Entomopathogenic Nematodes-Maize Interactions: Implications for Sustainable Agriculture

Inaugural dissertation
of the Faculty of Science,
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presented by
Arletys María Verdecia Mogena

Supervisor of the doctoral thesis:
Prof. Dr. Christelle Robert,
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Accepted by the Faculty of Science

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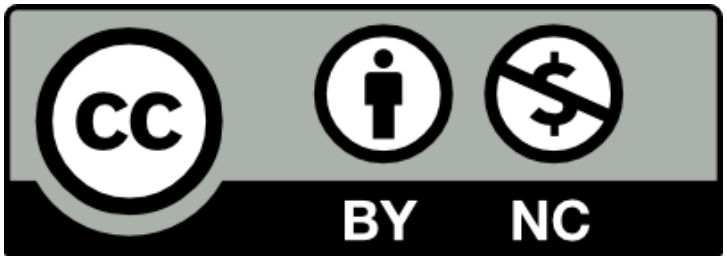

Table of contents

Abstract

Natural enemies of herbivores provide a biocontrol tool for insect pest management. Previous studies focused on understanding the interactions between plants, herbivores and herbivore natural enemies. However, the direct interactions between plants and herbivore natural enemies remains underexplored despite their potential relevance for agroecology. This work aimed to provide a better understanding of the interactions between plants and insect natural enemies and the role of bacteria associated with insect natural enemies in these interactions.

The model system was maize *Zea mays* L. and a species of entomopathogenic nematodes (EPNs) *Heterorhabditis bacteriophora* Poinar. **Chapter 1** provides a comprehensive characterization of maize local response to EPN exposure using transcriptomic and metabolomic analyses. The results indicates that maize plants respond locally to the presence of EPNs in the soil inducing a mild stress response that resulted in altered metabolomic and signalling pathways. However, this EPN-induced shift in the maize metabolome does not affect herbivore performance nor survival of *Diabrotica* spp. larvae. **Chapter 2** presents a characterization of systemic maize response to EPN presence in the soil after an observation in the field trial where EPNs reduce aboveground maize plants infestation by the herbivore *Ostrinia nubilalis*. The chemical cues underlying the insect oviposition preference remain unknown, although preliminary data suggest the involvement of wax or lipid derivatives. **Chapter 3** confirms that EPNs carry a rich microbiota other than the well-known symbiont genus *Photorhabdus* quite conserved across environmental conditions and insect hosts. The findings also suggest that EPN surface bacteria can alter soil bacterial community. **Chapter 4** provides a broad description of the effect of members of the core microbiota of EPNs on plants, insects and EPN fitness and survival. The results confirm that some members of the core microbiota of EPNs are involved in EPN entomopathogenicity and influence EPN survival in *in vitro* conditions.

This work highlights the context-dependent nature of plants-EPNs-microbes interactions providing a better understanding of multitrophic interactions. The findings present new aspects for improving the use and the potentiality of EPNs and their associated microbes in sustainable agriculture.

General Introduction

Tritrophic interactions and sustainable agriculture

Tritrophic interactions are the interactions amongst multiple trophic levels (Price et al., 1980). Plants are the first trophic level of the ecological food chains. Herbivores are the second trophic level and herbivore natural enemies the third trophic level. A better understanding of the complexities of tritrophic interactions is necessary to control herbivorous pests effectively (Agrawal, 2000). Chemical ecologists have made already significant progress in understanding the complex mechanisms that direct tritrophic interactions and the cascading effects on larger ecological systems (Turlings & Erb, 2018). However, plant direct responses to member of third trophic level is still less investigated.

Interactions between plants and insect natural enemies gained attention because of their potential for using them as biological control (Hajek & Eilenberg, 2018). Biological control or biocontrol refers to the use of organisms to control plant pests and diseases (Van Driesche & Hoddle, 2009). Plant diseases and pests still account for 20%-40% of crop production losses (Sharma et al., 2017). Biocontrol offers a viable alternative to chemical pesticides, especially considering that the use and risk of chemical pesticides should be reduced by 50% by 2030 according to the European Union Commission (Silva et al., 2022). Biocontrol supports sustainable agriculture by efficiently using natural resources to feed people and protect biodiversity (Reganold et al., 1990).

The enemies of my enemies are my friends: entomopathogenic nematodes

Entomopathogenic nematodes (EPNs) are soil nematodes that live in symbiosis with bacteria that they carry in their intestine, they infect a wide variety of insect hosts and kill them rapidly, typically within 48 hours (Poinar, 1975). EPNs are also characterized by their ability to ensure symbiotic bacteria are passed on to future generations (Dillman et al., 2012). EPNs from the families Steinernematidae and Heterorhabditidae have been identified in various countries worldwide and widely proposed as biocontrol agents of insect pests (Bhat et al., 2020).

Heterorhabditis bacteriophora, a species of EPNs, establish this highly co-evolved symbiotic interaction with bacteria from the genus *Photorhabdus* (Ciche et al., 2008). The life cycle of *Heterorhabditis bacteriophora* begins with the Infective

Juvenile (IJs) stage, the only free-living stage (Kaya & Gaugler, 1993). The juveniles penetrate the host insect and release symbiotic bacteria from their intestines into the host's hemocoel (Forst et al., 1997). These bacteria multiply in the insect's hemolymph, release toxic molecules and in combination with the EPNs cause the death of the insect within 24-48 (Abd-Elgawad, 2021; Koppenhöfer et al., 2020). Post-infection, EPNs feed on the bacteria cells and the host tissue, mature, and reproduce (Kaya & Gaugler, 1993). IJs of *Heterorhabditis* nematodes become hermaphroditic adults but individuals of the next generation produce both male and females (Sagun et al., 2015). The progeny develops through four stages to adulthood, and depending on available resources, multiple generations may occur within the host cadaver (Dillman & Sternberg, 2012). Many IJs are eventually released into the environment to infect other hosts (Figure 1).

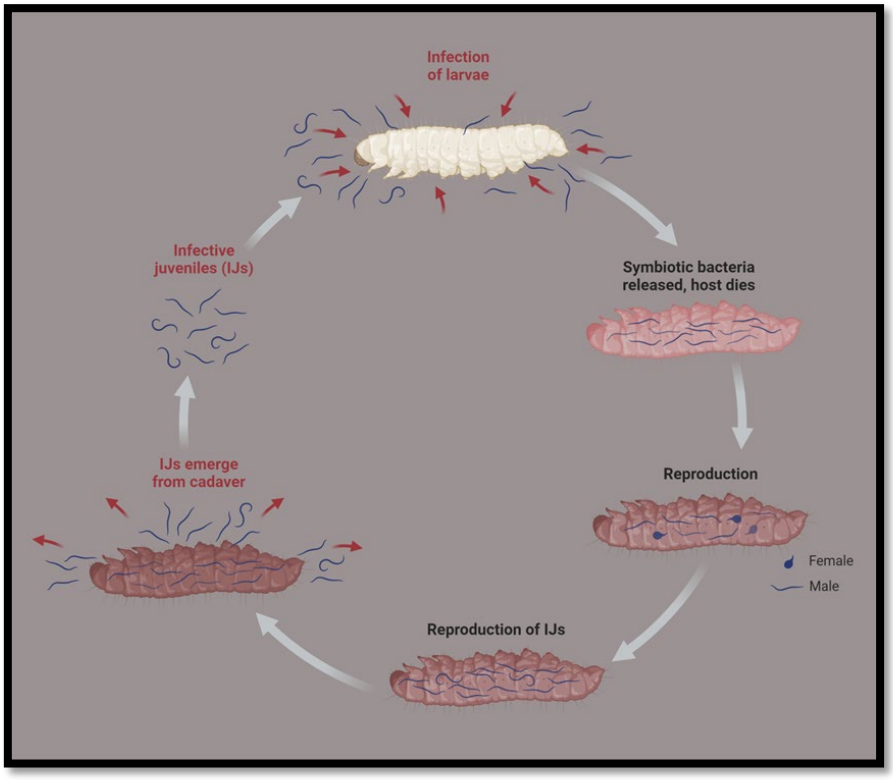

Figure 1. Life cycle of *Heterorhabditis bacteriophora*. Diagram created with BioRender.

EPNs are already used as biocontrol of different insect pests including the western corn rootworm (WCR) *Diabrotica virgifera virgifera* LeConte (Koppenhöfer et al., 2020; Shapiro-Ilan et al., 2023; Toepfer et al., 2014). WCR larvae are a major damaging soil insect pest in maize (*Zea mays* L.) fields, costing around 2 billion USD every year just in the United States (Wechsler & Smith, 2018). Maize is one of the largest-produced

cereals worldwide and is under constant threat by the WCR and other insect pests. Hence, EPNs represent a cost-effective strategy for pest management (Figure 2). However, the impact of EPNs on plants and other components of the soil food web remains largely unexplored.

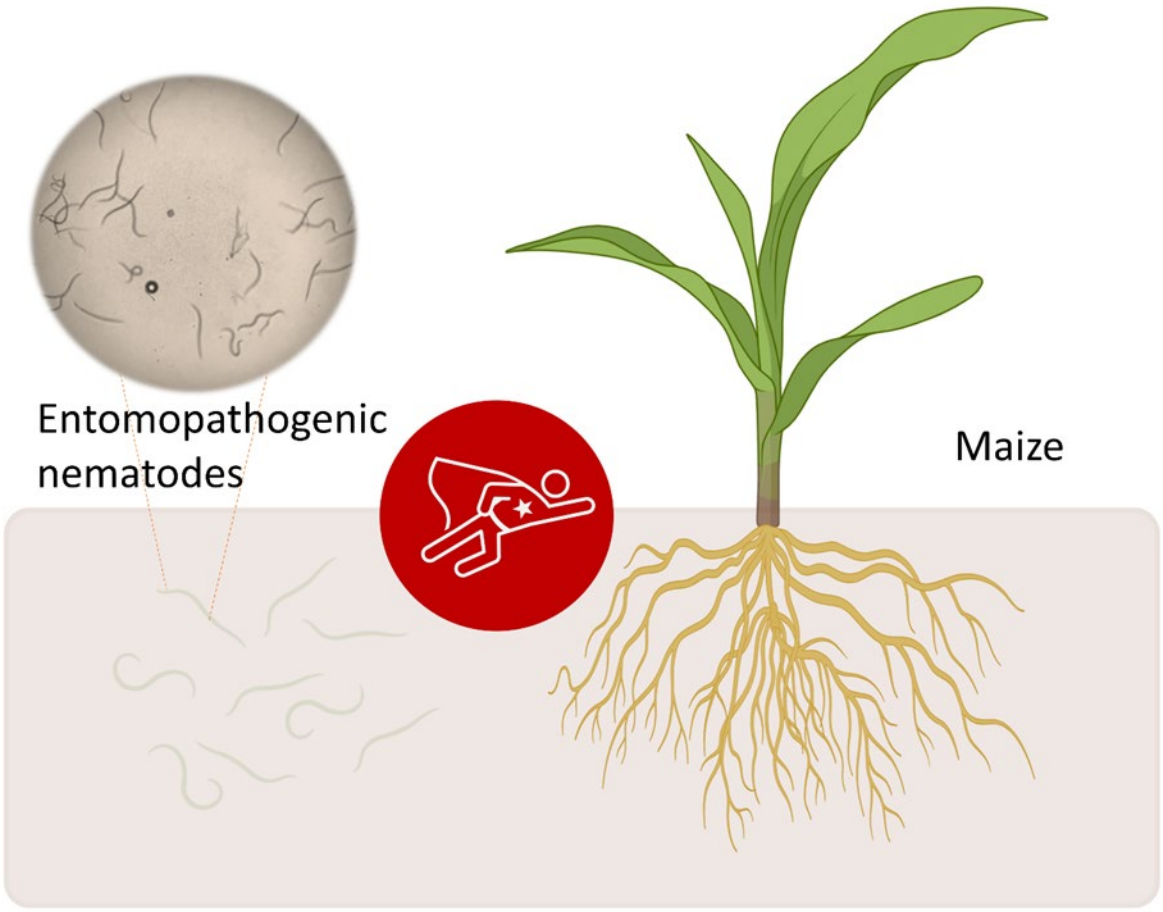

Figure 2. Biocontrol is a tool for sustainable agriculture. Diagram created with BioRender.

EPNs induce plant responses

Nearly a century after the discovery of EPNs, initial reports suggest that plants respond to EPNs, revealing new implications for tritrophic interactions (Jagdale et al., 2009; Kamali et al., 2022; Li et al., 2020). The first report showed that *Arabidopsis thaliana* plants significantly increased catalase activity in leaves after *Steinernema carpocapsae* IJs nematodes application compared to the control (Jagdale et al., 2009). Arabidopsis stimulation of catalase activity and the expression of genes as *PR1* upon *S. carpocapsae* exposure suggested that EPNs can trigger systemic plant defense (Jagdale et al., 2009). Furthermore, EPNs capacity of triggering systemic plant defence has been demonstrated even using EPN-infected larvae indicating the

presence of EPN-associated cues that shape tritrophic interactions (An et al., 2016; Helms et al., 2019; Jagdale & Grewal, 2008; Wang et al., 2025).

Research continued focusing on EPNs induction of systemic response and the implications for plant defence against pests and pathogens, until advances in molecular techniques allowed to explore also the local response of plants to EPNs. A recent study exposed that *S. carpocapsae* activated polyphenol oxidase and guaiacol peroxidase activity in tomato roots, reducing the aboveground preference of tomato leafminer *Tuta absoluta* (Kamali et al., 2022). Additionally, transcriptomic data suggested that *S. carpocapsae* modify hormone signaling and metabolite biosynthesis in tomato plants (Kamali et al., 2022). However, no published study has been conducted on maize local response to EPNs.

Microbes mediating tritrophic interactions

The nematode microbial community, including the endosymbionts and surface-associated bacteria, influence multitrophic interactions. The hypothesis that bacteria attached to the nematode surface could mediated nematode interactions emerged few years ago (Topalović & Vestergård, 2021). Plant parasitic nematodes (PPNs) recruit passively for specific soil microbes to their cuticle or surface coat in their way to the plant roots (Elhady et al., 2017). Another study showed that bacteria attached to PPNs surface induce pathogen-associated molecular pattern-triggered immunity (PTI) in plants (Topalović et al., 2020).

On EPNs, most of the research have focused on the symbionts bacteria located in the EPN intestine. Interestingly, a study showed that other nematode-associated bacteria are also involved in insect infectivity by EPNs (Ogier et al., 2020) (Figure 3). The role of this second bacterial circle of EPNs in the nematode-induced responses could have relevant implications for sustainable agriculture (Ogier et al., 2023). However, the impact of the EPN surface microbes on soil food webs interactions have not been studied yet.

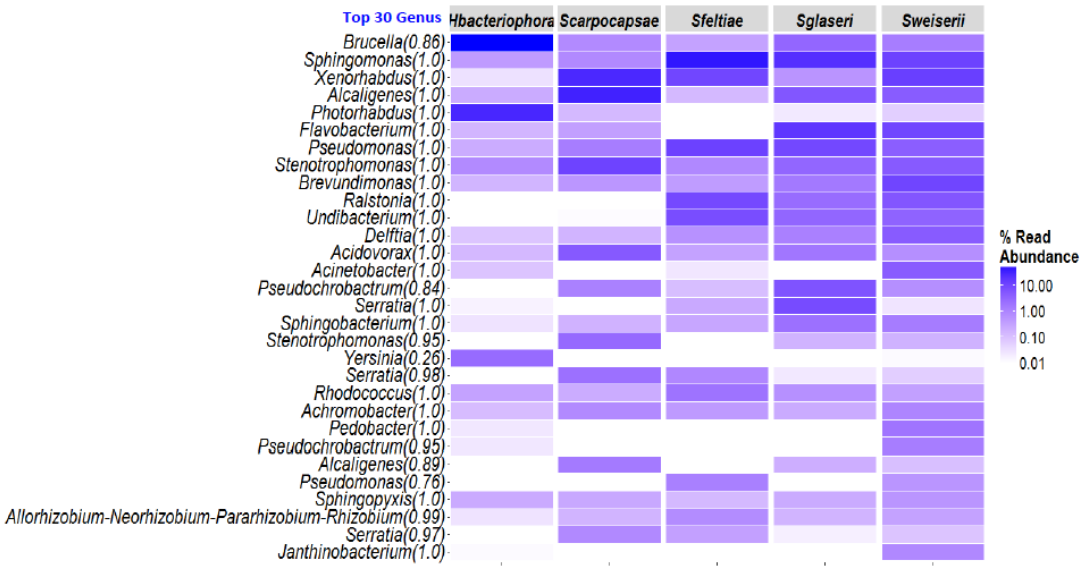

Figure 3. There are different bacteria genera associated with infective juveniles (IJs) of different EPNs species: *Steinernema* and *Heterorhabditis*. Heatmap showing the microbiota composition of IJ samples based on the V3V4 region of the 16S rRNA gene. Each column represents an IJ species. The 30 most abundant Operational Taxonomic Units (OTUs) across the samples at the genus affiliation level (Top30 Genus) are listed on the left. The percentage relative abundance is indicated by the gradient of blue hues (Adapted from Ogier et al. (2020)).

EPNs host a broad spectrum of bacteria that have been overlooked until recently despite their potential implications for agroecosystems. The main hypotheses of this study include the role of EPN-associated bacteria to shape tritrophic interactions and the impact of members of the second bacteria circle of EPNs in EPNs performance.

Aims and scope

This thesis encloses the findings of different randomized experiments that aimed to provide a better understanding of EPN-maize interactions and the implications for sustainable agriculture. The study system involves laboratory-reared IJs of *H. bacteriophora* and maize plants (var. B73). The results include a molecular characterization of EPN-induced local and systemic responses in maize and the effects of these responses in herbivore performance. Moreover, this thesis also includes an integrated approach to decipher the location, origin, occurrence and transmission of EPN-associated bacteria and exploring their impact on soil food web components.

In **Chapter 1**, a time series experiment was performed to investigate EPN-induced response in maize roots in greenhouse conditions. Chapter I included a transcriptomic analysis and several targeted analyses comparing control against EPN-treated plants. EPN exposure was achieved by adding 2’000 IJs per plant in the soil. The targeted

analysis was repeated once collecting the root samples 72 hours upon EPNs exposure because in the transcriptomic analysis the high number of differentially expressed genes (DEGs) was achieved after 72 hours in EPN-treated plants. The results suggested that EPNs induce a mild and dynamic but still significant response in maize roots. Ecological relevance of the findings of this chapter was also considered recording *Diabrotica* spp. larvae performance after previous exposition of maize seedling to EPNs.

In **Chapter 2**, observations from a field experiment suggested that presence of EPNs in the soil reduces *Ostrinia nubilalis* infestation aboveground. Despite measuring amino acids, phytohormones, sugars and volatiles concentrations in maize leaves from field and greenhouse experiments, more research is needed for elucidating the mechanisms involved in repelling moths by EPN-treated plants. Nevertheless, the transcriptomic analysis of maize leaves supports some hypotheses that could explain how the presence of EPNs in soil induces maize resistance to aboveground herbivore infestations.

In **Chapter 3**, the aims included to isolate bacteria from IJs of *H. bacteriophora* and IJ supernatant and perform amplicon sequencing of IJs to decipher what are the bacteria associated with *H. bacteriophora*, what is their origin, how much recurrent they are, where are they located in the nematode body and how they transmit. Besides, Chapter 3 explore the effect of EPN-associated bacteria in soil bacterial communities. **Chapter 4** contains *in vitro* and *in vivo* experiments exposing the impacts of EPN-associated bacteria in maize plants, insect larvae and EPNs as representative of soil food webs. The findings consist of a holistic view of EPN-associated bacteria and their implications for sustainable agriculture.

This thesis presents the results of extensive research on new aspects of EPNs-plant interactions. Hence, the end of this thesis contains a deeper discussion of the findings of this study and their relevance for biocontrol use in agriculture. To guide future researchers, several questions are posed to enhance our understanding of the interactions between EPNs, plants and soil ecosystems.

Chapter 1 - The presence of entomopathogenic nematodes in soil modulate the maize root metabolism

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Abstract

Entomopathogenic nematodes (EPNs) are key biological control agents in sustainable agriculture, but their direct effects on plant metabolism and resistance to herbivory remain underexplored. By combining transcriptomic, metabolomic, and herbivore assays, this study aimed at providing a holistic description of maize root responses to EPNs and to assess their potential relevance for plant-herbivore interactions. EPNs triggered a dynamic shift in root metabolism, suggesting a reallocation of primary resources towards chemical defences. After 72 hours, pathways related to ethylene signalling and protein folding and turnover were downregulated, while pathways for protein export were enriched. Amino acid levels, particularly glutamate and aspartate, decreased, while glucose levels were elevated. In parallel, enrichments in alpha-linolenic acid metabolism, glycan biosynthesis, and, albeit not significantly, cutin, suberine, and wax biosynthesis pathways suggested enhanced barrier functions and lipid signalling. Secondary metabolite concentrations, such as benzoxazinoids, were transiently increased. Despite these shifts, the overall plant response remained of relatively modest magnitude, as illustrated by a low number of differentially expressed genes exceeding 200 reads across the time points. Consistently, EPN exposure did not enhance resistance to subsequent herbivory by the root herbivores *Diabrotica balteata* or *Diabrotica virgifera*. However, the plant responses might influence other belowground interactions, such as those involving plant-microbes or plant-parasitic nematodes, calling for further investigations. This study provides valuable insights into plant responses to EPNs, which might ultimately inform pest management strategies and promote sustainable agriculture.

Introduction

Direct interactions between plants and herbivore enemies, such as predators and parasitoids, are pivotal components of terrestrial ecosystems (Poelman et al., 2012; Price et al., 1980; van der Putten et al., 2004). These interactions have usually been examined from the perspective of how plants attract natural enemies of herbivores through mechanisms like volatile emissions, creating an indirect defence mechanism for the plant (Aljbory & Chen, 2018; Turlings & Erb, 2018). Yet, how the presence of natural enemies on plant physiology and growth have long remained overlooked, despite evidence suggesting significant changes in plant physiology, and potential use for integrated pest management (Kansman et al., 2024).

Plant direct responses to predators have been documented in ants (Blüthgen & Wesenberg, 2001) and mirids (Calvo et al., 2009; Pappas et al., 2015; Pérez-Hedo & Urbaneja, 2016; Zhang et al., 2018). Plant responses included the modulation of the plant metabolism, in turn affecting herbivore performance and subsequent trophic interactions. For instance, the presence of the green lacewing *Chrysoperla carnea* larvae induced a reconfiguration of the primary and secondary metabolism of tomato, triggering plant resistance to herbivores such as the spider mite *Tetranychus urticae* and the aphid *Myzus persicae* (Errard et al., 2016). Similarly, the presence of the mirid *Macrolophus pygmaeus* induced jasmonic acid (JA)-related defence pathways in sweet pepper plants, *Capsicum annuum*, which coincided with a reduced performance of the spider mite *Tetranychus urticae* and the western flower thrips *Frankliniella occidentalis*, but not the aphid *Myzus persicae* (Zhang et al., 2018*)*. The presence of the mirid predatory tomato bug *Nesidiocoris tenuis* induced the upregulation of the abscisic acid (ABA) and JA signalling pathways in the apical part of tomato plants *Solanum lycopersicum* (Naselli et al., 2016). As a result, tomato plants were less attractive to the whitefly *Bemisia tabaci* and more attractive to the parasitoid *Encarsia formosa* (Naselli et al., 2016). However, the plant direct responses to these predators remains debated, as the latter also feed on- or oviposit in plants, potentially triggering plant responses due to the damage inflicted rather than due to their presence alone.

Plant responses to parasitoids, which do not damage the plants, however demonstrated that plants can perceive and respond to the presence of herbivore enemies (Kansman et al., 2024). Entomopathogenic nematodes (EPNs) in particular, can modulate plant physiology and subsequent trophic cascades. These small

roundworms exist as free-living infective juveniles in the soil and invade soil-dwelling insect hosts, where they reproduce for multiple generations until resource depletion (Dillman et al., 2012). Interestingly, the presence of EPNs in soil is sufficient to induce systemic responses in plant leaves. For instance, *Steinernema carpocapsae* presence in soil increased peroxidase and catalase activity in leaves of *Arabidopsis thaliana* (Jagdale et al., 2009) and reduced guaiacol peroxidase activity in tomato leaves (Kamali et al., 2022).

EPNs can enhance plant resistance against leaf pathogens and herbivores (An et al., 2016; Helms et al., 2019; Jagdale et al., 2009; Jagdale et al., 2002; Kamali et al., 2022; Molina et al., 2007; Perry et al., 1998; Somasekhar et al., 2002). For instance, EPN presence in soil reduced the attractiveness and fitness of the leaf-mining insect *Tuta absoluta* (Kamali et al., 2022). Additionally, EPN volatile cues induced systemic resistance in potato *Solanum tuberosum* against the Colorado potato beetle *Leptinotarsa decemlineata* (Helms et al., 2019). Similarly, EPN-infected insect cadavers trigger systemic responses (Helms et al., 2019; Jagdale et al., 2009; Wang et al., 2024; Zhang et al., 2019). For example, *S. carpocapsae*-infected insect cadavers induces systemic resistance in tomato plants *Solanum lycopersicum* against the beet armyworm *Spodoptera exigua*, sweetpotato whitefly *Bemisia tabaci*, and the bacterial pathogen *Pseudomonas syringae* (An et al., 2016). However, the local effect of the herbivore natural enemies on root physiology has received less attention to date. A recent study showed that EPNs application in soil boost polyphenol oxidase (PPO) and guaiacol peroxidase (GP) activity in roots of tomato plants (Kamali et al., 2022). Moreover, the EPN-induced defence mechanisms decreased root knot nematode (RKN) populations in the soil (Kamali et al., 2022). EPNs are commonly used in as biocontrol agents of herbivorous insects in agriculture and released at high density (250’000-1 million IJs/m²) into the soil (Shapiro-Ilan et al., 2006). Thus, in-depth characterization of plant responses to EPNs presence and the consequence for subsequent trophic interactions belowground is crucial to ensure sustainable agriculture.

Maize is one of the most economically important crops globally, serving as a staple food for humans, a key source of animal feed, and a raw material for industrial products like biofuels, starch, and sweeteners (Ranum et al., 2014). Yet, maize suffers significant yield reductions due to insect pests such as the fall armyworm, *Spodoptera frugiperda*, aboveground, and the western corn rootworm (WCR), *Diabrotica virgifera*

virgifera belowground (Bažok et al., 2021). For instance, WCR alone is responsible for annual management costs and yield losses exceeding $2 billion in the US only (Wechsler & Smith, 2018). While EPNs have emerged as a sustainable biological control method against insect pests, a comprehensive assessment of their cascading effects on belowground interactions between maize and herbivores remain unknown. This study aimed at providing the first transcriptomic and metabolomic (for both polar and non-polar metabolites) characterization of maize root response to EPN presence after 6, 24, and 72 h. Further targeted analyses were conducted to evaluate EPN-induced changes in primary and secondary metabolisms. Finally, the ecological relevance of the plant responses to EPNs was assessed by measuring subsequent root herbivore performance and survival. This study provides critical insights into the molecular and ecological dynamics of maize-EPN interactions, offering a foundation for future studies aiming at developing more effective and sustainable EPN-based pest management strategies.

Materials and methods

Biological material

Maize seeds (*Zea mays* L.) of the variety B73 were kindly provided by the Maize Genetics and Genomics Database (MaizeGDB, www.maizegdb.org) and bred by Delley (Delley seeds and plants Ltd, Delley, Switzerland). Seeds of the variety “Akku” were bought at Delley (Delley seeds and plants Ltd, Delley, Switzerland).

Maize seeds (var. B73) seedlings were grown in 100 mL cylindric pots (Semadeni, Ostermundigen, Switzerland) filled with 80 % sand (LANDI Schweiz AG, Dotzingen, Switzerland) and 20 % soil on top (Selmaterra Schweizer Schwererde torfreduziert, Bigler Samen AG, Thun, Switzerland). The plants were grown in greenhouse conditions at 23 ± 2°C, approx. 60 % humidity and 16:8 dark/light cycle, with daily watering and Plataktiv® Typ K (Hauert HBG Duenger AG, Grossaffoltern, Switzerland) fertilizer added weekly according to the manufacturer instructions. Entomopathogenic nematodes (*Heterorhabditis bacteriophora* Poinar, strain EN01) were obtained from in-house colonies established with EPNs bought in 2021 from Andermatt Biocontrol (Grossdietwil, Switzerland) and reared *in vivo* in greater wax moth larvae (*Galleria melonella* Lepidoptera: Pyralidae, Fischereibedarf Wenger, Bern, Switzerland). EPNs

infective juveniles (IJs) were stored at 4°C in tap water until use. Plants with three fully developed leaves were used for all experiments.

Eggs of the banded cucumber beetle (BCB, *Diabrotica balteata* LeConte) were initially provided by Syngenta and reared in the laboratory since 2021. The larvae were reared on roots of young maize plants (var. “Akku”). Larvae in the L2 stage were used for the experiments. Eggs of the western corn rootworm (WCR, *Diabrotica virgifera virgifera* LeConte) were kindly provided by Chad Nielson and Sharon Schneider (US Department of Agriculture-Agricultural Research Service-North Central Agricultural Research Laboratory (USDA-ARS-NCARL), Brookings, USA). The hatched larvae from these eggs were reared on young maize plants (var “Akku”) and used for experiments at L2 stage.

EPN exposure

IJ suspensions of about 500 IJs/ml were prepared in tap water and were added into two 2 cm deep holes in the soil (2’000 EPNs per pot). Same volumes of water were added to control plants. After 6, 24 and 72 h maize roots were collected, gently washed under tap water, softly dried with tissue paper and snap-frozen in liquid nitrogen. Root samples were grinded to a fine powder in liquid nitrogen using a mortar and pestle.

Transcriptomic analysis

Total RNA was extracted from 100 ± 2 mg ground maize roots using a RNeasy Plant Mini Kit (QIAGEN Naamloze Vennootschap (N. V.), Venlo, The Netherlands) according to the manufacturer’s instructions. The RNA integrity was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc, Santa Clara, California, USA). Samples with an RNA Integrity Number (RIN) ≥ 7 were used for subsequent analyses. The libraries were sequenced in a DNBSEQTM platform by Beijing Genomics Institute (BGI) (BGI Hong Kong Company Limited, Hong Kong, China) which generated 100 bp paired-end reads and 30 M reads per sample. Low quality reads, reads with adaptor sequences, and reads with high levels of N base were removed from the raw data with the filtering software SOAPnuke (Version v1.5.2). Hierarchical Indexing for Spliced Alignment of Transcripts (HISAT; Version v2.0.4) was used to map RNA-seq reads (Reference Genome version: GCF_902167145.1_Zm-B73-REFERENCE-NAM-5.0) and the Bowtie2 tool was used to align the clean reads to the reference genes.

Metabolomic analyses

Root metabolomic profiling was conducted primarily on polar metabolites, as well as on primarily non-polar, hydrophobic metabolites using two different methods.

The polar metabolomic profile was conducted with an Acquity Ultra High-Performance Liquid Chromatography (UHPLC) system coupled to a G2-XS Quadrupole Time-Of-Flight (QTOF) mass spectrometer (MS) equipped with an electrospray source (Waters, Milford, Massachusetts, USA). First, the chromatography was performed using a Hydrophilic Interaction Liquid Chromatography (HILIC) column (100 × 2.1 mm i.d., 1.7 µm particle size) using the following elution profile: 2–50 % B over 4 min, 50–100 % B over 2 min, 100 % B for 2 min, followed by re-equilibration with 2 % B for 2 min, where A = 0.1 % formic acid (FA) (Fisher Scientific AG, Reinach, Switzerland) in ultra-pure MilliQ water and B = 0.1 % formic acid (FA) in 10 mM acetonitrile (ACN) ammonium formate. The flow rate and temperature of the column were respectively maintained at 0.4 $mL.min^{-1}$ and 40°C during the analysis. The QTOF-MS operated in positive mode. The electrospray capillary and sampling cone voltages were set to 2 kV and 20 V, respectively. The source temperature was kept at 140°C and the desolvation gas temperature at 400 °C. The desolvation and cone gas flows were 1000 $L.hr^{-1}$ and 100 $L.hr^{-1}$, respectively. Centroided data were acquired over a range of 50-1200 m/z in MSE mode, using alternating scans of 0.1 s at low collision energy of 6 eV and 0.1 s at high collision energy ramped from 10 to 30 eV. The resulting chromatograms were aligned and normalized with Progenesis QI (Waters, Milford, Massachusetts, USA).

Non polar and hydrophobic metabolomic analysis was conducted by UHPLC coupled to mass spectrometry as above, using a method adapted from (Robert et al., 2017). The chromatography was performed using an CM C18 column (50 × 2.1 mm i.d., 1.7 µm particle size, Waters, Milford, Massachusetts, USA, p/n: 186002350) using the following elution profile: 1-27.5 % B over 3.5 min, 27.5–100 % B over 1 min, 100 % B for 1 min, followed by re-equilibration with 1 % B for 1 min, where A = 0.1 % FA in ultra-pure MilliQ water and B = 0.1 % FA in ACN (Biosolve Chimie, Dieuze, France). The flow rate and temperature of the column were maintained at 0.4 $mLmin^{-1}$ and 40°C, respectively. The QTOF-MS operated in negative mode. The electrospray capillary and sampling cone voltages were set to 2 kV and 20 V, respectively. The source temperature was kept at 140°C and the desolvation gas temperature at 400°C.

The desolvation and cone gas flows were 1000 Lhr-1 and 100 Lhr-1, respectively. Centroided data were acquired over a range of 50-1200 m/z in MSE mode, using alternating scans of 0.15 s at low collision energy of 4 eV and 0.15 s at high collision energy ramped from 10 to 40 eV. The resulting chromatograms were aligned and normalized with Progenesis QI (Version 2.4, Waters, Milford, Massachusetts, USA). Benzoxazinoids (BXs) were identified and quantified with the use of external standards containing DIMBOA, DIMBOA-Glc, HDMBOA-Glc, HMBOA, MBOA, HMPMA and BOA in QuanLynx (Version 4.1, Waters, Milford, MA, USA). Double methylated BXs were quantified by using the related single methylated compound (e.g. DIMBOA-Glc for DIM2BOA-Glc), DIBOA-Glc was quantified with DIMBOA-Glc standards, and HMBOA- Glc was quantified with the aglucone HMBOA. These compounds were chosen according to their similar ionisation levels in the mass spectrometry and therefore similar detected ion abundances at the same concentration.

Soluble sugar analysis

D-glucose, D-fructose and sucrose concentrations of maize roots were quantified a colorimetric assay kit (Megazyme, Bray, Ireland) according to the manufacturer's instructions. The analysis of the samples was performed in transparent, flat-bottom 96-well plates (Greiner Bio-One GmbH, Kremsmünster, Upper Austria) and measurements performed with a Tecan Infinite M200PRO plate reader (Tecan Group Ltd., Männedorf, Switzerland). Mega-Calc™ software tool from Megazyme was used for transforming the data from absorbance values to concentration values.

Amino acid analysis

Free amino acids were quantified using the AccQ-Tag Ultra Derivatization Kit (Waters, Milford, Massachusetts, USA) according to the manufacturer instructions. Amino acids from the tissues were extracted by mixing 100 mg of tissue powder with 1 mL extraction buffer (50 % ethanol (EtOH) + 0.1 % FA). After vortexing, the samples were centrifuged at 15'000 g for 10 min. The supernatant was kept overnight in vacuum under 45°C in a CentriVap (Labconco Corporation, Kansas City, USA). Dry powder was resuspended in 400 µL of MilliQ water. Derivatized samples were analyzed with an Acquity UHPLC system coupled to a quadrupole mass spectrometer (QDa-MS) equipped with an electrospray source and an UV/Vis-Detector (Waters, Milford,

Massachusetts, USA). The chromatography was operated with a BEH C18 column (100 × 2.1 mm i.d., 1.7 µm particle size, Waters, Milford, Massachusetts, USA, p/n: 186002352) using the following elution gradients: 0.1 % B for 0.54 min, 0.1-9.1 % B over 5.2 min, 9.1-21.2 % B over 2 min, 21.2-59.6 % B for 0.3 min, followed by flushing the column with 90 % B for 0.6 min and re-equilibration at 0.1 % B for 0.89 min (A = H_20:ACN 99:1 + 0.1 % FA, B = ACN + 0.1 % FA). The flow rate and temperature of the column was kept constant at 0.7 ml/min and 55°C. The QDa-MS was operated in positive, single ion recording mode. The electrospray voltage was kept at 0.8 kV, while the cone voltage was adjusted according to the measured ions. The source and probe temperature were kept at 120°C and 600°C, respectively. The sampling frequency was set to 8 Hz. The chromatographic data obtained were processed in the Quanlynx software (Waters, Milford, Massachusetts, USA) and the amino acid concentrations were quantified using a mixture of 17 amino acids as external standards: Alanine, Arginine, Aspartic acid, Cystine, Glutamic acid, Glycine, Histidine, Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Proline, Serine, Threonine, Tyrosine, Valine (Waters, Milford, Massachusetts, USA, p/n: WAT088122).

Statistical analyses

All statistical analyses were conducted using R (Version 4.3.1), MetaboAnalyst (Pang et al., 2024), and ShinyGo 0.77. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was conducted by mapping candidate genes to the Gene Ontology (GO) database (www.geneontology.org), calculating the number of genes per entry, applying hypergeometric tests, and correcting P values by multiple testing with Bioconductor package (Version 4.3) as Q value. Pathways with a final Q value ≤ 0.05 were defined as those significantly enriched in differentially expressed genes (DEGs). The DEGs were further analysed, including the GO term and KEGG enrichment pathways analyses, using Dr. Tom, a BGI customized data mining system (biosys.bgi.com). Network analyses were conducted on ShinyGo 0.77, a graphical gene-set enrichment online tool (Ge et al., 2019). RT-qPCR data were analysed using Student's t-tests or Kruskal-Wallis test depending on the normality of the data and the equality of residual variance.

Metabolomic data were first aligned, normalized, and centroid-scaled and compared to a pooled sample using Progenesis QI software (Version 2.4, Waters, Milford,

Massachusetts, USA). Features with a retention time lower than 1 min were removed from the analyses. Functional analyses were performed using the mummichog algorithm on MetaboAnalyst. The potential compound matches were obtained using the KEGG pathway for *Z. mays* (zma.kegg) library from December 2023 with a mass tolerance of 5 ppm. When several features were identified as the same compound, only the feature with highest abundance in the pool was kept, and the mummichog analysis was repeated to prevent overweighing of multiple features of the same compound. The m/z data were uploaded as m/z ranked by p-value for analysis. A hypergeometric p-value for each metabolic pathways with more than three entries was calculated. This step was repeated multiple times to calculate the null distribution of p-values for all pathways and was modelled as a Gamma distribution. Significant m/z features were used to calculate the p-values for each pathway. The resulting p-values were then adjusted for the permutations. The enrichment factor of a pathway was calculated as the ratio between the number of significant pathway hits and the expected number of compound hits within the pathway. Principal component analyses (PCAs) were performed on grouped features. Grouped features were obtained by removing background noise from samples using a signal to noise (S/N) ratio of 5, subtracting blank signals, removing artefact masses (first decimal place >0.8), and clustering features into groups based on similar retention times (± 0.02 min) and strong correlations (Pearson > 0.8) in their intensity profiles across all samples, suggesting they may represent fragments, isotopes, or adducts of the same compound. The most abundant feature of each group was selected for further analyses. Data was log 10 transformed to account for high differences in ion abundances. PCAs were created and visualized in MetaboAnalyst. PERMANOVAs were conducted based on 999 permutations. P-values were adjusted for multiple comparisons by False Discovery Rate (FDR) correction. Soluble sugars, amino acids, insect performance and survival, were tested for heterodasticity and analysed accordingly using Student’s t-tests and Mann Whitney U-tests. Benzoxazinoid concentrations were analysed using two-way ANOVAs on ranks.

Results

EPNs shifted the root transcriptome towards energy conservation.

EPN presence triggered a dynamic and time-dependent reprogramming of the root metabolism, transitioning from a rapid defense activation (at 6-24 h, Supplementary Information S1 and S2) to energy conservation (Figure 1). The presence of EPNs in soil for 72 h triggered the differential expression of 3’968 transcripts, among which 1’879 (including 718 annotated genes) where uniquely expressed in control plants and 2’098 (717 annotated genes) uniquely expressed in EPN-exposed plants (Figure 1A). Differentially Expressed Genes (DEGs) with more than 200 reads in at least one of the treatments can be found in Supplementary Table S2.

