DEDICATION

I dedicate this thesis

To my parents, my brothers, my friends

To their constant support and unconditional love

To Raquel, your belief in my potential and capabilities was the only reason that kept me improving

To all the people who care about me

To me

To my self-esteem to my overthinking

To my weaknesses, to my oversights, to my despair

To the knowledge, to my future

I am very grateful for it

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ABSTRACT

Earthworms are ecological engineers; they displace, disseminate, and interact with biological control agents such as the entomopathogenic nematodes (EPNs), fungi (EPF), and nematophagous fungi (NF) while displacing, feeding, or releasing their cutaneous excreta or even regeneration. In laboratory experiments, we first evaluated the killing ability of EPNs and EPF against *Galleria mellonella* (Lepidoptera: Pyralidae) larvae when inoculated in autoclaved soil alone or in combination with earthworms or their excreta. We also evaluated EPN and EPF efficacy and reproduction when exposed to CEx while at two concentrations, EPN (1.5 and 10 IJs/cm^2) and EPF (1-2 x 10^7 conidia/mL and 1-2 x 10^8 conidia/mL). In addition, we grew EPF and NF in two different earthworm extract media (EDG) and (FE) at different concentrations to evaluate the growth parameter.

In our first trials, the presence of earthworms or their cutaneous excreta significantly reduced the larval mortality caused by some of the steinernematids depending on the species ($P< 0.015$), while heterorhabditids were mainly not affected. The EPN progeny production was also significantly reduced ($P < 0.01$) when exposed to CEx for some species. Similarly, depending on the species, EPF pathogenicity was significantly altered ($P < 0.01$) by the presence of earthworms or their CEx. Our results showed that earthworms and their CEx could have positive, deleterious, or neutral impacts on entomopathogens that often coinhabit soils and that we must consider the species specificity of these interactions for mutual uses in biological control programs depending on the anticipated outcome.

Keywords: earthworm, entomopathogenic nematodes, entomopathogenic fungi, nematophagous fungi, cutaneous excreta.

RESUME

Les vers de terre sont des ingénieurs écologiques ; ils déplacent, disséminent et interagissent avec des agents de contrôle biologique tels que les nématodes entomopathogènes (EPNs), les champignons (EPF) et les champignons nématophages (NF) tout en déplaçant, en se nourrissant, en libérant leurs excrétions cutanées (CEx) ou même leur régénération. Dans des expériences de laboratoire, nous avons d'abord évalué la capacité de tuer des EPN et EPF contre les larves de *Galleria mellonella* (Lepidoptera : Pyralidae) lorsqu'elles sont inoculées dans un sol autoclavé, seules ou en combinaison avec des vers de terre ou leurs excréments. Nous avons également évalué l'efficacité et la reproduction de l'EPN et de l'EPF lorsqu'ils sont exposés au CEx à deux concentrations, l'EPN (1,5 et 10 IJs/cm^2) et l'EPF (1-2 x 10^7 conidies/mL et 1-2 x 10^8 conidies/mL). De plus, nous avons cultivé EPF et NF dans deux milieux d'extraits de vers de terre (EDG) et (FE) à différentes concentrations afin d'évaluer les paramètres de croissance.

Dans nos premiers essais, la présence de vers de terre ou de leurs excréments cutanés a réduit de manière significative la mortalité larvaire causée par certains des stéinernematidés selon l'espèce ($P< 0,015$), tandis que les hétérohabditidés n'étaient principalement pas affectés. La production de progéniture d'EPN a également été significativement réduite ($P < 0,01$) lors de l'exposition au CEx pour certaines espèces. De même, selon les espèces, la pathogénicité des EPF était significativement modifiée ($P < 0,01$) par la présence de vers de terre ou de leurs CEx. Nos résultats montrent que les vers de terre et leurs CEx peuvent avoir des impacts positifs, délétères ou neutres sur les entomopathogènes qui cohabitant souvent dans les sols et que nous devons considérer la spécificité des espèces de ces interactions pour des utilisations mutuelles dans les programmes de contrôle biologique en fonction du résultat attendu.

Mots-clés : ver de terre, nématodes entomopathogènes, champignons entomopathogènes, champignons nématophages, excrétions cutanées.

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ABBREVIATIONS:

ANOVA: Analysis of variance

BHI: Brain Heart Infusion

CBD: Convention on Biological Diversity described

CEx: Cutaneous excreta.

CFS: conventional farming system

COI: Cytochrome c oxidase subunit I gene

EDG: Earthworms devoid of intestinal contents

Efet*: Eisenia fetida*

EPF: Entomopathogenic fungi.

EPN: Entomopathogenic nematodes.

EW: Earthworm

FAO: Food and Agriculture Organization

FE: Fresh earthworms

GLM: Generalized linear models

HSD: Honestly significant difference

HTS: High-Throughput Sequencing

ICM: Integrated Crop Management

IJ: The infective juvenile

IPM: Integrated Pest Management

LEAF: Linking Environment and Farming

Lter: *Lumbricus terrestris*

NC: Negative control

NF: Nematophagous fungi.

PDA: Potato dextrose agar

Pexc: *Perionyx excavatus*

SNM: Sustainable Nutrition Management

SOM: Soil organic matter

SSM: sustainable soil management

GENERAL INTRODUCTION

Understanding and preserving soil biodiversity (organisms and their activities) is critical to preventing loss and enhancing beneficial services. Soil is a complex matrix composed of unconsolidated mineral material, organic matter, air, water, and a large number and variety of organisms. Soil communities are highly complex and diverse, including numerous organisms ranging from microscopic size (bacteria, archaea, oomycetes, fungi) to others of medium and large size (nematodes, mites, collembolan, earthworms, snails, moles, etc.) (Perry, 1995; Barot et al., 2007; Lavelle et al., 2016). Valuable ecosystem services, such as soil structure maintenance and organic matter decomposition, are supported and modulated by soil biota (Wall, 2012; Bardgett and van der Putten, 2014).

Earthworms (Annelida: Oligochaeta), the dominant soil organism biomass (Karaca, 2011), commonly present in both natural and agricultural areas (Hendrix et al., 2008), are considered, in a broad sense, beneficial soil organisms. On one side, earthworms are known as 'ecological engineers' thanks to their feeding activity and ability to move through the soil, enhancing the turnover of the organic matter, and favoring the structure, aeration, and fertility of the soil (Eisenhauer and Scheu, 2008; Wall, 2012). Besides, earthworm guts contain bacteria that can modify soil properties that stimulate certain plant functions and change micro and mesofauna communities (Brown et al., 2000; Aira et al., 2016, 2015). Thus, earthworms can reduce the number of plant-parasitic nematodes (Dash et al., 1980; Boyer et al., 2013) and enhance the movement of other beneficial soil organisms such as entomopathogenic nematodes and fungi (Shapiro-Ilan and Brown, 2013). Depending on their drilling capability, feeding activity, and oxygen availability, there are three main groups of earthworms: epigeic, anecic, and endogeic (Bouch, 1977; Römbke et al., 2005; Guhra et al., 2020; Sapkota et al., 2020). Overall, epigeic species such as *Eisenia fetida* or *Perionyx excavatus* live in the topsoil, mainly feeding on decomposition material (Edwards et al., 1998; Birundha et al., 2013). Conversely, vertical movements that reach three meters in depth characterized anecic species as *Lumbricus terrestris* (included in an intermediate category by Bottinelli et al., 2020, named epi-anecic). Finally, endogeic species move randomly across the soil, making horizontal burrows all over the lower part of the ground (Bouch, 1977; Lavelle, 1988).

