep). Overall we detected different species of EPN, all of which had already been described for Swiss soil (Steiner, 1996; Kramer et al., 2001; Hominick, 2002), with the exception of newly detected *S. poinari.* described by Mracek et al. (2014). The very low number of EPN in Swiss soil and, more particularly, in Swiss agroecosystems may highlight a lack of hosts. As Switzerland does not suffer from massive pest invasions, EPN hosts are limited to naturally occurring native insects and the EPN population is likely regulated by stable populations of insect hosts. Nevertheless, we cannot exclude that the soil proprieties and other environmental conditions are not suitable to EPN. Most of the farming lands have been exploited for decades and may have been modified in ways that limit the settlement and survival of EPN.

The study presented in this thesis investigated the prevalence of EPN depending on different farming systems and management practices (conventional, organic, and biodynamic) in the DOK field. We revealed that EPN populations were not affected by the type of farming system. We did not detect any difference in EPN abundance nor activity between organic, conventional and biodynamic managed plots. This is encouraging in the perspectives of using EPN in IPM programs as our results suggest that EPN application might work just as well in conventional as in biological farming. But it cannot be excluded that the scarce abundance of EPN prevents the detection of differences between the different farming systems. Investigating the effects of same parameters but with applied EPN can bring more information on the dynamics of EPN populations at higher densities in different farming systems.

We did find that EPN numbers were influenced by the type of crop (maize, wheat, grass-clover ley). During the year of sampling (2013), we detected significantly more EPN in wheat plots. As the field that was investigated is subjected to crop rotation, it is difficult to assess whether the previous crop (potatoes) or the crop at the sampling time was responsible for the observed pattern. But this result is an interesting first step towards understanding which crops maybe most suitable for the settlement of EPN and whether the use of crop rotation may help to increase EPN prevalence. The presence of insect hosts in the initial crop (wireworms in potatoes; Paul Mäder personal communication) may also have enhanced the abundance of EPN in the wheat plots. Our results are in line with those reported by Susurluk and Elhers (2007). These authors investigated

the persistence of applied *H. bacteriophora* in different crops. The higher persistence was of 23 months, found in beans followed in rotation by wheat with rye clover as cover crop, whereas their half-life in the lab in absence of insect host was of 24.8 days. The presence of the pea weevil *Sitona lineatus* allowed EPN to recycle and to persist into the field and highlighted the importance to have insect host for EPN persistence. Further studies in the DOK field should include a survey of the potential hosts that are present in the field, in order to assess the effect of host densities on EPN prevalence.

*Enhancing EPN shelf-life in formulation*

In the perspective of increasing the shelf life of EPN in formulations, we investigated the use of a natural quiescence factor (QF) in the exudate of roots that induces a state of quiescence in EPN. Following the first discovery of this phenomenon by Hubbard et al. (2005), we first showed that several species of EPN are sensitive to pea root-cap exudates, and found that the exudate triggered a state of quiescence in all of the tested species (*Steinernema feltia, S. carpocapsae, H. bacteriophora, H. megidis*) (Hiltpold et al., 2014). We further showed that QF induced quiescence ensured that the EPN better preserved their lipids, survived longer, and were more infectious after awakening them, as compared to EPN that had not been exposed to QF (Hiltpold et al., 2014). Interestingly, EPN that were exposed to a very low dose of QF displayed higher activity, being more motile and infectious than control EPN (Hiltpold et al., 2014).

The identification of the QF has been a challenge and working with the low quantities in the exudate proved to be very tedious. Collecting sufficient amounts was very difficult and it was impossible, while fractionating the exudate to isolate the active compound(s), to distinguish between a loss of activity due to dilution and the loss of the active compound. To obtain larger quantities of the active material we developed a method of extraction from deep-frozen roots of both maize and pea. This resulted in a very potent extract, active at a dilution of 0.33mg/ml. We showed that the QF was not only present at the tip of the root were it is secreted, but also in the rest of a freshly germinated root, allowing us to collect considerably more material for experiments and the eventual identification of QF. Even after exposing EPN to a very high dose of QF (10 mg/ml of extract) for 24h they were still able to recover and be infectious both against the highly susceptible larvae of *Galleria mellonella*, as well against the more resistant larvae of *Tenebrio molitor*. Their infectiousness was no different from EPN that had only been exposed to water. These results were assessed for an exposure of only 24h (enough to put all EPN fully quiescent) and should be confirmed for a longer exposure period and the different concentration of QF.

During our investigations, we noticed that under certain condition the QF extract did not trigger quiescence. It was found that quiescence is triggered only when EPN are slightly deprived of oxygen. Under high oxygen conditions, the extract was ineffective. This should be considered

when developing EPN formulations, as these specific conditions need to be respected to reach a good quiescence.