EPN exposure led to a shift away from growth-related processes. After only 6 h, EPN exposure decreased the expression of domain involving sugar phosphate transporters and glycolysis-related proteins such as gcn5-related N-acetyltransferase (Supplementary Information S2A). After 24 h, the downregulation of glycogen synthase kinase 3 and glycogen-related domains suggests a shift away from energy storage. Since glycogen-related annotations were inferred from homologous sequences and plants do not produce glycogen, this may indicate a shift away from starch synthesis in plants.

In parallel, EPN presence triggered a time-dependent reprogramming of the sugar metabolism (Figure 1, Supplementary Information S1 and S2). The expression of marker genes involved in sugar transport and metabolism showed an overall increase, albeit not significant, in gene expression at 6 and 24 h, but an inverted response at 72 h (Figure 1B, Supplementary Information S1-S3). Marker genes, including the invertase-encoding INCW3, INCW4, IVR1, and IVR2, exhibited increased expression after 6 and 24 h, suggesting an early boost in sugar metabolism and transport capacity (Supplementary Information S3). MTRANS, a sugar transporter gene, followed a similar expression pattern (Supplementary Information S3).

Additionally, EPNs induced the downregulation of sterol biosynthesis and mevalonate kinase pathways, crucial for membrane formation and cell division, (Supplementary Information S2). The downregulation of the glucose starvation response at 72 h further indicated that the plant was not actively mobilizing stored reserves for energy

production (Figure 1D). Consistently, the presence of EPNs in the soil led to a shift in the protein metabolism, lowering cellular maintenance processes, including actin-mediated functions, and upregulating some specific myosin activity and vesicle trafficking (Figure 1B-E, Supplementary Information S1 and S2). The effect was already noticeable 6 hours after EPN application (Supplementary Information S1 and S2) and extended until 72 hours. At this stage, the reduction in RNA splicing, protein import into the nucleus, and cytoplasmic translational initiation (biological processes), RNA binding (molecular function), RNA polymerase II activity, and polysomal RNA activity (cellular components) suggested that the protein synthesis machinery was suppressed (Figure 1C).

Biological processes such as glycophosphatidylinositol (GPI) anchor biosynthetic processes and protein export were further reduced, affecting transport of proteins (Figure 1B, C). The downregulation of actin motor activity, actin binding, and actin filament binding highlighted a lower ability for vesicle trafficking (Figure 1C). Similarly, the downregulation of Sec7 and Mon2 proteins, involved in vesicle trafficking and cellular signalling, alongside with the suppression of genes with cyclic guanosine monophosphate-specific phosphodiesterases (GAF) and ovarian Tumor domain (OTU)-like cysteine protease domains, involved in protein degradation and turnover, indicated that EPN-exposed plants limited certain aspects of cellular maintenance and protein turnover (Figure 1D). The downregulation of components related to myosin complexes, vesicles, and the ribosome (cellular components) further supported a shift away from routine cellular maintenance (Figure 1C).

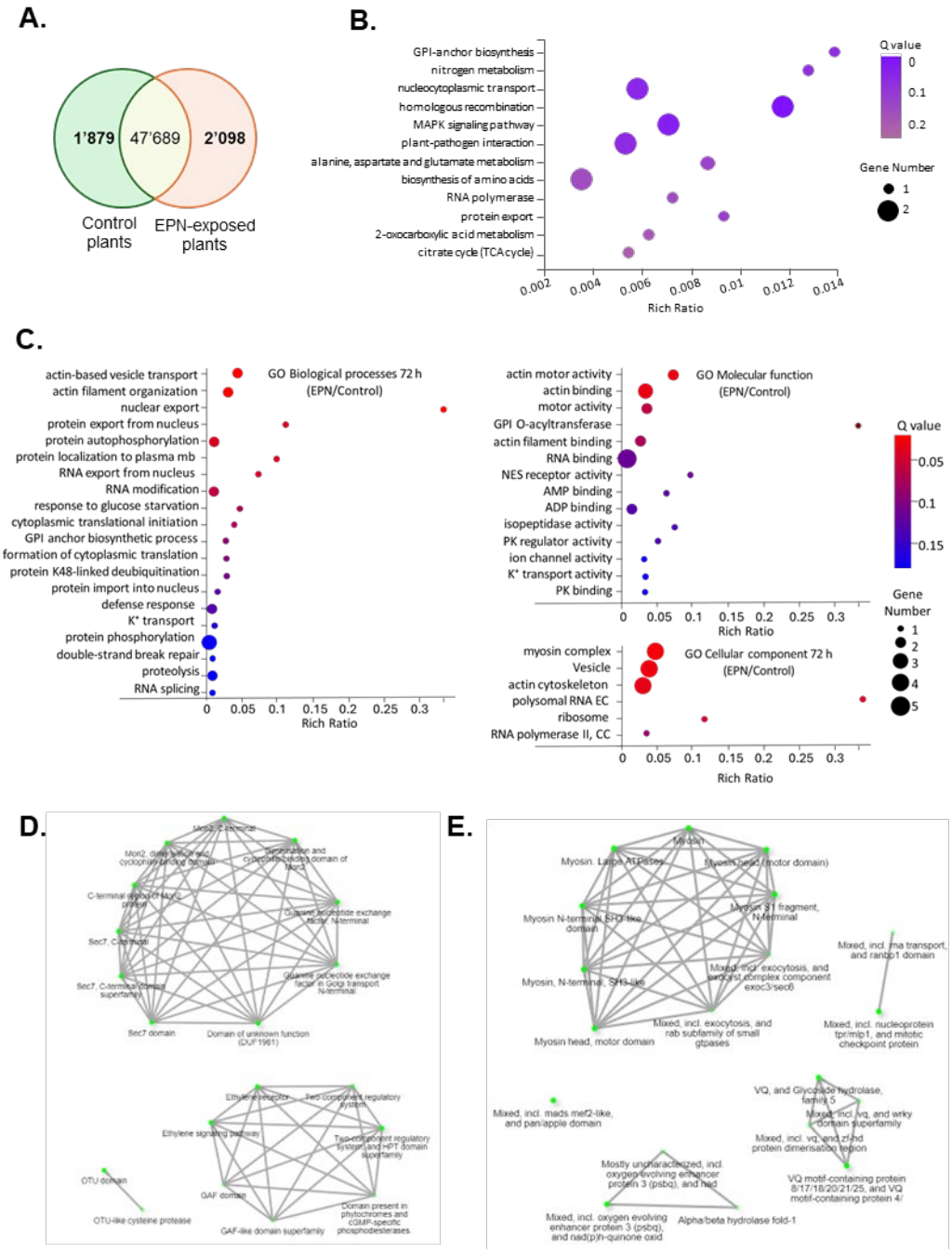

Figure 1. **Entomopathogenic nematodes (EPNs) modulate maize primary metabolism**. (A). Venn diagram illustrating the number of differentially expressed genes in control and EPN-exposed plants in transcriptomic analyses. (B). Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway enrichment analysis, where the size and colour of the bubbles represent the gene number and Q-value, respectively. (C) Gene Ontology (GO) term enrichment analyses of biological processes, molecular functions, and cellular components, respectively. The bubble plots show various enriched terms with their respective rich ratios and Q-values. Network analyses of (D) down- and (E) upregulated genes, respectively. EPN exposure was achieved by adding 2'000 *Heterorhabditis bacteriophora* nematodes for 72 h in the soil (n=5 per treatment). Mon2: domain of a specific protein involved in endomembrane trafficking. SC7: domain that is found in some pathogenesis-related proteins (PRs) in plants. DUF3475: Domain of Unknown Function 3475. OTU: Ovarian Tumor domain. cGMP: cyclic guanosine monophosphate. GAF: cGMP-specific phosphodiesterases, adenylyl cyclases, and FhlA. HPT: Histidine Phosphotransfer domain. MEF2: Myocyte Enhancer Factor. VQ: Valine-Glutamine. SH3: Src Homology 3 domain. EXO3/SEC6: Exocyst Complex Component 3. ZF-HD: Zinc Finger-Homeodomain protein. S1: Subfragment 1.

The downregulation protein autophosphorylation further reflected a strategy of energy conservation by lowering biosynthesis (Figure 1C). However, the upregulation of specific myosin-related domains, including the myosin head (motor domain) and myosin N-terminal SH3-like domains, and of exocytosis-related genes, such as those in the Rab subfamily of small GTPases and Exocyst complex components, suggested increased vesicle trafficking and cytoskeletal remodelling (Figure 1E), possibly facilitating the secretion of defence-related proteins or reinforcement cell wall defences (Figure 1E). The EPN-induced reduction protein kinase activity suggested a potential shift in regulation of growth and defence (Figure 1C).

EPNs induced a rapid, yet transient, defence response. After 6 h exposure to EPNs, the numerous lectin domains were upregulated (Supplementary Information S2). After 72 h, their expression reverted to control levels, in favour of genes encoding for stress-responsive enzymes, such as glycoside hydrolases, and proteins related to Valine-Glutamine (VQ) motifs (Figure 1E). Interestingly, genes involved in ethylene pathways and components of the two-component regulatory system were then downregulated (Figure 1D).

The EPN-induced response was time-dependent and highly dynamic. While EPN presence induced 2004, 1893, and 2098 uniquely expressed in EPN-exposed plants at 6, 24, and 72 h respectively, only one DEG (LOC100193455, nuclear-pore anchor) was consistently found through the three time points, demonstrating a highly dynamic and rapidly changing response (Supplementary Information S1). The expression of LOC100193455 was not changed after 72 h EPN exposure in an independent experiment (Supplementary Information S1).

EPNs induced metabolic shifts in primary and secondary metabolisms.

The presence of EPNs altered primary metabolism components in maize (Figure 2). EPNs induced a slight shift in metabolomic profiles of polar compounds (HILIC, $p=0.061$; Figure 2A, Supplementary Information S4). No individual compound showed significant differences between treatment after FDR correction. Yet, the beta-alanine metabolism, with hits for L-aspartate, spermidine, 5,6-dihydroucail, 3-ureidopropionate, 1,3-diaminpropane, and 4-aminbutyraldehyde, was enriched (Figure 2B). The cutin, suberine, and wax biosynthesis, including hits for 9,10-epoxy-

18-hydroxystearate, 10,16-dihydroxyhexadecanoic acid, and 16-feruloyloxypalmitic acid, tended to be enriched (p=0.051) (Figure 2B). EPN presence in soil induced a rapid increase in soluble sugar concentrations (Supplementary Information S5). After 72 h exposure, only glucose concentrations were more elevated in roots of EPN-exposed plants (Figure 2C, Supplementary Information S5). EPN exposure transiently increased total amino acid contents 6 h after application (p=0.054), an effect that disappeared after 24 h, and was even reversed for glutamic acid (Glu) and aspartate (Asp) levels after 72 h (Figure 2D, Supplementary Information S5).

Furthermore, the presence of EPNs in soil increased benzoxazinoids (BXs) levels in maize (Figure 2E) although did not induce an overall shift in non-polar and hydrophobic metabolomic profiles in maize (Supplementary Information S6). After EPN exposure, HMBOA and HMBOA-Glc levels led to the total increase in BXs levels in maize, with significant differences at 6 h and 24 h (Supplementary Information S5). Besides, EPN exposure also increased HMBOA-Glc concentration in maize root exudates after 72 h EPN exposure in an independent experiment (Supplementary Information S7). However, the time-dependent nature of the response, coupled with its initial progression in one direction followed by a reversal, introduced complexity and variability that made replicability challenging (Supplementary Information S8).

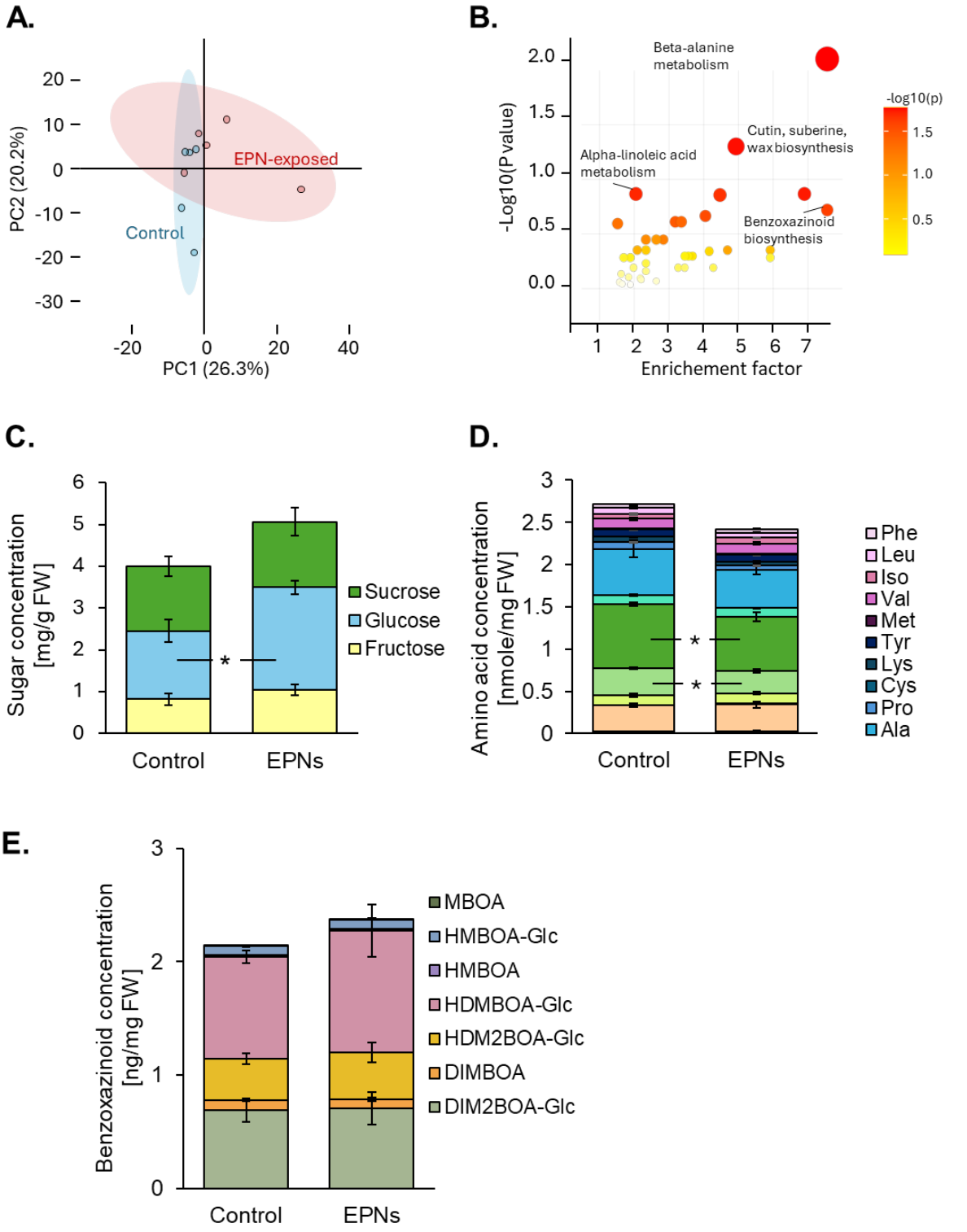

Figure 2. **Entomopathogenic nematodes (EPNs) altered maize soluble sugars and amino acids.** (A) Principal Component Analysis (PCA) of control and EPN-exposed roots based primarily on polar metabolites (p= 0.104). (B) Mummichog pathway activity profile of roots based primarily on polar metabolites. The colour and size of each circle corresponds to its p-value and enrichment factor, respectively. (C) Soluble sugar concentrations (Mean ±SEM) in control and EPN-exposed (EPNs) roots. (D) Free amino acid concentrations (Mean ±SEM) in in control and EPN-exposed (EPNs) roots. (E) Soluble sugar concentrations (Mean ±SEM) in control and EPN-exposed (EPNs) roots. EPN exposure was achieved by adding 2'000 *Heterorhabditis bacteriophora* nematodes for 72 h in the soil (n=5 per treatment). FW: fresh weight. Stars indicate significant differences (*: p<0.05).

Prior exposure to EPNs did not influence plant resistance to subsequent root herbivory.

Herbivore performance and survival of *D. virgifera* and *D. balteata* after six days of feeding was not affected on maize with a prior three-day long exposure to EPNs (Figure 3A-B). Herbivory by *D. virgifera* larvae increased BX levels (Figure 3C). Interestingly, a previous exposure to EPNs did modulate HMBOA, DIMBOA, and DIM_2BOA-Glc levels in roots (Figure 3C) in this experiment. While herbivory by *D. balteata* larvae did not increase BX levels (Figure 3D). No interaction between a previous EPN exposure and herbivory was observed (Figure 3).

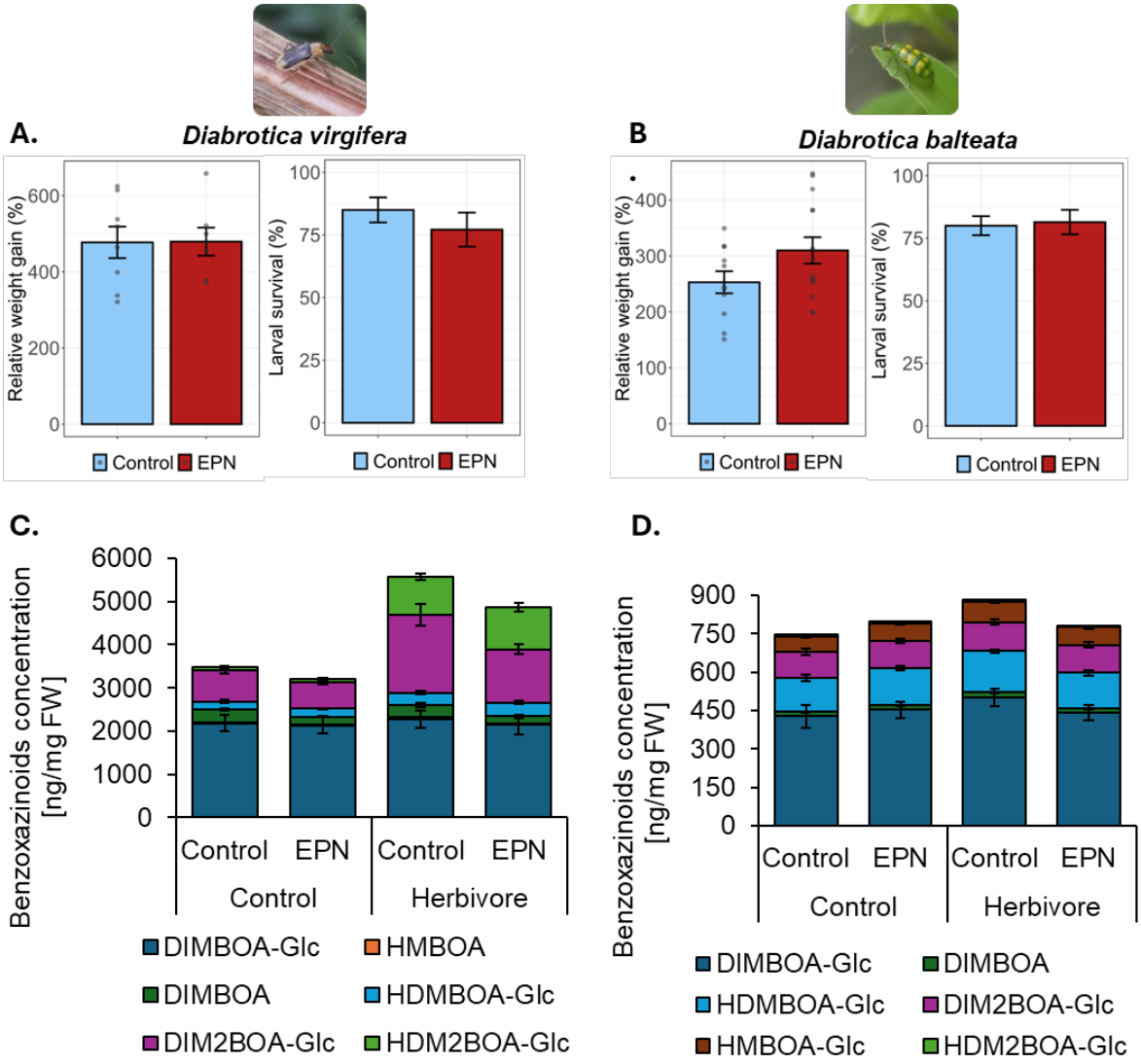

Figure 3. Herbivore performance and survival of *Diabrotica virgifera virgifera* (WCR) and *Diabrotica balteata* (BCB) is not affected on maize with prior exposure to entomopathogenic nematodes (EPNs). (A, B) Bar plots representing relative weight gain and larvae survival of *D. virgifera* larvae (n = 8) and *D. balteata* larvae (n = 15), respectively. Photos show adults of *D. virgifera* and *D. balteata*. (C, D) Stacked bar plots illustrating benzoxazinoids levels in maize roots. Maize seedlings were exposed to 2'000 *Heterorhabditis bacteriophora* nematodes for 72 h, then EPNs were removed, and root herbivores allowed to feed for 6 days. Bars indicate mean +/- standard error.

Discussion

This study demonstrates that maize plants exhibit a mild, yet highly dynamic, metabolic local response to EPN exposure, characterized by altered carbohydrate and amino acid metabolisms, increased BXs concentrations alongside with structural changes in cell walls. While the observed changes did not affect the performance nor survival of two root herbivores, their potential ecological relevance and implications for sustainable agriculture are discussed below.

The presence of EPNs in soil elicited a reconfiguration of the maize root metabolism, marked by slower growth-related processes, and defense activation. At 6 h, EPN presence induced resource mobilization, reflected by enriched pathways such as glycolysis/gluconeogenesis and oxidative phosphorylation, and increased sugar and amino acid contents in the roots. Interestingly, EPNs further tended to increase total BX contents, with significantly higher levels of HMBOA and HMBOA-Glc.

At 24 h, the response shifted toward the activation of secondary metabolic pathways and structural adjustments. EPN presence further enriched phenylpropanoid biosynthesis, suggesting a potential increase in phenolic compound production and/or lignin biosynthesis and potential reinforcement of cell walls. Consistently, total benzoxazinoid contents were increased, mostly driven by increases in HMBOA-Glc, HDMBOA-Glc, HDM_2BOA-Glc and DIM_2BOA-Glc.

At 72 h, EPN-induced responses transitioned to long-term structural and metabolic adjustments. Pathways related to protein biosynthesis, folding, and turnover and ethylene signalling were downregulated, while pathways for protein export and catalysis were enriched. Notably, amino acid and sugar metabolism showed an opposing trend compared to 6 h, with significant decreases in glutamate and aspartate levels, reduced expression of sugar-metabolism marker genes, despite elevated glucose levels. In parallel, benzoxazinoid levels returned to control baseline. Yet, enrichments in alpha-linolenic acid metabolism, glycan biosynthesis, and, albeit not significantly, in cutin, suberine, and wax biosynthesis pathways suggested enhanced barrier functions and lipid signalling. Despite the evident metabolic shift from primary to secondary processes, the overall plant response remained relatively modest, as evidenced by the low number of DEGs exceeding 200 reads across the time points.

Several hypotheses can explain the adaptive value of plant responses to EPNs. First, maize plants might use the presence of EPNs as an indicator of nearby herbivores and increase their investment in defence accordingly. Since EPNs emerge from herbivore hosts and have limited mobility of just some cm per day (Bal et al., 2014; Labaude & Griffin, 2018), their presence could signal the proximity of soil-dwelling herbivores. However, our results did not support that hypothesis given that EPN-induced plant response did not enhance resistance to root herbivory.

Second, plants may optimize top-down control of herbivores by investing in the recruitment or maintenance of EPNs as beneficial organisms. While EPN juveniles do not feed, the root exudates may act directly on EPN behavior to maintain them around the roots (Zhang et al., 2021) or indirectly by creating a favorable rhizosphere environment (Maushe et al., 2023). However, the increased BXs levels upon EPN exposure observed in an independent experiment did not supported this hypothesis given that BXs are toxic for EPNs (Robert et al., 2017). Additionally, the transient increase in sugar metabolism and transport might lead to an increased exudation into the soil. EPN-induced root exudation and its consequences on EPN behaviour and infectivity should be further investigated.

Alternatively, plants may respond to conserved nematode signals present in EPNs, but also in phytopathogenic nematodes (PPNs). Both EPNs and PPNs are soil-dwelling nematodes that interact with roots, and plants may perceive common nematode-associated molecular patterns (NAMPs) such as Ascaroside 18, Ascr#18 (Manosalva et al., 2015). The perception of unspecific signals could trigger non-specific defence responses. For instance, plants often activate pathways related to glycan biosynthesis, cell wall modification, and secondary metabolite production, reflecting a strategy to reinforce root defences upon PPN exposure (Meresa et al., 2024). Similarly, EPN exposure in maize led to the upregulation of genes involved in carbohydrate biosynthesis and binding, and increased benzoxazinoid concentrations in roots and in root exudates. It is thus tempting to speculate that EPN-induced plant responses might underlie some of the EPN-mediated PPN population reduction in the field (Kamali et al., 2022; Sayedain et al., 2021; Sushma et al., 2024). However, EPNs did not induce significant enrichment but rather a reduction in pathways associated with nematode feeding sites, such as auxin and ethylene regulation (Gheysen &

Mitchum, 2011). This divergence might reflect the absence of certain cues specific to PPN-host interactions, such as effector-triggered responses, or mechanical signals from feeding sites. Finally, EPNs may manipulate plants to facilitate predation. The impact of the EPN-induced reconfiguration of the plant primary metabolism on the insect susceptibility to infection would allow to test this hypothesis.

This study provides valuable insights into EPN-induced plant responses and offers promising avenues for integrated pest management strategies strategies. Despite the dynamic nature of the plant responses and the associated challenges in reproducibility, assessing EPN-induced plant responses may provide valuable insights into key belowground interactions and their ecological significance. For instance, testing the cell wall fortification and its consequences for PPNs might provide critical insights into plant-mediated EPN-PPN interactions. Additionally, refining the timing of the response and its duration will help characterizing potentially long-lasting effects. Finally, testing the effects in the field will further shed light on the ecological relevance of the effect. By leveraging EPNs as biological control agents and understanding their interactions with plant defences, it may be possible to finetune current biocontrol strategies and to improve root stress resilience. Future studies should aim to replicate these findings under field conditions, explore the broader impacts of EPN-induced exudate changes, and investigate the long-term implications of such responses for crop health and soil ecosystem dynamics. These efforts will be critical for integrating EPN-based strategies into sustainable agricultural practices.

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Data availability statement

The data will be deposited on the DRYAD repository (Dryad | Home - publish and preserve your data). All raw data can be provided upon reasonable demand to the corresponding author.

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Conflict of interest

The authors declare no conflict of interest.

Author contribution

AMVM designed and conducted the experiments, analysed the data, and wrote the chapter. PAH designed and conducted the experiments, analysed the data, and wrote the chapter. PM established, validated, and ran samples for chemical analyses. KD conducted experiments. MH ran samples for chemical analyses. RRC and CP analysed transcriptomic data. CAMR acquired funding, supervised the project, analysed data, and wrote the chapter.

List of figures

Supplementary Information

Supplementary Figure S1: Transcriptomic profiles of maize roots after 6, 24, and 72 h EPN exposure.

Supplementary Figure S2: Network analyses on transcriptomic profiles of maize roots after 6, 24, and 72 h EPN exposure.

Supplementary Figure S3: Gene expression of marker genes involved in sugar metabolism after 6, 24, and 72 h EPN exposure.

Supplementary Figure S4: Polar metabolomic profiles (HILIC) of maize roots after 6, 24, and 72 h EPN exposure.

Supplementary Figure S5: Sugar, amino acid, and benzoxazinoid concentrations in maize roots after 6, 24, and 72 h EPN exposure.

Supplementary Figure S6: Hydrophobic and non-polar metabolomic profiles (C18) of maize roots after 6, 24, and 72 h EPN exposure.

Supplementary Figure S7: Benzoxazinoid concentrations in maize root exudates after 72 h EPN exposure.

Supplementary Figure S8: Metabolomic profiles of maize roots after 72 h EPN exposure from an independent repetition experiment.

Figure S1. Transcriptomic profiles of maize roots after 6, 24, and 72 h EPN exposure. (A) KEEG pathways enrichment bubble charts of maize roots after 6 h EPN exposure. (B) KEEG pathways enrichment bubble charts of maize roots after 24 h EPN exposure. (C) KEEG pathways enrichment bubble charts of maize roots after 72 h EPN exposure. Venn diagrams represent the number of transcripts expressed in control and EPN-exposed plants (n=5 per treatment). (D) Box plot representing the gene expression of the only common differentially expressed gene (DEG) *Zm*193455 in maize roots after 6, 24, and 72 h EPN exposure in an independent experiment where roots were collected just after 72 h EPN exposure (n=6). EPN exposure was achieved by adding 2'000 infective juveniles of *Heterorhabditis bacteriophora* nematodes in the soil.

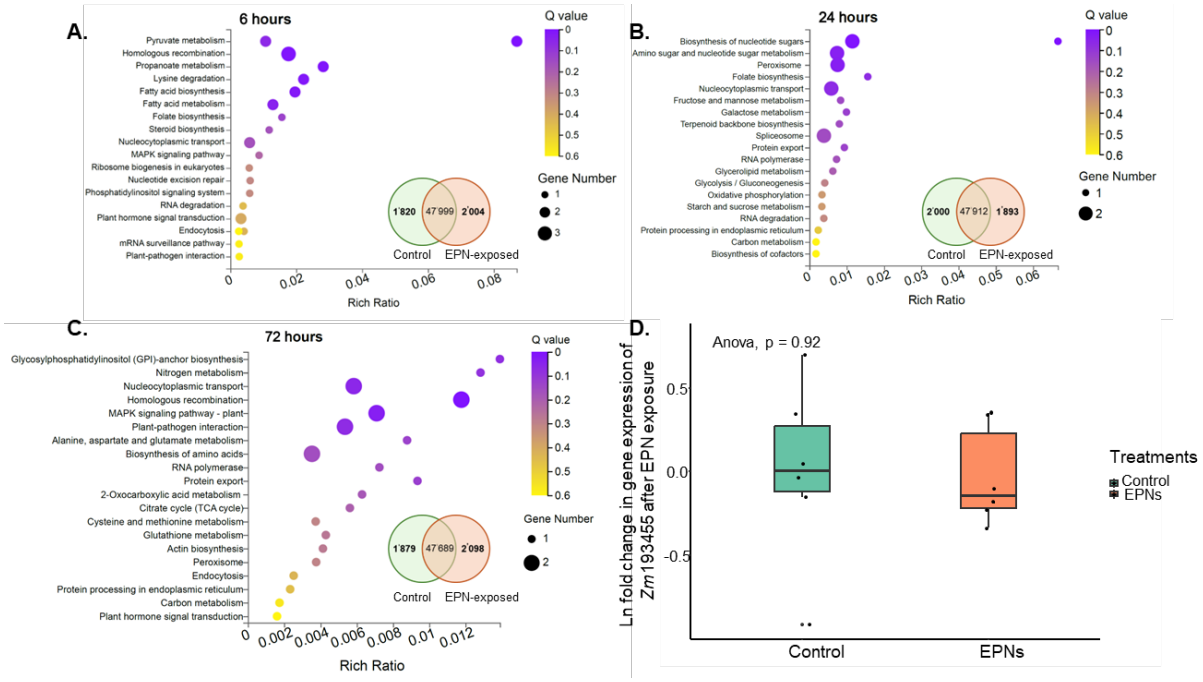


Figure S2. Network analyses on transcriptomic profiles of maize roots after 6, 24, and 72 h EPN exposure. (A). Network analyses of down- and (B) upregulated differentially expressed genes (DEGs), respectively after 6 h EPN exposure. (C). Network analyses of down- and (D) upregulated DEGs, respectively after 24 h EPN exposure. (E). Network analyses of down- and (F) upregulated DEGs, respectively after 72 h EPN exposure. EPN exposure was achieved by adding 2'000 *Heterorhabditis bacteriophora* nematodes for 72 hours in the soil (n=5 per treatment).

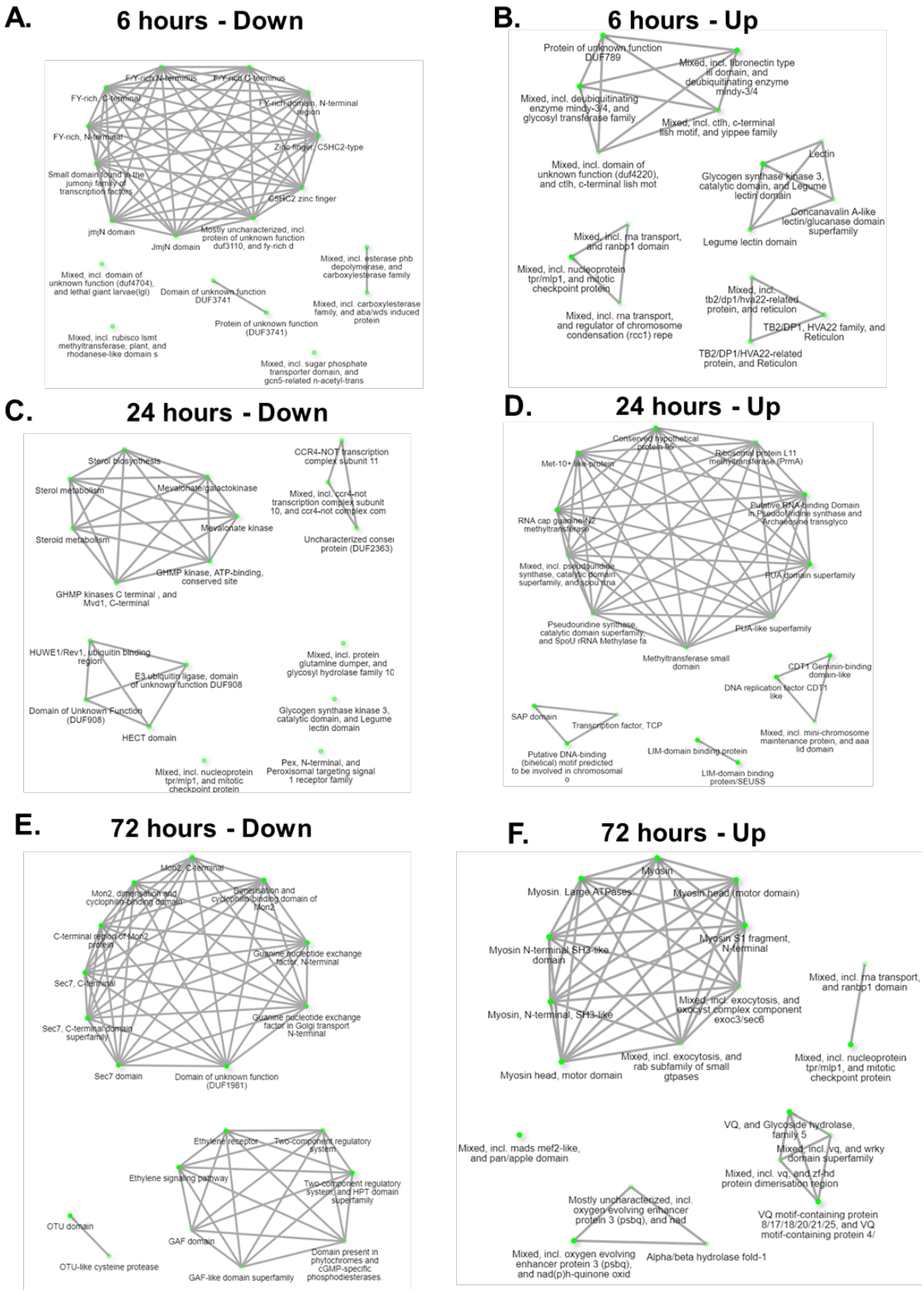


Figure S3. Gene expression of marker genes involved in sugar metabolism after 6, 24, and 72 h EPN exposure (n=5). (A) Bar plot representing Ln-transformed fold-change values (mean ± SE) in the expression of marker genes involved in carbohydrate reallocation in maize roots after EPN exposition for 6 h. (B) Bar plot representing Ln-transformed fold-change values (mean ± SE) in the expression of marker genes involved in carbohydrate reallocation in maize roots after EPN exposition for 24 h. (C) Bar plot representing Ln-transformed fold-change values (mean ± SE) in the expression of marker genes involved in carbohydrate reallocation in maize roots after EPN exposition for 72 h.

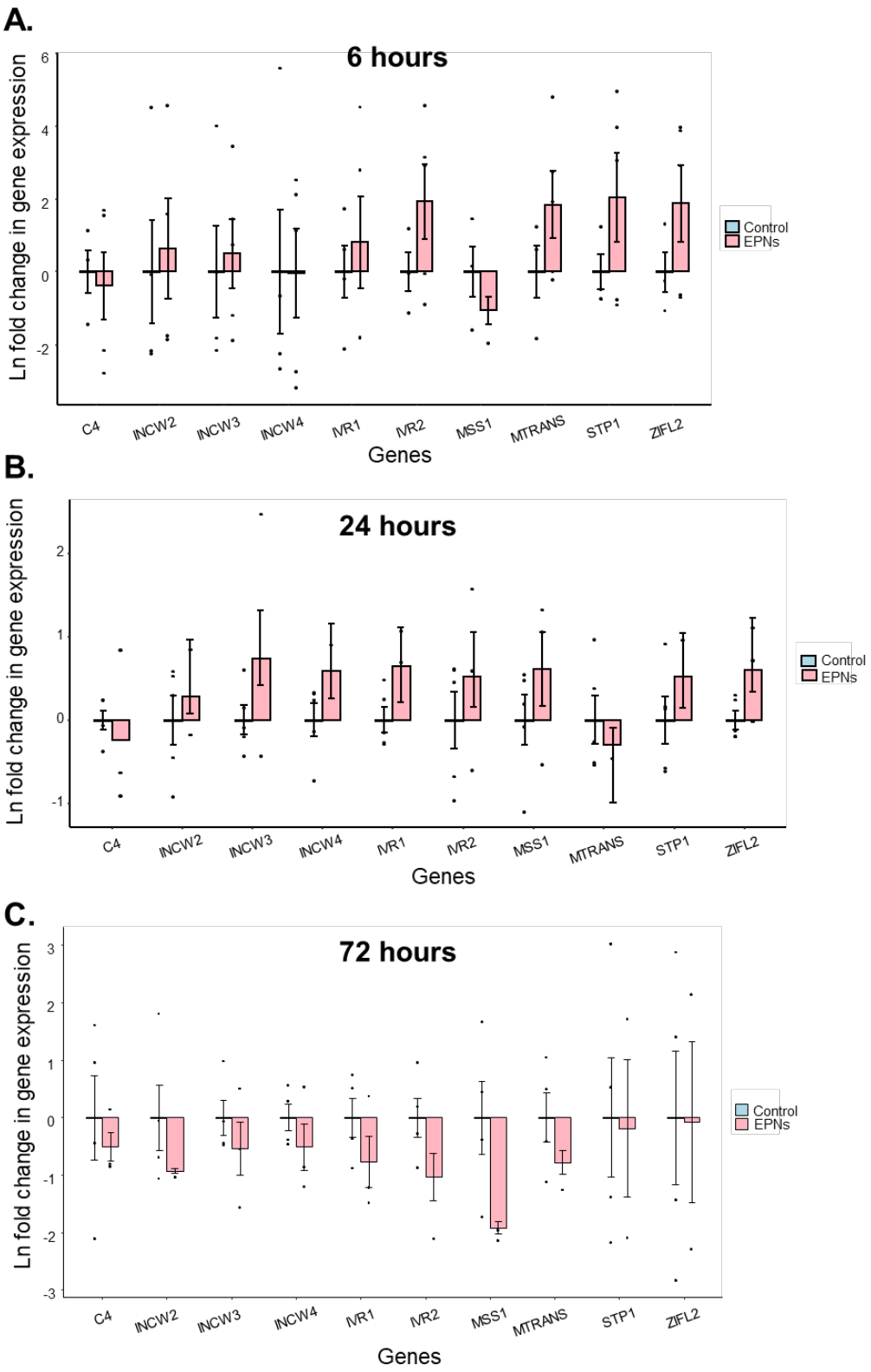


Figure S4. Polar metabolomic profiles (HILIC) of maize roots after 6, 24, and 72 h EPN exposure. (A), (C) and (E) PCAs of control and EPN-exposed roots based primarily on polar metabolites after 6, 24 and 72 h EPN exposure, respectively. (B), (D) and (F) Mummichog pathway activity profile of roots based primarily on polar after 6, 24 and 72 h EPN exposure, respectively. The colour and size of each circle corresponds to its p-value and enrichment factor, respectively. EPN exposure was achieved by adding 2'000 *Heterorhabditis bacteriophora* nematodes for 72 h in the soil (n=6 per treatment).