Moreover, the cutaneous excreta (CEx) of earthworms are secreted by the micropores of the glandular cells of the epidermis during earthworm movement through the soil and also contains urine excreted from urinary pores, coelomic fluid from coelomic pores, and mucus from the micropores of their epidermal glandular cells (Homa et al., 2008; Santocki et al., 2016). The composition of the coelomic fluid, produced under high-stress conditions, is species- specific and comprises immune cells called coelomocytes, which play an essential role in defense reactions mediated by phagocytosis (Bilej et al., 1991; Dales and Kalac, 1992; Kasschau et al., 2007). Furthermore, other compounds of antibiotic nature (Dales and Kalaç, 1992; Bilej et al., 1995, 1991; Kasschau et al., 2007; Fiołka et al., 2012) play an essential role in the antibacterial immune system of earthworms (Wang et al., 2011; Bityutskii et al., 2012; Guhra et al., 2020).

These compounds may have an impact on the surrounding organisms such as nematodes and fungi. For example, the CEx of *Dendrobaena vineta* and *E. fetida* reduced the growth of the phytopathogenic fungi *Fusarium oxysporum* (Hypocrealeas: Nectriaceae) (Plavšin et al., 2017), and the CEx of *E. fetida* was able to kill and decrease the reproduction capability of Caenorhabditis elegans (Yu et al., 2019). Similarly, Fattore et al. (2020) observed that *H. megidis* IJs did not move toward maize roots attacked by *Diabrotica balteata* (Coleoptera: Chrysomelidae) larvae and watered with the CEx extracted from the earthworm species *Allolobophora icterica*, despite the high increase of the (E)- β-caryophyllene (Eβc) production, a potent volatile organic compound described as an attractant for EPNs (Rasmann et al., 2005).

Entomopathogenic nematodes (EPNs), fungi (EPF), and nematophagous fungi (NF) are natural inhabitants of most terrestrial ecosystems, including natural and agricultural areas (Bidochka et al., 1998; Adams et al., 2006; Meyling and Eilenberg, 2006). Since they are considered excellent biological control agents of arthropod pests (Faria and Wraight, 2007; Roy et al., 2010; Lacey et al., 2015), many commercial products based on EPN, EPF or NF are also available to implement in IPM programs and organic production (Campos-Herrera, 2015; Lacey et al., 2015).

For the entomopathogenic nematodes (EPNs), The infective juvenile (IJ) is the free-living, non-feeding stage that locates a suitable host, penetrates the hemocoel, and defends itself against the immune reaction of the insect (Boemare, 2002; Stock, 2015). Once inside the hemocoel, EPNs in the genera Steinernema and Heterorhabditis (Nematoda:

Rhabditida) selectively search for their insect hosts and kill them within 2–3 days with the aid of mutualistic bacteria in the genera Xenorhabdus and Photorhabdus, respectively (Dillman et al., 2012; Lacey et al., 2015). Both nematodes and bacteria reproduce until the nutrients are depleted, and excretory products become limiting. At this point, a new generation of IJs develop, incorporate the bacterial symbiont, and emerge to reinitiate the life cycle (Adams et al., 2006; Stock, 2015). The IJs can persist in soil for weeks or even months, depending on species longevity, soil properties, and natural enemies (Gaugler, et al., 1997; Helmberger et al., 2017). Recent studies showed that in a controlled experiment, the survival of the EPN was higher in media with high water holding capacity (Abate et al., 2019). In addition, EPN soil food webs are related primarily to abiotic factors such as soil pH and moisture (Campos-Herrera et al., 2013, 2016, 2019; Jaffuel et al., 2018). Therefore, activities that modify the physical and chemical characteristics of the soil could potentially affect the biocontrol efficiency of EPNs.

Similarly, entomopathogenic fungi (EPF) such as the species *Beauveria bassiana* and *Metarhizium anisopliae* (Ascomycota: Hypocreales) (Hibbett et al., 2007) can kill the host in 4-5 days (Zimmermann, 2007; Roy et al., 2010), they act as parasites and saprophytes of arthropod hosts (Charnley and Collins, 2007). When the EPF spores contact the host and penetrate the cuticle, the EPF is in the parasitic phase (Oreste et al., 2012). Inside the insect body, the fungus produces specific metabolites and antibiotics to kill the insect and avoid the proliferation of undesired organisms, respectively (Strasser et al., 2000; Charnley and Collins, 2007; Donatti et al., 2008). Once the insect is dead, the EPF change to the saprophytic phase, starts active hyphal growth from reproductive structures and generates aerial mycelia for dispersion to restart the life cycle again.

Nematophagous fungi (NF) are predominantly natural inhabitants in the soil environment (Gray, 1987). They are carnivorous fungal species sometimes used as biological control agents (Nordbring-Hertz et al., 1989, 2006; Liu et al., 2009; Herrera-Estrella et al., 2016). They act as natural enemies of nematodes, using spores or mycelia as traps or hyphal tips to kill all nematode life stages including eggs and cysts (Nordbring-Hertz, 2004). Nematode-trapping fungi such as *Arthrobotrys musiformis* (Orbiliales: Orbiliaceae) are saprophytes that can form a hyphal net structure to trap nematodes by adhesion or mechanically (Nordbring-Hertz et al., 2006). *Purpureocillium lilacinum* (Hypocreales: Ophiocordycipitaceae) is an egg-parasitic fungus active against root-knot

and cyst nematodes (Luangsa-Ard et al., 2011).

Few studies have explored the interaction between earthworms and entomopathogenic/nematophagous organisms in soils. The first studies of this kind evaluated the indirect impact of earthworms on the dispersal and biocontrol potential of EPNs and EPF, based mainly on the ability to mix the soil while earthworms move while feeding (Shapiro et al., 1993, 1995; Shapiro-Ilan and Brown, 2013). Other possible interactions may be related to the feeding activity of earthworms, which can reduce the number of nematodes present in soils, including EPNs (Campos-Herrera et al., 2006). This thesis reveals some hitherto unknown interactions between earthworms and biological control agents.

1. Thesis outline, hypothesis, and objectives

Increasing the knowledge about the soil as a three-dimensional regulatory natural ecosystem needs a clear understanding of its multiple functions to generate new strategies and establish the ecosystem service based on ecological engineers and biocontrol agents (Blum, 2005; Lavelle et al., 2016). In this thesis, we evaluated the multitrophic impact of the presence of live and dead earthworms and their CEx on other members of the soil: EPN, EPF, and NF. The general hypotheses were:

1. The presence of earthworms and their CEx might have a sub-lethal effect on the IJs of EPN, thereby reducing their fitness and biocontrol potential,

2. The impact of the earthworms on the entomopathogens (EPN and EPF) will depend on the combination of earthworm/CEx and entomopathogen species, and

3. Earthworm extract can enhance the growth of EPFs and NF by changing the underground nutrient availability.

The overall objective of this thesis was to expand the knowledge on the impact of earthworms or their derivate (CEx, extract) in selected members of soil biota (EPN, EPF, and NF) that can contribute to modulating natural biocontrol of pest and pathogens. In this thesis, we have addressed several specific objectives to achieve a comprehensive understanding of those complex interactions, each described in the document.