During this thesis, we attempt to identify the active compound(s). We successfully isolated the compound(s) using semi-preparation liquid chromatography. We performed NMR on the active fraction, but it still contained several compounds, such as sugars and amino-acids that interfered with the identification of the active substance, which apparently is present only in small quantities. Current efforts to remove the interfering compounds seem to have been successful and new chemical analyses will be performed. We have also performed further studies to improve the alginate based formulation of EPN-containing beads, by characterizing the more suitable polymerization time and concentration of the different reagents to increase EPN shelf life, but also ensuring that the EPN can eventually escape of from the beads. The addition of glycerol proved to be efficient to induce EPN quiescence in beads (Kim et al., in prep). Future investigations will aim to replace the glycerol, which has to be used at the very high concentration of 18% in bead formulations, by the much more potent QF.

*Enhancing the prevalence of EPN*

Agriculture practices are continuously changing and some of the changes focus on the conservation of beneficial soil properties through reduced tillage, and the inclusion of inter crops or cover crops, as well as a reduction of chemical applications. Conservation tillage is increasingly adopted and is recognized as a promising method to enhance soil quality, which can be further enhanced by planting cover crops. We investigated the possibility to also use cover crops during the winter season to enhance the persistence of EPN as compared to bare soil plots. Under the conditions that were used, the presence of a cover crop during winter only marginally influenced the prevalence of applied or naturally occurring EPN. Yet, one of the results showed some promise; in December, 1 month after EPN application, the prevalence of EPN was higher in plots with pea and mustard as cover crops (90±32 IJs in 100g soil) compare to bare soil plots (48±18 IJs in 100g soil). But this trend did not hold over time and the difference was no longer detected in March, four month after EPN application. Nevertheless, the interaction among the augmentation treatment, cover crop treatment and sampling period was significant. It would be necessary to repeat the same kind of experiment over several seasons and various conditions, to confirm a potential beneficial effect of cover crop on EPN prevalence. Overall, we found that the prevalence of EPN decreased rapidly over time. Four months after application, the total EPN abundance was reduced by 71% compare to one month after application. But, interestingly, four month after EPN application, the infectiousness of the nematodes (EPN + FLN) was just as good as one month after application. This shows that the plots where EPN were applied were still very active after winter even though EPN were less abundant. These trials also highlighted that the choice of EPN species for application is a very important factor and this choice should be based on which

species/population is best adapted to the local conditions. At every time point, the plots with the highest number of EPN detected with the qPCR method were the plots that had been augmented with the commercial population of *H. bacteriophora*. However, the highest activity (infection rate of baiting larvae) was consistently recorded for plots augmented with the native S. *feltiae*, and the qPCR analyses after the emergence of the cadavers confirmed that S. *feltiae* was the species responsible for the infection. Hence, the species that was the most abundant was not the most infective. This is another factor should be considered when choosing the most suitable EPN species, as abundance may not correlated with infectivity. Several studies have shown that the application of EPN trigger a trophic cascade resulting in a higher abundance of their natural enemies and competitors, thereby suppressing the population of EPN (i.e. Duncan et al., 2007; Jabbour and Barbercheck, 2011; Campos-Herrera et al., 2012, 2013). In the CC27 field trial, we failed to detect an effect of the EPN application on the abundance of NF and FLN. The specific conditions of a Swiss winter may have prevented the NF and FLN to build up their populations in response to EPN application.

In the CC29 trial, the natural presence of EPN was very low as it was found earlier by Campos-Herrera et al. (2015a). Cover crops did not enhance this presence of EPN and throughout the season their numbers were low in both bare soils and soils covered with pea. However, NF and FLN were abundant and the results confirmed a negative effect of tillage on FLN that was also in a previous study at the same field site. It also confirmed a preference of FLN and NF for heavy soil (Campos-Herrera et al., 2015a). The very too low number of EPN makes it difficult to draw solid conclusions about the effects soil texture or tillage on EPN populations, but other studies have shown a negative effect of tillage on EPN (reviewed in Stuart el al., 2015).

*Intra- and interspecific competition*

During our Swiss-wide surveys of EPN, we exposed a large number of soil samples to larvae of *G. mellonella*. We quickly noticed that several species of nematodes were produced by the cadavers. We confirmed our visual observation by analyzing the DNA of the nematodes progeny by qPCR. The majority of the cadavers produced a mixed progeny. The first round of baits revealed the presence of several species of EPN, but also mixes of EPN and FLN, including blends of several species of EPN and several species of FLN. The qPCR data confirmed that, one or several species of EPN could share cadavers with nematodes from the *Acrobeloides*-group and *Oscheius* spp. The Kosh's postulate larvae (2nd generation of bait) also presented mixed progeny. But for the second generation only EPN and *Oscheius* spp. were found, and *Acrobeloides*-group were apparently not able to reproduce. *Oscheius* spp. are poorly described and their biology and competition with EPN was not well understood. Follow up experiments showed that the prevalence of *Oscheius* spp. in relation to EPN plays a key role in the success of EPN to reproduce inside a cadaver (Campos-Herrera et al., 2015b). When *Oscheius* spp. prevalence is largely superior

compared to EPN, they had a negative effect on EPN reproduction. However, if their prevalence compare to EPN was low, EPN reproduction was not impaired. The fact that we found very low number of EPN in the collected soil samples may be explained by the high abundance of *Oscheius* spp., which we found in almost every isolate. Yet, the number of larvae infected did not differ when *Oscheius* spp. and EPN were found together (Campos-Herrera et al., 2015b). It remains to be determined if *Oscheius* spp. interfere with the effectiveness of EPN biological control agents.