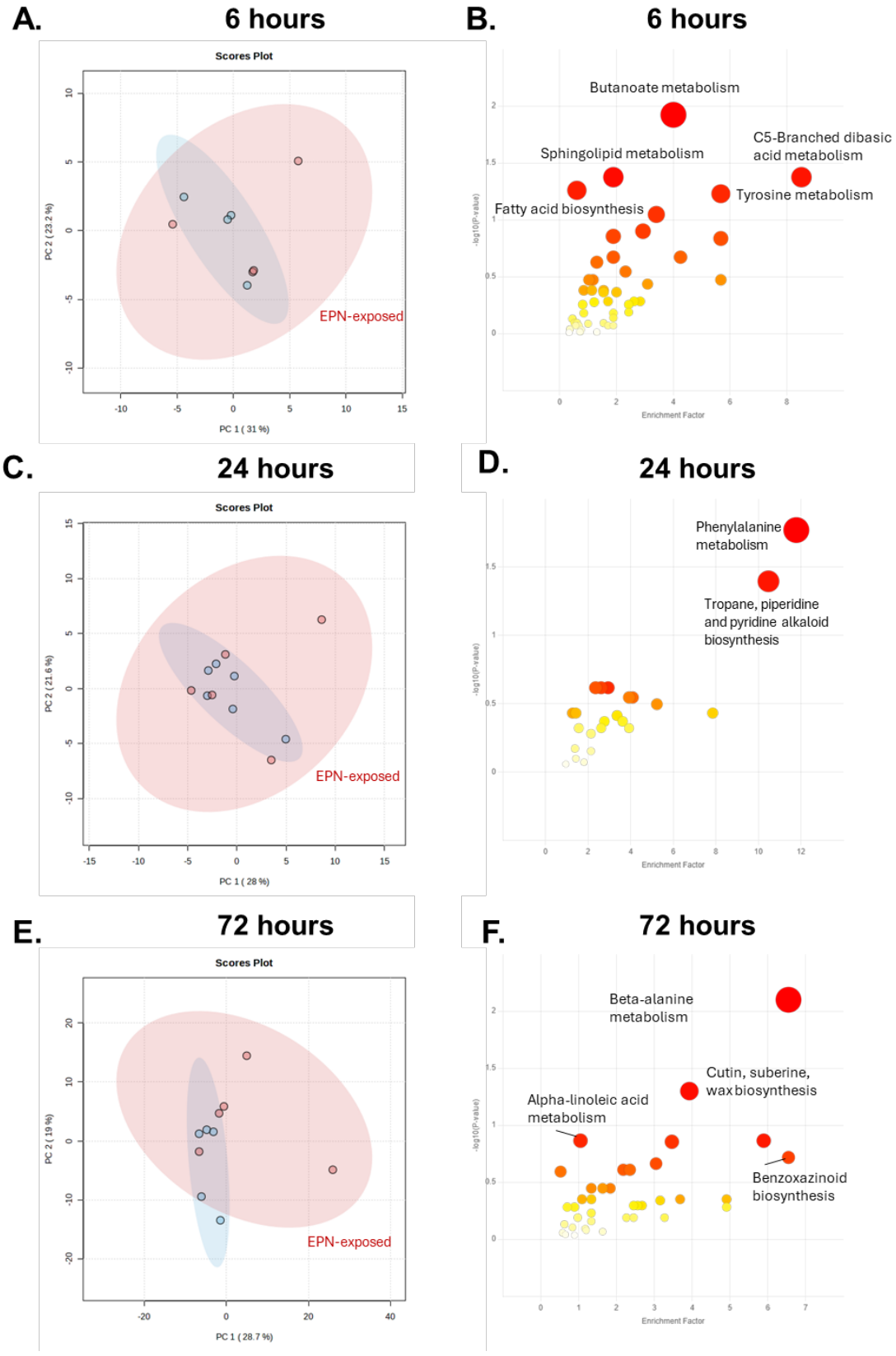


Figure S5. Amino acid, sugar and benzoxazinoid concentrations in maize roots after 6, 24, and 72 h EPN exposure. (A), (B) and (C) Stacked bar plots representing the concentrations of amino acids, sugars and benzoxazinoids, respectively, in maize roots after 6 h EPN exposure. (D), (E) and (F) Stacked bar plots representing the concentrations of amino acids, sugars and benzoxazinoids, respectively, in maize roots after 24 h EPN exposure. (G), (H) and (I) Stacked bar plots representing the concentrations of amino acids, sugars and benzoxazinoids, respectively, in maize roots after 72 h EPN exposure. EPN exposure was achieved by adding 2'000 *Heterorhabditis bacteriophora* nematodes for 72 h in the soil (n=5-6 per treatment). FW: fresh weight. Stars indicate significant differences (º: p<0.10; *: p<0.05).

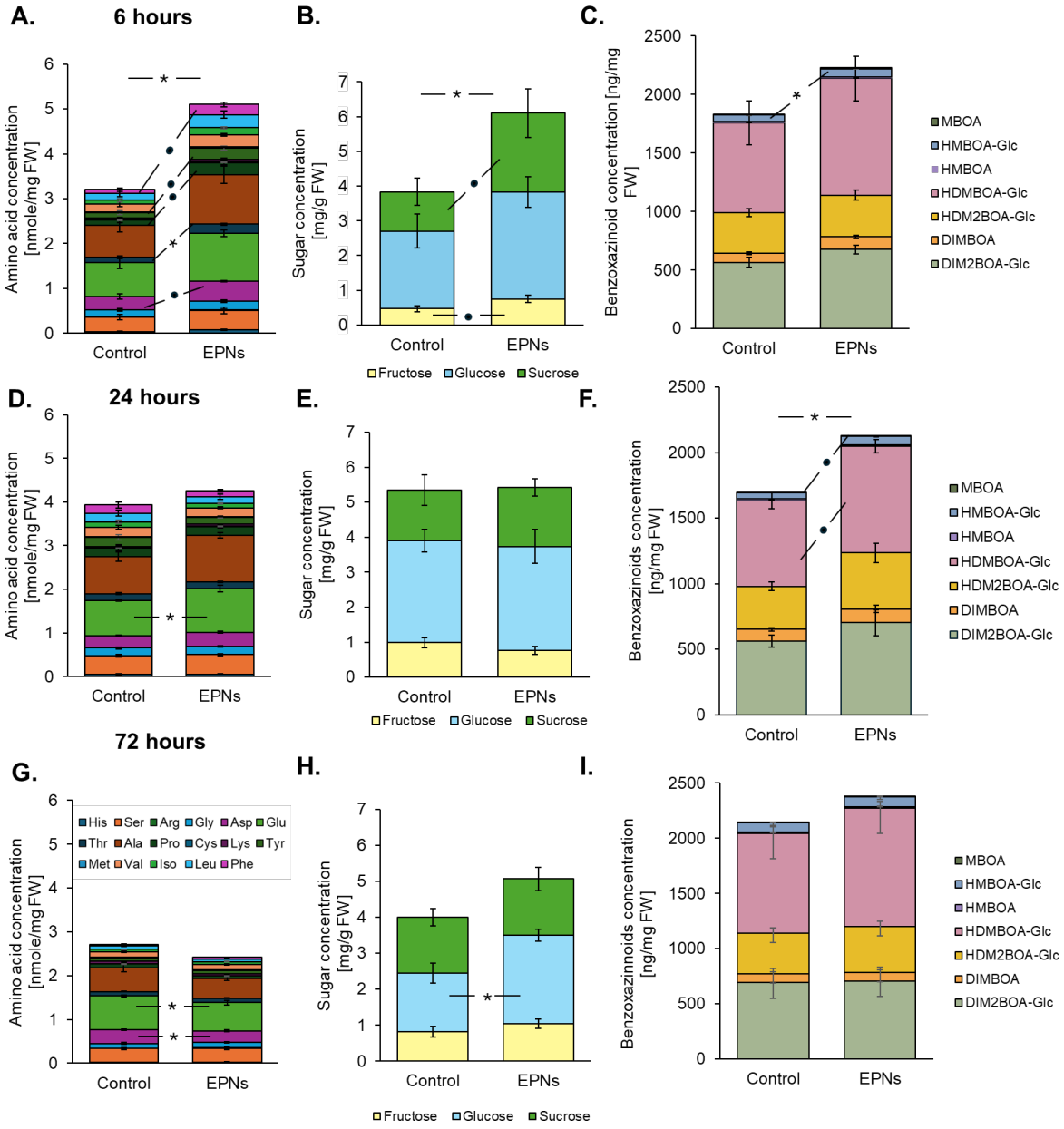


Figure S6. Hydrophobic and non-polar metabolomic profiles (C18) of maize roots after 6, 24, and 72 h EPN exposure. (A), (C) and (E) PCAs of control and EPN-exposed roots based primarily on hydrophibic and non-polar metabolites after 6, 24 and 72 h EPN exposure, respectively. (B), (D) and (F) Mummichog pathway activity profile of roots based primarily on hydrophibic and non-polar after 6, 24 and 72 h EPN exposure, respectively. The colour and size of each circle corresponds to its p-value and enrichment factor, respectively. EPN exposure was achieved by adding 2'000 *Heterorhabditis bacteriophora* nematodes for 72 h in the soil (n=6 per treatment).

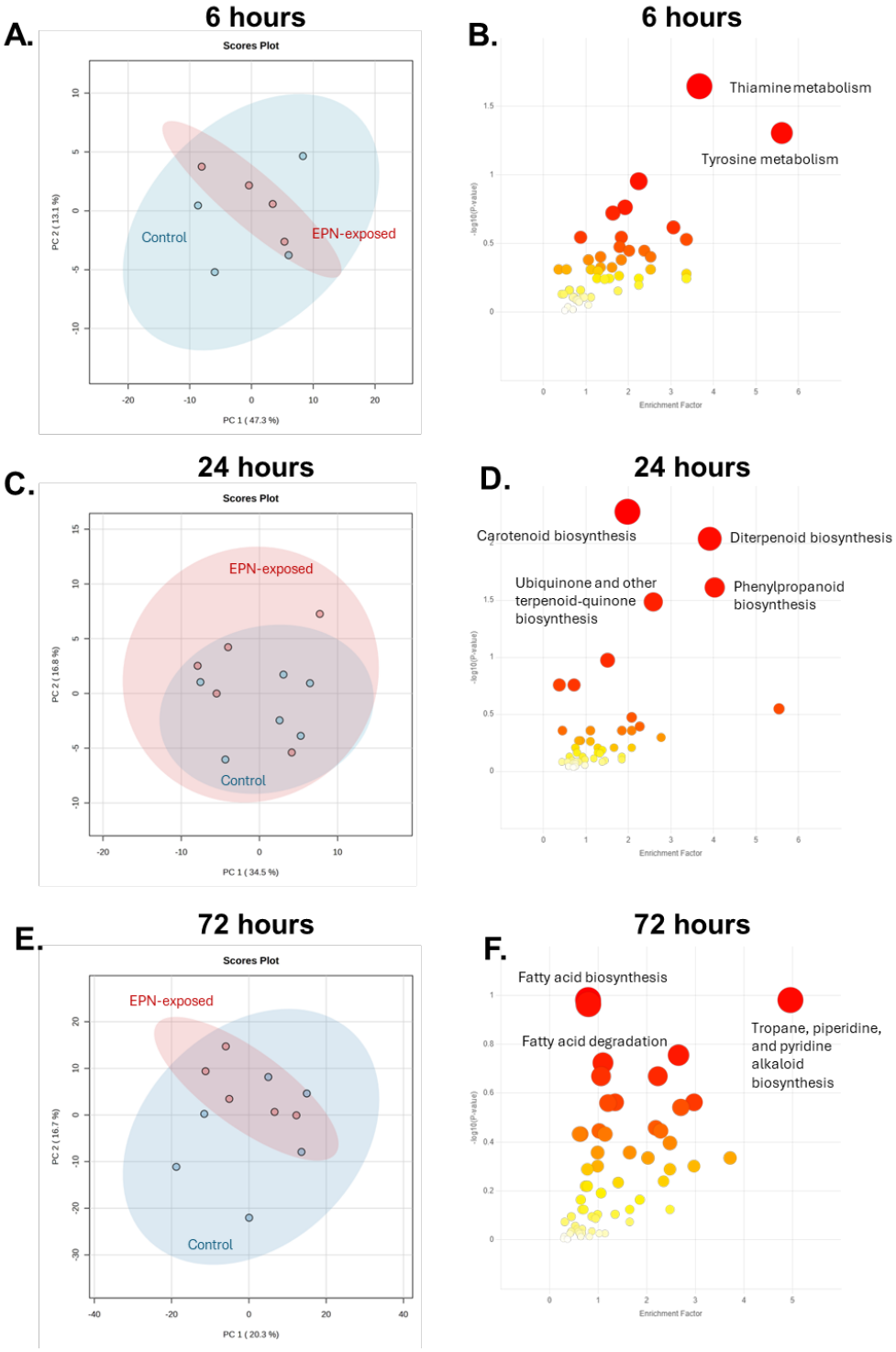


Figure S7. Benzoxazinoid concentrations in maize root exudates after 72 h EPN exposure. EPN exposure was achieved by adding 2'000 infective juveniles of *Heterorhabditis bacteriophora* nematodes for 72 hours in the soil (n=6 per treatment). Bars indicate mean +/- standard error. Star indicates significant differences (*: p<0.05).

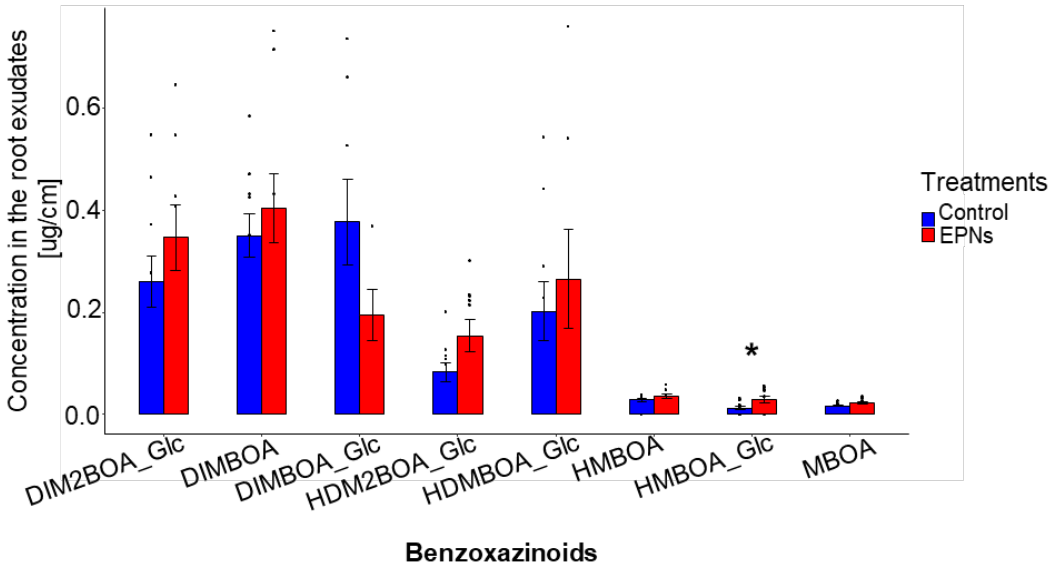
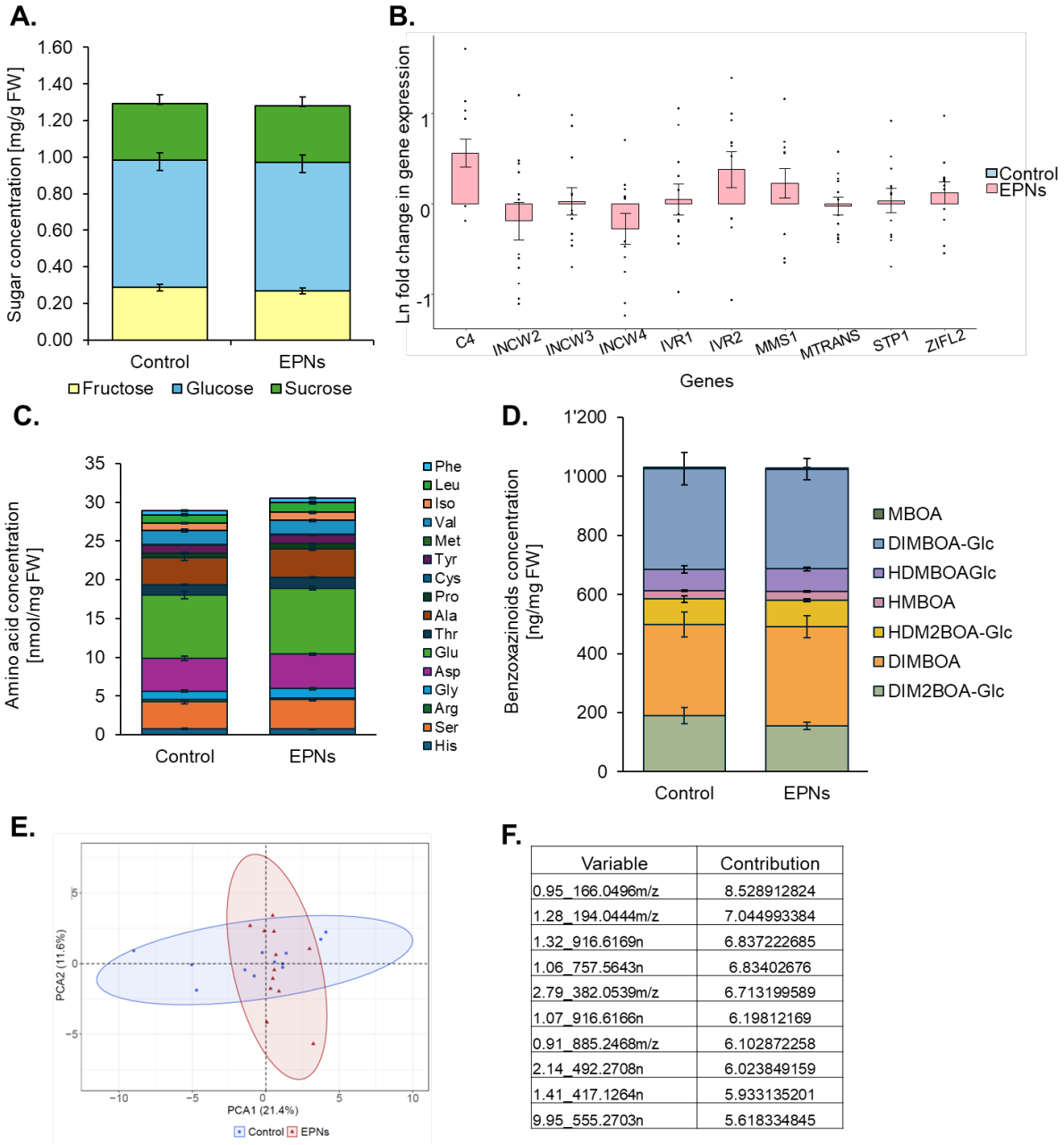


Figure S8. Metabolomic profiles of maize roots after 72 h EPN exposure from an independent repetition experiment. (A) Stacked bar plot representing fructose, glucose and sucrose concentration in maize roots upon EPN exposure. (B) Bar plot representing Ln-transformed fold-change values (mean ± SE) in the expression of marker genes involved in carbohydrate reallocation in maize roots upon EPN exposure. (C) Stacked bar plot representing amino acids concentration in maize roots upon EPN exposure. (D) Stacked bar plot representing benzoxazinoids concentration in maize roots upon EPN exposure. (E) Principal components analysis (PCA) including powered partial least squares – distribution analysis (PPLS-DA) of control and EPN-exposed plants. (F) Table showing the mass/charge ratios and contributions of the 10 top compounds that influence PC1. EPN exposure was achieved by adding 2'000 *Heterorhabditis bacteriophora* nematodes for 72 hours in the soil (n=12 per treatment). Bars indicate mean +/- standard error. FW: fresh weight.



F.

Variable	Contribution
0.95_166.0496m/z	8.528912824
1.28_194.0444m/z	7.044993384
1.32_916.6169n	6.837222685
1.06_757.5643n	6.83402676
2.79_382.0539m/z	6.713199589
1.07_916.6166n	6.19812169
0.91_885.2468m/z	6.102872258
2.14_492.2708n	6.023849159
1.41_417.1264n	5.933135201
9.95_555.2703n	5.618334845

Supplementary Tables

Supplementary Table S1: Primers used for RT-qPCR analysis in this study.

Supplementary Table S2: Differentially Expressed Genes (DEGs) in maize roots after 6, 24, and 72 h exposure to entomopathogenic nematodes (EPNs).

Table S1. Primer list for RT-qPCR used in the evaluation of the carbohydrate allocation patterns in this study adapted from previous study (Robert et al., 2012).

Gene name	Putative function	Forward primer (5'-3')	Reverse primer (5'-3')
ZmACTIN1	Actin	CCATGAGGCCACGTACAACT	GGTAAAACCCCACTGAGGA
ZmUBI1	Ubiquitin	TAAGCTGCCGATGTGCCTGCG	CTGAAAGACAGAACATAATGAGCACAG
ZmC4	Carbohydrate transporter	GGTGGGCGTACACGTTCCCG	TCGGAGCTGGACGAGCGGAA
ZmINCW2	Cell wall invertase	GACCCTACCAAGTCGTCCCTGA	CGACCGGTCGATCAGGCTTC
ZmINCW3	Cell wall invertase	GACGATCGCGCTGAGGACAC	TAGCTACTGCGCCGGCACG
ZmINCW4	Cell wall invertase	TGCGGGGAGAAGGGCG	CGTCTCCGCGTGCTCAGG
ZmIVR1	Invertase	TCTCCCGTGATCCTGCCCCG	GGCCCGCGCAAAGTGTTGTG
ZmIVR2	Invertase	GGGCGTCGCTGCAGGGTATC	CCTCCTCCACGGGCCACTGA
ZmMSS1	Hexose transporter	GGCTGCCACAGGCGGTTTGA	GTCAGCCCCGCGAGGTACAG
ZmMTRANS	Mannitol transporter	GCGTTGCTAGAAACAGCTACCG	GATGGAGGCACTCTTCGCCGCC
ZmSTP1	Carbohydrate transporter	TTCGCCAACCAGTCCGTGCC	CAGCCGCCCCTGATCTTGGC
ZmZIFL2	Carbohydrate transmembrane transporter	GGGAGCCACTGCTGGCGAAG	CGGCAGGGTGCAGGTGATGG

Table S2. Significant differentially expressed genes (DEGs), filtered to more than 100-200reads, of local response of maize plants to entomopathogenic nematodes (EPN) exposure. EPN exposure was achieved by adding 2'000 infective juveniles of *Heterorhabditis bacteriophora* nematodes for 72 h in the soil (n=5 per treatment). SAUR: Small Auxin Up RNA. WAT1: Walls are thin 1.

Symbol	log 2 (EPNs/Control)	Q value	Description	Molecular Function	Time Point
LOC100191931	-0.585	0.036	SAUR56-auxin-responsive SAUR family member	response to auxin	6 h
LOC100191759	-0.487	0.0437	uncharacterized LOC100191759	acid phosphatase activity	24 h
LOC100275282	-1.354	0.047	uncharacterized LOC100275282	integral component of membrane	72 h
LOC100279365	1.940	0.035	WAT1-related protein	transmembrane transporter activity	72 h
LOC100279369	0.454	0.008	uncharacterized LOC100279369	nucleotide binding	72 h
LOC100280503	-0.204	0.017	uncharacterized LOC100280503	translation initiation factor activity	72 h
LOC100284097	3.754	0.019	xyloglucan endotransglucosylase/hydrolase protein 1	hydrolase activity, hydrolyzing O-glycosyl compounds	72 h
LOC118474731	1.256	0.005	uncharacterized LOC118474731		72 h

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Chapter 2 - The presence of entomopathogenic nematodes in soil induces plant resistance to the leaf herbivore *Ostrinia nubilalis*

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Abstract

Tritrophic interactions between plants, herbivores and herbivore natural enemies are key drivers of shaping natural and agroecosystems. Several interactions have been well explored over the past decades, but one remains severely understudied: The direct plant response to entomopathogenic nematodes (EPNs), and how it may impact the plant metabolome and resistance towards aboveground herbivory. This study investigated how maize response to EPNs influence plant-herbivore interactions aboveground. The results showed that EPN presence in the field decreased the abundance of the European corn borer (*Ostrinia nubilalis*). The observed difference was due to reduced oviposition on EPN-exposed maize plants, mediated by contact cues. Transcriptomic analysis exposed that EPN presence induced a shift in fatty acid metabolism, potentially indicating the remodelling of leaf cuticular waxes or cuticular contents. Moreover, the data suggested that EPN presence induced plant defence markers in maize leaves. Therefore, further research is needed to further elucidate the underlying mechanisms of the response in field to improve the use of EPNs as a tool of sustainable pest management in agriculture.

Introduction

Tritrophic interactions between plants, herbivores, and the enemies of the herbivores are critical in ecosystem functioning and diversity (Price et al. 1980; Abdala-Roberts et al. 2019; Colazza et al. 2023). Interestingly, plants can perceive and respond to the presence of the third trophic level (Jagdale et al. 2009; Helms et al. 2019; Li et al. 2020). However, the ecological impact of these direct plant-predator interactions remains overlooked.

Studies on how plants react to herbivore natural enemies have been sparse until now. Nevertheless, a previous study found that lacewing larvae cause a shift in the plant metabolome of tomato plants in the absence of herbivores (Errard et al. 2016). Besides, predatory mirid bugs have been shown to induce resistance in tomato plants against thrips and spider mites (Puysseleyr et al. 2011; Pappas et al. 2015; Zhang et al. 2018). However, mirid bugs are omnivorous and most likely induced the plant response by feeding. Even the carnivorous lacewing larvae were observed to feed on the plants, most likely due to absence of prey. Therefore, natural enemies in these studies may have simply induced a wound response in the plant like a herbivore.

Plants also respond to entomopathogenic nematodes (EPNs), roundworms that infect and kill soil-dwelling insect herbivores but do not attack plants. EPNs belong mostly to the genera *Heterorhabditis* and *Steinernema* and are found worldwide (Kaya and Gaugler 1993; Koppenhöfer et al. 2020). They live freely in the soil as infective juveniles (IJs) and look for potential hosts to infest (Zhang et al. 2021). Once they have found a suitable host, they enter their body through the mouth, anus, or weak spots in the outer cuticle. Inside the insect, the IJs start to excrete endosymbiotic bacteria into the hemolymph. The bacteria release proteinogenic toxins into the insect, which leads to its death within 1-3 days (Gaugler 1990; Rodou et al. 2010). Afterwards, the bacteria grow on the carcass and EPNs start feeding on the decaying flesh and multiply. As soon as the resources of the carcass are depleted, IJs emerge from the body in search for another host. EPNs and cues associated to them elicit stress responses by increasing salicylic acid content and induce activity of ROS-scavenging enzymes in Arabidopsis (Jagdale et al. 2009). Application of two EPN species reduced the concentration of nicotine in the leaves in tobacco, while only the presence of *Heterorhabditis bacteriophora* increased glucose levels (Li et al. 2020). Furthermore, the presence of EPNs in the soil reduced the number of aphids present on the plants

(Li et al. 2020). In tomato, *Steinernema carpocapsae* presence decreased the activity of guaiacol peroxidase and polyphenol oxidases in leaves like the presence of plant pathogenic nematodes (Kamali et al. 2022). Additionally, application of three different EPN species in the soil lowered the oviposition rate by *Tuta absoluta* moths (Kamali et al. 2022). However, the plant response to EPNs has rarely been investigated in the field. So far, we still lack information on the ecological relevance of plant response to EPNs.

This study evaluated the impact of EPN presence on maize growth and resistance to natural herbivory in the field. The results showed that the presence of EPNs induced plant resistance against the European Corn Borer (*O. nubilalis*), *Ostrinia nubilalis*. The possible underlying mechanisms of EPN-induced plant resistance to aboveground herbivores are still under investigation. Moreover, this study includes the implications of these results and highlights further questions to be addressed to further elucidate the ecological implications of systemic plant responses to EPNs.

Materials and methods

Biological resources

Maize seeds (*Zea mays* L.) of the variety B73 were kindly provided by Maize GDB (www.maizegdb.org) and bred by Delley (Delley seeds and plants Ltd, Delley, Switzerland). Maize seeds var. Amaveritas were bought from Agromais GmbH, Everswinkel, Germany.

Plants were sown in cylindrical 100 mL plastic pots (11 cm height x 4 cm diameter, Semadeni, Ostermundigen, Switzerland), 1 L or 10 L plastic pots (Lambrecht-Verpackungen GmbH, Göttingen, Germany) depending on the duration of the experiment. All plants were grown in soil (Selmaterra Schweizer Schwererde torfreduziert, Bigler Samen AG, Thun, Switzerland) and kept in a greenhouse at 23 +/- 2°C, approx. 60 % humidity and a day:night cycle of 16:8 hours. Fertilizer (Plantaktiv® Typ K, Hauert HBG Dünger AG, Grossaffoltern, Switzerland) was added once a week (0.1-0.3 % w/v) following the manufacturer instructions.

Larvae of *Galleria melonella* were bought at a local fishery shop (Fischereibedarf Wenger, Bern, Switzerland) and stored at 8°C until use for EPN rearing. Eggs of *Ostrinia nubilalis* were bought from BTL Bio-Test Labor GmbH Sagerheide (Groß Lüsewitz, Germany) and reared on general purpose lepidoptera diet (Frontier

Agricultural Sciences, DE, USA) and young maize leaves (var. “Akku”, DSP, Switzerland).

Entomopathogenic nematodes (*Heterorhabditis bacteriophora* Poinar, EN01) were bought from Andermatt Biocontrol (Grossdietwil, Switzerland) and reared *in vivo* in greater wax moth larvae (*G. melonella* Lepidoptera: Pyralidae, Fischereibedarf Wenger, Bern, Switzerland)). For the field trial, *H. bacteriophora* (EN01) were kindly provided by e-nema GmbH (Schwentinental, Germany) and used directly without prior rearing in *G. melonella*. Infective juveniles (IJs) were used for all experiments.

Field study

A field study was conducted in 2021 at Tachenhausen at the agricultural facilities of the Nürtingen-Geislingen University, Germany (48°39'08.9"N 9°23'20.7"E). A week before planting, the field was treated with the herbicides Stomp and Spectrum (BASF, Ludwigshafen am Rhein, Germany) at a concentration of 2.8 and 1.4 L/hectare, respectively. In May, Amaveritas maize seeds were grown as buffer plants at 8 plants per m^2 with 75 cm row spacing. B73 maize seeds were grown every third row of the hybrid variety at a 1.5 m distance between plants, to ensure that EPNs cannot travel between B73 plants. One month later, randomly chosen B73 plants across the field were inoculated with 10’000 live EPN IJs (n=8). The inoculation was performed by adding 50 mL of tap water containing IJs into a 5 cm deep hole, dug 5 cm away from the stem. Control plants underwent the same procedure but received 50 mL of tap water without IJs. EPN inoculation was repeated monthly over three months. Plant height was measured bimonthly. The presence of *O. nubilalis* larvae and associated damage were recorded in August at the flowering stage.

Insect performance

Pre-germinated seedlings were transferred into 1 L pots with soil. After five to six weeks of growth under greenhouse conditions, 2’000 EPN IJs were applied in 10 mL tap water by creating a 2–3 cm deep hole in the soil approximately 4 cm away from the stem to minimize root damage (n=5). Control plants received water only (n=5). After 24 h, three pre-weighed third-instar *O. nubilalis* larvae were placed into the leaf whorl of each plant. The plants were then covered with air- and humidity-permeable fleece (Pflanzen-Winterschutz-Vlies, 17 g/m², Tebo AG, Windisch, Switzerland) to prevent larval escape. Seven days later, the larvae were collected and weighed. Leaf

tissue was harvested and immediately frozen in liquid nitrogen for subsequent metabolomic, phytohormone, sugar, and amino acid analyses.

Insect oviposition preference

Oviposition preference was assessed in two independent experiments. In a first experiment, two-week-old maize plants were opposite each other within a dome-shaped cage 80 × 60 × 60 H × L × W cm, mesh size 0.23 × 0.33 mm (Mathe Textil Ltd, Budapest, Hungary). One of the plants was treated with 2'000 EPNs in 10 mL of tap water applied at 3 cm from the stem, while the second plant received 10 mL of tap water without IJs. A dish containing cotton soaked in honey water (concentration 3 %) was placed between the plants to provide nutrients for the *O. nubilalis* moths. How long later, three female moths were released into each cage and allowed to oviposit for 72 h. The number of egg clutches and eggs per clutch were then recorded for each plant (n=3). Because some cages did not have any eggs after 72 h, the experiment was repeated two times to ensure sufficient replicate number.

In a second experiment, the plants offered to *O. nubilalis* females were covered with perforated plastic bags to prevent *O. nubilalis* females from landing on the plant and using contact cues (Figure S5). Because some cages did not have any eggs after 72 hours, the experiment was repeated four times to ensure sufficient replicate number.

Transcriptomic analysis

Total RNA was extracted from 100 ± 2 mg ground maize leaves using a RNeasy Plant Mini Kit (QIAGEN Naamloze Vennootschap (N. V.), Venlo, The Netherlands) according to the manufacturer's instructions. The RNA integrity was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc, Santa Clara, California, USA). Samples with an RNA Integrity Number (RIN) ≥ 7 were used for subsequent analyses. The libraries were sequenced in a DNBSEQTM platform by Beijing Genomics Institute (BGI) (BGI Hong Kong Company Limited, Hong Kong, China) which generated 100 bp paired-end reads and 30 M reads per sample. Low quality reads, reads with adaptor sequences, and reads with high levels of N base were removed from the raw data with the filtering software SOAPnuke (Version v1.5.2). Hierarchical Indexing for Spliced Alignment of Transcripts (HISAT; Version v2.0.4) was used to map RNA-seq reads (Reference Genome version: GCF_902167145.1_Zm-B73-REFERENCE-NAM-5.0) and Bowtie2 tool was used to align the clean reads to the reference genes.

Gene Ontology (GO) functional significant enrichment analysis gave the GO functional entries (terms) that are significantly enriched in candidate genes compared to the full genetic background of the species. For this analysis, first they mapped all candidate genes to each entry in the Gene Ontology database (www.geneontology.org), calculates the number of genes per entry, and then applies a hypergeometric test to find the GO function that is significantly enriched in candidate genes compared to all background genes of the specie. Then, they calculated the p-value using stats package (Version 4.4.0) of R. The p-value was corrected by multiple testing with Bioconductor package (Version 4.3) as Q value. Finally, Q value (corrected p-value ≤ 0.05 was used as the threshold), and the GO term that satisfied this condition was defined as the GO term that was significantly enriched in candidate genes.

Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was done with the same methodology as the GO functional enrichment analysis, as described above for GO enrichment. Pathways with a final Q value ≤ 0.05 were defined as those significantly enriched in differentially expressed genes (DEGs), and significant enrichment by pathway identified the most important biochemical metabolic and signalling pathways involved in candidate genes. The DEGs were further analysed, including the GO term and KEGG enrichment pathways analyses, using Dr. Tom, a BGI customized data mining system (biosys.bgi.com). Network analyses were conducted on ShinyGo 0.77, a graphical gene-set enrichment online tool (Ge et al., 2019).

Metabolomic analyses

Metabolomic profiles of leaf samples were analysed with an Acquity Ultra-High Performance Liquid Chromatography (UHPLC) system coupled to a G2-XS quadrupole Time-Of-Flight Mass Spectrometer (qTOF-MS) equipped with an electrospray source (Waters, Milford, MA, USA) as previously described (Sun et al., 2022). Briefly, the chromatography was performed using a Ethylene Bridged Hybrid (BEH) C18 column (100 × 2.1 mm i.d., 1.7 µm particle size) using the following elution profile: 2–50% B over 4 min, 50–100% B over 2 min, 100% B for 2 min, followed by re-equilibration with 2% B for 2 min, where A = 0.1% formic acid (Fisher Chemical, Waltham, MA, USA) in ultra-pure MilliQ water and B = 0.1% formic acid in acetonitrile (Biosolve Chimie, Dieuze, France). The flow rate and temperature of the column were maintained at 0.4 mL.min^{-1} and 40°C, respectively. The qTOF-MS operated in positive mode. The electrospray capillary and sampling cone voltages were set to 2 kV and 20

V, respectively. The source temperature was kept at 140°C and the desolvation gas temperature at 400 °C. The desolvation and cone gas flows were 1000 L.hr-1 and 100 L.hr-1, respectively. Centroided data were acquired over a range of 50-1’200 m/z in MSE mode, using alternating scans of 0.1 s at low collision energy of 6 eV and 0.1 s at high collision energy ramped from 10 to 30 eV. The resulting chromatograms were aligned and normalized with Progenesis QI v.2.4 (Waters, Milford, USA).

Phytohormone analysis

The phytohormones in leaf tissue were extracted and analysed according to an adapted protocol described in Glauser et al. 2014. In short, fresh frozen leaf tissue was ground to a fine powder in liquid nitrogen. Then, 50 mg of powder were mixed with 500 µL of extraction solvent (10 % Methanol [MeOH, Fisher Chemical] + 0.2 % FA) and 10 µL of internal standard solution (10 ng/mL d_5-JA, d_6-ABA, d_6-SA, 1 µg/mL d_5-IAA [CDN Isotopes Inc., Point-Claire, Canada] and 10 ng/mL $^{13}C_6$-JA-Ile, kindly provided by Gaétan Glauser). The mixture was extracted for 3 min at 30 Hz in a mixer mill (Retsch, Haan, Deutschland). After centrifugation at 15’000 *g*, the supernatant is removed and mixed with 500 µL of MilliQ water. This mixture is loaded onto an Oasis MAX SPE column (30 mg, 1cc, Waters, Milford, MA, USA) after equilibration of the column using MeOH and 95:5 H_2O:MeOH + 0.1 % FA. Then, columns were washed with a 95:5 H_2O:NH_3 (25 %, Fluka Chemie AG, Buchs, Switzerland) solution and MeOH + 0.2 % NH_3 (25 %). Afterwards, the phytohormones were eluted from the columns with a 98:2 % MeOH:FA solution. Finally, the eluent was evaporated in a CentriVap (Labconco Corp, MO, USA) and phytohormones were redissolved in 200 µL of 35 % MeOH. Samples were analysed using a UHPLC-MS/MS targeted approach (Glauser et al. 2014). The analysis was performed on an Acquity UHPLC system equipped with a BEH C18 column (50 x 2.1 mm, 1.7 µm) coupled to a QTRAP 6500+ LC-MS/MS (AB Sciex LLC, Framingham, MA, USA) operating in negative mode.

Sugar analysis

Soluble sugars from maize roots and root exudates were quantified using a Sucrose/D-Glucose/D-Fructose assay kit (Megazyme, Bray, Ireland) according to the manufacturer instructions (n=10 per treatment). Sugars from leaf tissue were extracted by mixing 50 mg of finely ground frozen tissue powder with 250 µL of 80 % Ethanol (EtOH, Fisher Chemical). This mixture was heated to 78°C while shaking at 800 rpm for 15 min. Afterwards, extracts were centrifuged for 10 min at 15’000 g and

supernatant removed. This step was repeated twice more with 50 % EtOH and supernatants pooled for the assay. The preparation of the samples was done in transparent, flat-bottom 96-well plates (Dynatec laboratories, El Paso, USA) and the measurements were performed on a Tecan Infinite M200PRO plate reader (Tecan Group Ltd., Männedorf, Switzerland).

Amino acid analysis

Free amino acids were quantified using the AccQ-Tag Ultra Derivatization Kit (Waters, Milford, USA) according to the manufacterer instructions (n= 10 per treatment). Amino acids were extracted by mixing 100 mg of tissue powder with 1 mL of extraction buffer (50 % EtOH + 0.1 % FA). The mixture was vortexed and centrifuged at 15'000 x *g* for 10 minutes. The supernatant was removed and evaporated to dryness in vacuum under 45°C in a CentriVap (Labconco Corporation, Kansas City, USA). Free amino acids were redissolved in 400 µL of sterile MilliQ water obtained from a Barnstead™ Nanopure™ (Thermo Fisher Scientific Inc, Waltham, USA). Derivatized samples were analysed with an Acquity UHPLC system coupled to a Quadrupole Dalton-based Mass Spectrometer (QDa-MS) equipped with an electrospray source and an UV/Vis-Detector (Waters, Milford, MA, USA). The chromatography was operated with a BEH C18 column (100 × 2.1 mm i.d., 1.7 µm particle size, REF provider) using the following elution gradients: 0.1 % B for 0.54 min, 0.1-9.1 % B over 5.2 min, 9.1-21.2 % B over 2 min, 21.2-59.6 % B for 0.3 min, followed by flushing the column with 90 % B for 0.6 min and re-equilibration at 0.1 % B for 0.89 min (A = H20:ACN 99:1 + 0.1 % FA, B = ACN + 0.1 % FA). The flow rate and temperature of the column was kept constant at 0.7 mL/min) and 55°C. The QDa-MS was operated in positive, single ion recording mode. The electrospray voltage was kept at 0.8 kV, while the cone voltage was adjusted according to the measured ions. The source and probe temperature were kept at 120°C and 600°C, respectively. The sampling frequency was set to 8 Hz. The chromatographic data obtained was processed in QuanLynx v.4.1 (Waters, Milford, USA) and amino acid concentrations were quantified using external standards (Waters, Milford, USA).