PART 1: Literature review

The Literature review covers the element related to the topics, such as the concept of sustainable agriculture and the soil as a live ecosystem. We completed a brief but complete description of the earthworms (phylogeny, morphology, ecology, and their importance in agriculture) regarding the target soil organisms. We described the three categories of biological control agents connected with the earthworms: entomopathogenic nematodes, entomopathogenic fungi, and nematophagous fungi.

PART 2: Practical part, Results and discussions that include the three studies.

2. **Cutaneous excreta of the earthworm *Eisenia fetida* (haplotaxida: lumbricidae) might hinder the biological control performance of entomopathogenic nematodes**

For the first time, it was investigated whether cutaneous excreta (CEx) derived from the earthworm *Eisenia fetida* (Haplotaxida: Lumbricidae) used as a model species can alter the biological control performance of entomopathogenic nematodes. Six EPN species were tested: two heterorhabditids (*Heterorhabditis zealandica* Btw population, and *H. bacteriophora* AM- 203 population) and four steinernematids (*Steinernema feltiae* AM-25 population, *S. khuongi* Arc population, *S. glaseri* NC population, and *S. carpocapsae* ALL population). The specific objectives were (i) to evaluate the interaction between the earthworm and/or their CEx and the EPN in two substrates (sand and soil) (ii) to determinate the effect of CEx on the biocontrol potential of two concentration of EPN, and (iii) to assess whether the EPN virulence and reproduction is affected by the earthworms/CEx, by using *Galleria mellonella* (Lepidoptera: Pyralidae) as a model insect.

3. **Earthworms and their cutaneous excreta can modify the virulence and reproductive capability of entomopathogenic nematodes and fungi**

We investigated how three species of earthworms and their CEx could alter the virulence and reproductive capability of EPNs and EPF. We expected that different earthworm/CEx species would affect the EPN and EPF virulence and reproduction differently. Moreover, we suppose that the exposure to the individual earthworm will impact more strongly on entomopathogens than the combination with the CEx. For it, we employed two epigeic (*E. fetida* and *P. excavatus*) and one anecic (*L. terrestris*) earthworm

species, two steinernematid (*S. feltiae* and *S. riojaense*) and one heterorhabditid (*H. bacteriophora*) EPN species, and two EPF species of broad distribution (*B. bassiana* and *M. anisopliae*). The specific objectives were:
(i) to assess the impact of the presence of earthworms or their CEx on the virulence and reproduction capability of EPNs and EPF in soils, and (ii) to investigate whether the CEx can affect the infectivity and reproductive success of EPNs and EPF applied in high or low concentrations.

4. **could earthworm extract promote the growth of nematophagous fungi (*Arthrobotrys musiformis, Purpureocillium lilacinum*) and entomopathogenic fungi (*beauveria bassiana*)?**

We investigated how earthworm extract could promote the mycelial growth and conidial germination of EPF and NFs. We speculate that earthworm body tissue macerate could serve as an integral culture media, and the presence or absence of its gut component will modulate the fungal growth. Also, we hypothesize that different concentrations of macerate of earthworm tissue could grow fungi. In this study, we employed two NF species: *Arthrobotrys musiformis* (trapping-fungi) and *Purpureocilium lilacinus* (opportunistic fungi), the EPF *Beauveria bassiana*, and one earthworm species *Aporrectodea molleri*. The specific objectives were: (i) to test the mycelia growth and conidia produced in solid media made from earthworm body tissue with or without gut components in different concentrations (ii) to investigate the effect of earthworm body tissue on the germination of conidia.

GENERAL DISCUSSION AND PERSPECTIVES

The section General Discussion and Perspectives is an updated and integrative chapter that summarizes the preceding findings and integrates them within the actual context of sustainable agriculture. It resulted in an innovative outlook on each chapter that allows readers to dive into the other studies required to advance in the gap of knowledge highlighted.

PART 1: LITERATURE REVIEW

I. Sustainable agriculture

Agriculture faces the challenge of producing food and other goods (textiles, biofuel, pharmaceutical) with quality guarantees and respecting the environment within the "*population growth and climate change*". The global challenge of the sustainability of resources has been reflected in the "2030 Agenda for sustainable development" promoted by the UN, where agriculture is a fundamental objective, both directly in objectives 2 "*Zero hunger*" and 12 "*Responsible consumption and production*", as well as in a transversal way in other five objectives (UN, 2015). Hence, we are moving toward the "*Sustainable agriculture*" paradigm, which implies securing the necessary resources for safeguarding global food production, biodiversity reserves, recreation needs, water quality, and well-developed rural areas that protect the wildlife reserves. In detail, the main sub-goals of sustainable agriculture are: (i) Farmers' income should be sufficient to provide an adequate standard of living in the rural area, (ii) Farmers should practice production methods that do not threaten human or animal health, nor degrade the environment, including means for biodiversity protection and preservation, (iii) Farmers should minimize the environmental and health problems as responsibility action for future generations, (iv) Non-renewable resources have to be replaced gradually by renewable resources, and recirculation of non-renewable resources should be maximized, (v) Sustainable agriculture will meet society's needs of food and other material goods, place for recreation and preserve the landscape, cultural values, and the historical heritage of rural areas at the same time that it contributes to creating stable, and well-developed, and secure rural communities, and (vi) The ethical aspects of agricultural production are secured (Modified from Andersin et al., 2003).

Human societies are implementing these aspects of sustainable agriculture thanks to national and international directives and regulations. The current situation is moving toward the mandatory adoption of Integrated Crop Management (ICM). There are many definitions of ICM, and those have evolved since the 90s to date. For example, ICM was defined in 1993 as"*The economic production of safe, wholesome food in an environmentally sensitive way*" (Linking Environment And Farming, LEAF, 1993). This has been updated to a "Whole farm policy aiming to provide efficient and profitable production, which is economical, viable and environmentally responsible" (Linking Environment and Farming, LEAF, 2000). More recent

definitions also include the relevance of agriculture practice *"...using natural resources and regulating mechanisms to replace polluting inputs and secure sustainable farming*" (Boller et al., 2004). ICM should tend to minimize the so-called "*conventional farming system*" (CFS), which, until today, is still dependent on fossil energy, in the form of diesel for soil tillage and transport and the form of nitrogen fertilizers (Rathke et al., 2006) and implies a widespread use of pesticide of organic synthesis.

A sustainable farming system would depend on the acreage available for the supply of production energy (Bailey et al., 2003). Such a farming system will minimize soil tillage to save energy, and crop rotation will be central to the potential to decrease the need for pesticides (Alford et al., 2007). Conserving and enhancing biocontrol will be an integral part of this farming system. Using cultural control methods and reducing agrochemical use is a crucial part of ICM philosophy. Hence, a critical part of the ICM is the strategies defined for the Integrated Pest Management (IPM), which is a system that protects the crops from pests and diseases while imparting profit, safeguards environmental and human health, encompasses cultural sensitivities, and ensures social acceptance (Finch et al., 2014).