**Outlook**

- The quiescence factor shows promise as an additive to be used in EPN formulations, but limitations should be considered, especially if the compound(s) are hard to obtain or synthesize. Moreover, the identification of the active compound(s) is challenging and still under investigation.
- EPN abundance in Swiss soil is very low, especially in agricultural soils. The consequence of the low EPN prevalence in case of pest outbreak will need to be assessed.
- Naturally occurring EPN abundance did not differ between the different farming systems that were investigated (organic, conventional, and biodynamic). However, this was only tested under conditions with very low numbers of EPN. Similar experiments should be conducted in soils with a higher abundance of EPN, following application for instance.
- EPN abundance was not significantly enhanced in plots with a winter cover crop, but there was a significant interaction among the sampling period, the cover crop and the application of EPN. This indicates that cover crops may help to maintain large EPN populations in agricultural soils and calls for more investigations to confirm an effect on EPN abundance.
- EPN in Swiss soil strongly compete with free-living nematodes for insect cadavers, and it should be further investigated if the interaction of free-living *Oscheius* spp. with EPN interferes with their potential to control insect pests.

**Reference of the conclusion**


Campos-Herrera, R., Ali, J. G., Diaz, B. M., Duncan, L. W., 2013. Analyzing spatial patterns linked to the ecology of herbivores and their natural enemies in the soil. *Front. Plant Sci.* 4 : 1–18.

Campos-Herrera, R., El-Borai, F. E., Duncan, L. W., 2012. Wide interguild relationships among entomopathogenic and free-living nematodes in soil as measured by real time qPCR. *J. Invertebr. Pathol.* 111: 126–135.

Campos-Herrera R., Jaffuel G., Chiriboga X., Fesselet M., Půža V., Blanco-Pérez R., Mascher F and Turlings T.C.J., 2015a. Traditional and molecular detection methods reveal intense interguild competition and other multitrophic interactions associated with native entomopathogenic nematodes in Swiss tillage soils. *Plant Soil.* 389: 237-255 doi:10.1007/s11104-014-2358-4.

Campos-Herrera, R., Puza, V., Jaffuel, G., Blanco-Pérez, R., Čepulytė Rakauskienė, R., Turlings, T. C. J., 2015b. Unraveling the intraguild competition between *Oscheius* spp. nematodes and entomopathogenic nematodes: implications for their natural distribution in Swiss agricultural soils. *J. Invertebr. Pathol.* 132: 216-227.

Duncan, L. W., Graham, J. H., Zellers, J., Bright, D., Dunn, D. C., El-Borai, F. E., and Porazinska, D. L., 2007. Food web responses to augmenting the entomopathogenic nematodes in bare and animal manure-mulched soil. *J. Nematol.* 39:176–89.

Hiltpold, I., Hibbard, B. E., French, B. W., and Turlings, T. C. J,. 2012. Capsules containing entomopathogenic nematodes as a Trojan horse approach to control the western corn rootworm. *Plant and Soil. 358*(1-2): 11–25. doi:10.1007/s11104-012-1253-0

Hominick W. M. 2002. Biogeography. In*: Gaugler R (ed),* Entomopathogenic Nematology*, CABI Publishing, Wallingford, UK*, pp. 115–43.

Hubbard, J. E., Yolanda F. L., Schmitt M., M., McCLure, M. A., Stock, S. P., Hawes, M. C., 2005. Increased penetration of host roots by nematodes after recovery from quiescence induced by root cap exudate. *Nematology*. 7(3): 321–331.

Kramer I., Hirschy O., Grunder J. M., 2001. Survey of baited insect parasitic nematodes from the Swiss lowland. *In: EUR 19696-COST ACTION 819. Developments in Entomopathogenic Nematode/bacterial Research. Proceedings of the Workshop at National University of Ireland Maynooth. 13-15 April 2000.*

Mráček, Z., Půža, V., Nermuť, J., 2014. Steinernema poinari sp. n. (Nematoda: Steinernematidae) a new entomopathogenic nematode from the Czech Republic. *J. Helminthol.* 90:249–251.

Steiner W. A. 1996. Distribution of entomopathogenic nematodes in the Swiss Alps. *Revue Suisse de Zoologie.* 103: 439–52.

Susurluk, A., and Ehlers, R. U., 2008. Field persistence of the entomopathogenic nematode *Heterorhabditis bacteriophora* in different crops. *BioControl.* 53:627–641.

Stuart, R. J., Barbercheck, M. E., Grewal, P. S., 2015. Entomopathogenic nematodes in the soil environement: distributions, interactions and the influence of biotic and abiotic factors. In: Campos-Herrera, R. (Ed.), *Nematode pathogeneses of insects and other pests*. Springer International Publishing, Switzerland, pp. 97-137.