Statistical analysis

Data obtained was analysed using R (www.r-project.org, version 4.2.2). Plant growth and targeted analysis data were tested for normality and homogeneity of variance. Height measurements over time were analysed using linear models. Field height data

were analysed using Type III ANOVAs, as the design was unbalanced. Infestation of *O. nubilalis* in the field and oviposition preference were analysed with a Chi-square test, assuming both conditions to be equal as a null hypothesis. Two-sample tests were performed using Welch's test for parametric data and the Wilcoxon-rank-sum test for non-parametric data. Assays on inducibility of plant response to herbivory and EPNs were analysed using Two-way ANOVA. Tukey HSD tests were used as post-hoc tests, but only when the interaction between the factors was significant, with p-values adjusted by the false discovery rate. The heatmap of amino acid fold change in leaf and stem tissue was generated with the heatmap.2 function (gplots, version 3.1.3), and statistical evaluation was performed with a Dunnett's test to compare the control to each condition only.

Metabolomic data were analysed using principal component analysis (PCA) and powered partial least squares – distribution analysis (PPLS-DA). For all analyses, background noise from samples was removed by discarding peaks present in blank measurements at a signal:noise ratio of 5:1. Retention time of measured features were rounded to 1/100 minutes and the most abundant ion feature at each retention time was selected for analysis. Data was log transformed and scaled to account for high differences in relative ion abundances. To create and visualize PCAs, the packages FactoMineR (Version 2.4), factoextra (Version 1.0.7) and ggplot2 (Version 3.3.6) were used. PPLS-DAs were calculated using the MVA.test and MVA.cmv function in the package RVAideMemoire (Version 0.9-81-2). Volcano plots were created with the use of the package EnhancedVolcano (Version 1.12.0).

Results

EPN presence in soil reduced oviposition by the stem borer, *Ostrinia nubilalis*, through changes in plant contact cues.

EPN presence in soil positively affected plant growth in the field (Figure S1). The difference in plant height between control and EPN-exposed plants increased until day 76, between the 2nd and 3rd EPN application, and disappeared later in the season (Figure S1) but was not observed under greenhouse conditions (Figure S2). EPN presence did not affect chlorophyll content nor yield under both field and greenhouse conditions (Figures S1 and S2). EPN-exposed plants were less frequently infested by naturally occurring *O. nubilalis* (Figure 1A-B), although similar damage levels were observed on the plants when present (Figure S3). Consistently, preference assays

conducted under laboratory conditions revealed that *O. nubilalis* females that could land onto the plant leaves preferentially oviposited on control plants rather than on EPN-exposed plants (Figure 1C). However, preference assays preventing *O. nubilalis* females to land on maize leaves was sufficient to abolish the selective behaviour (Figure 1C), suggesting that contact cues were necessary for the oviposition selection of control over EPN plants. This observation was consistent with the fact that no difference in maize volatile emissions was detected upon EPN exposure (Figure S4). In both preference assays, the number of eggs per clutch remained similar between control and EPN-exposed plants (Figure 1D). *O. nubilalis* larval performance and survival was not affected by the presence of EPN in soil (Figure 1E-F).

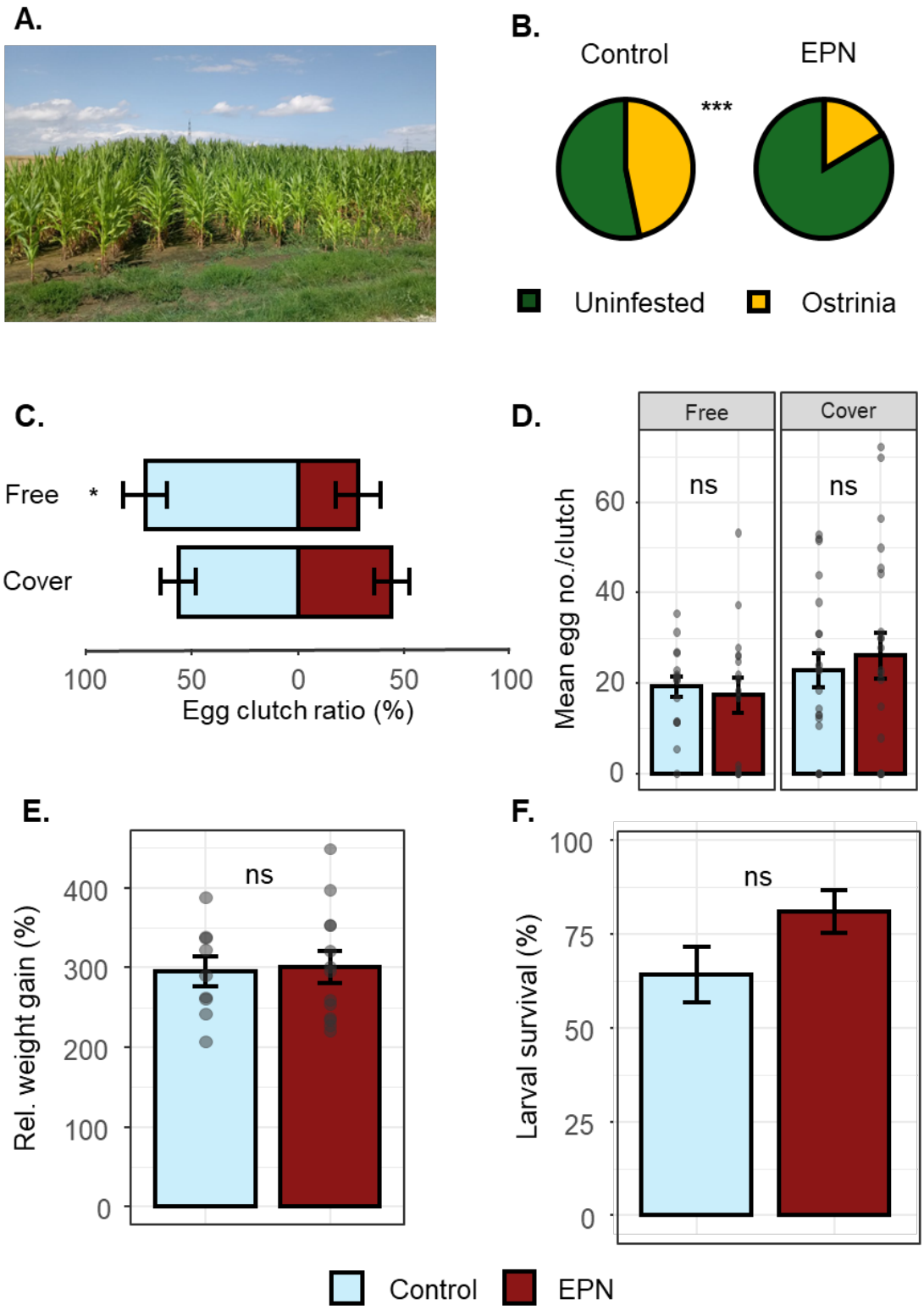

Figure 1. Entomopathogenic nematodes (EPNs) presence in the soil reduced *Ostrinia nubilalis* oviposition in maize plants. (A). Photo of maize field used in this study. (B) Pie charts representing the rate of infestation by *O. nubilalis* in control and EPN exposed plants in the field (n=8). (C) Ratio of egg clutches on maize plants without ("Free") or with ("Bagged") covers (n=5). (D) Mean number of eggs per clutch (n=13). (E) Relative weight gain of 3rd to 4th instar *O. nubilalis* larvae after one week of feeding in maize plants (n=13). (F) Survival of *O. nubilalis* larvae after one week of feeding in maize plants (n=13). Error bars indicate mean +/- standard error. Stars indicate significant differences (***= p-value < 0.001, ns: not significant).

EPN presence in soil altered the maize transcriptomic profiles

The presence of EPNs in soil induced the differential expression of 4’173 transcripts, including 2’144 transcripts uniquely expressed in control plants and 2’029 uniquely expressed in EPN-exposed plants (Figure 2A). The KEGG enrichment analysis highlighted enrichment in pyruvate metabolism, propanoate metabolism, fatty acid biosynthesis and metabolism, and sesquiterpenoid and triterpenoid biosynthesis (Figure 2B). The observed enrichment may reflect EPN-induced enhanced energy production, cell membrane integrity and the adaptation of defence-related pathways.

Consistently, the GO term enrichment analyses showed that the presence of EPNs in the soil influenced several cellular and metabolic processes in plants, potentially affecting plant energy production and cell wall biosynthesis. Biological process enrichment in processes such as mitochondrial transcription, mRNA processing, and para-aminobenzoic acid biosynthetic process highlighted changes in cellular maintenance and in metabolic pathways (Figure 2C). Molecular function enrichment included pathways of nucleotide binding, NEDD8 activating enzyme activity, 4-amino-4-deoxychorismate synthase activity, and gamma-glutamylamine/cysteinylserine synthase activity, potentially pointing towards lower signalling in the presence of EPNs (Figure 2C). Cellular component enrichment, such as in mitochondrial DNA-directed RNA polymerase, dehydrodolichyl diphosphate synthase complex, and cellulose synthase complex pathways, indicated decreased cellular activity related to energy production and cell wall biosynthesis (Figure 2C) (Table S2).

The network analysis also suggested that EPN presence in the soil lower some signalling process in maize leaves. The results exposed that differentially expressed genes (DEGs) related to endoribonuclease, and gamma-glutamylaminecyclotransferase and TOPLESS (TPL)-binding domain in jasmonate signalling and RNA recognition motif activities were downregulated (Figure 2D). However, other processes also relevant for plant immunity were upregulated such as receptors, transporters and Lazarus 1(LAZ1)-domain (Figure 2E).

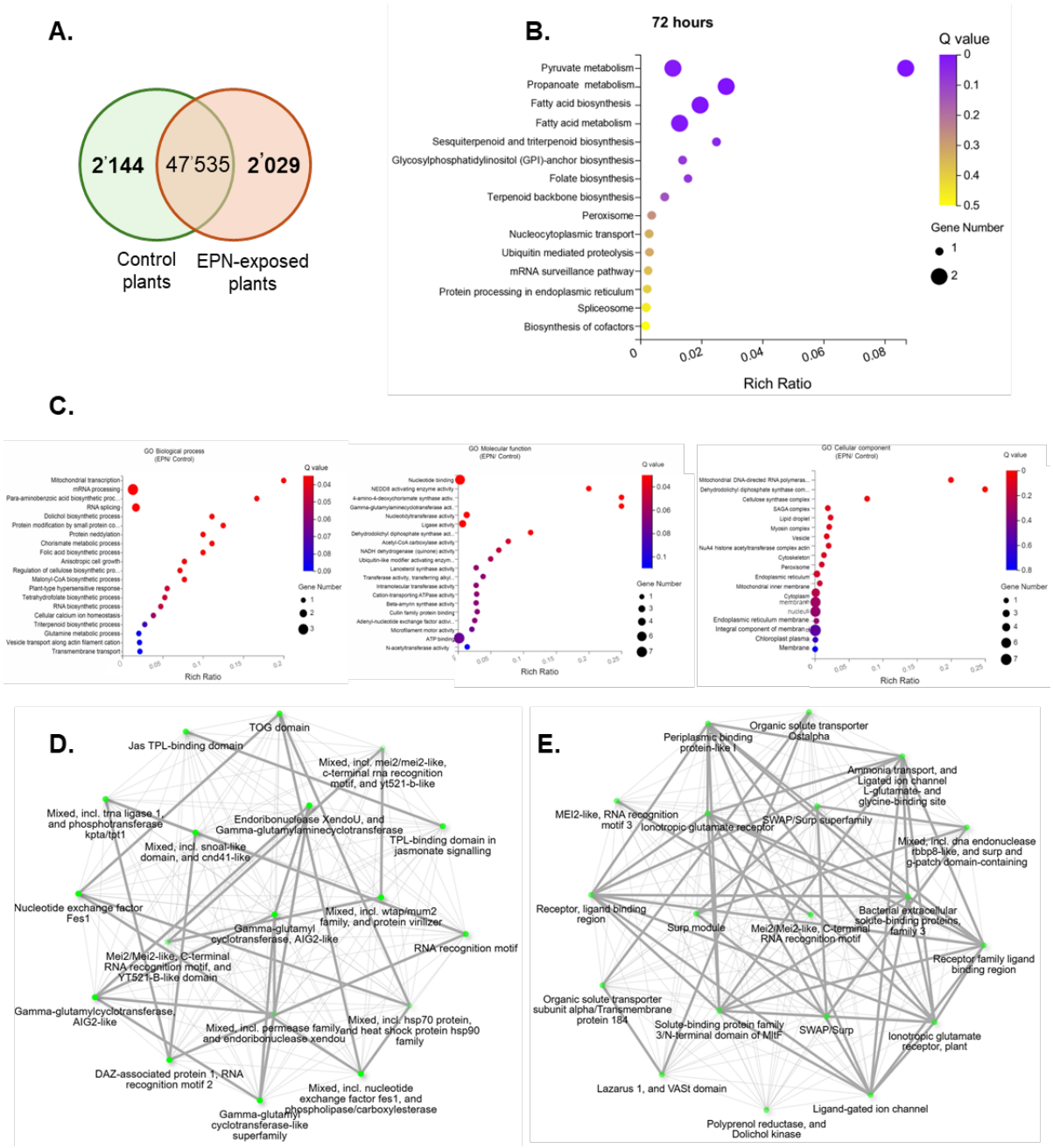

Figure 2. **Entomopathogenic nematodes (EPNs) modulated aboveground immunity**. (A). Venn diagram illustrating the number of differentially expressed genes in control and EPN-exposed plants in transcriptomic analyses. (B). Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway enrichment analysis, where the size and colour of the bubbles represent the gene number and Q-value, respectively. (C) Gene Ontology (GO) term enrichment analyses of biological processes, molecular functions, and cellular components, respectively. The bubble plots show various enriched terms with their respective rich ratios and Q-values. Network analyses of (D) down- and (E) upregulated genes, respectively. EPN exposure was achieved by adding 2'000 *Heterorhabditis bacteriophora* nematodes for 72 h in the soil (n=5 per treatment). TOG: Tumor Overexpressed Gene. TPL: TOPLESS. Jas TPL-binding domain: TPL-binding domain in jasmonate signalling. Mei2: Meiotic 2. YT521-B: YT521-B Homolog. XendoU: Xenopus Endoribonuclease U. KPTA: Kinase-Phosphotransferase A. TPT1: tRNA Phosphotransferase 1. snoaL: Small Nucleolar RNA-Like. CND41: Chloroplast Nucleoids DNA-binding protein 41. Fes1: Functionally Essential for Stress response 1. AIG2: AvrRpt2-Induced Gene 2. WTAP: Wilms Tumor 1-Associating Protein. MUM2: Methyltransferase-like protein 2. DAZAP1: Deleted in Azoospermia-Associated Protein 1. HSP70: Heat Shock Protein 70. HSP90: Heat Shock Protein 90. SWAP: Suppressor of White Apricot. SURP: Serine/Arginine-Rich Protein. rbbp8: Retinoblastoma Binding Protein 8. VASt: Vps27, Hrs, and STAM domain.

EPN presence in soil did not modulate the leaf metabolomic profiles.

EPN presence in soil did not modulate the leaf metabolomic profiles of maize plants in field conditions although altered primary metabolism in greenhouse conditions. The results exposed that the metabolomic profile of the leaf was not affected by EPNs in the field nor the greenhouse (Figure 3A, PPLS-DA, CER = 60.8 %, $p = 0.791$). Besides, phytohormone levels of plants in the greenhouse showed also no induction after EPN exposure (Figure 3B).

Nevertheless, amino acid levels in leaves were similar in control and EPN plants in the field but in the greenhouse, amino acids levels were increased in plants growing under EPN presence (Figure 3C). Although the total amount of amino acids was not altered, the levels of serine (Welch's Test, $p<0.05$), and methionine (Welch's Test, $p < 0.05$) were significant increased by EPN presence in the soil. Moreover, the levels of soluble sugars remained constant across the treatments in field and greenhouse as well, but sucrose levels in plants growing in the greenhouse showed a trend of being reduced in EPN plants (Figure 3D, Welch's Test, $p = 0.053$).

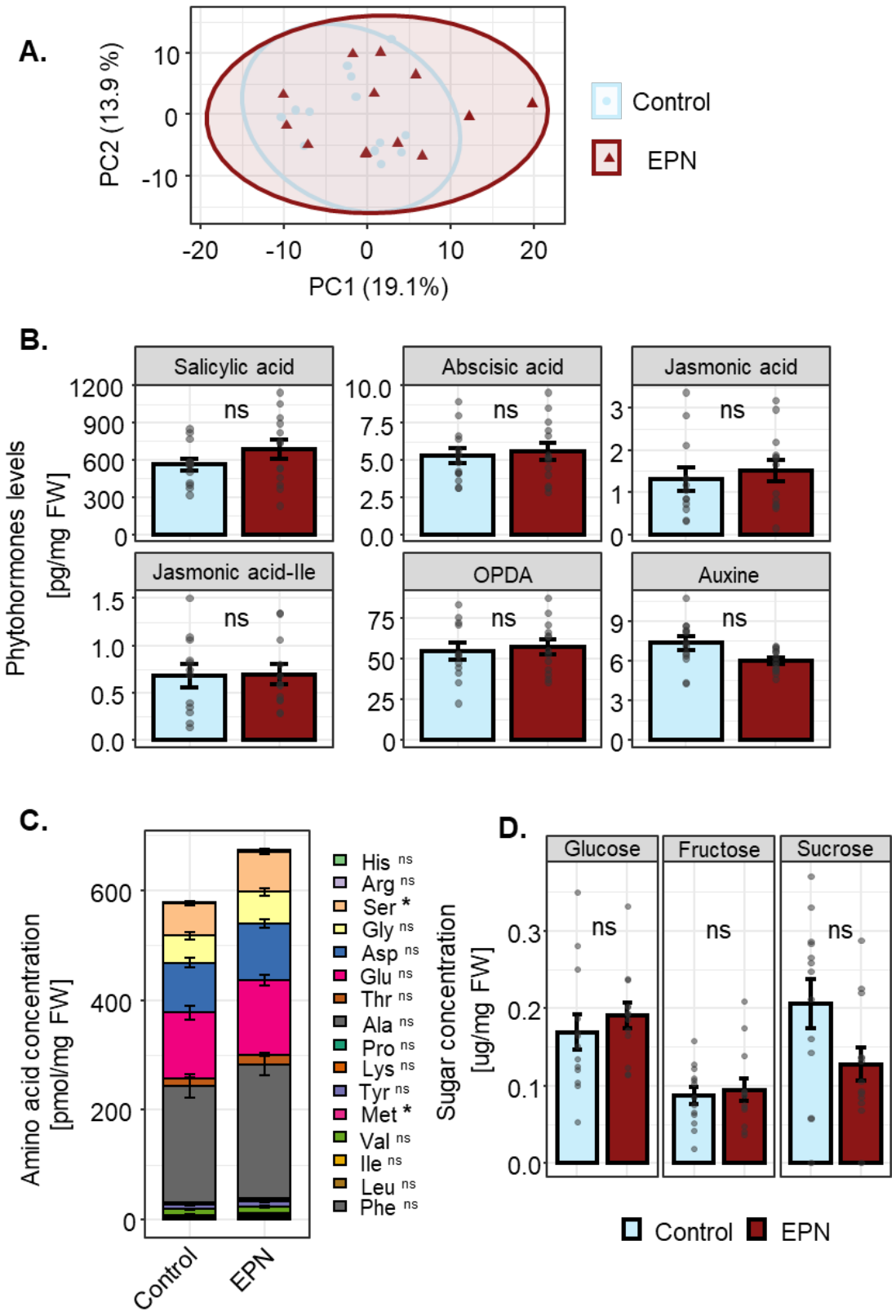

Figure 3. Entomopathogenic nematodes (EPNs) presence in soil did not affect the leaf metabolome of maize plants. (A) Principal component analysis (PCA) of the metabolomic profile of maize leaves (n=12). (B) Levels of phytohormones present in maize leaves (n=10). (C) Concentrations of free essential amino acids present in maize leaves (n = 12). (D) Concentrations of soluble sugars present in maize leaves (n=12). Bars indicate mean +/- standard error. ns: not significant. Stars indicate significant differences (*= p-value < 0.05, ns: not significant). FW: Fresh weight.

Discussion

EPN presence in the soil reduced the infestation of maize plants by *O. nubilalis* in the field. Furthermore, *O. nubilalis* moths preferred to oviposit on plants in the absence of EPNs. Besides, EPNs altered maize biochemical and signalling pathways in greenhouse conditions. However, the metabolic profile of maize leaves did not reinforce an explanation for the underlying mechanism of moth repellence. Nevertheless, the findings suggest that EPN presence in the soil induce systemic resistance in plants against aboveground herbivores in the field. Thus, this section discusses how EPN presence could induce aboveground insect resistance and its agroecological implications.

The exposure of maize plants to EPNs reduced the rate of infestation by natural populations of *O. nubilalis* in the field. EPNs have been reported to benefit plants by reducing aboveground herbivory of sweet potato whitefly, beet armyworm, and tomato leaf miner in tomato plants and of Colorado beetle in potato plants (An et al. 2016; Helms et al. 2019; Kamali et al. 2022). A group of molecular markers found in nematodes called ascarosides, reduces the growth of leaf pathogens, when applied to the plant (Manosalva et al. 2015; Klessig et al. 2019). Ascarosides are conserved signalling molecules in the nematode kingdom (Choe et al. 2012). In EPNs, their concentration is especially high during the emergence from the cadaver and mediates dispersal behaviour and IJ recovery (Noguez et al. 2012; Wang et al. 2022). The plants may perceive ascarosides as a signal associated to plant pathogenic nematodes and therefore react equivocal to EPNs. However, this remains to be confirmed, as the bouquet of ascarosides is specific to nematode species, and the plant response to plant pathogenic nematodes is different to that of EPNs (Kamali et al. 2022). Nevertheless, this study confirmed that EPN presence in the soil increased plant resistance towards an aboveground herbivore.

Transcriptomic analysis showed that EPN presence induced a shift in fatty acid metabolism, potentially indicating the remodelling of leaf cuticular waxes or cuticular contents. Besides, the data exposed that EPN presence not just altered biochemical pathways but also signalling pathways that can influence plant response to herbivory. EPN presence reduced the expression of genes related to jasmonic acid (JA) and salicylic acid (SA) pathways such as the Jas TPL-binding domain and the AIG2-like proteins, respectively. Extensive research has provided evidence that JA and SA

signalling pathways interact to coordinate plant defences against a variety of biotic stresses, including herbivore attacks (Bari & Jones, 2009; Caarls et al., 2015; Kawazu et al., 2012; Schweiger et al., 2014; Tamaoki et al., 2013). Thus, the downregulation of JA and SA signalling in maize leaves upon EPN exposure do not explain the oviposition preference observed in the field. However, oviposition preference can be better understood looking at the upregulated genes in maize upon EPN exposure.

EPN presence in the soil induced the expression of genes with receptors functions that play key role in detecting herbivore presence and triggering defence response (Chow & McCourt, 2006; WU & BALDWIN, 2009). Furthermore, EPN exposure induced LAZ1 expression, which is a regulator of resistance to pathogens and herbivores. Recent studies demonstrated that LAZ1 expression is involved in systemic acquired resistance protecting the plants against future attacks (Chen et al., 2024). Upregulation of LAZ1 also suggested the induction of hypersensitive response in plants (Malinovsky et al., 2010). Thus, transcriptomic data confirmed that EPNs induce aboveground plant response in maize. *O. nubilalis* preference for plant not exposed to EPNs could be caused for this induction of systemic response in maize in the field. Although, these findings also suggest that EPN-induced response is context-dependent reinforcing the importance of field studies.

EPNs infest and kill insects and can induce systemic plant defence, protecting against both belowground and aboveground herbivores. However, EPN-induced responses in maize appears to be very context-dependent because the enrichment of metabolic pathways observed in the transcriptomic data was not observed in the metabolic profile analysed later in independent experiments. Additional studies are required to understand what influences the induction of plant responses by EPNs.

The presence of EPNs in the plant vicinity reduced the oviposition rate by *O. nubilalis* females, although it did not affect the survival and growth of the larvae, nor the amount of damage they inflicted to the plant. A similar observation was made in tomato, EPN presence in the soil reduced oviposition by *Tuta absoluta* moths (Kamali et al. 2022). Kamali et al (2022) did not investigate into the plant cue that mediated the reduced oviposition. However, one of the hypotheses of this study was that plants could exhibit a volatile or tactile cue during EPN presence, which affects herbivore preference. But EPNs trigger a minor response in the leaf parameters measured in this study, including

phytohormones, soluble sugars and amino acids as well volatiles released from leaves.

EPN exposure increased plant amino acids in maize leaves. Long term plant growth parameters under *O. nubilalis* infestation and concurrent EPN presence were not investigated, thus the role of amino acids in tritrophic interactions needs further evaluation. Amino acid content in plants may be linked to insect oviposition. However, reduced oviposition by *O. nubilalis* moths was unlikely due to alteration of amino acid content, as the plants were healthy. Potentially, the combination of oviposition and EPN presence may modulate the plant to be less favourable, but that remains to be checked. Another hypothesis is that altered volatile emissions in EPN-treated plants possibly lowered the oviposition rate. However, the profile of volatiles emitted by plants exposed to EPNs was no different than from control plants. To entangle the volatile or tactile cues that drives the reduced *O. nubilalis* oviposition on EPN-treated maize, it would require more analyses of volatile and non-volatile compounds affecting oviposition behaviour.

In summary, the plant response to EPNs influenced plant metabolism and defence in the field under herbivory. The plant gains an advantage by reduced oviposition and may be affected in its performance under the stress of herbivory. This study shows that plants do respond to EPNs as herbivore natural enemies in a context-dependent manner. The results support the existing evidence for a systemic plant response to EPNs which impact the tritrophic interactions. The mechanisms behind this interaction remain to be confirmed and are an exciting topic to be explored. Direct plant responses to natural enemies are increasingly significant in the context of sustainable agriculture.

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Data availability

The data will be deposited on the DRYAD repository (Dryad | Home - publish and preserve your data). All raw data can be provided upon reasonable demand to the corresponding author.

Conflict of interest

The authors declare no conflict of interest.

Author contribution

PAH designed and conducted laboratory and field experiments, analysed the data, and wrote the chapter. AMVM designed and conducted laboratory experiments, analysed the data, and wrote the chapter. SK and MF conducted the field experiment. PM established, validated, and ran samples for chemical analyses. CAMR acquired funding, supervised the project, analysed data, and reviewed the chapter.

List of figures

Supplementary information

Supplementary Information S1: EPN presence on maize growth and yield in the field.

Supplementary Information S2: EPN presence on maize growth and yield in the greenhouse.

Supplementary Information S3: *O. nubilalis* damage in the field.

Supplementary Information S4: Leaf volatile emissions of control and EPN-exposed plants.

Supplementary Information S5: Diagram representing plants covered and uncovered with plastic bags during the second oviposition assay.

Figure S1. EPNs presence improves maize growth in the field. The growth rate of control and EPN-treated plants was assessed in the field. (A) Plant height over time of one field plot in the season 2021, Germany (n = 11-80). Arrows indicate time point of EPN treatment. (B) Yield of plants growing in the field expressed as dry weight of kernels (n = 36). Line plots indicate mean +/- standard error. DW: Dry weight.

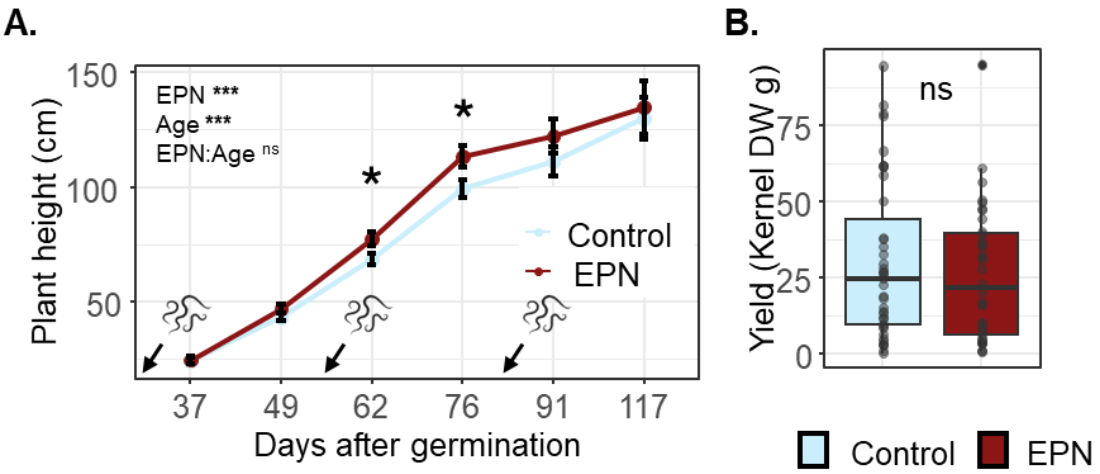

Figure S2. EPN presence did not affect maize growth in the greenhouse. (A) Plant height over time (n = 12-72). (B) Leaf chlorophyll content expressed as SPAD value over time (n = 12-72). (C) Fresh and (D) dry weight, respectively, of the entire plant (n = 6). (E) Yield of plants grown in the greenhouse expressed as cob yield index, calculated by multiplying the number of kernels in a row around the cob middle with the length of the cob in cm (n = 6).

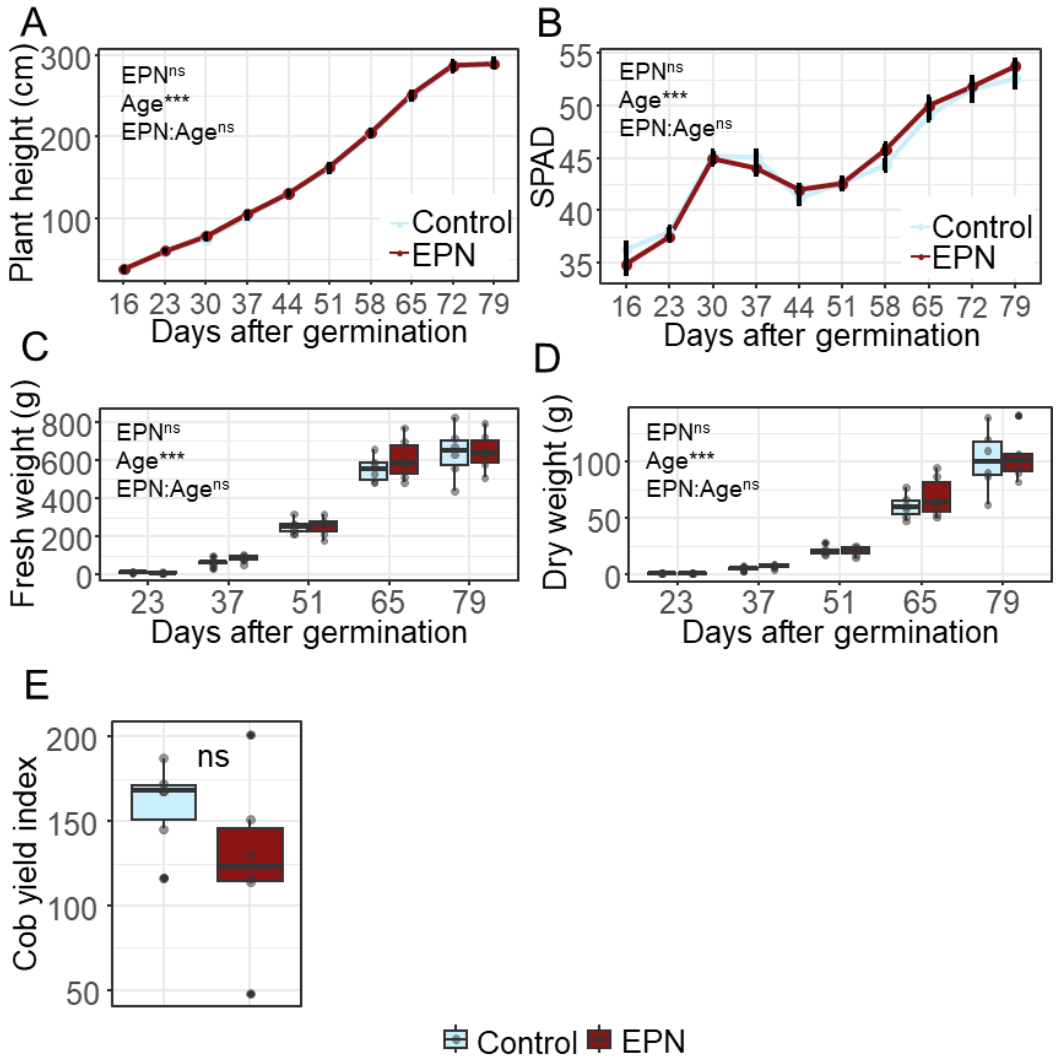

Figure S3. Plant damage by *O. nubilalis* infestation in the field. Maize plants exposed to EPNs were infested with *O. nubilalis* neonates above and below the ear. The plant damage was recorded at the end of the season (n = 40). (A) Number of feeding tunnels from *O. nubilalis* larvae in maize stems. (B) Length of feeding tunnels caused by *O. nubilalis* larvae in maize stems. Bars indicate mean +/- standard error.

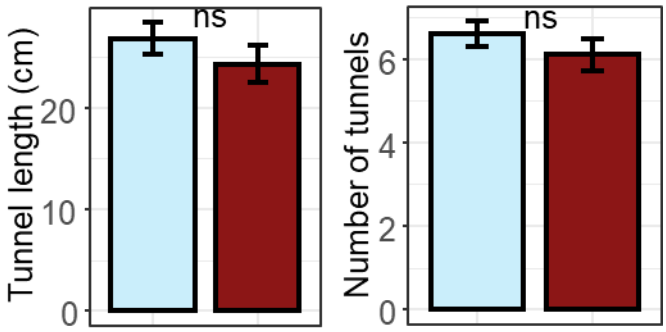

Figure S4. Leaf volatile emissions of control and EPN-exposed plants. Volatiles emissions were measure in a puss pull system in greenhouse conditions.

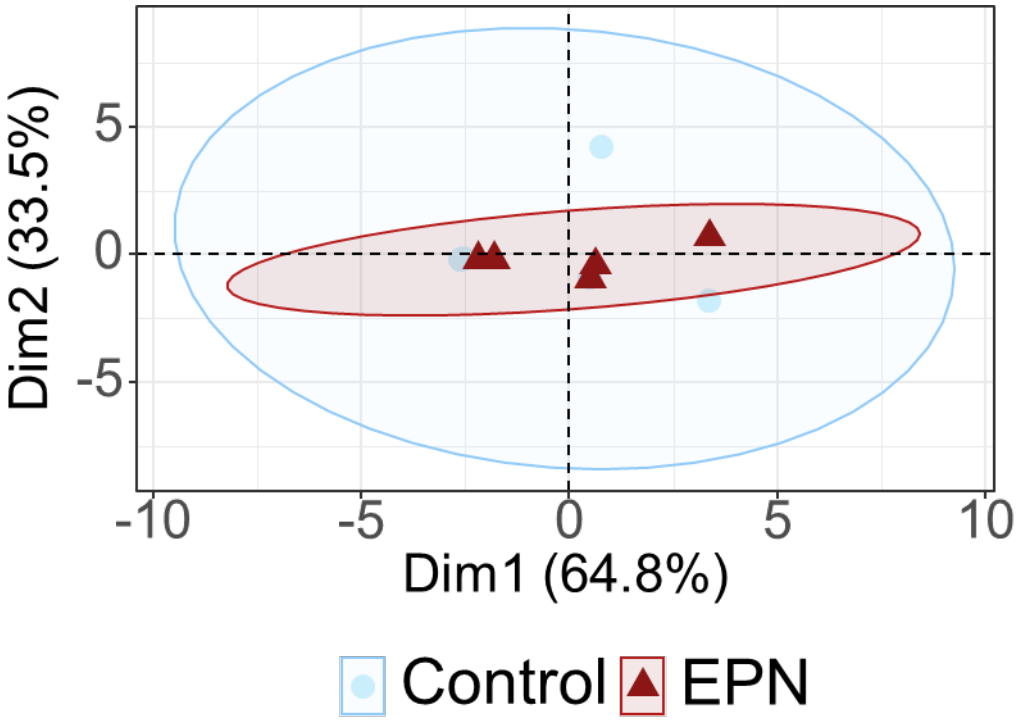

Figure S5. Diagram representing plants covered and uncovered with plastic bags during the second oviposition assays (created with BioRender).

Supplementary Tables

Supplementary Table S1: Differentially Expressed Genes (DEGs) in maize leaves after 6, 24, and 72 h exposure to entomopathogenic nematodes (EPNs).

Table S1. Significant differentially expressed genes (DEGs), filtered to more than 200reads, of systemic response of maize plants to entomopathogenic nematodes (EPN) exposure. EPN exposure was achieved by adding 2'000 infective juveniles of *Heterorhabditis bacteriophora* nematodes for 72 h in the soil (n=5 per treatment).

Symbol	log 2 (EPNs/Control)	Q value	Molecular Function	Time point
LOC541873	0.379	0.046	methyltransferase activity	6 h
LOC542607	0.179	0.044	pyruvate dehydrogenase (acetyl-transferring) activity	6 h
LOC100276501	0.278	0.005	mediator complex binding	6 h
LOC100282721	-0.358	0.022	prenyltransferase activity	6 h
LOC100283462	0.452	0.003	sarcosine oxidase activity	6 h
LOC103625889	0.629	3.12E-05	sulfate adenylyltransferase (ATP) activity	6 h
LOC541809	0.280	0.028	carbohydrate:proton symporter activity	6 h
LOC107546758	0.606	0.012	trehalose-phosphatase activity	6 h
LOC100275801	0.267	0.011	integral component of membrane	6 h
LOC103627895	-0.424	0.008	iron ion binding	6 h
LOC100194392	0.367	0.037	protein kinase activity	6 h
LOC100283380	-0.329	0.027	guanyl-nucleotide exchange factor activity	6 h
LOC100382229	-0.368	0.048	nucleotide binding	24 h
LOC100283559	0.200	0.022	nucleotide binding	72 h
LOC103629626	0.463	0.015	calcium ion binding	72 h
LOC103631855	-0.388	0.001	amino acid transport	72 h

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Chapter 3 - Entomopathogenic Nematodes-Associated Bacteria: Characterization, Origin, and Consequences for the Soil Microbiome

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Abstract

Recent evidence demonstrated that *Heterorhabditis bacteriophora*, a species of entomopathogenic nematodes (EPNs), harbour other bacteria genera than *Photorhabdus*. But the origin of these bacteria associations and their interactions with the soil microbial community remains unexplored. In this study, we characterized the second bacteria circle associated with *Heterorhabditis bacteriophora*. The results confirmed the presence of more than ten genera on the nematode surface, including 20 cultivable strains. Additionally, comparing five different strains of *H. bacteriophora* revealed that most of the present genus were shared, including *Brevundimonas*, *Comamonas*, *Pseudomonas*, *Sphingopyxis* and *Variovorax*. Besides, the exposure of EPNs to soil or insect host microbes showed no major alterations of the EPN-associated bacteria, as *Achromobacter, Acinetobacter, Brevundimonas, Dyadobacter, Leucobacter, Luteimonas* and *Variovorax* genera were present through all conditions. The mode of bacterial transfer—whether horizontal, pseudo horizontally or vertical—remains under investigation. Furthermore, our results suggest that EPN washing solutions, but not the EPNs themselves, significantly reduced the number of Amplicon Sequence Variants (ASVs) in soil samples, indicating that the EPNs and their supernatant influence the soil bacterial community in different ways. These findings enhance our understanding of the complex interactions between EPNs, their associated microbiome, and the soil microbial environment.

Introduction

Microbes are associated with a wide range of organisms, establishing interactions that influence the fitness and behaviour of the host organisms and vice versa (Berg et al., 2020). When interactions between different species are close and for long-term established, these interactions are called symbiotic (Frank, 1997). Symbiosis can take various forms, including mutualism, commensalism, and parasitism (Paracer & Ahmadjian, 2000), thereby affecting ecological communities and population dynamics. In mutualistic interactions, both organisms benefit. For example, the intestinal microbiota and host, where bacteria contribute to the production and conservation of nutrients and protection against pathogens (Malard et al., 2021). Legume plants roots and soil bacteria as *Rhizobium* also establish a mutualistic interaction that is essential for the nitrogen fixation process (Mahmud et al., 2020). Furthermore, the mutualistic interaction between symbionts and parasitoids, such as entomopathogenic nematodes (EPNs), where bacteria are involved in insect infectivity (Campos-Herrera, 2015). However, despite evidence that these interactions are not monoxenic, the interactions between EPNs and other microbes remains largely overlooked, limiting the understanding of their ecological impact.