As part of the ICM, there is a social consensus on the relevance of sustainable soil management (SSM). This concept is based on the use of minimum cultivation or '*No till*' has the benefit of reducing the amount of energy used to establish a crop and therefore meets the aim of reducing farm energy inputs. There are also additional benefits to invertebrates and wildlife. For example, the slicing and inversion associated with tilling kill earthworms resulting in fewer earthworms/m² than minimum cultivation and direct drilling (Finch et al., 2014).

Another essential concept within the frame of the ICM and the overall sustainable agriculture is Sustainable Nutrition Management (SNM). This approach is expected to provide nutrients at the correct rate, considering the appropriate timing to grow the crops to their full potential. However, by applying at a too high rate or at times of the year when they are not utilized, fertilizers can lead to losses and pollution of water and air, exacerbating those health and environmental problems. Alternatively, using organic manures in cropping systems and assessing their nutrient contribution is a more environmentally friendly approach that will require characterizing the manure to assess the nutrient content and subtracting the quantity of available nitrogen, phosphate, and potash (Finch et al., 2014).

Organic farming is evolving as a "Sustainable Management" approach that maximizes natural resources by optimizing nutrient recycling, chemical fertilizers, and reducing pesticides. In organic farming systems, managing organic matter to enhance the soil's chemical, biological, and physical properties is critical to optimizing crop production (Spiertz, 2010). Furthermore, organic farming recognizes the complex relationships between different system components and that the system's sustainability depends on the functioning of a whole integrated and inter-related system (Watson et al., 2002). Thus, those strategies highlight the urgent need to understand and provide the best management of bio-fertilizers and biological control tools to reveal their potential and increase efficiency.

II. Soil

Soil is a critical component of the crops because all the roots' systems are linked. Hence, it should be a central topic to consider in achieving sustainable agriculture. Different scientists have defined soil in different ways, and these definitions show an evolution from the classical description to the modern concepts of soil.

In 1879, Dokuchaev provided the first definition is: "*Soils are applied solely to those superficial or nearly superficial horizons of rocks, that have been modified naturally by the interaction of water, air and various kinds of organisms, either living or dead; this being reflected in a certain manner in the composition, structure, and color of such formations. Where these conditions are absent, there are no natural soils, but artificial mixtures of rock*". Here, the concept of soil focused just on the mineral composition, neglecting the life part of the soil. Later, Hilgard (1914) gives a description that linked the soil and the plants: "*Soil is, more or less, loose, friable material in which, through their roots, plants may or do find a foothold, nourishment as well as all other conditions of growth*". In the s. XXI, the definition by USDA (2003), the soil is already seen as complex systems, in which the three major composed of soil (solids, liquid, and plants) are combined: "*Soil is a natural body comprised of solids (minerals and organic matter), liquid, and gases that occurs on the land surface, occupies space and is characterized by one or both of the following: horizons, or layers, that are distinguishable from the initial material as a result of additions, losses, transfers, and transformations of energy and matter or the ability to support rooted plants in a natural environment*".

Nowadays, the soil is defined as a complex ecosystem comprising diverse organisms such as bacteria, fungi, protists, micro-arthropods, nematodes, and earthworms, held in a mixture of minerals, water, and air conforming to the edaphic environment (Ruiz et al., 2008). These organisms are interrelated in complex food webs, with different levels of influence that interact in time and space. Hence, soil biodiversity is fundamental for developing soil functions, as Benckiser (1997) already recognized. Moreover, the soil is considered a non–renewable resource with high socio-economic and natural value. Intensive agriculture, industry, and urban development are significantly impacting the soil.

Soil biodiversity is extremely sensitive to changes in soil usage (Cortet et al., 2002), and those actions are translated into the loss of functionality and diversity (Pulleman et al., 2012). Soil degradation produces severe changes in this ecosystem which are irreversible on a human scale. Hence, the concept of "*Soil health*" gained relevance, being defined as "*the capacity of soil to function as a living system, with the ecosystem and land-use boundaries, to sustain plant and animal productivity, maintain or enhance water and air quality, and promote plant and animal health. Healthy soils maintain a diverse community of soil organisms that help to control plant disease, insect and weed pests, form beneficial symbiotic associations with plant roots, recycle essential plant nutrients, improve soil structure with positive repercussions for soil water, and nutrient holding capacity, and ultimately improve crop production*" (FAO, 2008).

The current view of the soil is a dynamic entity that depends on the activity of soil organisms, and they produce changes that impact soil fertility and crop productivity. Understanding these complex interactions could provide a model for monitoring soil disturbances and optimizing ecological scenarios to enhance crop production by favouring biological control of pests and diseases and increasing plant health. Traditionally, such interactions have been studied one–by–one, and there is a need for a multidisciplinary approach to study such relationships in a broader context.

II.1. The functional domains of soil

Seven main spheres of influence (functional domains) in soil have been identified according to their origin and significance in regulating major soil processes and functions such as aggregation, organic matter decomposition, nutrient cycling, microbial populations and

activity, and plant production. These are: (1) the litter system or detritusphere; (2) rhizosphere; (3) porosphere; (4) aggregatusphere; (5) the drilosphere; (6) termitosphere; and (7) myrmecosphere (Brown et al., 2000) In detail, the Detritusphere and Rhizosphere referred to plant and animal associated to the soil surface and plant roots and represent the two primary sources of soil organic matter additions to soil. The nature, quality, quantity, and spatio-temporal distribution of these inputs is critical to the amount of decomposition and mineralization as well as the final biomass (Beare et al., 1995). The Porosphere consists of the arrangement of cavities and solids of various sizes (macro 20-2.0 mm, meso 2.0-0.1 mm, micro < 0.1 mm) in the soil (Figure 1), and it is extremely related to the soil organism's size.

Then, these pores, filled with air and/or water, can be occupied by soil- organisms inhabiting the aerial portions, also named macro, meso, and microfauna (Figure 1). The aggregatusphere is the groups of soil particles bound together, forming a more substantial unit than the surrounding particles. These aggregates can range in size from micro (50–250 μm diam.) to macro (>250 μm diam.) and are often associated with the soil organic matter (SOM), the microbial and faunal activities, and C and N cycles (O'brien and Stout, 1978, Beare et al., 1995). The drilosphere is the zone of earthworm influence, including midden litter and the soil volume descending along the burrow wall (Lavelle et al., 1989). Drilosphere soils are enriched in N, P, and humified organic matter compared to the surrounding grounds. They are also estimated to contain a high percentage of the whole soil's N2-fixing and denitrifying bacteria (Beare et al., 1995). Termitosphere and Mermycosphere are the own functional domain of termites and ants, respectively (Lavelle, 2002).