Biocontrol of insects involves the use and manipulation of organisms such as parasitoids, to manage pest populations (DeBach & Schlinger, 1964). Biocontrol presents several benefits such as reducing chemical pesticide use, supporting biodiversity, and being cost-effective for long-term pest management, representing an environmentally friendly alternative to control invasive insect pests. (Omkar & Kumar, 2016). In the past decades, biocontrol agents have made significant progress in protecting crops from pests worldwide (Omkar & Kumar, 2016).

Biocontrol agents range from insects to microorganisms. Generally used entomopathogenic microorganisms include bacteria as the well-known *Bacillus thuringiensis* and fungi as *Beauveria bassiana* (Bravo et al., 2011; Mascarin & Jaronski, 2016). In addition, EPNs, such as *Steinernema* and *Heterorhabditis* species are also parasitoids used as biocontrol agents (Peters, 1996). EPNs are soil nematodes that in symbiosis with luminescence bacteria that they carry in their intestine, infect and kill insect larvae within 48 h (Poinar et al., 1980). EPNs are a clear example of the tool that host-microbe symbioses represent for crop pests management (Boemare et al., 1996).

Heterorhabditis bacteriophora nematodes establish this highly co-evolved interaction with bacteria from the genus *Photorhabdus* (Ciche Todd et al., 2008). Once that they are inside the host body, many metabolomic and molecular processes start including the production of antimicrobial compounds that allow the establishment of infective juveniles (IJs) and the symbiotic bacteria (Tarasco et al., 2023). A stable EPN association requires nematodes to infect and kill an insect, produce progeny, and ensure this progeny exit the insect host with the bacteria (Dillman et al., 2012). Hence, *Photorhabdus* is vertical transmitted from the mother to the progeny (Ciche Todd et al., 2008). *Heterorhabditis* spp. and *Photorhabdus* spp. have co-evolved so closely that those nematodes just feed and reproduce on this bacteria genus (Enright & Griffin, 2005).

Previous research was mostly focused on the implication of *Heterorhabditis* spp. and *Photorhabdus* spp. interaction for agriculture. But evidence of varied EPN-bacterial interactions suggest EPNs specialization with one genus of bacteria is less frequent than previously assumed (Dillman et al., 2012). EPNs carry other bacteria in their body and some of them are related to EPNs pathogenicity (Ogier et al., 2020). Recently, the monoxenic concept changed to pathobiome and a second bacterial circle gained more attention (Ogier et al., 2023).

EPNs are primarily associated with the symbiotic bacteria in their intestine but there are cases of EPN-bacteria symbioses in other anatomical region (Dillman et al., 2012). For example, *Paenibacillus nematophilus* associates on the cuticle of *Heterorhabditis* species (Enright et al., 2003). Some studies suggested that these other host-microbe symbioses could also influence EPNs fitness and survival (Enright & Griffin, 2005; Ogier et al., 2023). Therefore, EPN-associated bacteria are a niche for new bioproducts for agriculture. However, many questions remain open about the implications of these EPN-bacterial symbioses for soil food webs.

Few studies had focused their research on understanding the implications of the second bacterial circle of EPNs in the nematode-induced responses. In plant parasitic nematodes (PPNs), the juveniles recruit passively for specific soil microbes to their cuticle or surface coat in their way to the plant roots (Elhady et al., 2017). Attached microbes to PPNs trigger plant defence (Topalović et al., 2020). Although, EPNs also

induce plant systemic defence (Kamali et al., 2022; Kansman et al., 2024), the effects of the EPN surface microbes on plant trophic interactions have not been studied yet.

The current study represents an integrated approach for the study of EPN-associated bacteria and their implications for sustainable agriculture. The main questions of this study were: (a) What is the second bacterial cycle associated with *Heterorhabditis bacteriophora* IJs? (b) Is there a core EPN-associated microbiota? (c) How is it acquired? And (d) What impact does this second bacterial cycle and each member of it have on the soil bacterial community? With a combination of traditional and novel research techniques, this study confirmed that EPN-bacteria interactions include other genera than just *Photorhabdus*. Furthermore, the current research is the first study that explored the effect of EPN-associated bacteria in components of the soil food webs.

Materials and methods

Biological resources

Maize seeds (*Zea mays* L.) of the variety B73 were used for all experiments. Seeds were kindly provided by the Maize Genetics and Genomics Database (MaizeGDB, www.maizegdb.org) and bred by Delley (Delley seeds and plants Ltd, Delley, Switzerland). Maize seedlings were grown in 100 mL cylindric pots (Semadeni, Ostermundigen, Switzerland) filled with 80 % river sand 4 mm (LANDI Schweiz AG, Dotzingen, Switzerland) and 20 % soil on top (Selmaterra Schweizer Schwererde torfreduziert, Bigler Samen AG, Thun, Switzerland). The plants grew in greenhouse conditions at 23 ± 2°C, approx. 60 % humidity and 16:8 dark/light cycle, with daily watering and Plataktiv® Typ K (Hauert HBG Duenger AG, Grossaffoltern, Switzerland) fertilizer added weekly according to the manufacturer instructions.

Entomopathogenic nematodes (*Heterorhabditis bacteriophora* Poinar) came from the nematode colony of the Biotic Interactions and Chemical Ecology groups of the Institute of Plant Science at the University of Bern. The EN01 strain of this in-house colony, was established with EPNs bought from Andermatt Biocontrol in 2021 (Grossdietwil, Switzerland) and reared *in vivo* in greater wax moth larvae (*Galleria mellonella* Lepidoptera: Pyralidae) bought at a local fishery shop (Fischereibedarf Wenger, Bern, Switzerland). The RM102, IT6e, HU2e and IR2e strains were stablished from EPNs were provided for free by research collaborators as previously described (Zhang et al., 2019). Infective juveniles (IJs) of EPNs (EN01, RM102, IT6e,

HU2e and IR2e) were collected from white traps using 25 µm sieves and stored at 8°C in tap water until use (White, 1927).

Tenebrio molitor larvae (Fischereibedarf Wenger, Bern, Switzerland) and *Ostrinia nubilalis* larvae were also used in the experiments. Eggs of *O. nubilalis* were bought from BTL Bio-Test Labor GmbH Sagerheide (Groß Lüsewitz, Germany) and reared on general purpose lepidoptera diet (Frontier Agricultural Sciences, Newark, USA). L3-L4 larvae were used in the experiment.

Preparation of different treatments used in this study

The treatments included in different experiments of this study are summarized below (Table 1). The same day of the inoculations, EPNs were counted and Nemawash collected. Nemawater, called WASH in this study, is the EPN supernatant collected from EPNs containing flasks kept in 60-70 mL of tap water at 8°C. EPN supernatant was separated from IJs using 25 µm sieves. EPNs from where the Nemawash was collected, were labelled as “washed EPNs” after washing them thoroughly with tap water and used as a different treatment in some experiments. Besides, Nemawash was used as a different treatment when WASH was filtered with 0.2 µm filters to eliminate the microorganisms of the WASH collected from EPN and label as “filtered WASH” treatment.

Table 1. Different treatments included to unravel the implications of the entomopathogenic nematodes (EPNs)-associated microbes in maize tritrophic interactions.

Treatments	Description
Control	Tap water
EPNs	IJs of *Heterorhabditis bacteriophora*
Washed EPNs	EPNs washed thoroughly with tap water
Sterile EPNs	Surface-sterilized nematodes: EPNs exposed to sterilization processes
Nemawater or WASH	EPN supernatant collected from the EPN-containing flasks

The sterilization process of EPNs was conducted to eliminate the microorganisms of the EPN surface and produce the “Sterile EPNs” treatment. This process was performed the same day that the inoculation was planned. First, EPNs were left 15 min of incubation in 0.4 % Benzethonium chloride, then washed thoroughly with sterile distilled water. After that, EPNs were placed in an antibiotic solution containing Streptomycin (200 mg/L) (Sigma-Aldrich, Massachusetts, USA) and Rifampicin

(25 mg/L) (Sigma-Aldrich, Massachusetts, USA) for 4 h. Then, the sterile EPNs were washed with 3 L of sterile distilled water and kept in this water until use.

Heterorhabditis bacteriophora EN01 surface microbiome

The bacterial community attached onto *H. bacteriophora* surface was characterized through 16S rRNA gene sequencing and microbial isolation and culture.

Microbiome analyses were conducted on washed and sterilized EPNs (n=4-5). Washed EPNs were obtained by rinsing the IJs thoroughly with tap water, as previously described (Ogier et al., 2020). Sterilized EPNs were obtained by exposing washed EPNs to 1 mL 1 % of bleach solution for 2 min following of five washing steps with autoclaved distilled water and 2 h of exposition to 1 mL of antibiotic solution containing Streptomycin (200 mg/L), Rifampicin (25 mg/L) and CellCultureGuard 10X (Axon Lab AG, Baden-Dättwil, Switzerland) (Kaya & Patricia Stock, 1997). DNA was extracted using an innuPREP DNA/RNA Mini Kit (Analytik Jena AG, Jena, Germany) following the manufacturer instructions. Independent replicates per treatment were sent for Illumina sequencing of the V3-V4 region of the 16S rRNA gene to the Next Generation Sequencing (NGS) Platform of the University of Bern. DNA quality was checked with Qubit 2.0 system. Sequenced data was analysed with the DADA2 pipeline based on amplicon sequence variants (ASVs) (Callahan et al., 2016). The generated sample-wise ASV abundance table was used for further analysis.

Culture-dependent methods were used to isolate bacteria from IJs of EPNs (EN01) and EPN supernatant in 2021 and 2024. Bacterial colonies were isolated using three different media: LB Broth (Luria/Miller) (Carl Roth, Baden-Wurttemberg, Germany), 10 % LB, and 50 % Tryptic Soy Broth (TSB) media (Sigma-Aldrich, Massachusetts, USA), to achieve different nutritional media compositions and concentrations (Gerhardt et al., 1994). Serial dilutions were used to pick individual colonies after inoculating 50 µL of WASH or 50 IJs in agar plate for 48 h at 25-28°C in aerobic conditions. Subsequently, individual different-like colonies were picked and streaked on LB agar plates to obtain monocultures. The resulting 100 isolates were further identified through 16S rRNA gene amplicon sequencing using previously established methods (Thoenen et al., 2023). Briefly, the reaction mix was composed of 15 µL buffer (DreamTaq Green PCR Master Mix (2x), Thermo Scientific, USA), 15 µL autoclaved MiliQ water, 1.5 µL of each primer PCR of the 16S rRNA gene with universal primers (Sequences: **27F** (5′-AGAGTTTGATCCTGGCTCAG-3′) and **1492R**

(5′-TACGGYTACCTTGTTACGACTT-3′)) and 2 µL of diluted bacteria cultured (1:10 dilution in autoclaved MiliQ water). PCR amplification was performed in a Biometra thermocycler (Analytik Jena AG, Jena, Germany). The standard amplification program included 95°C for 5 min, 95°C for 15 s, 60°C for 15 s and 72°C for 30 s (35 x Step 2) and 72°C for 5 min. Amplified DNA from bacteria isolates were sequenced by Microsynth (Microsynth AG, Balgach, Switzerland). Isolates sequences were blasted using the BLAST tool of the National Center for Biotechnology Information (NCBI, Rockville Pike, USA).

Bacteria specificity among *Heterorhabditis bacteriophora* strains

To assess the specificity of the second bacterial circle, five *H. bacteriophora* strains (EN01, RM102, IT6e, HU2e, and IR2e) were used for full-length 16S rRNA gene sequencing (n=4 per strain). DNA was extracted using QIAamp DNA Micro Kit (50) (QIAGEN N.V., Venlo, the Netherlands) according to manufacturer instructions with the following modifications: (i) 100 µL of EPNs at a concentration of >50K EPNs/ml were added to 180 µL of buffer ATL, (ii) homogenization was carried out by adding eight 1.4 mm zirconium oxide beads and shaking in a bead beater at 30 X for 3 min, and 20 µL of proteinase K were added afterward. Elution was performed using 55 µL of elution buffer after a five-minute incubation period. Samples were stored at -20°C until further processing at the Next Generation Sequencing (NGS) facility of the University of Bern.

Origin of EPN-associated bacteria

To assess the horizontal and/or vertical transfer origin of the EPN-associated microbiome, three independent assays were conducted.

First, horizontal transfer from the soil microbial community to *H. bacteriophora* surface was evaluated in EN01. Infective juveniles were collected from white traps and stored at 8°C in tap water for 0 and 7 days. Nematodes from the same collection were inoculated in soil for 21 days and then recovered using *Galleria* larvae. DNA from IJs collected from those different environmental conditions, *Galleria* larvae and soil samples was extracted and sent for 16S rRNA gene sequencing (n=6 per condition). Soil DNA was extracted using DNeasy® PowerSoil® Pro Kit according to manufacturer instructions (QIAGEN N.V., Venlo, The Netherlands). Insect DNA was extracted with an innuPREP DNA/RNA Mini Kit (Analytik Jena AG, Jena, Germany) following the manufacturer instructions with some modifications: (a) samples were

vortexed for 3 min during the lysis step, (b) incubated for 1 h at 56°C and 750 rpm, and (c) centrifuged at 13,000 rpm instead of 11,000 x g. Additionally, the elution step was performed with 30 µL of MilliQ water and repeated once. EPN DNA was extracted using QIAamp DNA Micro Kit (QIAGEN N.V., Venlo, the Netherlands) according to manufacturer instructions with the modifications previously mentioned.
PacBio HiFi sequencing of the 16S rRNA gene in EPN, insect, and soil samples were performed by the NGS Platform of the University of Bern. The amplification of bacterial full-length 16S gene was conducted with barcoded primers according to manufacturer instructions (Pacific Biosciences, Menlo Park, USA). Bioinformatic analyses of the generated demultiplexed fastq-files were performed on the central Linux High Performance Computing (HPC) cluster at the University of Bern following the Nextflow pipeline (GitHub - PacificBiosciences/HiFi-16S-workflow: Nextflow pipeline to analyze PacBio HiFi full-length 16S data) based on ASVs. The generated sample-wise ASVs abundance table was used for further analysis.

Second, horizontal transfer from the host (insect) microbial communities to *H. bacteriophora* surface was assessed in EN01 EPNs grown in *T. molitor* and *G. mellonella*. Healthy insect hosts and EPN emerging from infected hosts were used for 16S rRNA amplicon sequencing (n=6 per insect species). Frozen insect larvae were grinded to a fine powder in liquid nitrogen using a mortar and pestle. Then, 40 mg of the powder were weighted for DNA extraction with an innuPREP DNA/RNA Mini Kit (Analytik Jena AG, Jena, Germany) for DNA isolation from tissue samples following the manufacturer instructions with some modifications: (a) samples were vortexed for 3 min during the lysis step, (b) incubated for 1 h at 56°C and 750 rpm, and (c) centrifuged at 13,000 rpm instead of 11,000 x g. Additionally, the elution step was performed with 30 µL of MilliQ water and repeated once. Amplicon sequencing and bioinformatic analysis was performed by the Beijing Genomics Institute (BGI, Hong Kong Company Limited, Hong Kong, China). In summary, qualified DNA template of 30 ng and 16S rRNA fusion primers were added for Polymerase Chain Reaction (PCR). The resulting PCR products were purified using Agencourt AMPure XP beads, dissolved in Elution Buffer, and labelled to complete library construction. The size and concentration of the libraries were determined using an Agilent 2100 Bioanalyzer. Qualified libraries were then sequenced on the DNB platform based on their insert size. Raw data were filtered to obtain high-quality clean data, and overlapping clean

reads were merged into tags and further clustered into Operational Taxonomic Units (OTUs). Taxonomic classifications were assigned to OTU representative sequences using the Ribosomal Database Project database. Analyses such as alpha diversity, beta diversity, differential species analysis, network analysis, and model prediction were conducted based on the OTU profile table and taxonomic annotation results.

Third, vertical transfer from EPNs to the following generations within an insect host was assessed by infecting *G. mellonella*, *T. molitor*, and *O. nubilalis* insect larvae with surface sterilized EN01 EPNs (n= 4 per insect species). The sequencing data are currently pending and will be added at a later stage.

Influence of the EPN surface-associated bacteria on the soil bacterial community

A randomized pot experiment was conducted to determine the influence of EPNs and EPNs-associated microbes on the soil bacteria community in greenhouse conditions. Maize plants were grown in 1 L pots filled with 100 % soil (Selmaterra Schweizer Schwererde torfreduziert, Bigler Samen AG, Thun, Switzerland). After 10 days, plants were inoculated with either 2’000 sterile or non-sterile EN01 IJs in 3.2 mL tap water, 3.2 mL WASH, or tap water (n=7-8 per treatment). Rhizosphere samples were collected 30 days after inoculation and DNA was extracted using DNeasy® PowerSoil® Pro Kit according to manufacturer instructions (QIAGEN N.V., Venlo, The Netherlands). Sequencing of the 16S rRNA gene was performed with PacBio HiFi technology at NGS platform and analysed with the Nextflow pipeline.

Statistical analysis

The statistical analyses of the sequencing data were conducted in the R Environment using dada2 package (Version 1.28.0). Bar plots of the EPN-ASVs relative abundance versus Sterile_EPN-ASVs were built with phyloseq package (Version 1.44.0). Alpha diversity measures of EPN-associated microbial assemblages were calculated per sample with this package as well. For the OTU analysis conducted by the BGI company, the following software were used: iTools Fqtools fqcheck (v.0.25), cutadapt (v.2.6), readfq (v1.0), Fast Length Adjustment of Short reads (FLASH) (v1.2.11), USEARCH (v7 .0.1090), UCHIME (v4.2.40), RDP classifier (v2.2) setting a sequence identity of 0.6, Venn Diagram of software R (v3.1.1). Furthermore, the following database were used by the BGI company: 16S (including bacteria and archaea): Greengene (default): V202210; RDP: Release19. Data obtained from the rest of the experiments of this study were analysed and plotted using R studio (Version 4.3.1).

ANOVA, Kruskal-Wallys and Dunn´s tests were conducted using stats package (Version 4.3.1).

Results and discussion

H. bacteriophora nematodes are associated with bacteria other than *Photorhabdus* across environmental conditions and different hosts.

The bacteria profiling of IJs of different strains of *H. bacteriophora* using 16S amplicon sequencing confirmed that EPNs carry other bacteria than just *Photorhabdus* (Figure 1). These results are like previous findings (Ogier et al., 2020), confirming the occurrence of a second bacteria circle in laboratory-reared EPNs. Moreover, the results from the control treatment showed that rinsing IJs thoroughly with water was not enough to remove bacteria attached to the surface of IJs (EN01) in the control treatment (Figure 1A). While in the surface-sterile IJs samples, three out of four replicates remained with just *Photorhabdus* bacteria after the sterilization treatment, suggesting that some EPN-associated bacteria are attached to the surface of EPNs and were removed by the sterilization treatment (Figure 1A) (Figure S1). Although, some of the identified bacteria associated with IJs could be attached not just to IJs surface but located in the intercuticular space (Ogier et al., 2023; Peters et al., 2017)

In this study, the first surface sterilization process involved exposing IJs to 1 % bleach for 2 min, which can remove the cuticle and affect surface or intercuticular bacteria, potentially impacting the results. (Ogier et al., 2020). In fact, a surface sterilization process that did not involve using bleach, provided same bacteria species identified from surface sterile and non-sterile nematodes (Gouge & Snyder, 2006).. For that reason, this study included washed IJs as control and bleach surface-sterile IJs for comparison. Moreover, the following experiment that included surface sterile IJs used benzenthonium chloride (0.4 %) instead of bleach.

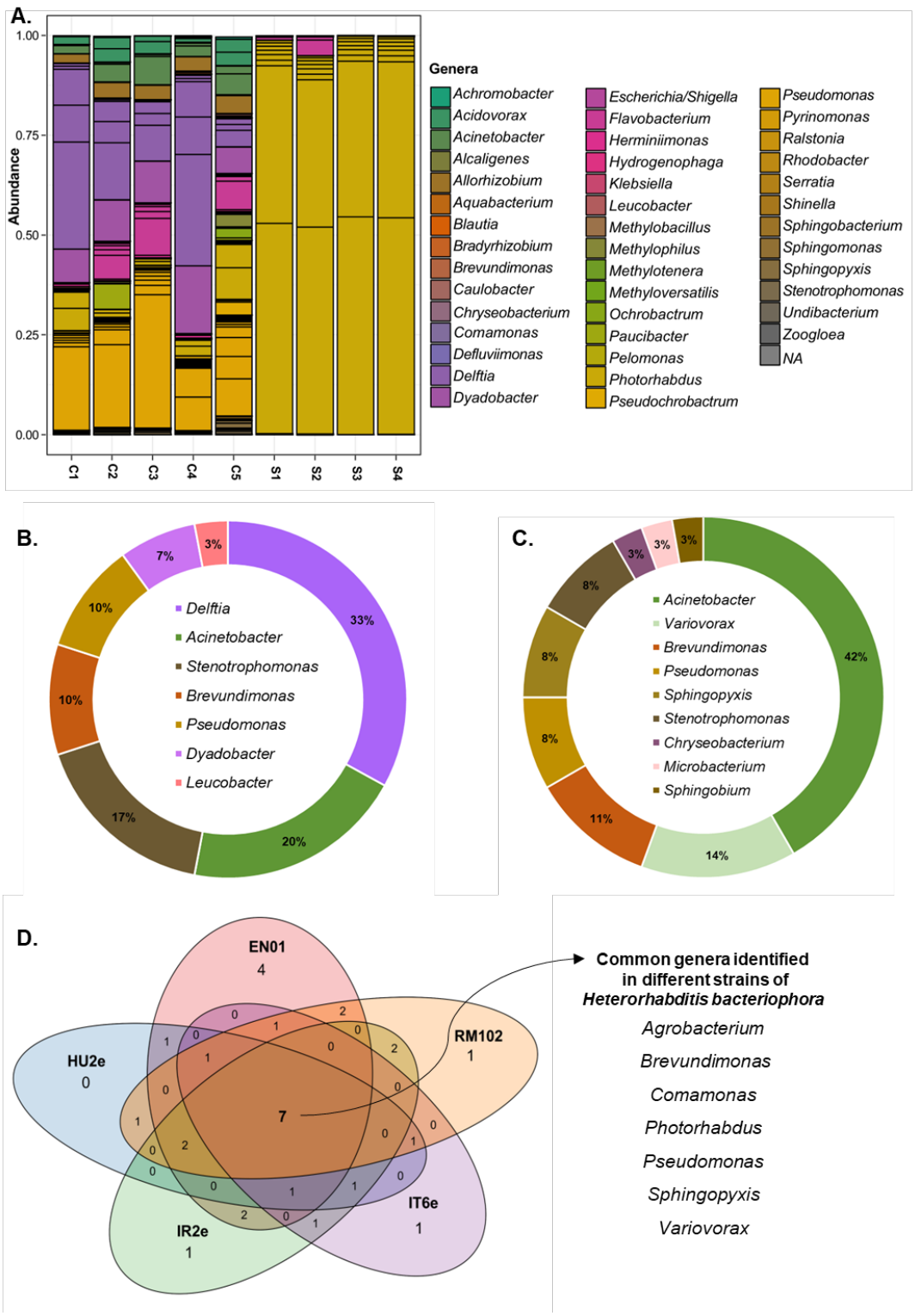

Figure 1. The entomopathogenic nematode *Heterorhabditis bacteriophora* harbours other bacteria genera than *Photorhabdus*. (A) Bar plot indicating the taxonomic distribution of the top 120 Amplicon Sequence Variants (ASVs) identified in infective juveniles (IJs) of EPNs (EN01) (n=4-5). (B) Donut chart showing the percentage distribution of identified isolates from IJs of EPNs (EN01) by genus. (C) Donut chart showing the percentage distribution of identified isolates from EPN supernatant of IJs (EN01) by genus. (D) Venn diagram illustrating bacteria genera present in IJs of different *Heterorhabditis bacteriophora* nematodes strains (n=4-5): EN01, RM102, IT6e, HU2e and IR2e. C: control samples (thoroughly washed IJs of EPNs (EN01) with tap water). S: Sterile EPNs (surface sterile IJs of EPNs (EN01) after treatment with 1 % bleach and antibiotic solution: Streptomycin (200 mg/L), Rifampicin (25 mg/L) and CellCultureGuard 10X).

Although, previous holistic approach of the EPN-bacteria symbioses used a multigenic metabarcoding including 16S (V3V4 region) and *rpoB* markers (Ogier et al., 2020), this study included just 16S (V3V4 region and full length) sequencing. Hence, future studies can consider monogenic metabarcoding of 16S sufficient to explore the second bacterial circle of EPNs. However, while more than 40 genera were found in IJs of EPNs through the first microbiome analyses, only seven were cultivable in the lab (Figure 1B).

Using culture-dependent methods, a total of 36 EPN-associated bacteria were isolated and identified from EPN supernatant and 30 from laboratory-reared IJs. Twenty different species were identified from the bacteria isolates (Table 2). The most abundant genera in the isolation from IJs of EPNs (EN01) were *Delftia*, *Acinetobacter*, *Stenotrophomonas* and *Brevundimonas* (Figure 1B). Besides, the most abundant genera isolated from EPN supernatant (WASH) were genera also isolated from EPNs including *Acinetobacter*, *Variovorax*, *Brevundimonas* and *Pseudomonas* (Figure 1C). The results exposed that there were common genera in both isolation sources, such as: *Acinetobacter*, *Brevundimonas*, *Pseudomonas* and *Stenotrophomonas* (Table 2). Altogether, the results suggest that most of the EPN-associated bacteria are on the surface of EPNs.

Table 2. Molecular identification through 16S region sequencing in lab conditions of bacteria isolated from entomopathogenic nematodes (EPNs), *Heterorhabditis bacteriophora* Poinar, strain EN01, and from EPN supernatant in comparison to previous studies (Ogier et al., 2020; Paddock et al., 2022; Topalović et al., 2019). WCR: Western Corn Rootworm, PPNs: Plant Parasitic Nematodes.

Bacteria species	Genera also found in PPNs (Topalovic et al., 2019)	Genera also found in IJs of EPNs (Ogier et al., 2020)	Genera reported on WCR (Paddock et al., 2022)	Genera identified in the first microbiome analysis of this study (2022)	Species also isolated from EPNs supernatant by Dr. Paul Himminghofen (2020)	Species isolated from EPNs supernatant in this study (2021)	Species isolated from IJs of EPNs in this study (2024)
Acinetobacter bereziniae						x	
Acinetobacter baumannii							x
Acinetobacter guillouiae	x	x	x	x		x	x
Pseudomonas monteilii			x	x	x	x	
Brevundimonas aurantiaca	x	x		x		x	
Brevundimonas diminuta							x
Sphingobium xenophagum				x		x	
Stenotrophomonas pavanii						x	
Stenotrophomonas maltophilia		x		x	x	x	x
Microbacterium oxydans	x		x		x	x	
Pseudomonas alloputida		x		x		x	
Pseudomonas putida							x
Variovorax boronicumulans						x	
Chryseobacterium mulctrae				x		x	
Sphingopyxis chilensis	x	x		x		x	
Delftia acidovorans		x		x			x
Delftia lacustris							x
Dyadobacter sp.				x			x
Leucobacter aridicollis				x			x
Flavobacterium microcysteis		x		x	x		

The data suggest a core microbiota associated to EPNs. For instance, *Stenotrophomonas* spp. was also previously isolated from *G. mellonella* cadavers killed by *Heterorhabditis* nematodes (Wollenberg et al., 2016). The recurrence of bacteria genera such us *Stenotrophomonas* spp. in different experiments of this study and in the previous study that profiled the bacterial community of IJs of EPNs (Ogier et al., 2020), confirms that this genus is part of the second bacterial circle of *Heterorhabditis* and *Steinernema* nematodes (Table2) (Ogier et al., 2020). However, some strain-specific variations could be detected.

Indeed, the findings point towards a core microbiota with some strain-specific variations (Figure 1D, Figure S2). When comparing bacteria associated with five laboratory-reared *H. bacteriophora* strains (EN01, RM102, IT6e, HU2e and IR2e), the results showed some conserved genera including *Brevundimonas*, *Comamonas*, *Pseudomonas*, *Photorhabdus*, *Sphingopyxis,* and *Variovorax*. However, *Achromobacter*, *Acidovorax*, *Luteimonas* and *Sphingobacterium* were just associated to IJs of EN01 strains.

The specific associative mechanisms between the bacterial community and the nematode are still uncertain. Previous study suggested that some bacteria have interspecific competition for attachment sites of the cuticle of the infective stage (J2) of PPNs (Topalović et al., 2019). Besides, attachment sites could have surface epitopes in PPNs and be different between nematodes species and/or strains (Elhady et al., 2017). A comparable hypothesis could be established for EPNs, and future research should aim to uncover the specific mechanisms of association between the second bacterial circle and EPNs.

Interestingly, the results suggested that IJs of EPNs (EN01) conserved the core bacteria community across environmental conditions and diets (Figure 2). Keeping the IJs in tap water for several days or inoculating them in soil for more than 20 days and then recovering EPNs with *G. mellonella* larvae did not affect the association with *Achromobacter*, *Acinotobacter*, *Brevundimonas*, *Dyadobacter*, *Leucobacter*, *Luteimonas* and *Variovorax* genera (Figure 2A, Figure S3). Although the soil used in the greenhouse experiments has more than 70 % of the ASVs classified as “unassigned”. Thus, further experiments should include field soil and surface sterile EPNs to elucidate the origin of the second bacterial circle of EPNs.

Furthermore, EPNs (EN01) reared on *G. mellonella* or *T. molitor* presented similar bacterial community showing that the diet did not significantly affect the occurrence of the frequently associated bacteria with IJs of EPNs (EN01) (Figure 2B). These results are opposite to one of the initial hypotheses because just the abundance of few genera such as *Brevundimonas* changed according to the host (Figure S4). To the date, there is no other study that investigated the influence of insect hosts on EPN-associated bacteria and could be compared with the findings of this experiment.

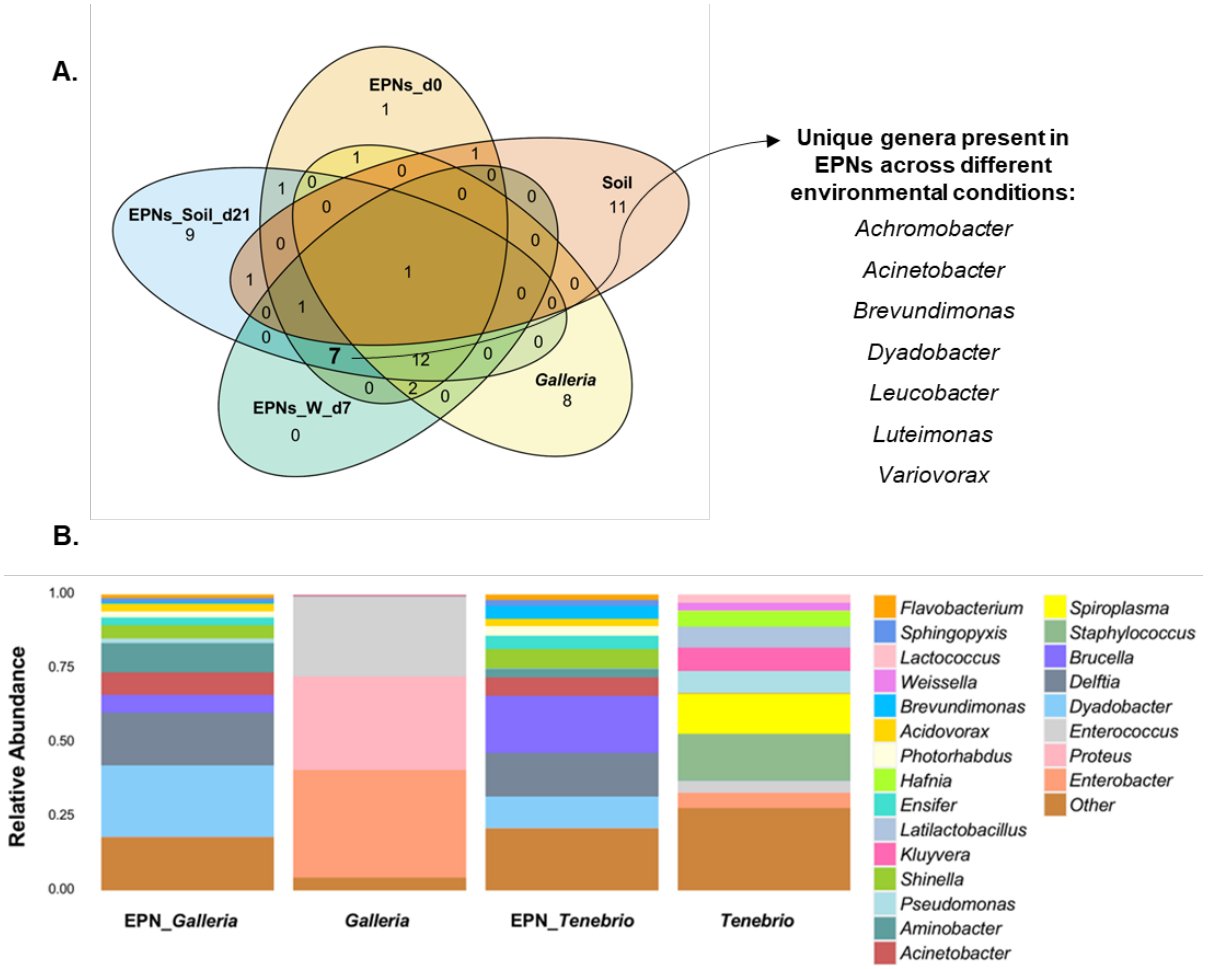

Figure 2. The entomopathogenic nematode *Heterorhabditis bacteriophora* harbours a core bacterial community across environmental conditions and diets. (A) Venn diagram illustrating bacteria genera present in IJs of EPNs (EN01) under different environmental conditions (n=6). (B) Bar plot indicating the taxonomic distribution of the Operational Taxonomic Units (OTUs) identified in IJs of EPNs (EN01) after rearing in different hosts (n=6). EPN_*Galleria*: IJs of EPNs (EN01) reared in *Galleria mellonella* larvae. *Galleria*: *Galleria mellonella* larvae. EPN_*Tenebrio*: IJs of EPNs (EN01) reared in *Tenebrio molitor* larvae. *Tenebrio*: *Tenebrio molitor* larvae. W: tap water. d: days.

Previous studies theorized that the second bacterial circle could be transmitted to EPNs horizontally, pseudo horizontally or vertically (Ogier et al., 2023). In summary, the results suggested that the second bacterial circle of EPNs is vertically transmitted to IJs. Future research should focus on confirming whether this transmission of bacteria to EPNs is just vertical.

EPN-associated bacteria can influence the soil bacteria community

Microbiome analysis of soil samples indicated that EPN supernatant from IJs of EPNs (EN01) application influenced the bacterial diversity and abundance in the soil of growing maize seedlings (Figure 3) (See Figure S5 for a complete description of the ASVs identified in soil samples in this experiment). The results highlighted that EPN supernatant application significantly reduced the number of ASVs detected per soil samples when compared to the control treatment (Figure 3A). Conversely, EPNs did not affect the number of ASVs detected although EPN supernatant contains bacteria that were also identified in EPNs. The results suggest that EPNs and EPN supernatant applications impact differently soil bacterial community. Future experiments should investigate why EPN supernatant although came from EPNs produced different effects than EPNs treatment.

Number of ASVs detected are usually referred as an indicator of microbial diversity present in the sample (Fasolo et al., 2024). But although there was a significant decrease in the number of ASVs in the EPN supernatant treatment, indicating a reduction in species richness, the Shannon index did not show a statistical difference (Figure 3B). This suggests that the overall diversity, considering both species richness and evenness, remained relatively stable. EPN supernatant application may have selectively reduced certain rare species without significantly affecting the abundance distribution of the remaining species.

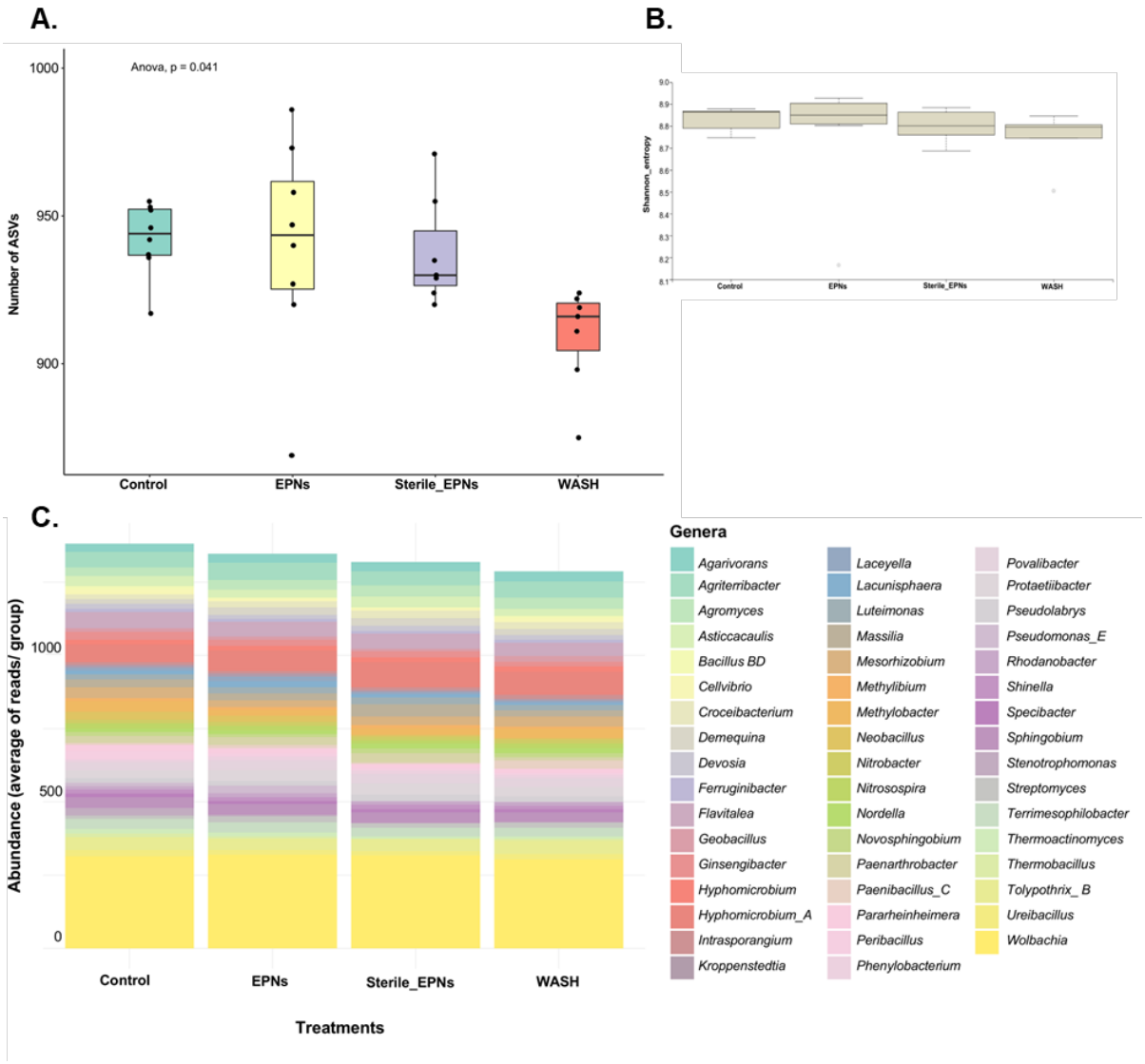

Figure 3. Surface bacteria from entomopathogenic nematodes (EPNs) influenced soil microbial community composition. (A) Box plot representing the number of Amplicon Sequence Variants (ASVs) in soil samples upon EPNs, Sterile EPNs and WASH exposure for 30 days. (B) Alpha diversity box plot representing the Shannon diversity index in soil samples upon EPNs, Sterile EPNs and WASH exposure for 30 days (Kruskal-Wallis, p-value=0.42). (C) Bar plot indicating the abundance of the top 50 ASVs in soil upon EPNs, Sterile EPNs and WASH exposure for 30 days. Sterile EPNs: surface sterile EPNs with 0.4% benzenthonium chloride and antibiotic solution (Streptomycin (200 mg/L) and Rifampicin (25 mg/L)). WASH: EPN supernatant. ns: no statistical difference. Stars indicate significant differences (**: p<0.01).

Nevertheless, EPN supernatant application also induced changes in the abundance of the soil bacteria community (Figure 3C). WASH application significantly increased the abundance of bacteria genera such as *Brevundimonas*, *Jiangella*, *Legionella* and *Thermomonas* (Table S1). Although, WASH application significantly reduced the number of ASVs detected in soil samples in other genera such as *Arthrobacter*, *Ginsengibacter*, *Hungateiclostridium* and *Neobacillus*.

Ensuring microbial diversity and balance is essential for optimal soil health and agricultural productivity (Chen et al., 2024). Hence, increasing and reducing the abundance of bacteria genera can cause multiple ecological and agricultural outcomes. For instance, *Brevundimonas* genus includes plant growth-promoting rhizobacteria (PGPR) that fix nitrogen and solubilize phosphorus (Zaim & Bekkar, 2023). While reduction of beneficial genera such as *Arthrobacter* can lead to less fertile soils and lower crop yields (Fu, 2014). But the consequences of altering some of these genera have not been fully explored in agroecosystems. Therefore, further research is needed to understand the agricultural implications of altering soil bacterial community upon WASH application.

This is the first study on the impact of WASH, as medium of EPN-associated bacteria, on soil bacterial community in greenhouse conditions. Although the soil used in this study has more than 70 % of the ASVs classified as “unassigned”. Nevertheless, WASH also significantly influenced the number of ASVs per samples in maize field soil but increasing the ASVs number in comparison with control treatment (Figure S6). Different effects of EPN supernatant in different conditions such as soil ecosystems suggest that WASH influence, as EPN performance, seems to be affected by diverse factors that contribute to this variable impact of soil bacterial community (Helmberger et al., 2017). Hence, more studies are needed to ensure a predictable field performance of WASH or specific bacteria members of the second bacterial circle of EPNs.

The impact of EPNs on the soil bacterial community have been poorly studied. Recently, a study presented that IJs application of *S. carpocapsae* nematodes altered bacterial communities and significantly changed fungal communities in *Solenopsis invicta* mound soils, decreasing beneficial microbes and increasing pathogenic ones, while boosting entomopathogenic fungi (Li et al., 2024). Obvious differences in experimental conditions, including EPN species and application dose can explain the opposite results between the study on the impact of *S. carpocapsae* on mound soils and the present study. For example, timing post-application is crucial for detecting these effects, as shown in a study where co-applying a different EPN genus, *S. feltiae*, and earthworms altered the soil bacterial community just after 30 days (Chelkha et al., 2025). However, in this study EPNs application did not modify significantly soil bacterial community 30 days after inoculation. Therefore, the impact of EPNs on the

soil bacterial community could be also EPN species dependent. Although, a microcosm study also found no effects of *S. carpocapsae* nematode application on soil microbial biomass (De Nardo et al., 2006). Overall, the findings suggest that soil types and ecosystems likely have the greatest influence on EPN performance, despite different measurement methods used in various studies (Chelkha et al., 2025; De Nardo et al., 2006). Additional studies are required to understand more the interactions among EPNs, EPN associated bacteria and soil bacterial communities, and the mechanisms of these interactions.

Conclusions and future perspectives

This study confirms the presence of a second bacterial circle associated with IJs of EPNs. The second bacterial circle has some member that are cultivable in laboratory conditions and frequently associated with the laboratory-reared EPNs in a strain-dependent manner. In addition, this study exposed the capacity of WASH, EPN supernatant, to induce changes in soil bacterial community. Further studies should focus on clarifying how stable are these influences in different environmental conditions. Future mechanistic studies could provide a better understanding on how these bacteria potentially enhance nematode fitness and survival. Additionally, field applications should be explored to evaluate the practical potential of these bacteria in improving nematode-based biocontrol strategies in agricultural settings. In conclusion, EPN-associated bacteria could be a source for developing potential bioproducts against plant disease and pests.

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Data availability statement

The data will be deposited on the DRYAD repository (Dryad | Home - publish and preserve your data). All raw data can be provided upon reasonable demand to the corresponding author.

Conflict of interest

The authors declare no conflict of interest.

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Author contribution

AMVM designed and conducted experiments, isolated and identified bacteria, analysed data, and wrote the chapter. PAH isolated some EPN-associated bacteria and conducted the field experiment. KD isolated some EPN-associated bacteria. CAMR acquired funding, designed and supervised the project, and commented on a previous version of the chapter.

List of Figures

Supplementary Information

Supplementary Figure S1: Alpha diversity indexes of washed and surface sterile EPNs (EN01).

Supplementary Figure S2: Visual representation of the core bacteria community of different *Heterorhabditis bacteriophora* strains.

Supplementary Figure S3: Taxonomic distributions of ASVs in EPNs (EN01) samples across environmental conditions.

Supplementary Figure S4: Core bacteria community of EPNs (EN01) across insect hosts.

Supplementary Figure S5: Taxonomic distributions of ASVs in soil samples after surface sterile and non-sterile EPNs and WASH application in greenhouse conditions.

Supplementary Figure S6: Taxonomic distributions of ASVs in soil samples after EPNs and WASH application in field experiment.

Figure S1. Sterilization process changes the microbial alpha diversity of entomopathogenic nematodes (EPNs). (A) Chart indicating the Shannon diversity index of EPN samples exposed or not to the sterilization process. (B) Chart showing the Simpson diversity index of EPN samples exposed or not to the sterilization process. C: control samples (thoroughly washed IJs of EPNs (EN01) with tap water). S: Sterile EPNs (surface sterile IJs of EPNs (EN01) after treatment with 1 % bleach and antibiotic solution: Streptomycin (200 mg/L), Rifampicin (25 mg/L) and CellCultureGuard 10X). Numbers indicate the number of replicates. Sa: sample.

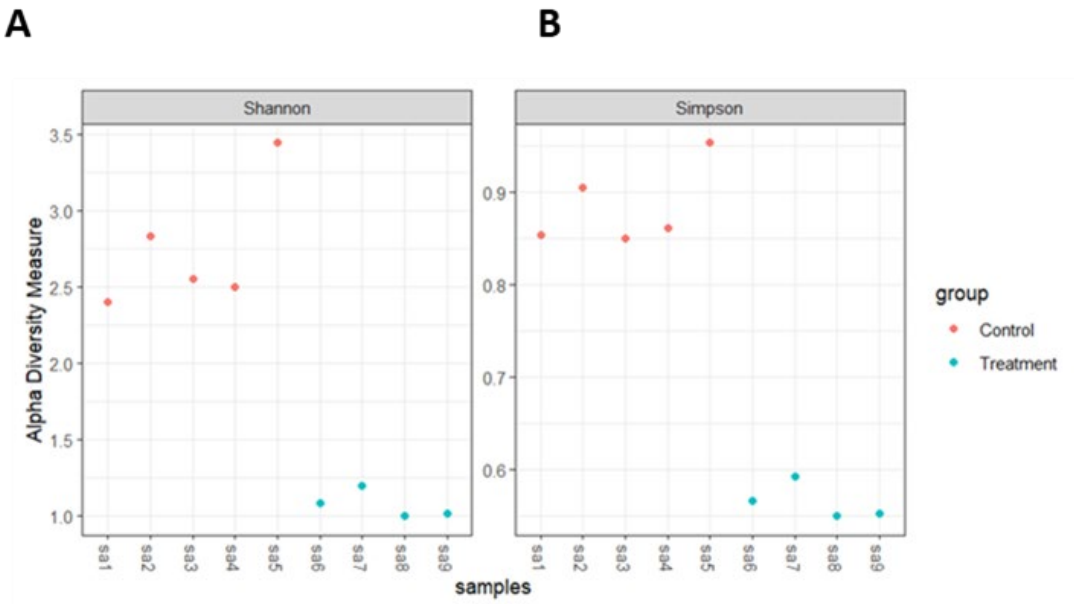


Figure S2. Visual representation of the core bacteria community of different *Heterorhabditis bacteriophora* strains. (A) Krona plot representing the taxonomic abundance in infective juveniles (IJs) of EPNs in one of the replicates of EN01 strain. (B) Venn diagram illustrating common bacteria genera present in laboratory-reared IJs of different *Heterorhabditis bacteriophora* nematodes strains, EN01, RM102, IT6e, HU2e and IR2e and in commercialized strain by Andermatt and *Galleria mellonella* larvae that was used as host (n=4-6).

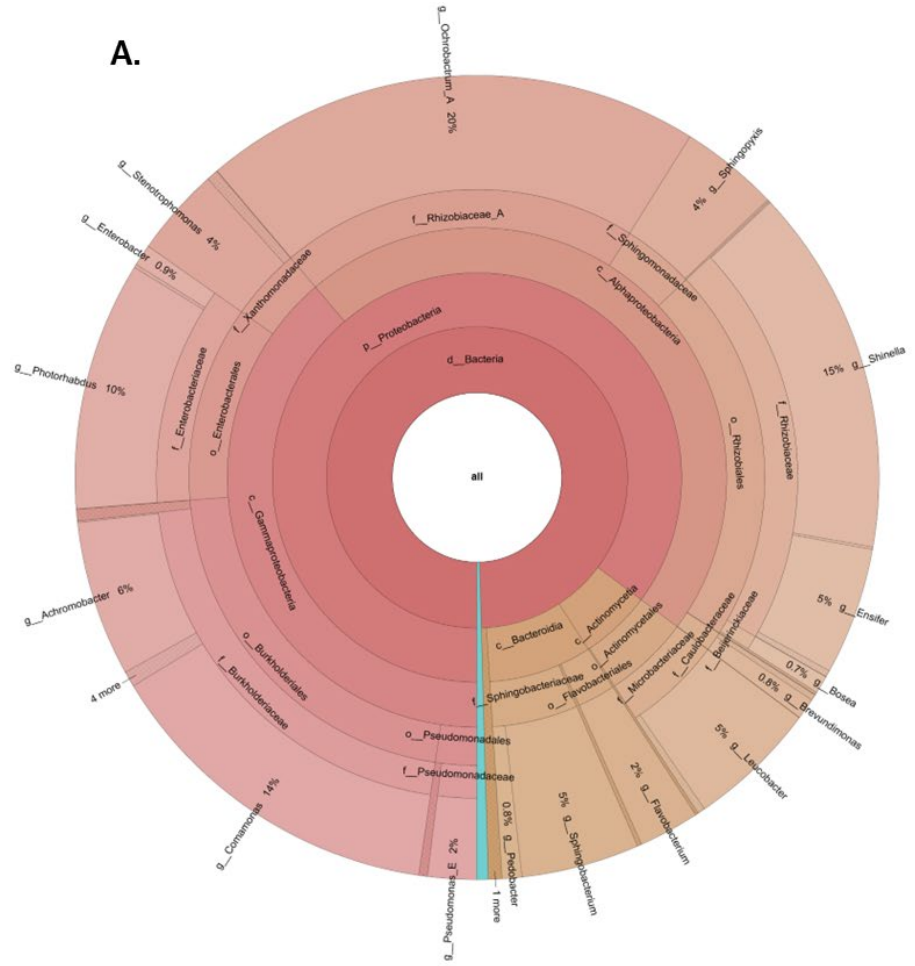


Figure S3. Bar plot indicating the taxonomic distribution by nb database of the Amplicon Sequence Variants (ASVs) identified in IJs of EPNs (EN01) under different environmental conditions.

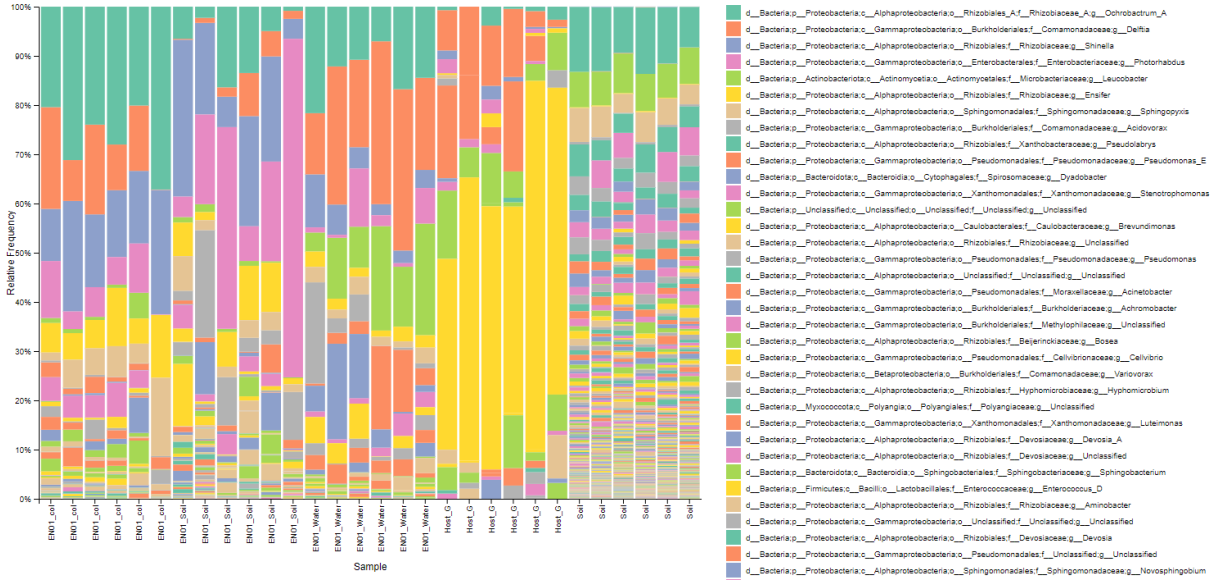


Figure S4. Core bacteria community of EPNs across insect hosts. (A) Heatmap representing relative abundance of bacteria genus identified per group. (B) Principal component analysis (PCA) based on Operational Taxonomic Units (OUT) level microbiota composition of all samples (n=6 per group). (C) Venn diagram representing number of identified OTU in EPNs reared in Galleria mellonella and Tenebrio molitor larvae (n=6 replicates per hosts). (D) and (E) Alpha and beta diversity indexes, respectively of EPNs and larvae hosts samples. *Galleria*: *Galleria mellonella* larvae. EPN_*Tenebrio*: IJs of EPNs (EN01) reared in *Tenebrio molitor* larvae. *Tenebrio*: *Tenebrio molitor* larvae.

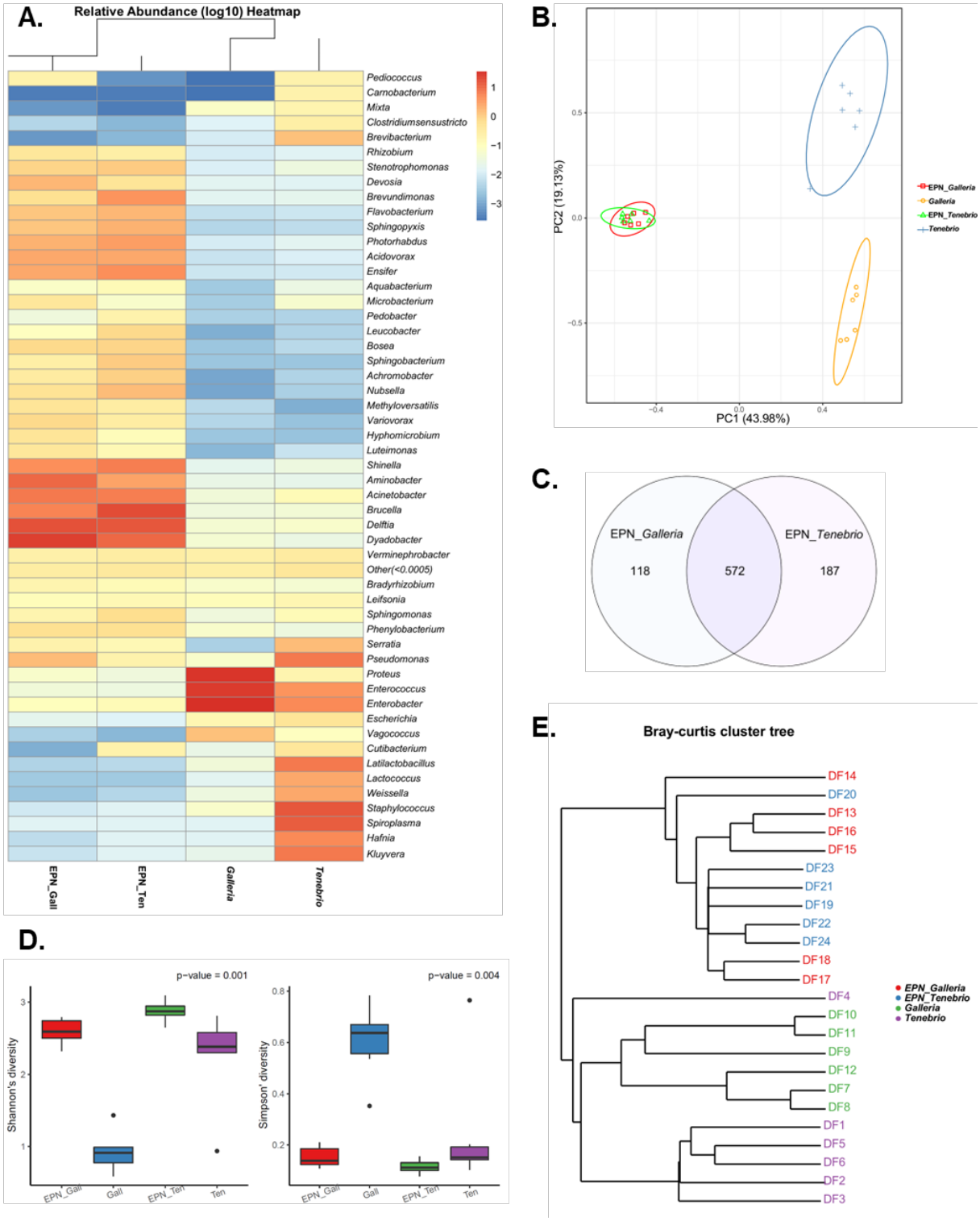


Figure S5. Bar plot indicating the taxonomic distribution by nb database of Amplicon Sequence Variants (ASVs) in soil upon EPNs, Sterile EPNs and WASH exposure for 30 days. Nematodes exposure consisted in the application of 2000 individuals per plant. Numbers indicate the number of replicates. C: Control treatment. E: IJs of EPNs (EN01) treatment. S: Sterile EPNs treatment. W: WASH treatment.

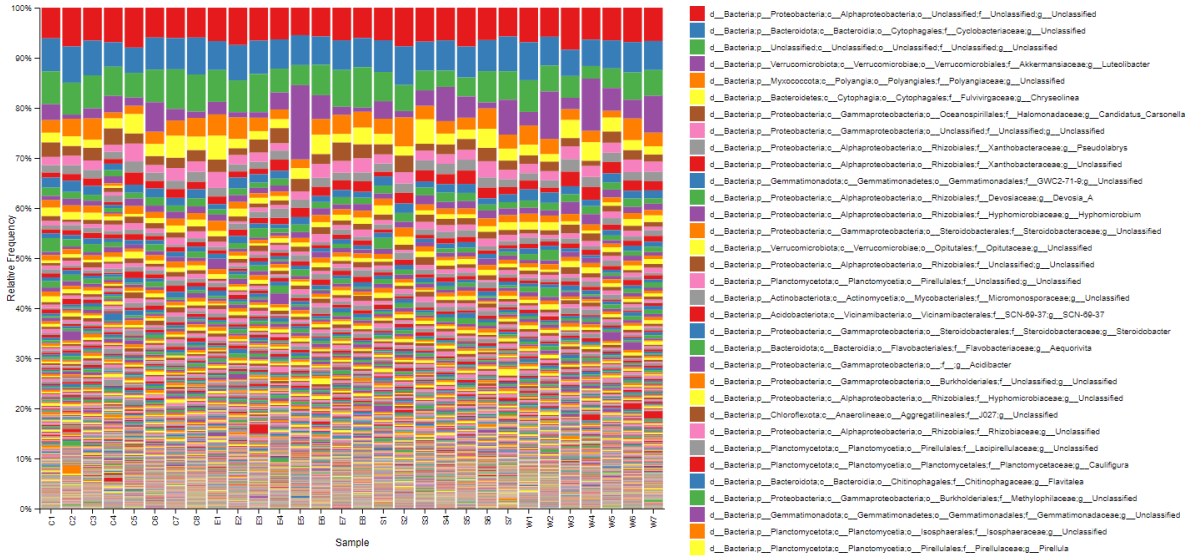
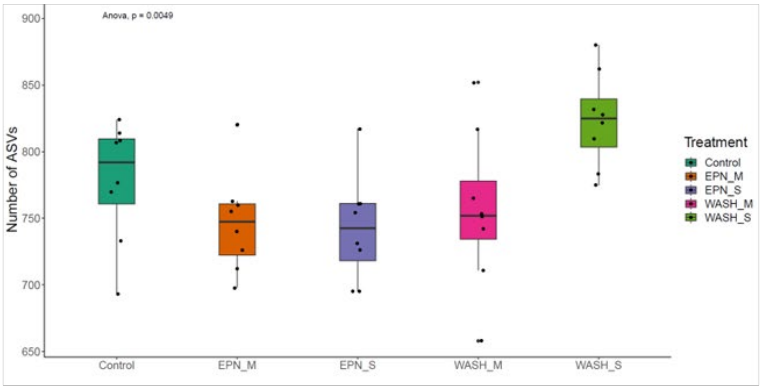
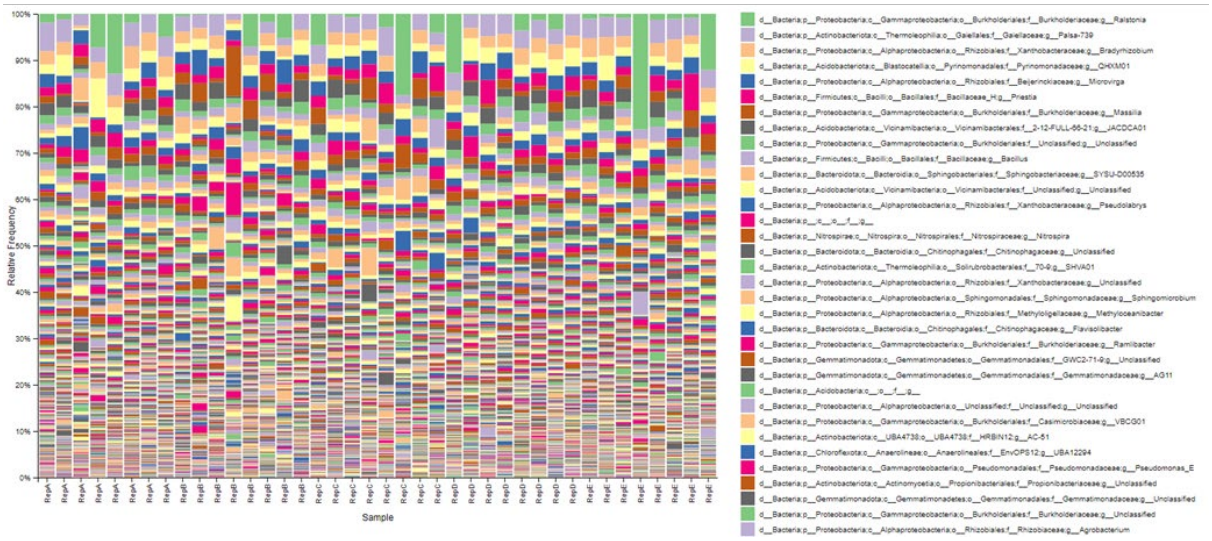


Figure S6. **WASH from entomopathogenic nematodes (EPNs) *Heterorhabditis bacteriophora* (EN01) contained bacteria and influenced soil microbial community composition in maize field soil**. (A) Box plot representing the number of Amplicon Sequence Variants (ASVs) in soil upon EPNs and WASH exposure in maize field soil for eight days. (B) Bar plot indicating the taxonomic distribution by nb database of ASVs in soil upon single and multiple EPNs and WASH application in maize field soil. S: Single treatment application. M: Multiple treatment application. RepA: Replicates of control treatment. RepB: Replicates of single EPNs application. RepC: Replicates of multiple EPNs application. RepD: Replicates of single WASH application. RepE: Replicates of multiple WASH application.

A.



B.



Supplementary Table

Supplementary Table S1: Statistical analysis of the abundance of ASVs between control and WASH treatments in soil samples

Table S1. Wilcoxon test results after comparing ASVs abundance of genera present in three or more soil replicates from the Control and WASH treatments.

Genus	p_value	Genus	p_value	Genus	p_value
Actinomadura_B	0.0206	*Actinotalea*	0.619	*Aeribacillus*	1
Afipia	0.413	*Agarivorans*	0.353	*Agriterribacter*	0.643
Agromyces	0.295	*Allorhizobium*	0.504	*Allosphingosinicella*	0.328
Altericroceibacterium_A	0.0807	*Ammoniphilus_A*	0.177	*Aquicella_A*	0.861
Arthrobacter_I	0.0450	*Asticcacaulis*	0.722	*Bacillus_BD*	0.858
Bacillus_O	0.647	*Bauldia*	0.00714	*Bradyrhizobium*	0.0980
Brevibacillus	0.261	*Brevundimonas*	0.0459	*Caenibacillus*	0.168
Cellulomonas	0.632	*Cellvibrio*	0.954	*Chitinophaga*	0.815
Clostridium_H	0.204	*Croceibacterium*	0.0818	*Cytobacillus*	0.710
Demequina	0.183	*Desertimonas*	0.679	*Devosi_ A_1*	0.0190
Devosia	0.562	*Devosia_A*	0.325	*Dokdonella_A*	0.229
Dongia	0.344	*Dyadobacter*	0.502	*Ferruginibacter*	0.726
Flavitalea	0.105	*Flavobacterium*	1	*Fluviicola*	0.764
Geobacillus	0.243	*Ginsengibacter*	0.0487	*Hamadaea*	0.334
Herbaspirillum	0.810	*Homoserinimonas*	0.344	*Hungateiclostridium*	0.0310
Hyphomicrobium	0.727	*Hyphomicrobium_A*	0.224	*Intrasporangium*	0.268
Jiangella	0.00685	*Knoellia*	0.905	*Kribbella*	0.125
Kroppenstedtia	0.241	*Laceyella*	0.802	*Lacipirellula*	0.488
Lacisediminihabitans	0.258	*Lacunisphaera*	1	*Legionella*	0.00761
Leifsonia_A	0.728	*Leptolyngbya*	0.768	*Limnobacter*	0.727
Luteimonas	0.602	*Massilia*	0.263	*Mesorhizobium*	0.296
Methylibium	0.415	*Methylobacter*	0.908	*Methylocaldum*	0.953
Methyloceanibacter	0.382	*Microbacterium*	0.672	*Micromonospora_E*	0.414
Miltoncostaea	0.503	*Mobilitalea*	0.677	*Mycobacterium*	0.729
Neobacillus	0.00139	*Nitrobacter*	1	*Nitrosomonas*	0.705
Nitrosospira	0.771	*Nordella*	0.322	*Novosphingobium*	0.772
Paenarthrobacter	0.246	*Paenibacillus_C*	0.348	*Paludisphaera*	0.0974
Paralcaligenes	0.331	*Pararheinheimera*	0.727	*Pararhizobium*	0.552
Parvibaculum	0.204	*Pedobacter*	0.131	*Pelagibius*	0.288

Pelotomaculum_C	0.213	*Peribacillus*	0.223	*Phenylobacterium*	0.816
Planifilum	0.449	*Polaromonas*	1	*Povalibacter*	1
Promicromonospora	0.597	*Protaetiibacter*	0.0716	*Pseudaminobacter*	0.861
Pseudolabrys	0.523	*Pseudolysinimonas*	0.228	*Pseudomonas_E*	0.679
Pseudomonas_H	0.508	*Pseudorhodoplanes*	0.130	*Pusillimonas_B*	0.599
Rhodanobacter	0.772	*Saccharococcus*	0.950	*Shinella*	0.954
Solibacillus	0.0776	*Specibacter*	0.162	*Sphingobium*	0.685
Sphingomicrobium	0.683	*Sphingomonas*	0.0184	*Spirillospora*	0.743
Stenotrophomonas	0.204	*Steroidobacter_A*	1	*Streptomyces*	0.561
Terrabacter	0.512	*Terricaulis*	0.858	*Terrimesophilobacter*	0.293
Thermoactinomyces	0.0863	*Thermobacillus*	0.200	*Thermobispora*	0.952
Thermomonas	0.0162	*Tolypothrix_B*	0.247	*Ureibacillus*	0.350
Vampirovibrio	0.155	*Wolbachia*	0.452		

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Chapter 4 - Entomopathogenic Nematode Pathobiome as Modulators of Belowground Tritrophic Interactions

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Abstract

The symbiosis between *Heterorhabditis bacteriophora*, a species of entomopathogenic nematodes (EPNs) and *Photorhabdus* spp. bacteria, relevant for insect pest control, was mostly considered monoxenic until 2020, when new evidence showed other EPN-associated bacteria are also insect pathogens, leading to the pathobiome concept in EPNs. But many questions remained open, including the impact of each member of the associated bacteria community on soil trophic interactions. EPN surface bacteria application changed maize the primary metabolism, notably in terms of amino acids and sugars concentration in tissues, although these induced responses in maize seems to be highly environment dependent. Furthermore, some members of the second bacterial circle of *H. bacteriophora* are insect pathogens and other members support EPN fitness and survival. This study reinforces the shift from the monoxenic idea in *Heterorhabditis bacteriophora* nematodes exposing new EPN-bacteria interactions. EPN-associated bacteria are a source of potential new bioproducts for sustainable agriculture and stakeholders must also evaluate the potential ecological consequences of their use.

Introduction

Microbes play a critical role in shaping the health, behaviour, and fitness of their host organisms, forming intricate relationships that can influence ecological dynamics (Kaiko & Stappenbeck, 2014). These microbial interactions can be classified as symbiotic when they are close and long-term (Frank, 1997), with symbiosis encompassing mutualism, commensalism, and parasitism (Paracer & Ahmadjian, 2000). In mutualistic interactions, both partners benefit, as seen in the human gut microbiota, where bacteria contribute to nutrient production, conservation, and protection against pathogens (Malard et al., 2021). Similarly, legume plants and nitrogen-fixing bacteria such as Rhizobium share a mutualistic relationship that is essential for the nitrogen cycle (Mahmud et al., 2020). While much research has focused on the role of microbes in plants, animals, and humans, the microbiomes of soil-dwelling parasitoids, such as entomopathogenic nematodes (EPNs), remain underexplored. Given the potential for these microbes to influence EPN entomopathogenicity and broader ecosystem interactions, understanding their role is essential to fully grasp the ecological impacts of these belowground organisms.

Microbes associated with parasitoids play crucial roles in various ecological processes (Dicke et al., 2020). Research on parasitoids and viruses, for instance, has shown that these microbes can affect the health and behaviour of plants and insects (Zhu et al., 2018). For example, viruses associated with parasitoids wasps can manipulate host immune responses, enhancing the ability of wasps to parasitize their hosts (Edson et al., 1981; Martinez et al., 2012). Aboveground, these interactions are well-documented, providing insights that can mirror and inform our understanding of belowground dynamics. The effects of these microbes on plant health, insect behaviour, and overall ecosystem functioning are profound, highlighting the need for a comprehensive overview of their roles in the formation and regulation of different ecological communities (Abdala-Roberts et al., 2019; Chomicki et al., 2022). Furthermore, host-microbe symbioses have rising interest for their potential in biocontrol of insect pests in pest management strategies (Popa et al., 2012).

Entomopathogenic nematodes (EPNs) are microscopic roundworms that parasitize insects, playing a significant role in natural pest control. Central to their pathogenicity is their association with specific symbiotic bacteria, such as *Xenorhabdus* and *Photorhabdus* species, which are essential for the infectivity of EPNs (Dillman et al.,

2012). These bacteria are transmitted vertically and reside within the nematodes, aiding in the infection process by producing toxins and enzymes that facilitate host tissue degradation (Clarke, 2020). This mutualistic relationship between EPNs and their symbiotic bacteria has been extensively studied, highlighting its importance in the lifecycle of nematodes and their effectiveness as biocontrol agents. Recent research has expanded the understanding of EPN-associated microbiota beyond the primary symbiotic bacteria. Studies have identified additional bacterial communities on the nematode cuticle, referred to as the “second bacterial circle” (Ogier et al., 2023).

These microbes, including species like *Pseudomonas protegens* and *P. chlororaphis*, have been implicated in enhancing the nematodes virulence and may play a role in the interactions of nematodes with their insect hosts. For instance, *P. protegens* has been shown to possess entomopathogenic activity, suggesting its involvement in the nematodes pathogenicity (Ruiu et al., 2022). Some studies suggested that these other host-microbe symbioses could also influence EPNs fitness and survival (Enright & Griffin, 2005; Ogier et al., 2023). The concept of the “pathobiome” in EPNs emphasizes the collective impact of these associated microbial communities on the parasitic lifecycle of nematodes. For example, *Paenibacillus nematophilus* associates on the cuticle of *Heterorhabditis* species (Enright et al., 2003). Thus, EPN-associated bacteria are a niche for new bioproducts for agriculture.

EPNs and EPN-infected insect cadavers also induce plant systemic response against plant pests and disease (An et al., 2016; Jagdale et al., 2009; Kamali et al., 2022; Kansman et al., 2024). For instance, the presence of EPNs in the soil induced plant response that resulted in a reduction of plant parasitic nematodes (PPNs) infestation (Kamali et al., 2022). Therefore, chemical cues from EPNs can shape tritrophic interactions (Kansman et al., 2024). Considering last evidence, these chemical cues could be produced by EPNs or EPN-associated microbiota. In PPNs, bacteria attached to nematodes cuticle induced plant defence (Topalović et al., 2019). However, the impact of the second bacterial circle of EPNs on multitrophic interactions remains unexplored.

This study aimed at assessing the impact of the second bacterial circle of *Heterorhabditis bacteriophora* on belowground interactions. The main question of this study was: What impact does this second bacterial cycle and each member of it have

on plant defence and growth, insect pathogenicity, and EPNs fitness and survival? This study confirmed that *Pseudomonas* species are also part of the *Heterorhabditis* pathobiome. Furthermore, the current research is the first study that explored the effect of EPN-associated bacteria in components of the soil food webs.

Materials and methods

Biological resources

Maize seeds (*Zea mays* L.) of the variety B73 were used for all experiments. Seeds were kindly provided by the Maize Genetics and Genomics Database (MaizeGDB, www.maizegdb.org) and bred by Delley (Delley seeds and plants Ltd, Delley, Switzerland). Maize seedlings were grown in 100 mL cylindric pots (Semadeni, Ostermundigen, Switzerland) filled with 80 % river sand 4 mm (LANDI Schweiz AG, Dotzingen, Switzerland) and 20 % soil on top (Selmaterra Schweizer Schwererde torfreduziert, Bigler Samen AG, Thun, Switzerland). The plants grew in greenhouse conditions at 23 ± 2°C, approx. 60 % humidity and 16:8 dark/light cycle, with daily watering and Plataktiv® Typ K (Hauert HBG Duenger AG, Grossaffoltern, Switzerland) fertilizer added weekly according to the manufacturer instructions.

Entomopathogenic nematodes (*Heterorhabditis bacteriophora* Poinar) came from the nematode colony of the Biotic Interactions and Chemical Ecology groups of the Institute of Plant Science at the University of Bern (Zhang et al., 2019). The EN01 strain of this in-house colony, was established with EPNs bought from Andermatt Biocontrol in 2021 (Grossdietwil, Switzerland) and reared *in vivo* in greater wax moth larvae (*Galleria mellonella* Lepidoptera: Pyralidae) bought at a local fishery shop (Fischereibedarf Wenger, Bern, Switzerland).

For infectivity assays *Tenebrio molitor* larvae (Fischereibedarf Wenger, Bern, Switzerland) were also used for EPNs inoculation. Eggs of *Ostrinia nubilalis* were bought from BTL Bio-Test Labor GmbH Sagerheide (Groß Lüsewitz, Germany) and reared on general purpose lepidoptera diet (Frontier Agricultural Sciences, Newark, USA). L3-L4 larvae were used in the experiment.

Preparation of different treatments used in this study

The treatments included in different experiments of this study are summarized below (Table 1). The same day of the inoculations, EPNs were counted and Nemawash collected. Nemawater, called WASH in this study, is the EPN supernatant collected from EPNs containing flasks kept in 60-70 mL of tap water at 8°C. EPN supernatant

was separated from IJs using 25 µm sieves. EPNs from where the Nemawash was collected, were labelled as “washed EPNs” after washed them thoroughly with tap water and used as a different treatment in some experiments. Besides, Nemawash was used as a different treatment when WASH was filtered with 0.2 µm filters to eliminate the microorganisms of the WASH collected from EPN and label as “filtered WASH” treatment.

Table 1. Different treatments included to unravel the implications of the entomopathogenic nematodes (EPNs)-associated microbes in maize tritrophic interactions.

Treatments	Description
Control	Tap water
EPNs	IJs of *Heterorhabditis bacteriophora*
Washed EPNs	EPNs washed thoroughly with tap water
Sterile EPNs	Surface-sterilized nematodes: EPNs exposed to sterilization processes
Nemawater or WASH	EPN supernatant collected from the EPN-containing flasks
Filtered WASH	EPN supernatant filter with 0.2 µm filters to eliminate microorganisms

The sterilization process of EPNs was conducted to eliminate the microorganisms of the EPN surface and produce the “Sterile EPNs” treatment. This process was performed the same day that the inoculation was planned. First, EPNs were left 15 min of incubation in 0.4 % benzethonium chloride, then washed thoroughly with sterile distilled water. After that, EPNs were placed in an antibiotic solution containing Streptomycin (200 mg/L) (Sigma-Aldrich, Massachusetts, USA) and Rifampicin (25 mg/L) (Sigma-Aldrich, Massachusetts, USA) for 4 h. Then, the sterile EPNs were washed with 3 L of sterile distilled water and kept in this water until use.

Influence of the second bacterial circle of EPNs on maize physiology

To investigate the effect of the second bacterial circle of EN01 IJs on maize plants two independent experiments were conducted.

Maize germination, growth and defence

First, two members of the second bacterial circle of the EPNs, *Acinetobacter guillouiae* and *Pseudomonas alloputida*, were selected based on preliminary data for seed treatment; distilled water and WASH were also included as treatments (n=33 seeds per treatment). Bacterial cultures were grown overnight in LB-medium at 25°C,

adjusted to an OD of 0.2, and centrifuged. Prior to seed treatment, all maize seeds (var. B73) were sterilized by soaking them in 15 % commercial bleach for 15 min, rinsed with distilled water for 2 min, and dried. Sterilized seed were placed in plastic cups with 50 mL of treatment solution and incubated in darkness at 25°C for 3 h. Then, treated seeds were placed in germination cups with wet filter paper, arranged in an outer ring (n=11 seeds per replicate). Data on germination and seminal roots were collected after four days.

Treated seeds (var. B73) were used in an herbivory experiment, where seedlings were exposed to *Spodoptera exigua* herbivory. Larvae of *S. exigua* (L3 stage) were placed on maize seedlings V2 stage (n=1 larvae per plant; n=10 plants per treatment) and led to feed for three days. The larvae were then collected and weighed to calculate weight gain. Additionally, damaged leaves were scanned and analysed using ImageJ (Health (NIH) and the Laboratory for Optical and Computational Instrumentation (LOCI) at the University of Wisconsin, Madison, USA) to measure the total damaged area.

Treated seeds were also placed in rhizoboxes (Vienna Scientific Instruments GmbH, Alland, Austria) to investigate the effect of seed treatments in root structure and plant growth (n=5). The A5 rhizoboxes (10.9 cm x 20.6 cm x 3 cm with plexiglass sides and drainage holes) were filled with 100 % soil. Seeds were planted near the transparent front of the boxes and grown in a greenhouse (Figure S1a). After nine days, the rhizoboxes were turned to protect roots from UV light. The experiment ended after 19 days when roots reached the bottom. Plants were photographed, washed, dried, and their shoots and roots were measured and weighed.

Maize primary metabolism

Second, maize seedlings (V2 stage) were inoculated with EN01 IJs, EPN supernatant and tap water as negative control (n=6). Suspensions of infective juveniles of about 500 IJs/mL were prepared in tap water and were added into two 2 cm deep holes in the soil (2’000 EPNs per pot). Same volumes of EPN supernatant and tap water were added accordingly. After 72 h maize roots and shoots were collected, gently washed under tap water, softly dried with tissue paper and snap-frozen in liquid nitrogen. Root and shoot samples were grinded to a fine powder in liquid nitrogen using a mortar and pestle. Samples were kept at -80°C until the content of amino acids and sugars in tissues were calculated. The experiment was repeated once including also filtered EPN supernatant, washed EN01 IJs and 2’000 surface sterile EN01 IJs as treatments.