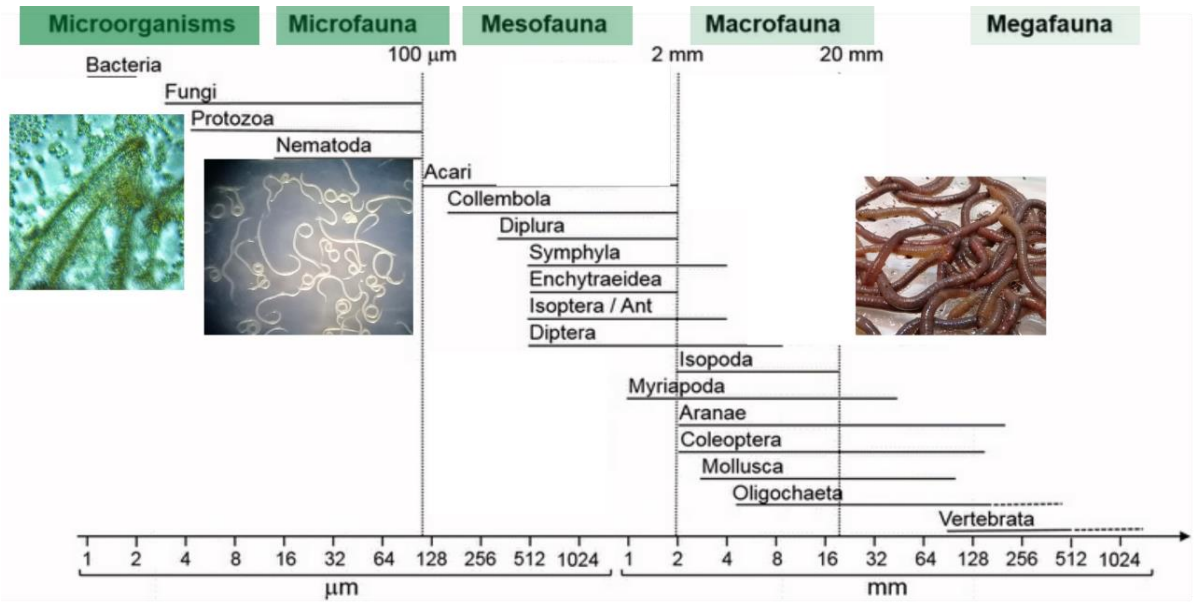


Figure 1: Classification of soil organisms according to their size (modified from Swift et al., 1979). Photo Credits: fungi: *Arthrobotrys musiformis*, Nematoda: *Heterorhabditis bacteriophora*, Earthworm: *E.fetida* (scheme modified from Guilland et al., 2018).

II.2. The ecological role of soil biodiversity

The soil supports a wide diversity of species, connected by complex food webs, and subjected to environmental oscillations that drive changes in the soil ecosystems in time and space (Bonkowski et al., 2000). In addition, organisms interact in different ways, including competitive, predatory, parasitic, and facilitative ways, and they are also responsible for pollination, seed distribution, and habitat provision. These underlying linkages among species and the environment comprise an interacting and ever-changing system known as an ecosystem.

The Convention on Biological Diversity described (CBD) defined the ecosystems as "*a dynamic complex of plant, animal and microorganism communities and their non-living environment interacting as a functional unit*" (Smith et al., 2009). These interactions sound simple in general outline, but they are enormously complex since each species has unique requirements for life, and each species interacts with both the physical and the biological environment. Hence, considering the soil ecosystem, understanding the interactions occurring among soil inhabitants will help elucidate mechanisms that govern fundamental ecological processes. Furthermore, this knowledge can build rational, ecologically sound agricultural

management methods that promote ecosystem services, such as biological control of soil-dwelling insect pests (Bommarco et al., 2013).

III. The soil inhabitants: selected beneficial soil organisms

Plants as primary producers capture energy by aerial leaf systems, and much of that energy is transported below ground to plant roots through the phloem, part of the plant's vascular system specialized for this purpose. Carbon molecules generated by roots serve as the principal source of energy for most heterotrophic soil organisms (Thies and Grossman, 2006).

Another primary energy source for soil heterotrophs is dead plant material (litter) and animal residues. Other primary producers that are present in surface soil are photosynthetic bacteria, cyanobacteria, and algae. However, their energy contribution to soil is comparatively tiny. They use light energy and generally require high moisture levels; hence, they are not active below the first few millimetres in soil. Variations in a photosynthetic capacity directly impact the amount of fixed C that reaches the soil and becomes available for heterotrophic soil organisms.

The soil biota has several important functional roles as consumers, which include C mineralization and OM turnover, nutrient cycling, important mutualisms with plants and each other, causing and suppressing plant and animal diseases, improving soil structure, bioremediating contaminated soil, and generating and consuming greenhouse gases (Susilo et al., 2004). The consumers are divided into several groups: The primary consumers, including Decomposers, Herbivores, Parasites, and Pathogens, they feed on root exudates and plant and animal residues, and numerous herbivores, parasites, and pathogens that feed on living root tissues. The secondary consumers could be Grazers, Shredders, and Predators, including the protozoa, bacterial and fungal feeding nematodes as nematophagous fungus, and microarthropods such as mites and collembola. The tertiary consumers or the higher trophic levels in the soil food web contain predatory nematodes and predatory arthropods, such as pseudoscorpions, centipedes, and species of spiders, beetles, and ants. The life history and functions of the predators are essential because they can help regulate substantial plant pest populations (Uphoff et al., 2006).

III.1. Earthworms

The earthworm derives its name from the fact that it burrows and eats its way into the earth. Aristotle called them the 'Intestine of the earth', and Charles Darwin described earthworms as the 'unheralded soldiers of mankind'. Phylogenomic evolution reveals that these terrestrial invertebrates belong to the Phylum Annelida, Order Oligochaeta, subclass Sedentaria which includes the Class Clitellata that is a large "class" of segmented worms, comprising about one third of all known annelid species characterized by the "clitellum" (Martinsson and Erséus, 2021) that include 9000spp (Capa and Hutchings, 2021) (Figure 2) (Struck et al., 2011; Purschke et al., 2014; Weigert et al., 2014). Otherwise, Rouse and Fauchald, 1997 consider that Annelida comprises the Polychaeta and Clitellata as sister groups. Nevertheless, the earthworm's classification is quite confusing, the deep-level evolutionary relationships of Annelida are still poorly understood (Purschke et al., 2014).

However, there has been a significant variation in the molecular methods used for species delimitation of clitellate worms during the last 40 years as the gel electrophoresis of proteins, the non-sequenced DNA, and Sanger sequencing of a limited number of DNA fragments also the High-Throughput Sequencing (HTS) of many DNA fragments. Those methodologies reveal more and more a better knowledge of the species taxonomy and characterisation by a population genetics approach rather than standard assessments of similarities and differences. This will improve the phylogeny and classification, and it may drive investigations on more practical elements of their biology and function in various ecosystems (Martinsson and Erséus, 2021).