Amino acids analysis

Free amino acids were quantified using the AccQ-Tag Ultra Derivatization Kit (Waters, Milford, Massachusetts, USA) according to the manufacturer instructions. Amino acids from the tissues were extracted by mixing 100 mg of tissue powder with 1 mL of extraction buffer (50 % ethanol + 0.1 % FA). After that samples were vortex, and then centrifuged at 15.000 x g for 10 min. The supernatant was transferred to a new 2 mL Eppendorf tube and kept overnight in vacuum under 45°C in a CentriVap (Labconco Corporation, Kansas City, USA). Dry powder was dissolved in 400 µL of MilliQ water. Derivatized samples were analysed with an Acquity Ultra High-Performance Liquid Chromatography (UHPLC) system coupled to a Quadrupole Dalton (QDa)-Mass Spectrometry (MS) equipped with an electrospray source and an UV/Vis-Detector (Waters, Milford, Massachusetts, USA). The chromatography was operated with an Ethylene Bridged Hybrid (BEH) C18 column (100 × 2.1 mm i.d., 1.7 µm particle size) using the following elution gradients: 0.1 % B for 0.54 min, 0.1-9.1 % B over 5.2 min, 9.1-21.2 % B over 2 min, 21.2-59.6 % B for 0.3 min, followed by flushing the column with 90 % B for 0.6 min and re-equilibration at 0.1 % B for 0.89 min (A = H20:ACN 99:1 + 0.1 % FA, B = ACN + 0.1 % FA). The flow rate and temperature of the column was kept constant at 0.7 mL/min) and 55°C. The QDa-MS was operated in positive, single ion recording mode. The electrospray voltage was kept at 0.8 kV, while the cone voltage was adjusted according to the measured ions. The source and probe temperature were kept at 120°C and 600°C, respectively. The sampling frequency was set to 8 Hz. The chromatographic data obtained were processed in the Quanlynx software (Waters, Milford, Massachusetts, USA) and the amino acid concentrations were quantified using a mixture of 17 amino acids as external standards: Alanine, Arginine, Aspartic acid, Cystine, Glutamic acid, Glycine, Histidine, Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Proline, Serine, Threonine, Tyrosine, Valine (Waters, Milford, Massachusetts, USA, p/n: WAT088122).

Soluble sugar analysis

D-glucose, D-fructose and sucrose concentrations of maize roots were quantified using a colorimetric assay kit (Megazyme, Bray, Ireland) was used according to the manufacturer's instructions. The analysis of the samples was performed in transparent, flat-bottom 96-well plates (Greiner Bio-One GmbH, Kremsmünster, Upper Austria) and measurements performed with a Tecan Infinite M200PRO plate reader (Tecan Group Ltd., Männedorf, Switzerland). Mega-Calc™ software tool from

Megazyme was used for transforming the data from absorbance values to concentration values as previously described (Robert et al., 2012).

Influence of the members of the second bacterial circle of EPNs on insects

To assess the effects of different bacterial strains on insect survival, *G. mellonella* were injected with members of the second bacterial circle of EPNs including an isolate per species (n=10). Control treatments were also included: a negative control with saline solution at a concentration of 10.5 g NaCl/L, a technical control where larvae were pierced without injection, and a control where larvae were injected with EPNs. Bacterial cultures with an OD of 0.8 were centrifuged at 3.5 g for 10 min to separate bacteria from the LB medium. The supernatant was discarded, and the bacterial pellet was resuspended in saline solution. Bacterial and EPNs suspensions were vortexed each time before use to ensure homogeneity. Uniformly sized *G. mellonella* larvae were selected and disinfected with 70 % ethanol on the injection site. Each bacterial suspension was drawn into an Injekt-F Luer Solo or Omnifix-F tuberculin syringe (0.01–1 mL) fitted with AGANI 0.55 x 25 mm needles. Larvae were injected with 20 µL of the different solution treatments at a consistent site—the second left proleg, counting from the head (Figure S2). Larvae for each treatment were placed individually in labelled petri dishes lined with filter paper and sealed with parafilm. Injected larvae were left in a carton box at 25°C. Survival rate and colour were recorded 24 h and 96 h after injection.

Influence of members of the second bacterial circle of EPNs on EPN infectivity and survival

EPN infectivity

IJs of EPNs (EN01) and Sterile EPNs were used to inoculate insect larvae for deciphering the role of EPN-associated bacteria in EPNs capacity to infect larvae in different environments as an indicator of EPN fitness. First, five *G. mellonella* larvae were placed in one petri dish with 40 g of autoclaved sand. Each petri dish constituted one replicate and 15 replicates per treatment were included in this first trial. Then, 8 mL of solutions with a concentration of 800 IJs/mL of EPNs or Sterile EPNs were added in the middle of the petri dish according to each treatment. Petri dishes were kept in dark conditions at room temperature for 7 days. Each replicate was watered with sterile distilled water to ensure certain humidity after 2 days. Number of alive and infected larvae was recorded 3, 5 and 7 days after inoculation.

A similar experiment was conducted later including larvae of *G. mellonella*, *O. nubilalis* and *T. molitor*. In this experiment, larvae from the different hosts were placed in petri dishes with filter paper. Four larvae *G. mellonella*, eight *O. nubilalis* larvae and six *T. molitor* larvae were included in each replicate according to each treatment. Four replicates were included in each treatment and each replicate was inoculated with 400 µL water solution containing about 400 IJs of EPNs or Sterile EPNs according to each treatment. Number of alive, dead and infected larvae was recorded 3 and 7 days after inoculation.

EPN survival

The effect of the bacteria isolated from WASH and EPNs was determined inoculating IJs on pre-inoculated Nematode Growth Medium (NGM) (Stiernagle, 2006). Different bacterial strains were refreshed from glycerol stocks on LB agar plates and grown in the BINDER incubator (BINDER GmbH, Tuttlingen, Germany) at 25°C for 2 days. A single colony was aseptically picked from each plate and transferred to 7 mL of LB liquid medium in sterile 14 mL tubes. The tubes were incubated at 25°C in an Infors HT Ecotron shaker incubator (Infors AG, Bottmingen, Switzerland) at 220 rpm for 36 h to allow bacterial growth. After incubation, each bacterial culture was diluted to an OD of 0.8–0.9 at 600 nm using a JENWAY 6705 UV/Vis spectrophotometer. Fifty µL of each diluted bacterial culture was evenly spread onto NGM petri dishes using sterile Delta Disposable cell spreaders. *Photorhabdus laumondii* strain was included as positive control. Plates were incubated at 25°C for 24 h to allow uniform bacterial growth across the agar surface. Then, a volume of 35 µL of IJs suspension, corresponding to approximately 50 IJs, was pipetted onto each plate and all plates were incubated at 25°C for five days. After the incubation period, the number of alive IJs on each plate was counted under a microscope (Kern & Sohn GmbH, Balingen Germany). The experiment was repeated twice.

Statistical analysis

Survival rates of EPNs and larvae were compared by first assessing normality using the Shapiro-Wilk test. In case of normality, ANOVA test was used. Moreover, due to non-normality in some experiments, a non-parametric approach was employed. The Kruskal-Wallis test identified significant differences among groups, followed by post-hoc pairwise comparisons using Dunn's test with Bonferroni correction to control for multiple comparisons. Adjusted p-values (p.adjusted) were reported to determine the

significance of differences between the control and treatment groups. These statistical analyses were performed using R software (Version 4.3.3), utilizing the *dunnTest* function from the FSA package for the Dunn's test with Bonferroni correction. Data obtained from the rest of the experiments of this study were analysed and plotted using R studio (Version 4.3.1). ANOVA, Kruskal-Wallys and Dunn´s tests were conducted using stats package (Version 4.3.1).

Results and discussion

The variable nature of EPN-associated bacteria impact on maize responses

Seed treatment with EPN-surface bacteria did not result in plant priming. The data exposed that when exposing maize seeds to seed treatment for 3 h, WASH and individual tested isolates did not affect the germination rate (Figure 1A). Besides, seed treatment with WASH and individual tested isolates did not influence herbivore performance in maize plants after 3 days (Figure 1B). Additionally, seed treatment did not increase roots and shoots length in maize plants (Figure 1C).

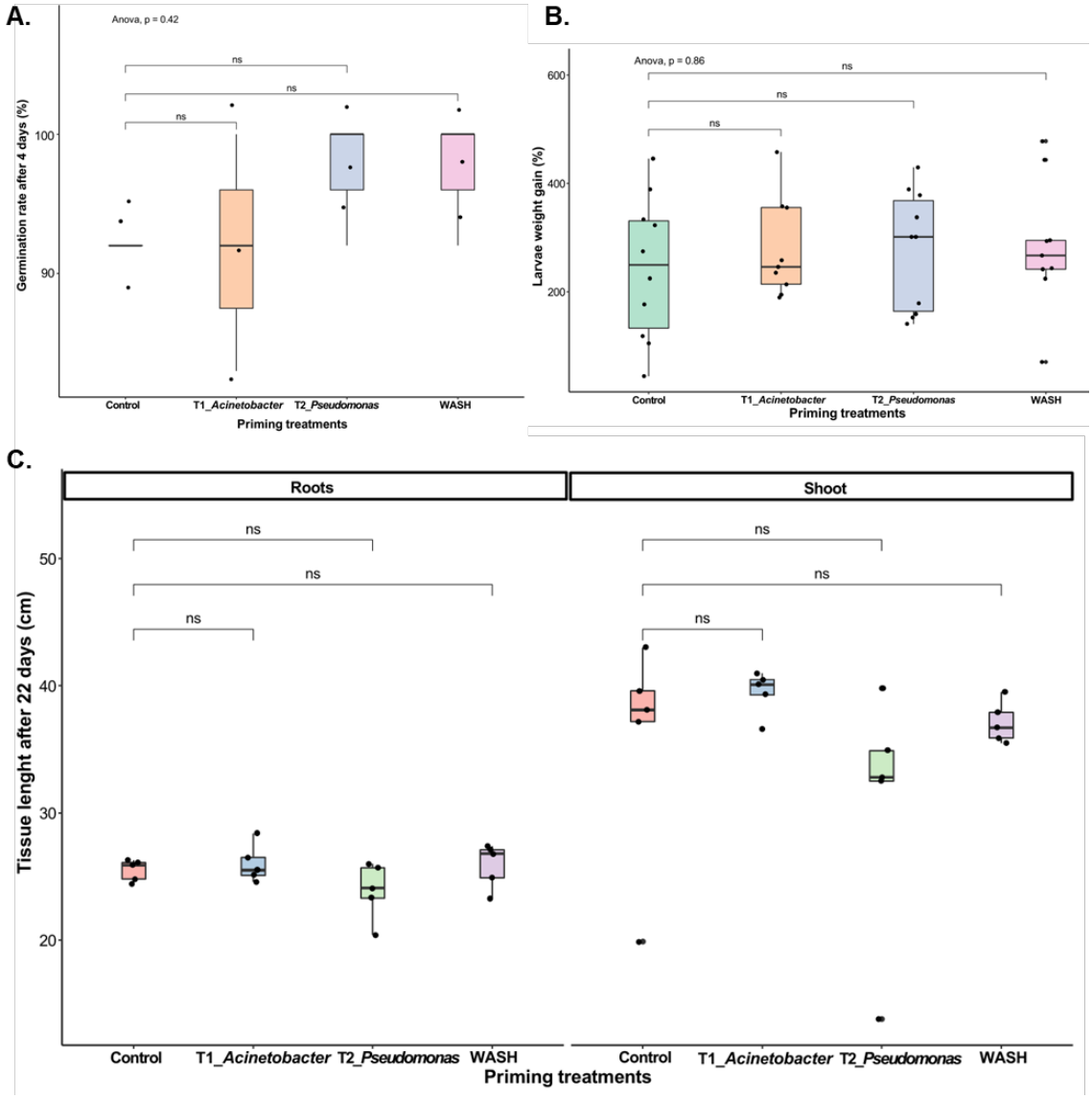

Figure 1. Seed treatment with selected EPNs surface bacteria do not increase plant germination, defence and growth. (A) Box plot representing germination rate of maize seeds upon seed treatment with *Acinetobacter*, *Pseudomonas* and WASH for 3 h. (B) Box plot representing larvae gain weight in percentage after exposing plants emerging from treated seeds with *Acinetobacter*, *Pseudomonas* and EPN supernatant for 3 h to *Spodoptera exigua* herbivory for 3 days. (E) Box plot representing tissue length in cm in plants emerging from treated seeds with *Acinetobacter*, *Pseudomonas* and EPN supernatant for 3 h. ns: no statistical difference.

Seed treatment included two of the most abundant bacteria isolated from EPN supernatant. *Acinetobacter* and *Pseudomonas* selection was also based on preliminary results (data not shown). However, considering that some EPNs surface bacteria are already reported as plant growth-promoting bacteria, other isolates must be tested. Moreover, many factors could influence seed priming including physiological state of the seeds and bacteria strain (Fiodor et al., 2023; Fu et al., 2024). More experiments are necessary to elucidate the potential of EPNs surface bacteria for seed priming and provide definitive conclusions.

Interestingly, when analysing the effect of EPN-associated bacteria on plants, the results showed that EPNs and WASH applications induce different response in maize seedlings (Figure 2, Figure S3). The inclusion of the WASH as a different treatment evidenced that EPN-associate patterns or microbes-associated patterns are sufficient for inducing plant response. EPN supernatant influenced the maize primary metabolic processes, leading to an increase in sugars and amino acids (Figure 2A-B, Figure S3, Figure S4). The results showed that maize plants increased sucrose content in leaves (Figure 2A), and amino acid content in roots (Figure 2B) after exposure to WASH for 72 h. Although, WASH did not induce changes in the completed metabolic profile or in phenotypic traits of maize plants (Figure S5). However, the experiment including filtered WASH and sterile EPNs as treatments confirmed that living microbes are not required in WASH to induce maize plant response (Figure 2C).

Previous studies reported that some bacteria also isolated in this study from EPN supernatant have plant growth promoting properties, including *Pseudomonas monteilii* (Alexander et al., 2019; Dharni et al., 2014; Passarelli-Araujo et al., 2021; Sun et al., 2018). But maize plants did not modify phenotypic traits, photosynthetic activity, or sugar content upon *Pseudomonas monteilii* inoculation after three weeks (Figure S6). The results of this study suggested that plant responses to EPNs and WASH is not stable and probably highly dependent on abiotic and biotic factors. EPN performance could be influenced by other members of the soil community such as annelids, arthropods, microorganisms and plants (Helmberger et al., 2017).

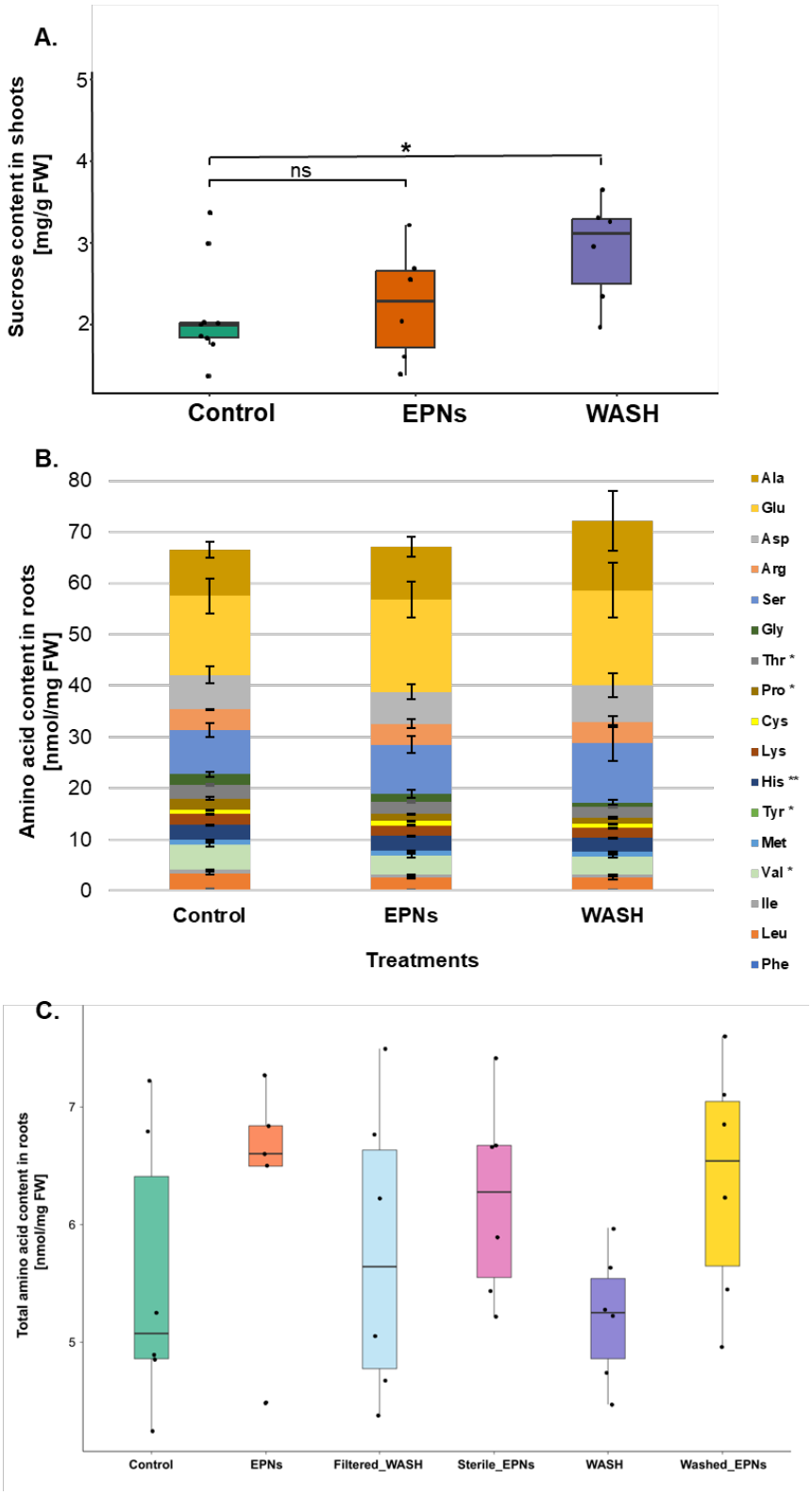

Figure 2. EPN supernatant inoculation can induce a response in plants but living microbes could be not essential to induce this response in maize seedlings. (A) Box plot representing the sucrose content in shoots upon EPNs and EPN supernatant exposition for 72 h. (B) Bar plot representing the representing amino acids content in maize roots upon EPNs and EPN supernatant exposition for 72 h (n=6). (C) Box plot representing the total concentration of amino acids in roots upon surface sterile, non-sterile and washed IJs of EPNs (EN01), EPN supernatant and filtered EPN supernatant (WASH) exposition for 72 h. FW: Fresh Weight. ns: no statistical difference. Stars indicate significant differences (*: $p<0.05$; **: $p<0.01$).

A preliminary experiment suggested that EPNs-induced effects in maize plants could be also influenced by the soil microbial community as EPNs induced different responses in sterile soil conditions compared to non-sterile soil conditions (Figure S7). Due to the implications for EPNs performance in the field, further studies are needed to better understand the interactions among EPNs, EPNs-associated bacteria, plants and soil bacterial community and the mechanisms of these interactions.

Previous studies also stated that the application of nematode supernatant induce plant responses (Atighi et al., 2020; Mendy et al., 2017; Przybylska & Obrępalska-Stęplowska, 2020; Tran et al., 2017). For example, WASH from different Plant-Parasitic Nematode (PPN) species resulted in global DNA hypomethylation in Arabidopsis plants (Mendy et al., 2017). However, this study in Arabidopsis and others focused mostly on pattern-triggered immunity (PTI) in plant-nematode interaction (Atighi et al., 2020). They affirmed that the plant response induce by WASH is due to the presence of nematode-associated molecular patterns (NAMPs) in WASH (Atighi et al., 2020). But they did not include EPNs in their studies.

The current study is the first report of the capacity of WASH from EPNs to induce plant response in certain conditions. These findings are relevant because WASH could potentially be used to enhance plant immunity against biotic stresses, thereby improving plant health and productivity. Moreover, they open a new window of possibilities to discover if different NAMPs as the ones described in PPNs are present in EPNs. Previous studies already suggested that plants response to EPNs exposure is different than plant response to PPNs, although both classes of nematodes are capable to trigger PTI (Kamali et al., 2022; Kansman et al., 2024).

Studies on nematode effectors are often performed in non-host plants or tissues, such as leaves, due to the absence of suitable root assays (Mitchum et al., 2013). Moreover, most of these studies escape the study of the role of EPN-associated bacteria in plant-EPNs interactions. Hence, the relevance of including EPNs, WASH and root assays in this study. Perhaps, plant response to WASH or components of the WASH trigger other defence markers that were not included in this study.

Some researchers hypothesised that those nematode-attached microbes could be direct antagonists of the nematodes and/or inducing plant defence response. For instance, a previous study showed that PTI marker genes *TFT1* and *GRAS4.1*

significantly increased in expression upon tomato root invasion by IJs of plant-parasitic nematodes (PPNs) with attached microbes compared to plants exposed to surface sterilized IJs (Topalović et al., 2020). Therefore, other approaches are required to fully understand the role of the second bacterial circle of EPNs in maize response to EPNs.

EPNs-associated bacteria could also induce plant defence markers under specific stress conditions, having potential for sustainable farming practices. Although EPN also induce plant systemic defence (Kamali et al., 2022), the effects of the EPN surface microbes on plant trophic interactions have not been studied yet. Future experiments should validate these hypotheses to develop new bioproducts from EPN-associated bacteria that can reduce the use of synthetic agrochemicals, thus mitigating health hazards to humans and the environment (Tan et al., 2022).

The second bacterial circle contributes significantly to the EPN pathobiome.

Endosymbiotic bacteria are crucial for EPN entomopathogenicity, and members of the second bacterial circle also impact the mortality rates of insect larvae. For instance, the results confirmed that *Pseudomonas* isolates significantly increased larvae mortality in *G. mellonella larvae* 24 h after injection (Kruskal-Wallis, $p<0.001$) (Figure 3). Interestingly, *Pseudomonas* isolates caused higher mortality in *G. mellonella* larvae than *Photorhabdus laumondii* within 24 h. Although, other bacteria isolates including *Delftia spp*., *Chrysobacterium mulctrae*, *Leucobacter aridicollis*, *Variovorax boronicumulans*, *Dyadobacter spp*., *Sphingobium xenophagum*, and *Sphingopyxis chilensis* did not show pathogenic effects in *Galleria* larvae 24 h after injection. Similarly, only the *Pseudomonas* strain, and no other bacteria isolated from *Steinernema carpocapsae*, another specie of EPNs, killed insect larvae within a comparable period post-injection (Ogier et al., 2020).

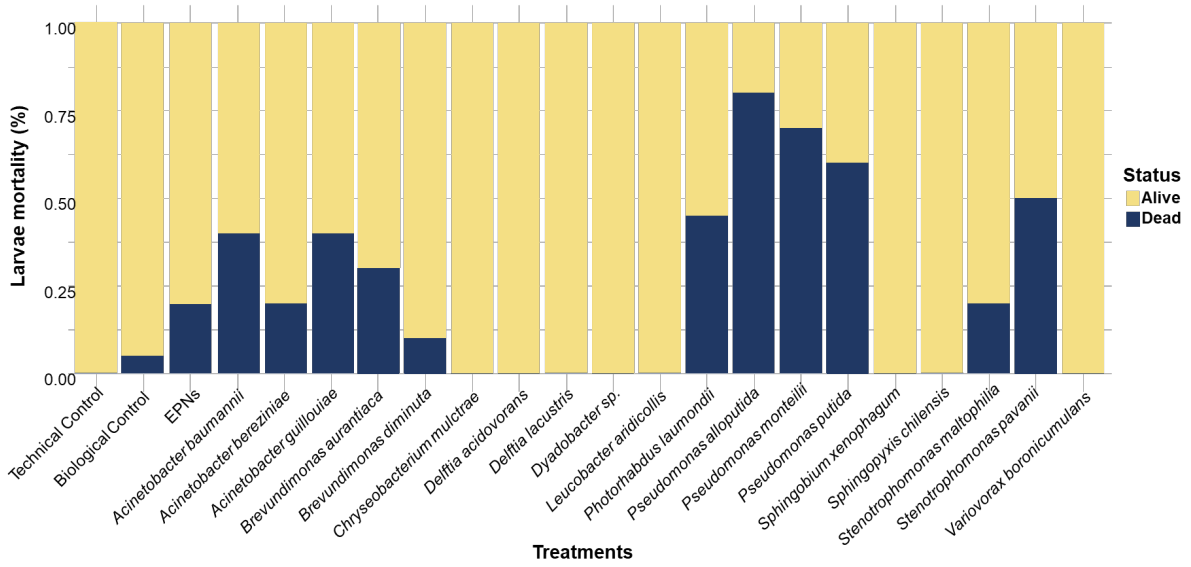

Figure 3. Associated bacteria with laboratory-reared infective juveniles of *Heterorhabditis bacteriophora* nematodes are also insect pathogens. Bar plot illustrating significantly different bacteria entomopathogenicity in *Galleria mellonella* larvae 24 h after injection with 20 µL of bacterial culture (n=10) (Kruskal-Wallis, $p<0.001$). Technical control: Injection without inoculating solution. Biological control: Injection of 20 µL of saline solution.

The results of the larvae mortality after 96 h confirmed the entomopathogenicity of *Pseudomonas* genus (Figure S8). Additionally, although *Pseudomonas* strains caused the highest mortality rates, other treatments also showed entomopathogenicity after 96 h. *Acinetobacter guillouiae*, *Photorhabdus laumondii*, and *Chryseobacterium mulctrae* significantly increased larvae mortality 96 h after injection (Figure S8).

Qualitative observations of the larvae highlighted changes in colour and activity. Some bacterial treatments caused melanization and/or lethargy, indicating a similar immune response activation as under entomopathogenic fungus infection or EPNs (Dubovskiy et al., 2013). These effects were consistent for pathogenic strains but absent in larvae injected with the biological control treatment. Larvae injected with *Photorhabdus laumondii* displayed the typical red colour seen also with EPN injections (Tarasco et al., 2023). Furthermore, larvae injected with some isolates such as *Pseudomonas* or *Acinetobacter* strains showed distinctive discolouration (Figure S9). The presence of these responses in larvae after injection with bacteria suggest that certain bacterial strains may activate specific immune pathways. Future experiments are required to elucidate the specific mechanisms of pathogenicity of this strain as well as the continuous challenge of developing the right formulation.

The findings highlighted the potential of *Pseudomonas* as biocontrol agent of pest insects due to their strong pathogenicity. The pathogenicity of *Pseudomonas* genus

on insect was previously reported on different hosts (Péchy-Tarr et al., 2013; Ruiu et al., 2022). However, the ecological risks, such as impacts on non-target organisms and potential antimicrobial resistance, need further analysis. While other *Pseudomonas* strains are already used in agricultural biocontrol for their ability to colonize plant roots and suppress pathogens (Weller, 2007), their virulence in insects can raise concerns about specificity. Hence, EPN-associated bacteria can be source of new biopesticides, but stakeholders must also be mindful of the potential ecological risks.

The second bacterial circle plays a vital role in maintaining nematode fitness.

The involvement of the second bacterial circle is crucial for EPN performance. The findings exposed that EPN-associated bacteria influenced EPNs fitness and survival (Figure 4). The inoculation of *Galleria* larvae with IJs of EPNs (EN01) and Sterile EPNs showed that the reduction of EPN-associated bacteria viability, significantly reduced EPNs capacity of infecting and killing insect larvae (Figure 4A). The results highlighted a significant reduction of the larvae mortality inoculated with Sterile EPNs (Figure 4A-B). Although the difference was more evident after 3 days, results after 5 and 7 days suggested same conclusion: The second bacterial circle plays a vital role in maintaining EPN fitness. These results supported the findings from the previous experiment where *G. mellonella* larvae were injected with different bacteria isolates exposing key role of some strains different than *Photorhabdus* in the entomopathogenicity of EPNs.

However, the results also suggested this role is host-dependent and highly influenced by environmental conditions (Figure 4C). When repeating the experiment in petri dishes with filter papers and including different hosts, although the mortality rate of different hosts larvae was always higher in the EPNs treatment, just the mortality rate of *Ostrinia* larvae was significantly higher in EPNs than in Sterile EPNs treatment. These discoveries suggest that the role of FAM could be even more significant in field soil conditions (Ogier et al., 2023).

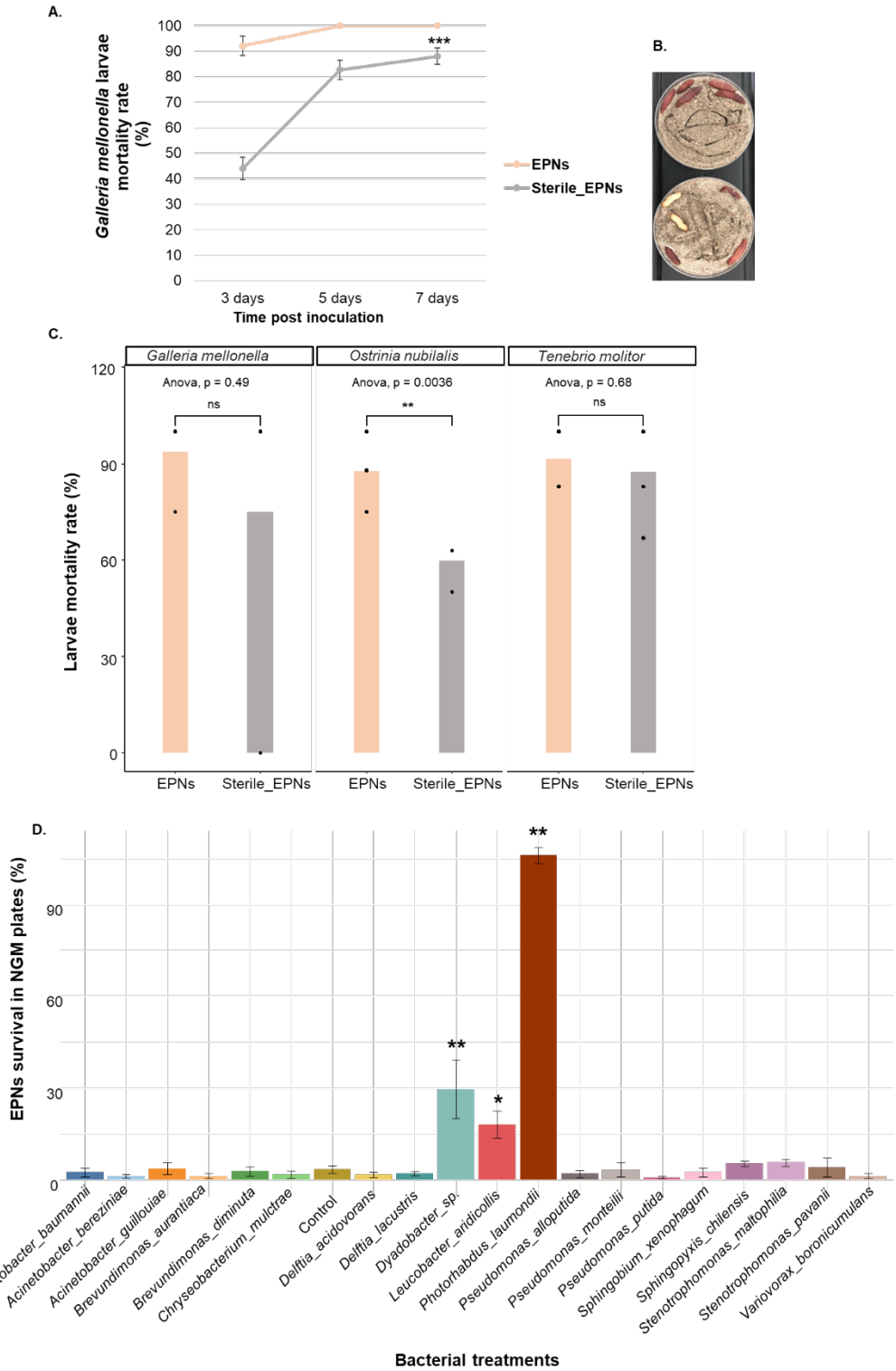

Figure 4. Bacteria associated to entomopathogenic nematodes (EPNs) influence EPN fitness in a host-dependent matter and survival in *in vitro* conditions. (A) Linear plot representing the mortality rate of *Galleria mellonella* larvae 3 days, 5 days and 7 days after inoculation with EPNs and Sterile EPNs (n=10). (B) Bar plot representing the mortality rate of *G. mellonella*, *Ostrinia nubilalis* and *Tenebrio molitor* larvae 3 days after inoculation with EPNs and Sterile EPNs (n=4). (C) Bar plot indicating EPNs survival in pre-inoculated plates with bacteria isolates after 5 days (n=5). Sterile EPNs: surface sterile EPNs with 0.4 % benzenthonium chloride and antibiotic solution (Streptomycin (200 mg/L) and Rifampicin (25 mg/L)). Stars indicate significant differences (*: p<0.05; **: p<0.01, ***: p<0.001).

Furthermore, visual observations of infected larvae indicated that FAM could also benefit EPNs competition against entomopathogenic microorganisms. Fungi usually colonized insect larvae inoculated with Sterile EPNs (Figure S10). A previous study exposed that *Alcaligenes faecalis*, symbiotic bacteria also associated with *Oscheius* spp. and *Rhabditis blumi* EPNs, have antifungal activity against entomopathogenic fungi, suggesting that *A. faecalis* may help EPNs fighting opportunistic soil fungi (Shan et al., 2019). However, the current study did not explore further the potential of each member of FAB of EPNs for controlling pathogenic fungi. Future studies should aim to integrate EPN-associated bacteria strains into pest management programs.

Finally, the results displayed that EPN-associated bacteria also influenced EPN survival in *in vitro* conditions (Figure 4D, Figure S11A). Interestingly, *Dyadobacter spp*. and *Leucobacter aridicollis* significantly enhanced nematode survival compared to the negative control. *Dyadobacter spp*. and *Leucobacter aridicollis* have been previously isolated from different environments including soil (Chabbi et al., 2024; Liu et al., 2006). Although the role of these bacteria species has not been previously studied in EPNs, similar benefits from bacterial symbionts are well-documented.

Secondary bacteria contribute to EPN success by enhancing nutrient acquisition, suppressing microbial competitors, and modulating host immune responses (Ogier et al., 2023; Tarasco et al., 2023). The absence of significant effects for other bacterial treatments indicates a more neutral or possibly antagonistic relationship, consistent with the diversity of bacterial impacts documented in entomopathogenic and plant-parasitic nematodes (Rosso et al., 2024).

Observations of the petri dishes plates confirmed that some treatments inhibited the growth of microbial competitors (Figure S11B). However, the beneficial effects of *Dyadobacter spp*. and *Leucobacter aridicollis* are probably due to other mechanisms because these strains did not inhibit the growth of other microorganisms. In several treatments, EPNs also moved from the agar to the walls of the petri dishes suggesting that some bacterial compounds could be toxic for EPNs. Additionally, the observations from one of the preliminary experiments exposed feeding of IJs (EN01) on *Leucobacter aridicollis* plates (data not shown). But EPNs feeding on *Leucobacter aridicollis* plates was not observed in other repetition. Further studies should be

conducted to elucidate the mechanisms of the interactions between EPNs and members of the second bacterial circle of EPNs.

Exploiting bacteria in EPN formulations or symbiotic systems can enhance EPNs robustness and effectiveness in biocontrol. Inoculating EPNs with beneficial bacteria during production or field application may boost pest suppression efficacy (Ogier et al., 2023; Tarasco et al., 2023). Researchers also suggest that improving microbial partnerships can enhance EPN stability and performance in the field, making bacterial symbionts a promising research area (Dillman et al., 2012). However, these applications need validation through studies on how these bacteria affect nematode fitness and survival in field conditions. In conclusion, EPN-associated microbes can significantly influence multitrophic interactions resulting crucial for the agriculture sector.

Conclusions and future perspectives

This study provides compelling evidence that certain members of the second bacterial circle associated with EPNs are also insect pathogens, while others support EPN survival under *in vitro* conditions in diverse ways. These findings underscore the complexity and potential of the EPN microbiome, highlighting that beyond the primary symbiotic bacteria, secondary bacterial communities could significantly contribute to the nematode's fitness and ecological role. Future mechanistic studies are critical to unravel the specific pathways through which these bacteria enhance EPN survival and virulence, offering insights into how microbial interactions within the nematode could be optimized for greater efficacy in biocontrol applications. Moreover, field-based research is necessary to explore the practical potential of these bacteria in enhancing the performance of EPNs in agricultural settings, particularly in pest management strategies that minimize the use of chemical pesticides. The exploration of EPN-associated bacteria offers a promising avenue for the development of novel bioproducts, which could provide sustainable solutions to combat plant diseases and pests, reducing dependency on synthetic chemicals and contributing to more resilient agricultural systems. In conclusion, the discovery and characterization of these microbial communities not only deepen our understanding of EPN biology but also open new frontiers in integrated pest management and the development of eco-friendly biocontrol agents.

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Data availability

The data will be deposited on the DRYAD repository (Dryad | Home - publish and preserve your data). All raw data can be provided upon reasonable demand to the corresponding author.

Conflict of interest

The authors declare no conflict of interest.

Author contribution

AMVM designed and supervised the study, conducted experiments, analysed data, and wrote the chapter. TMS conducted experiments. MH and PM conducted amino acid and untargeted metabolomic analyses. CAMR acquired funding, designed and supervised the project, and commented on a previous version of the chapter.

List of Figures

Supplementary Information

Supplementary Figure S1: Setting of rhizoboxes experiment

Supplementary Figure S2: Injection in *Galleria mellonella* larva

Supplementary Figure S3: Maize seedling response to EPNs and WASH application (sugars).

Supplementary Figure S4: Maize seedling response to EPNs and WASH application (amino acids).

Supplementary Figure S5: Maize seedling response to EPNs and WASH application (phenotypical traits and metabolic profile).

Supplementary Figure S6: Maize inoculation with *Pseudomonas monteilii* isolate in greenhouse conditions.

Supplementary Figure S7: Difference of maize response to EPNs in sterile and non-sterile soil.

Supplementary Figure S8: Larvae mortality rate upon injection with bacteria associated with EPNs after 96 h.

Supplementary Figure S9: Colour of *Galleria mellonella* larva upon injection with bacteria associated with EPNs.

Supplementary Figure S10: Fungi colonization in *Galleria mellonella* larva upon infection with surface sterile EPNs.

Supplementary Figure S11: Influence of members of the second bacterial circle of EPNs in EPN survival (preliminary experiments).

Figure S1. Setting of the rhizoboxes experiment. (a) Pre-treated seed in rhizoboxes. (b) Arrangement of the rhizoboxes at the start of the experiment. (c) Maize seedlings in rhizoboxes 10 days after sowing.

a b c

Figure S2. Injection site in *Galleria mellonella* larva.

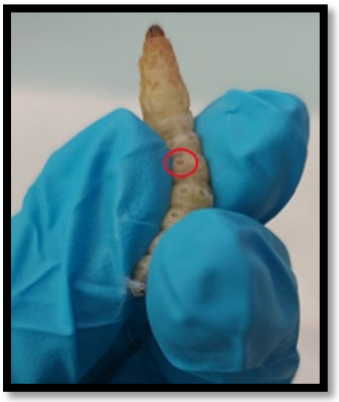


Figure S3. Maize plants modify the content of soluble sugars in the shoots and roots upon exposure to entomopathogenic nematodes (EPNs) and WASH. (A) Box plot representing the glucose concentration in shoots and roots upon EPN and WASH exposition for 72 h. (B) Box plot representing the fructose concentration in shoots and roots upon EPN and WASH exposition for 72 h. (C) Box plot representing the sucrose concentration in shoots and roots upon EPN and WASH exposition for 72 h. (D) Bar plot representing Ln-transformed fold-change values (mean ± SE) in the expression of marker genes involved in carbohydrate reallocation in maize roots after EPN and WASH exposition for 72 h. Relative expression was calculated as previously described (Livak & Schmittgen, 2001). Stars indicate significant differences (*: p<0.05).

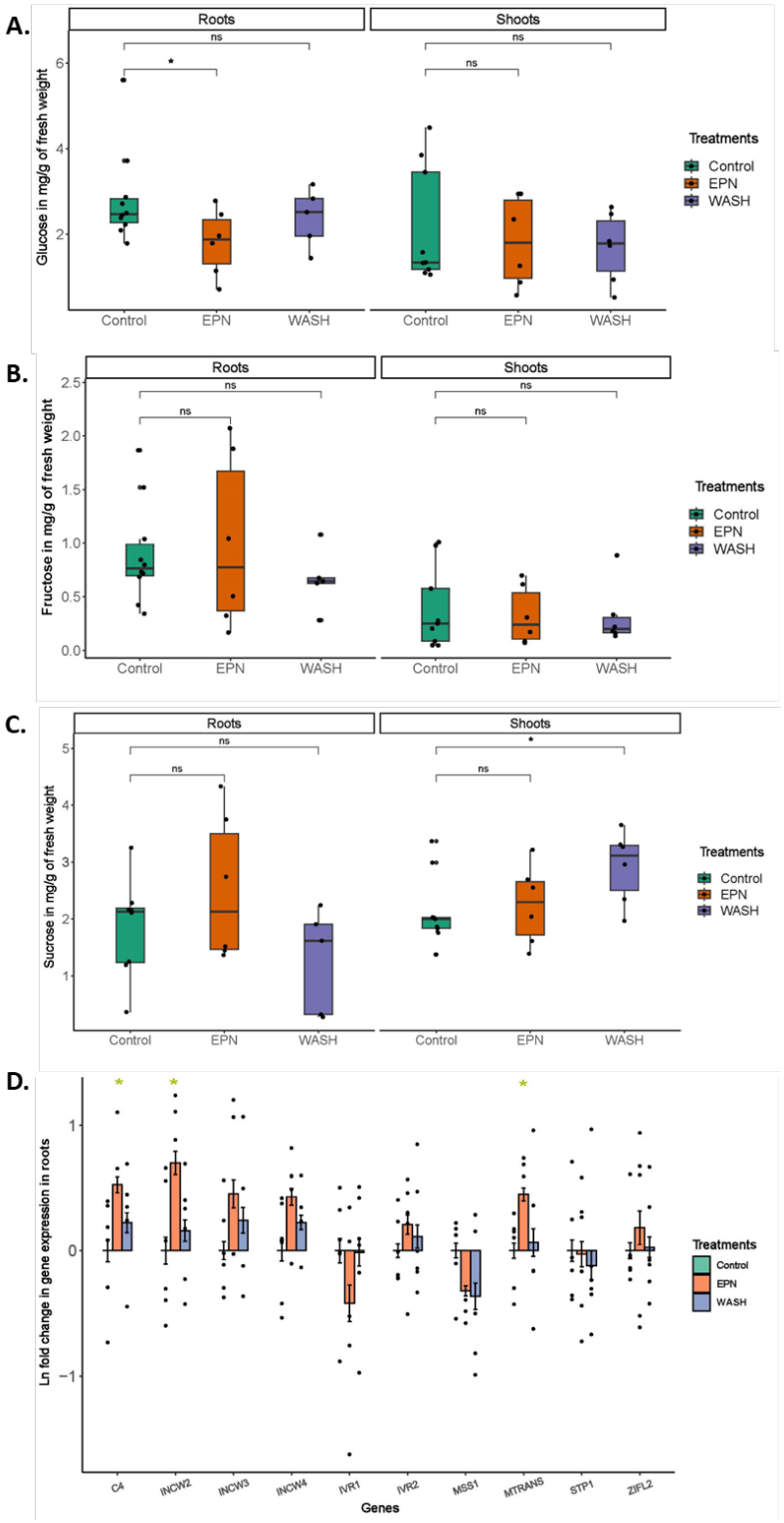


Figure S4. Maize plants reduce the concentration of amino acids in the roots upon exposure to entomopathogenic nematodes (EPNs) and WASH. (A) Box plot representing the total concentration of amino acids in shoots and roots upon EPN and WASH exposition for 72 h. (B) Box plot representing the histidine concentration in shoots and roots upon EPN and WASH exposition for 72 h. (C) Box plot representing the threonine concentration in shoots and roots upon EPN and WASH exposition for 72 h. (D) Box plot representing the proline concentration in shoots and roots upon EPN and WASH exposition for 72 h. (E) Box plot representing the tyrosine concentration in shoots and roots upon EPN and WASH exposition for 72 h. (F) Box plot representing the valine concentration in shoots and roots upon EPN and WASH exposition for 72 h. (G) Box plot representing the tyrosine concentration in shoots and roots upon EPN and WASH exposition for 72 h. C: Control. E: EPN. W: WASH. Stars indicate significant differences (*: p<0.05; **: p<0.01).

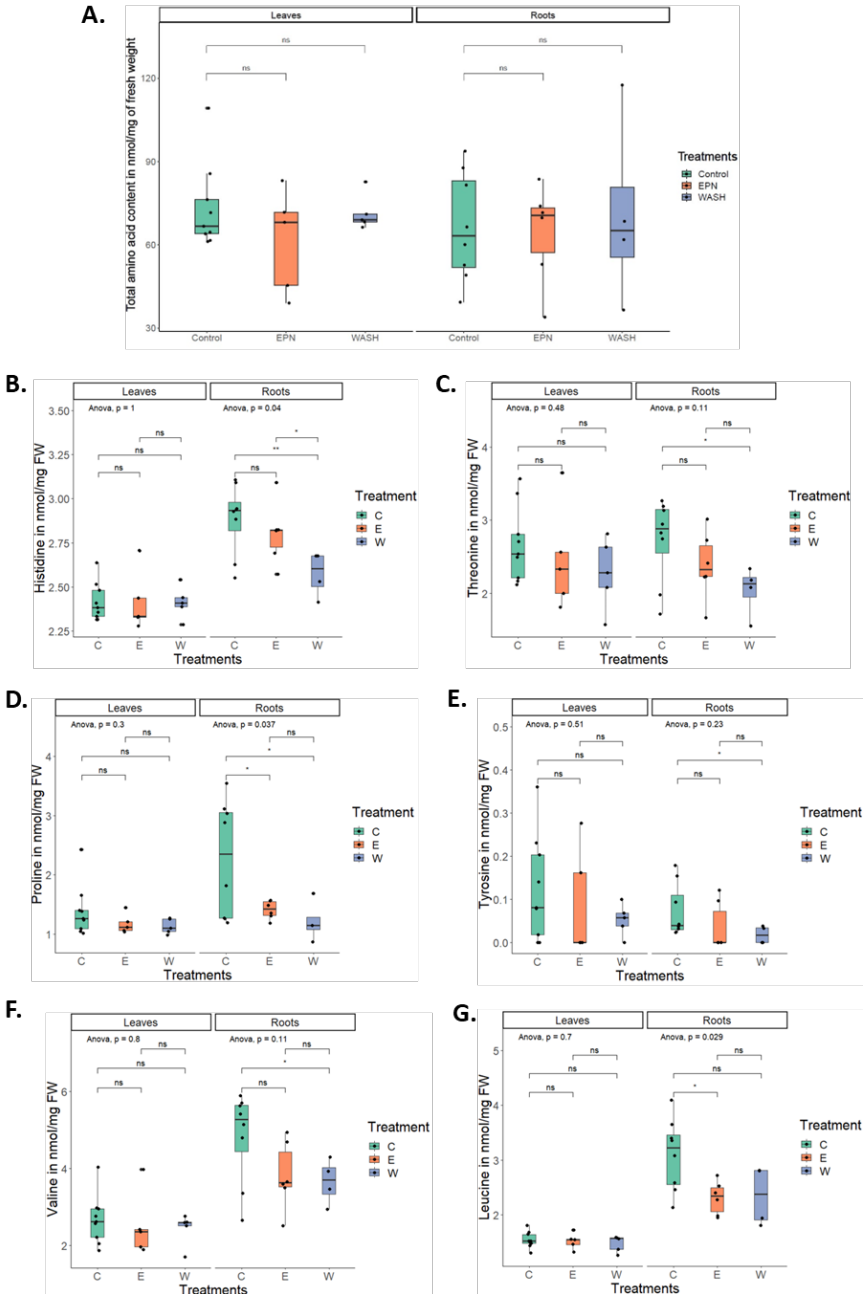


Figure S5. Maize plants do not change their phenotype or metabolic profile of root exudation, shoot and root tissues upon exposure to entomopathogenic nematodes (EPNs) or WASH. (A) Shoot weight (in g) of maize plants exposed to EPN or WASH for 72 h. (B) Crown root weight (in g) of maize plants exposed to EPN or WASH for 72 h. (C) Principal components analysis (PCA) including powered partial least squares – distribution analysis (PPLS-DA) of the root exudation of plants exposed to EPN or WASH for 72h. Dim: Dimension. (D) Principal components analysis (PCA) including powered partial least squares – distribution analysis (PPLS-DA) of shoot tissues of plants exposed to EPN or WASH for 72h. (E) Principal components analysis (PCA) including powered partial least squares – distribution analysis (PPLS-DA) of shoot tissues of plants exposed to EPN or WASH for 72h.

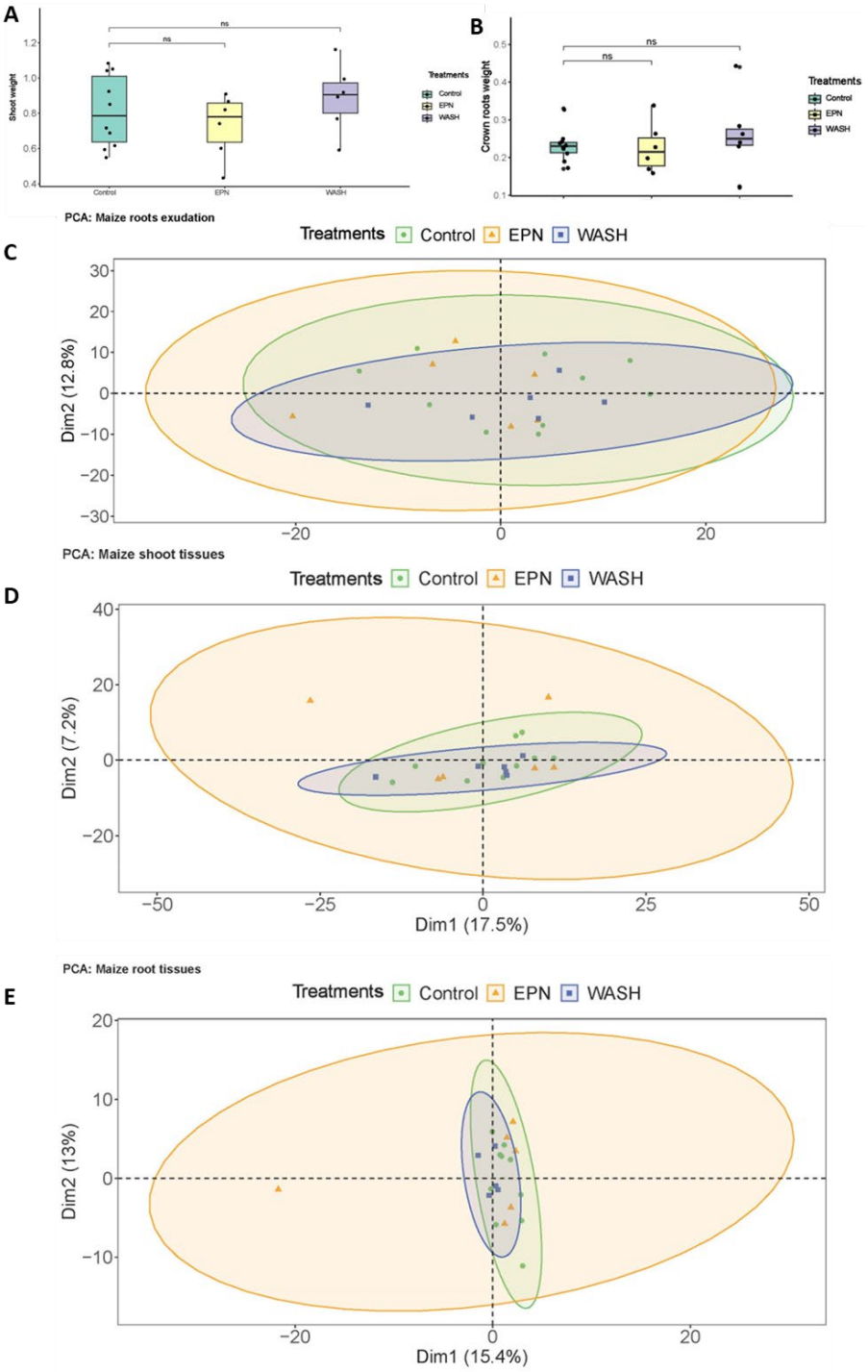


Figure S6. Maize plants do not induce modifications in the phenotypic parameters or soluble sugar concentrations in their tissues upon exposure to *Pseudomonas monteilii*, symbiont bacteria strain of infective juveniles of *Heterorhabditis bacteriophora* (EN01) nematodes. (A) Picture of 5 weeks old maize plants used in this study growing in greenhouse conditions. (B) Box plot representing shoot weight (g) of maize plants upon exposure to *Pseudomonas monteilii* for 3 weeks. (C) Box plot representing the chlorophyl content in the leaves of maize plants exposed to *Pseudomonas monteilii* for 3 weeks. (D) Box plot representing the glucose content in the crown roots of maize plants exposed to *Pseudomonas monteilii* for 3 weeks. FW: fresh weight. 5C: 5 mL of distilled water applied as control 1. 10 C: 10 mL of distilled water applied as control 2. 5T: 5 mL of bacterial solution of *Pseudomonas monteilii* applied as treatment 1. 10T: 10 mL of bacterial solution of *Pseudomonas monteilii* applied as treatment 2. Dots represent the replicates in each treatment.

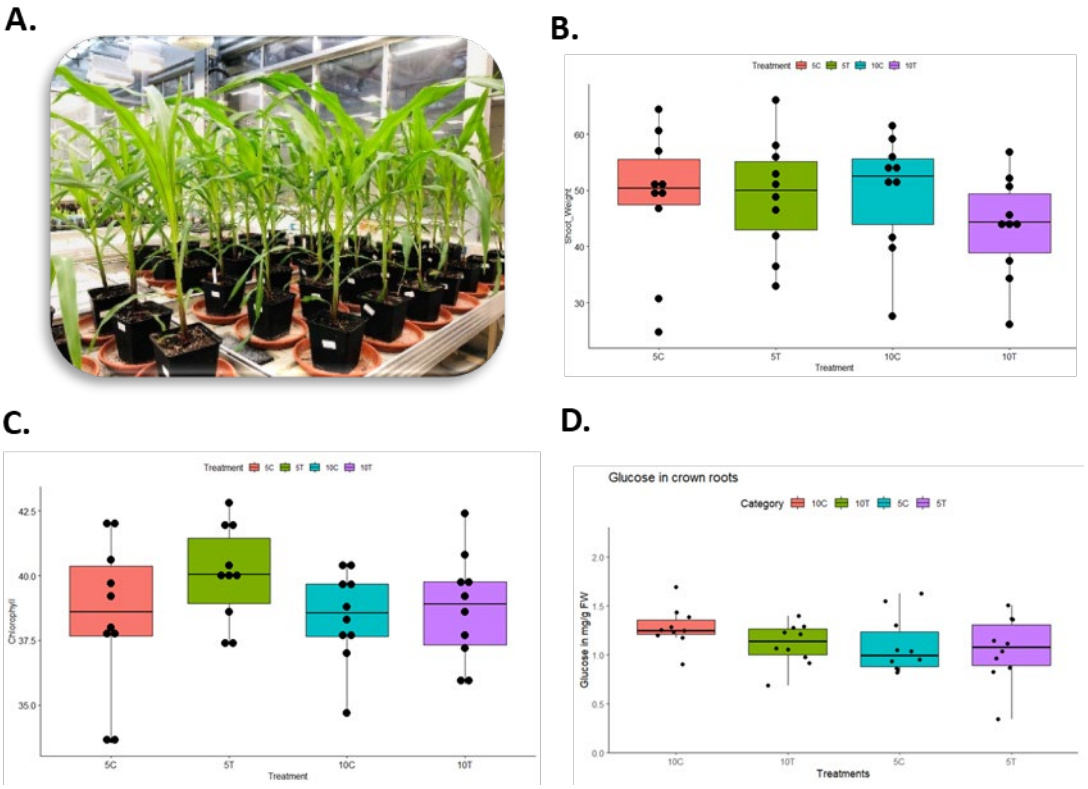


Figure S7. Maize plants induce changes in the amino acids content of roots and benzoxazinoids concentration of root exudates when exposed to entomopathogenic nematodes (EPNs) in sterile soil. (A) Box plot representing the valine concentration in roots upon EPNs exposition for 72 h in nonsterile and sterile soil. (B) Box plot representing HDM2BOA concentration in maize root exudates upon EPNs exposition for 72 h in nonsterile and sterile soil. FW: fresh weight. ns: no significant difference. Stars indicate significant differences (*: p<0.05).

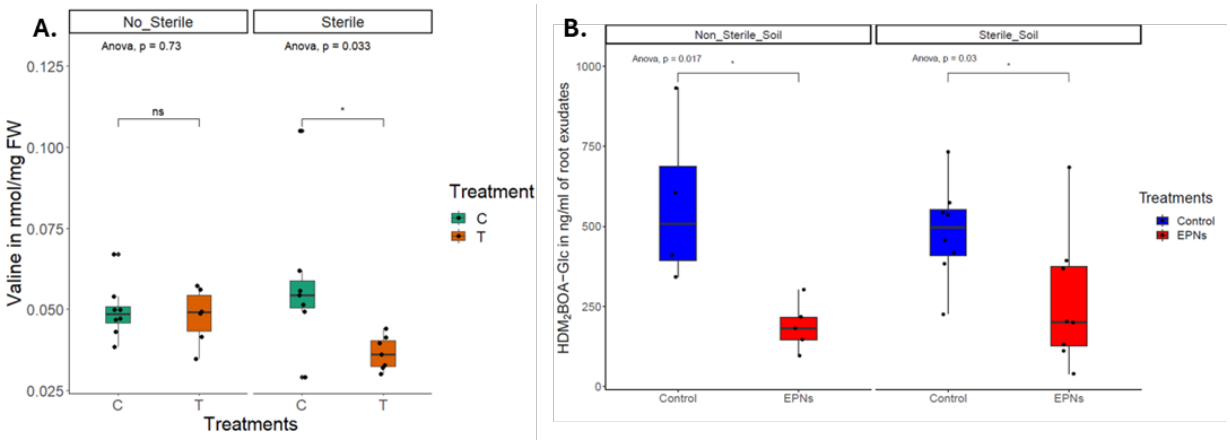


Figure S8. Bar plot illustrating bacteria entomopathogenicity in *Galleria mellonella* larvae 96 h after injection with 20 µL of bacterial culture (n=10) (Kruskal-Wallis, p<0.001). Technical control: Injection without inoculating solution. Biological control: Injection of 20 µL of saline solution.

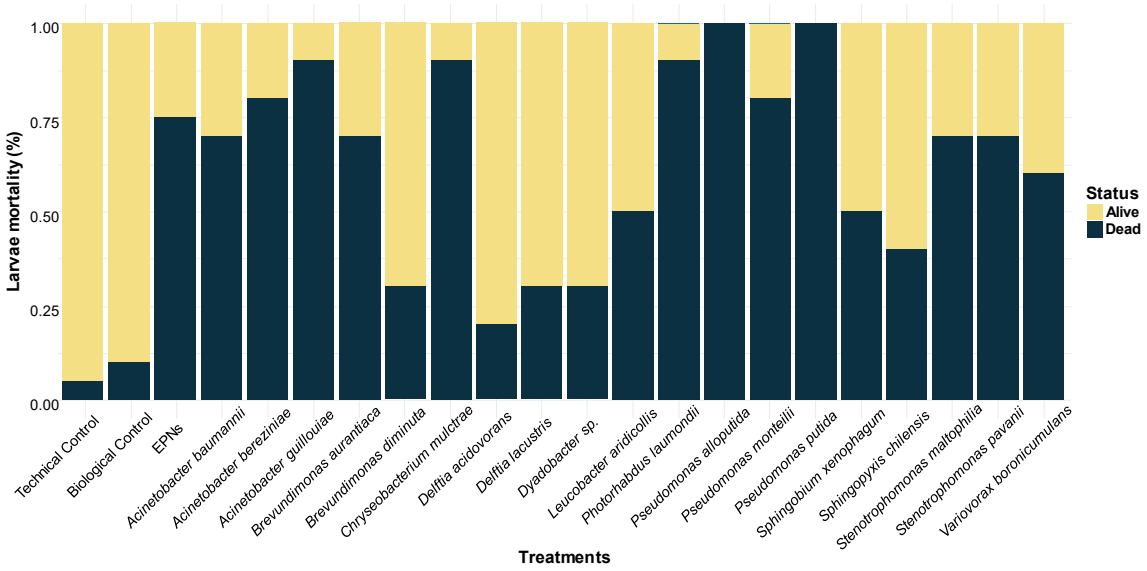
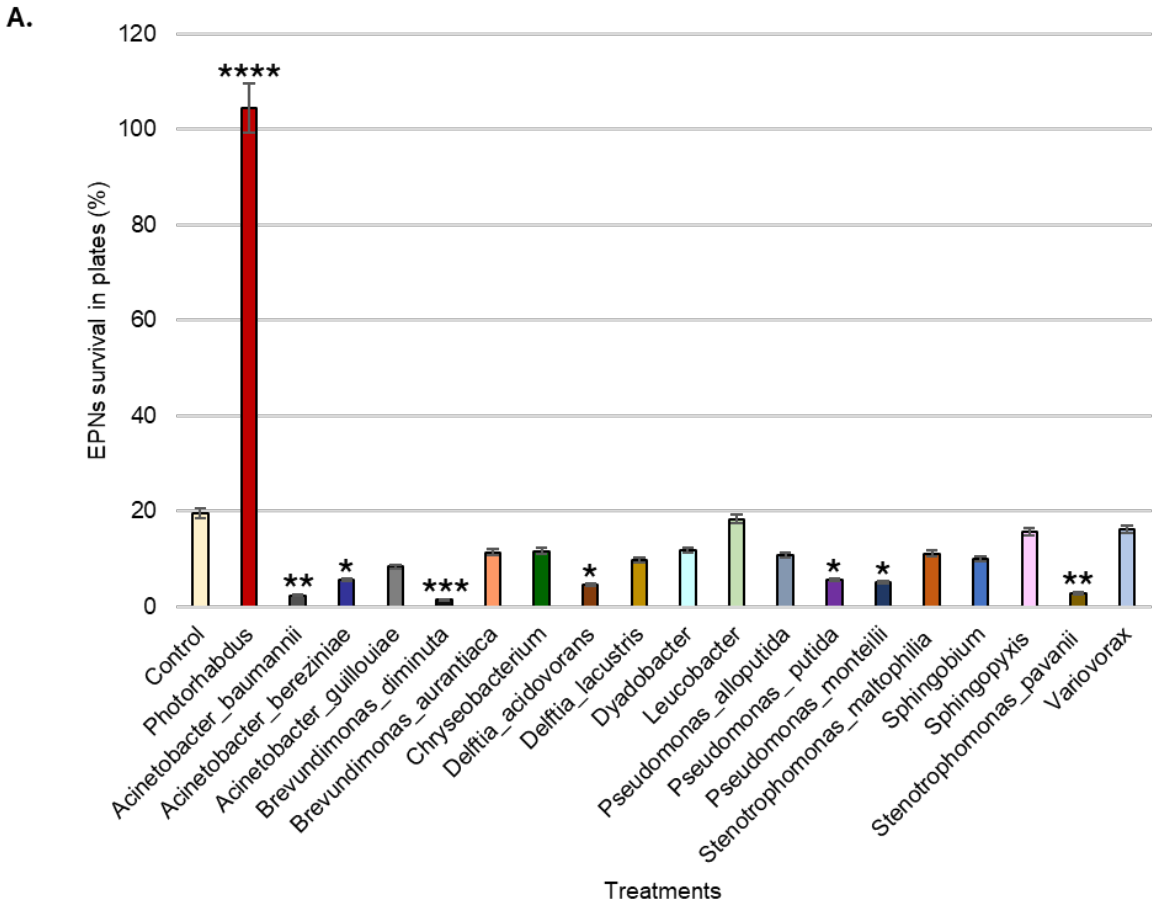
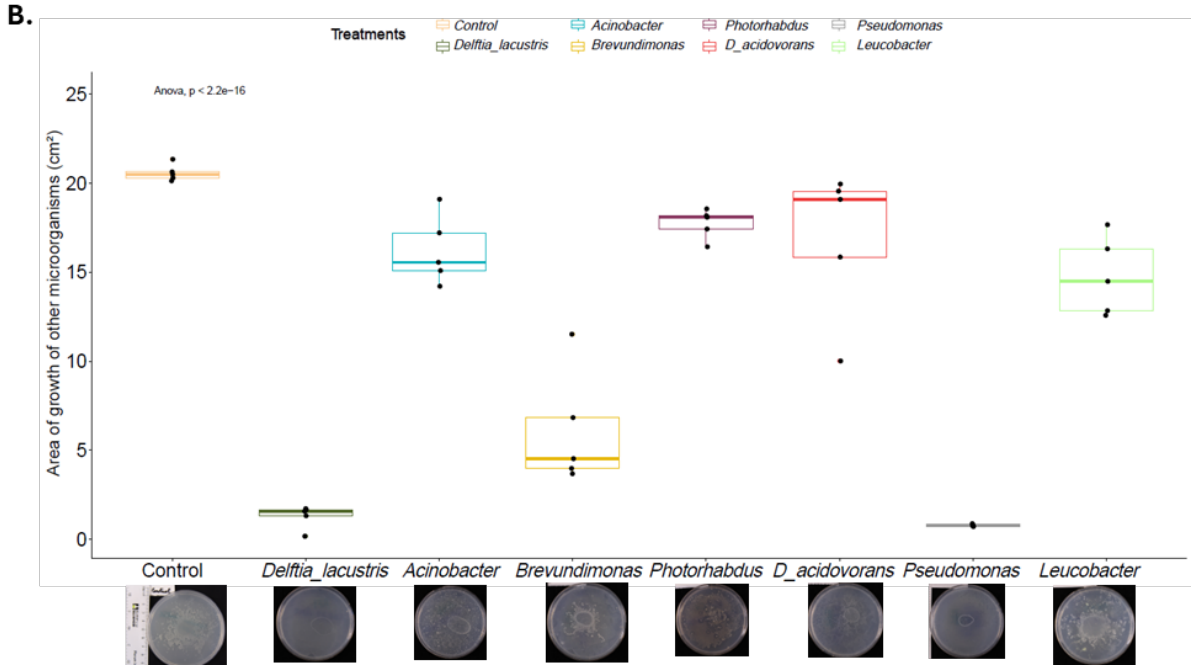


Figure S9. *Galleria mellonella* larvae presented discolouration after injection with *Acinetobacter* strain.

Figure S10. Fungi colonized insect larvae inoculated with surface-sterile infective juveniles of *Heterorhabditis bacteriophora* nematodes.

Figure S11. Results from preliminary experiments: Bacteria associated to infective juveniles of *Heterorhabditis bacteriophora* (EN01) affected the survival of infective juveniles and inhibited the growth of other microorganisms in *in vitro* conditions. (A) Bar plot indicating EPNs survival in pre-inoculated plates with bacteria isolates after 5 days. (B) Box plot representing the area of growth of other microorganisms in pre-inoculated plates with bacteria isolates and EPNs after 5 days. Stars indicate significant differences (*: p<0.05; **: p<0.01, ***: p<0.001).





Supplementary Table

Supplementary Table S1: Primers used for RT-qPCR analysis

Table S1. Primer list for RT-qPCR used in the evaluation of the carbohydrate allocation patterns in this study (Robert et al., 2012).

Gene name	Putative function	Forward primer (5'-3')	Reverse primer (5'-3')
ZmACTIN 1	Actin	CCATGAGGCCACGTACAACT	GGTAAAACCCCACTGAGGA
ZmUBI1	Ubiquitin	TAAGCTGCCGATGTGCCTGCG	CTGAAAGACAGAACATAATGAGCAC AG
ZmC4	Carbohydrate transporter	GGTGGGCGTACACGTTCCCG	TCGGAGCTGGACGAGCGGAA
ZmINCW2	Cell wall invertase	GACCCTACCAAGTCGTCCCTGA	CGACCGGTCGATCAGGCTTC
ZmINCW3	Cell wall invertase	GACGATCGCGCTGAGGACAC	TAGCTACTGCGCCGGCACG
ZmINCW4	Cell wall invertase	TGCGGGGAGAAGGGCG	CGTCTCCGCGTGCTCAGG
ZmIVR1	Invertase	TCTCCCGTGATCCTGCCCCG	GGCCCGCGCAAAGTGTTGTG
ZmIVR2	Invertase	GGGCGTCGCTGCAGGGTATC	CCTCCTCCACGGGCCACTGA
ZmMSS1	Hexose transporter	GGCTGCCACAGGCGGTTTGA	GTCAGCCCCGCGAGGTACAG
ZmMTRANS	Mannitol transporter	GCGTTGCTAGAAACAGCTACCG	GATGGAGGCACTCTTCGCCGCC
ZmSTP1	Carbohydrate transporter	TTCGCCAACCAGTCCGTGCC	CAGCCGCCCCTGATCTTGGC
ZmZIFL2	Carbohydrate transmembrane transporter	GGGAGCCACTGCTGGCGAAG	CGGCAGGGTGCAGGTGATGG

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General discussion

Entomopathogenic nematodes (EPNs) *Heterorhabditis bacteriophora* presence in the soil altered metabolic and signalling processes in maize roots and shoots introducing a soft stress response in plants that resulted in resources reallocation and an induction of systemic defence. In consequence, EPNs presence in the soil reduced aboveground *Ostrinia nubilalis* oviposition in maize plants. Moreover, this study confirmed that EPNs harbour different bacteria and they altered soil microbial community. EPN surface bacteria also induced plant primary metabolism. Furthermore, the results confirmed that the second bacterial circle of EPNs is involved in EPN entomopathogenicity and impact EPN fitness and survival. This section delves deeper into the implications of the key findings displayed in this study.

Heterorhabditis bacteriophora nematodes induce maize defence: implications for integrated pest management programs.

Entomopathogenic nematodes induce plant responses influencing multitrophic interactions. For example, EPNs induced benzoxazinoids (BXs) accumulation in maize roots and in root exudates. BXs are regulators of plant defence and in particular of plant-herbivore interactions (Erb & Kliebenstein, 2020). Besides, some studies suggested that BXs, although still debated, are toxic for some EPNs and PPNs species (Desmedt et al., 2020; Meyer et al., 2009; Robert et al., 2017; Sikder et al., 2021). Therefore, EPN capacity of triggering defence compounds such as BXs, could explain how EPN presence in the soil supress also PPN infestations in some studies (Jagdale et al., 2002; Kamali et al., 2022; Lewis & Grewal, 2005). Further studies are needed to examine to what extend does BX induction affect EPN performance in maize fields and determine the cost-benefit for integrated pest management of EPN induction of BXs in plants.

The findings of this study showed that EPNs presence in the soil induce maize responses and exposed that these responses are variable. EPN exposure produced different results across experiments and varied from mild responses to no response. Therefore, given the variability in the results, it is reasonable to question whether the EPN-induced response observed in maize is influenced by ecological factors and still relevant for agriculture.

Plant response induction by EPN-related cues have been previously documented in *Arabidopsis*, tomato, potato, tobacco and maize (An et al., 2016; Helms et al., 2019;

Jagdale et al., 2009; Kamali et al., 2022; Kansman et al., 2025; Wang et al., 2025). Although previous studies did not always mention whether the experiments were repeated obtaining the same results, they suggested that various ecological factors could explain why plants respond to EPNs. Some studies suggested that plants are ambiguously responding to EPNs after a recognition of some molecular patterns that plants recognize from pathogens such as PPNs (Helms et al., 2019; Kamali et al., 2022; Kansman et al., 2025). For instance, EPNs and PPNs produce a conserved family of nematode pheromones such as ascarosides and these signalling molecules can induce plant response (Manosalva et al., 2015). But although EPN- and PPN-induced responses could be similar, they are not the same (Kamali et al., 2022). Therefore, plants may detect specific EPN cues, which require further experiments to identify.

Another hypothesis is that plants respond to EPNs because plants could have evolved to identify EPNs as a signal of the presence of belowground herbivores. Herbivore-associated chemical cues trigger defence in plants (Hu et al., 2019). Other experiments are also required to test this hypothesis. Furthermore, EPNs harbours also different bacteria in their body introducing more complexity to these multitrophic interactions. Thus, plant could be detecting chemical cues from the microorganisms that EPNs carry and not from the EPNs itself. Plants detect microbe-associated molecular patterns (MAMPs), leading to a mild yet effective activation of systemic defence (Van Wees et al., 2008). Nevertheless, whether EPNs and/or EPN microbiota induce plant response, these findings are relevant for the efficient use of EPNs in agriculture and the possible reasons that could have caused these dynamics response should be also discussed.

The presence of EPNs in the soil induce variable responses in plants exposing the context-dependent nature of the performance of biocontrol agents. But experimental reproducibility is key in scientific research (Baker, 2016; Diaba-Nuhoho & Amponsah-Offeh, 2021). Besides, a variable performance reduces the use of EPNs as biocontrol agents for pest control by farmers (Helmberger et al., 2017). Unfortunately, many experimental moderators still affect the efficacy of biocontrol agents (Serrão et al., 2024). Some of the factors that could affect reproducibility are small sample size and difference in protocols (Chalmers & Glasziou, 2009). But this study followed the reproducibility practices in all the experiments. Hence, another hypothesis is that environmental conditions could explain the lack of experimental reproducibility.

EPNs are significantly affected by environmental conditions such as temperature and humidity (Maushe et al., 2023; Patil et al., 2024). Hence, plant response to EPNs could be influenced by abiotic and biotic factors such as the soil microbial community (Helmberger et al., 2017). Although, the factors inducing this unpredictable response are not fully understood yet, the possibility that soils ecosystems could influence EPN performance is a strong hypothesis (Helmberger et al., 2017).

Multitrophic interactions are highly influenced by the interactions between soil, microbes, and plants, with the environment playing a crucial role in these processes (Aqeel et al., 2023). Moreover, EPNs and their associated microbiota are not just interacting with the plant roots but with the dynamic soil network that encompass a high biodiversity (Bender et al., 2016; Pathan et al., 2020). While this study has shed light on some aspects of maize seedlings response to EPNs, it has also opened new avenues for further research. Further experiments are required to decipher the optimal conditions for achieving the optimal EPN-maize interaction, beneficial for sustainable agriculture.

The second bacterial circle of *Heterorhabditis bacteriophora* influences soil food webs.

EPNs have been widely advertised as beneficial nematodes that can be used as biocontrol agents of insect pests (Koppenhöfer et al., 2020; Piedra-Buena et al., 2015). However, whether the effect of EPNs in soil food webs is always beneficial remains overlooked. Recent studies suggested that EPN application alone or in combination with other organisms such as earthworms influence soil bacterial communities (Chelkha et al., 2025; Li et al., 2024). Moreover, new players were introduced as a second bacterial circle of EPNs and potential influencers of soil food webs (Ogier et al., 2020; Ogier et al., 2023).

This study confirmed that EPNs carry a diverse array of bacteria on their surface and that those bacteria could shape EPN interactions with other members of the soil ecosystem. First, the EPN surface bacteria induced changes in microbial diversity and abundance in the field and the greenhouse soils. The influence of rhizosphere dynamics for biotic factors have been discussed before but there is still limited knowledge to fully decode the high complexity of soil network interactions (Pathan et al., 2020). Nevertheless, the formation of new interactions between microorganisms in the soil after EPN surface bacteria application could explain the observed results. Further research is needed to determine whether mutualism and/or competition and/or

other bacteria interactions are established in the soil after EPN surface bacteria exposure.

Second, EPN surface bacteria altered maize primary metabolism although this effect is variable. Plant-microbe interactions are shaped by multiple factors, such as the composition of the soil, the availability of nutrients, and environmental conditions like temperature and moisture (Pathan et al., 2020). Furthermore, different mechanisms regulate plant-microbe interactions and influence both parties (Berg, 2009). In PPNs, microbes attached to the surface of the nematodes play a crucial role in inducing plant immunity in tomato roots (Topalović et al., 2020). This study showed that EPN surface bacteria altered sugars and amino acids concentration in maize probably because EPN-associated bacteria are taking resources from maize plants. But this hypothesis was not confirmed yet. Thus, Additional studies are needed to investigate the effect of the second bacterial circle of EPNs in plant growth and defence.

This study also confirmed that the second bacterial circle of EPNs include entomopathogenic species. A crucial aspect of the life cycle of EPNs is the infectivity of insect hosts (Dillman & Sternberg, 2012). Hence the significance of considering the pathobiome concept in EPN ecology (Ogier et al., 2020). Furthermore, EPNs are used in pest management with limited information of the effect on non-target organisms (Piedra-Buena et al., 2015). Based on this new pathobiome perspective in EPN ecology, this study reinforces previous recommendations for more research to accurately assess the risks of using EPN in soil applications (Ogier et al., 2023).

Finally, this study exposed that the second bacterial circle of EPN influence EPN fitness and survival. Whether the influence of the second bacterial circle of EPNs is beneficial or detrimental for EPNs continues to be debated. The findings suggested the second bacterial circle of EPNs influence EPN fitness in a context-dependent matter suggesting that the role of the second bacterial circle of EPNs could be more crucial in *in vivo* conditions when insect host and soil characteristics also influence EPN performance. Previous studies exposed that those other EPN-associated bacteria than the symbionts reduced EPN fitness traits such us mobility (Enright & Griffin, 2005), infectivity and reproduction success (Bonifassi et al., 1999). However, a recent study discussed the context-dependent nature of EPN-bacteria interactions and reasoned that core microbiota of EPNs validated through NGS approaches, and not just suggested contaminants as in previous studies, establish neutral or positive influence with EPNs (Ogier et al., 2023). This study validates that there is core EPN

microbiota associated with *H. bacteriophora* nematodes through NGS approaches. Moreover, some members of this core microbiota of *H. bacteriophora* nematodes protect them against other competitors as entomopathogenic fungi and increased EPN survival in *in vitro* conditions. Although, the mechanism of these new described EPN-bacteria associations remains key question. Hence, further studies could investigate if members of the second circle complement the role of *Photorhabdus*, contributing to the effectiveness of the EPN parasitic cycle in *in vivo* conditions and if they establish mutualistic interactions with EPNs.

In summary, the relevance of this study extends beyond plant-nematode interactions, significantly contributing to our understanding of multitrophic interactions and complex ecological systems. These findings have profound implications for agroecosystems, highlighting the potential of EPNs and their associated microbiota as crop protection products. This biocontrol approach supports global efforts to reduce chemical pesticide use and promote sustainable agriculture. However, stakeholders should also consider the potential ecological consequences of using EPNs and their associated microbiota as biocontrol agents. The journey to fully understand these complex interactions continues.

Outlook and future perspectives

Plants respond locally and systemically to EPNs leading to increase resistance against aboveground herbivores. Besides, EPNs carry different bacteria that influence soil food webs components. While the potential applications of the findings of this study are various, many questions remained open and new questions appeared, such as:

- What factors can researchers, producers and/or farmers control to ensure a positive outcome of the plant response to EPNs in the field?
- Do plants respond to cues from EPNs, EPN-associated microbiota, or both?
- What environmental conditions support beneficial associations between EPNs and microorganisms?
- How stable is the core microbiota of EPNs across the world?
- What would enhance the efficacy of EPNs in fields: applying EPNs with a consortium of bacteria attached or applying surface-sterile EPNs?

This study offers new perspectives in the exploration of plant-EPNs interactions pointing towards the need of conducting more field studies and evaluating also the

ecological implications of the use of EPNs in agriculture. Besides, this study provides a list of bacteria associated with EPNs and propose them as a new source of bioproducts for agriculture. Future research should include conducting *in vitro* and *in vivo* assays to evaluate the effectiveness of the isolated microbial strains in controlling different targets such as fungi, bacteria, and insects. Additionally, performing genomic and transcriptomic analyses will be crucial to uncover the mechanisms of action of these microbial strains, identifying key genetic and molecular traits that enhance their biocontrol capabilities.

The extensive use of chemical fertilizers over the past few decades has resulted in numerous environmental and health problems. The reliance on fertilizers has also led to the cultivation of crops with low nutrient use efficiency, meaning they require more inputs to achieve optimal growth. Hence, bioproducts represent alternative solutions to increase crop production. By leveraging natural processes and beneficial microorganisms, bioproducts can reduce the need for chemical inputs, thereby minimizing environmental harm. Moreover, they can improve soil health, enhance nutrient uptake, and increase crop resilience to pests and diseases. The adoption of bioproducts not only supports sustainable agriculture but also contributes to food security by improving the quality and safety of food. As these products are derived from natural sources, they are less likely to leave harmful residues in the environment or in the food we consume. This aligns with global efforts to promote environmentally friendly farming practices and reduce the ecological footprint of agriculture. Bioproducts can contribute to guarantee a healthier environment and global food security for future generations.

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Declaration of consent

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