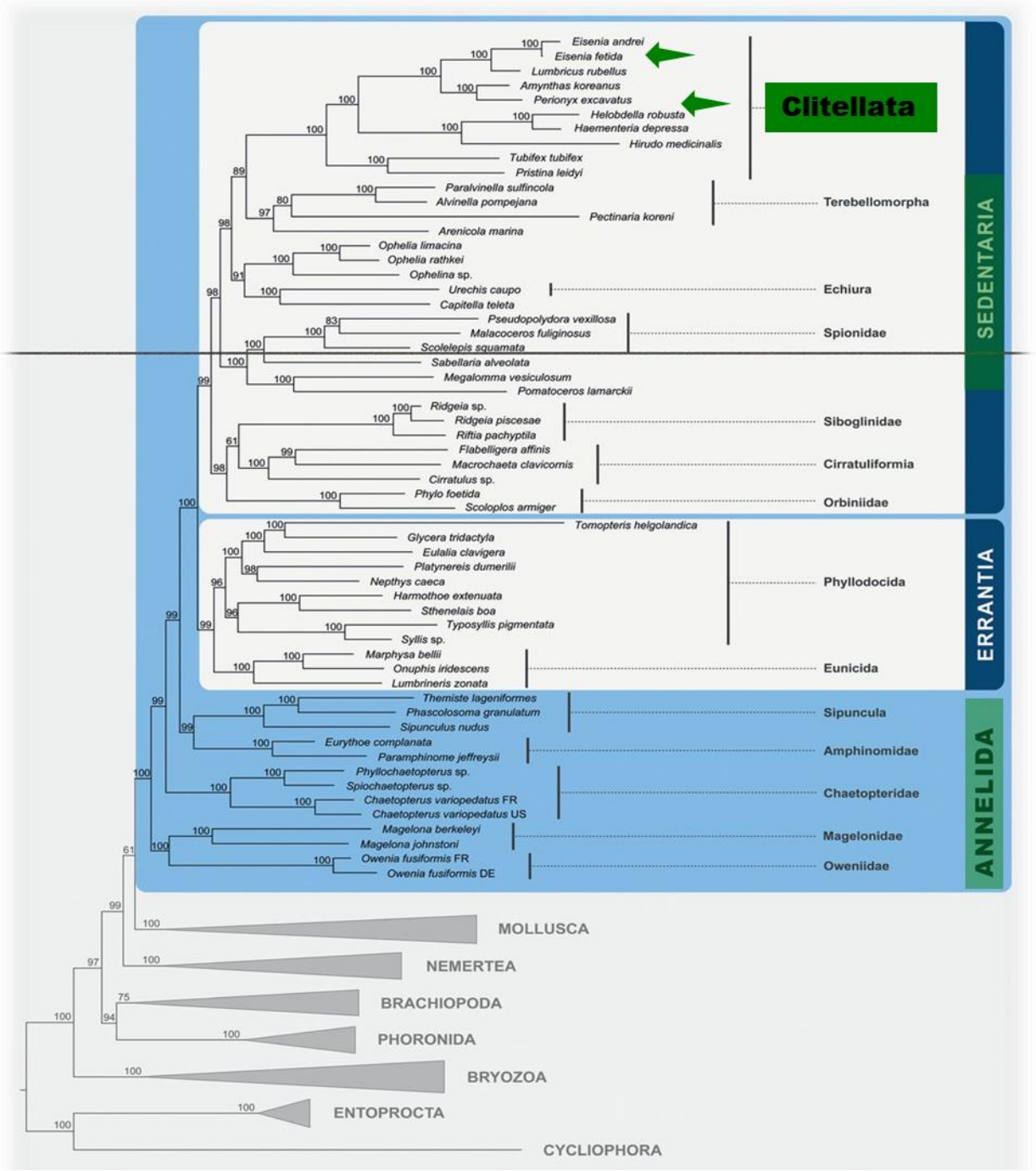


Figure 2: Phylogenetic relationships within Annelida (following Weigert et al., 2014, modified).

III.1.1. Morphology

Earthworms contribute to invertebrates' total weight or biomass in soil, particularly in temperate regions (Phillips et al., 2019). Earthworms range in size from a fraction of 1 cm to exceptional individuals as Megascolides australis, which may measure 2.75 m in length and 3 cm in diameter (Edwards and Lofty, 1977). Earthworms have segments, which are external divisions along the length of the body by furrows or intersegmental grooves, which coincide with the positions of the septa dividing the body internally (Edwards and Lofty, 1977). The number and size of the segment are characteristics of each species. For example, redworms (*Eisenia fetida)* have about 95 segments, while nightcrawlers (*Lumbricus terrestris*) have about 150.

Earthworms are hermaphrodites and have male and female genital openings to the exterior, as paired pores on the ventral or ventrolateral side of the body. In lumbricid worms, the male pores are situated ventrolaterally on the 15th or occasionally on the 13th segment. Usually, earthworms have two or more spermathecal pores, with a maximum of seven pairs in some species, but in others, they are absent. Spermathecal pores are usually intersegmental and are often in the ventral or lateroventral position, but they are sometimes close to the mid-dorsal line. The female pores are commonly a single pair, situated either in an intersegmental groove or on a segment. Their position often being diagnostic of a particular family. These pores communicate with the body cavity and the coelomic fluid.

The other openings in the body wall are the nephridiopores. They are situated just posterior to the intersegmental grooves on the lateral aspect of the body and usually extend in a single series along the body on either side. The nephridiopores are the external openings of the nephridia (the earthworm's excretory organs) and are closed by sphincter muscles.

The clitellum (Figure 3) is the glandular portion of the epidermis associated with cocoon production. It usually appears swollen, although sometimes it can only be differentiated externally by colour. When swollen is in mature lumbricids, the intersegmental grooves are often indistinct or entirely obscured, particularly on the dorsal surface. The position of the

clitellum and the number of segments over which it extends differs considerably among oligochaetes.

The coelom is a large cavity that extends through the length of the body and is filled with coelomic fluid. It is surrounded on the outer side by the peritoneum of the body wall and the inner side by the peritoneum covering the alimentary canal. Transverse septa divide it into segmental portions. The septa (Figure 3) usually correspond to the external segmental grooves but often do not occur in the first few segments of the body. The septa (Figure 3) usually correspond to the external segmental grooves but often do not occur in the first few segments of the body. The septa are perforated by pores, allowing the coelomic fluid to pass freely between segments (Edwards and Lofty, 1977). Peritoneal cells are modified in the intestine region as chloragogen cells, forming chloragogenous tissue. Chloragogen cells are characterized by yellow or greenish-yellow globules, the chloragosomes.

The alimentary canal or gut of earthworms is a tube extending from the mouth to the anus, and it is differentiated into a buccal cavity, pharynx, oesophagus, crop, gizzard, and intestine. The short buccal cavity begins at the mouth and occupies only the first or the second segments, with one or two diverticula or evaginations. The epithelium lining the buccal cavity is not ciliated, except occasionally in a small dorsal diverticulum, but very young earthworms do have patches of ciliated epithelium on the ventral surface. Overlying the epithelium is a thick cuticle, except in the ciliated portions.

The pharynx (Figure 3), which is not always differentiated from the buccal cavity, extends back to the sixth body segment. The dorsal surface of the pharynx is thick, muscular, and glandular and contains the pharyngeal glands, which appear as a whitish lobed mass. Earthworms use the pharynx as a suction pump so that muscular contractions of their walls draw particles through from the mouth, and some species evert the pharynx in this process.

The nephridia are the main organs of nitrogenous excretion in oligochaetes and are paired in each segment except the first three and the last. The internal opening from the coelom into each nephridium is just in front of a septum and is a funnel-shaped nephrostome, leading to a short preseptal canal that penetrates the septal wall into the segment behind, where the central part of the nephridium lies. The nephridium continues as a long postseptal canal with three loops, which can be distinguished as four sections: a very long 'narrow tube'; a shorter ciliated 'middle tube'; a 'wide tube'; and finally, the 'muscular tube' or reservoir, which opens to the

exterior at the nephridiopore. Most of the post-septal part of the nephridial tube is secretory, except for the last portion (the muscular tube), and the lumen of the whole of the secretory section is intracellular, also with 'drain-pipe cells' (Edwards and Lofty, 1977).

The central nervous system has a concentration of nerve cells that form a central nervous system with a brain and nerve cords. The brain is only the first and largest of a series of compact masses of nerve cells, or ganglia, in each body segment. The sense organs are of two types: photoreceptor organs and epithelial sense organs. The photoreceptor organs of lumbricids occur at the ends of the nerves to the skin. They are smaller than those in the epidermis and are aggregated (Hess, 1925). One or sometimes two nerves pass into each cell, and large neural fibers also extend from the nerves into the cells. Small nerves continuous with the neural fibers extend throughout the protoplasmic contents of the cells as a neurofibrillary network. In each cell, there is an ellipsoidal or elongated rod-shaped body, the optic organelle, with an outer surface consisting of interconnecting fibrillae, the retinella, and inside, a transparent hyaline substance. The epithelial sense organs are groups of thirty-five to forty-five elongate cells, broader at their bases, with distal ends terminating in sensory hairs projecting through thin regions of the cuticle. The bases of the cells end as processes, one of which extends as a nerve fibre along the basal membrane and joins the nearest epidermal nerve. Proprioceptor cells situated in the muscle layers act as tension or stretch receptors (Edwards and Lofty, 1972).

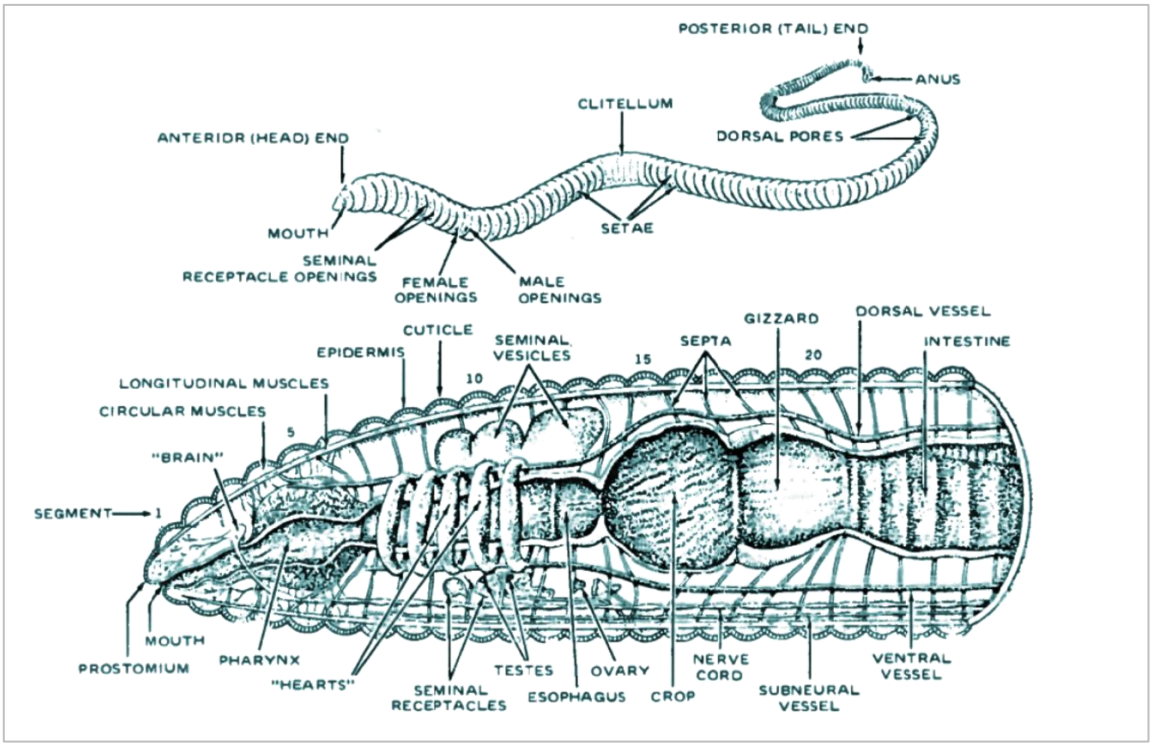


Figure 3: Major elements of earthworm (*L. terrestris*) reproductive circulatory, nervous, digestive, excretory, and muscular system (from Gaddie and Douglas, 1975).

III.1.2. Biology

Earthworm life duration varies between 3 and 7 years depending upon the species and ecological situation. There is a correlation between the number of cocoons produced and the exposure to adverse environmental factors such as desiccation, extremes of temperature, and predation (Satchell and Lowe, 1967). Earthworms' time to reach sexual maturity from hatching differs significantly between species. Furthermore, the incubation periods of different species of earthworms differ significantly. Satchell (1967) suggested that the life duration of mature lumbricids in the field is probably relatively short, often no more than a few months, although their potential longevity was 4–8 years as they were exposed to many risks. The growth rate of earthworms strongly depends on the type and quality of the substrate (Ton et al., 2009). For example, the growth cycle of *E. fetida* takes 40–60 days for the juvenile to develop into a mature or adult worm (Fadaee, 2012). Adult worm weight is approximately 1.5g at 50 to 55 days. Adult worms can create a cocoon every three days on average, and after 23 days, one-third of newborns come out of the cocoon (Figure 4).

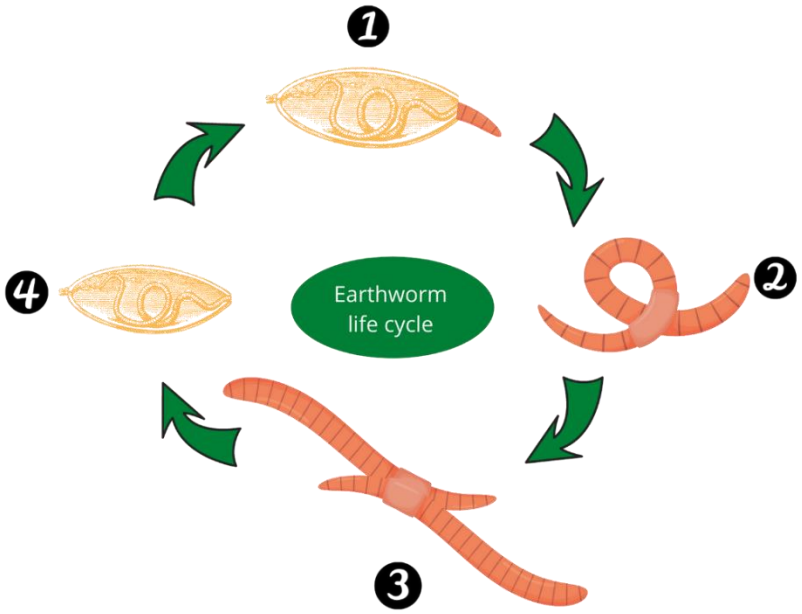


Figure 4. Growth cycle of earthworm *Eisenia fetida* (1) earthworm hatching (2) a matured earthworm (3) mating and fertilization (4) cocoon (Venter and Reinecke, 1988).

III.1.3. Earthworm's burrows and casts

Earthworms develop tunnels by eating their way through the ground and pushing through holes. Earthworm burrows that occur in the drilosphere can serve as favourable pathways for root development (Ehlers 1975; Edwards and Lofty 1980; Kirkham 1981; Ehlers et al. 1983; Wang et al. 1986; Kladivko and Timmenga 1990; Hirth et al. 1997; Jiménez 1999), particularly in compacted deeper soil zones. The distribution of roots in soil is often intimately linked to the zones of earthworm activity (Edwards and Lofty 1978, 1980). Estimates of burrows in temperate zone soils go as high as 100–800 m−2 (Lavelle 1988). These macropores can significantly alter the circulation of air, water, and solutes. Earthworm tunnels operate as water channels. The number and size of burrows determine the hydrological conductivity. This, in turn, has effects on the leaching of chemicals, including pesticides and their residues and particles from bulk soil, as water will infiltrate through the burrows preferentially (Bohlen et al., 2004; Domínguez et al., 2004; Edwards, 2020). Besides, the earthworm's gut is well known for breaking down the organic matter into finer particles by microbial decomposition that

stimulate fast mineralization of organic matter, resulting in nutrient release in their castings that can be easily absorbed by the plants (Shefali et al., 2017).

Earthworms produce "casts," a biologically active mass that generates many bacteria, enzymes, and remains of plant materials and animal wastes that the earthworm has not digested (Lavelle and Martin, 1992). Essentially, there are four types of casts (Lavelle 1988; Edwards and Bohlen 1996): (1) Globular, consisting of coalescent round or flattened units, generally produced by the larger earthworm species (anecic and endogeic species), (2) Pastelike slurries, mainly produced by endogeic or anecic species and excreted as single masses of soil without a distinct shape, but that take on irregular shapes once dried, (3) Tall vertical heaps or columns of variable shapes, usually deposited on the soil surface where they are most visible by endogeic or anecic species, and (4) Granular, typically in the form of pellets, produced mainly by smaller earthworm species (epigeic, small endogeic, and some anecic species) and distributed on or beneath the soil surface (Brown et al., 2004).

The positive effects of earthworm casts on vegetation were attributed to high concentrations and availability of soil nutrients, the improved physical structure of the soil, enzyme activities, and an effect of microbial inoculation (Lavelle and Martin, 1992; Edwards, 1995; Bohlen et al., 2004; García-Palacios et al., 2014; Lv et al., 2020). Inoculated fresh surface casts into artificial soil burrows and found that available N in casts was initially at levels several times higher than that in the bulk soil, while total C was also significantly higher than that in the soil (Mariani et al., 2007). In addition, higher microbial population densities were observed in casts than in the surrounding mineral soil (Scheu, 1987; Pashanasi et al., 1992; Nechitaylo et al., 2010).

III.1.4. Digestive Mechanism

Earthworms obtain their nutrients from organic materials, in the form of plant material, living protozoa, rotifers, nematodes, bacteria, fungi, and other microbes, as well as decomposing remnants of large and small animals. Some species eat directly on leaves, and even some exhibit a preference for particular species and circumstances. Earthworms are known physically as aerators, crushers, and mixers; chemically as degraders and biologically as stimulators in decomposer systems (Aira et al., 2008; Dendooven et al., 2011; Rodriguez-Campos et al., 2014; Edwards, 2020; Siddiqui, 2020). Earthworms' foregut acts as mechanical blenders, modifies the physical status of ingested organic wastes, and consequently increases

the surface area for digestive enzyme actions. These worms have an in-house supply of amylase, cellulose, nitrate reductase, acid, and alkaline phosphatases (Kiyasudeen et al., 2016). Enzymes are produced jointly by worms and gut microflora, which play a central role in the digestion and humification of soil organic matter (Brown et al., 2000).

III.1.5. Fluids of the earthworm

Cutaneous mucus of earthworms comprises 69% proteins and peptides and 31% carbohydrates (Cortez and Bouché, 1987), but the specific amount or quality may vary according to ecological earthworm categories and ingested substrates. Earthworm mucus proteins exhibit efficient innate immune mechanisms, which determined by the pattern and quantity of amino acids. Table I shows the amino acid composition of epidermal mucus of three earthworm species (Kauschke et al., 2007, Zhang et al., 2016) (Table I). In addition, the pattern of N output, a significant component of mucus, differs according to earthworm species (Needham, 1957). Moreover, the cutaneous mucus of earthworms can neutralize the pH of the immediate environment through its buffering capacity (Raouane and el Harti, 2016; Salmon, 2001).

Table I: Amino acid composition of epidermal mucus of three earthworm species (Zhang et al.,2016).

Composition	Amino acid concentration (mg 100 g^{-1})		
	E. fetida	***A. trapezoides***	***A. pingi***
Aspartic acid	28.45	40.47	11.29
Threonine	23.97	22.97	6.64
Serine	13.39	17.64	5.34
Glutamic acid	28.37	54.53	18.14
Proline	15.36	40.35	7.96
Glycine	16.50	34.11	7.28
Alanine	16.55	22.68	12.05
Valine	32.38	35.94	9.67
Methionine	0.42	3.52	1.13
Isoleucine	13.07	14.03	4.45
Leucine	16.87	23.84	8.36
Tyrosine	2.02	14.06	4.57
Phenylalanine	10.19	11.64	3.76
Histidine	39.73	26.23	3.99
Lysine	11.20	18.35	5.59
Arginine	10.30	14.94	3.91
Total	278.77	395.30	114.13

The traditional, physical perspective is that cutaneous mucus in earthworms, released through gland cells in the epidermis, avoids desiccation, promotes respiration, and provides lubrication for movement through the abrasive ground (Edwards and Bohlen, 1996). However, considering the ubiquity and constant mucus production in all grounds inhabited by earthworms (Zhang et al., 2016), mucus is also likely to be a significant agent of biochemical processes in soils and trophic interactions with other organisms, including plants. Earthworm mucus is believed to enormously increase microbial activities and accelerate soil organic matter turnover by adding labile carbon sources (Huang and Xia, 2018). It has recently been proposed that earthworm mucus contains signalling molecules that can modify the development and growth of plants (Puga-Freitas et al., 2012). Interestingly, it has been suggested that earthworm mucus

may influence the toxicity and bioavailability of polluted soils due to its high affinity for organic contaminants (Pan et al., 2010). There are numerous ecological roles of earthworm mucus, and still, our knowledge of its chemical composition is minimal (Cortez and Bouché, 1987). Better knowledge would enhance our mechanistic understanding of biochemical processes in soil involving earthworm mucus and interactions with other soil fauna, microorganisms, plants, and contaminants (Shutenko et al., 2020).

Earthworms eject **coelomic fluid** through the dorsal pores, in response to mechanical or chemical irritation, or when subjected to heat and cold extreme. This fluid differs from the circulatory system of vertebrates in that it is not circulated by a heart or equivalent organ but only by body movements. The coelomic fluid is in contact with different tissues such as muscle, intestine, gonads, and nerves. The composition comprises proteins and various cell types involved in reproduction and defence mechanisms as the denominated "coelomic cells". Among these compounds have been described several bioactive proteins, which exhibit a variety of antibacterial (Lassègues et al., 1989; Valembois,' et al., 1982), hemolytic (Andrews and Kukulinsky, 1975), cytotoxic (Kauschke and Mohrig, 1987), hemagglutinating (Valembois, 1982; Valembois et al., 1986), and proteolytic (Tučková et al., 1986). Other inclusions in the coelomic fluid include breakdown products of the corpuscular bodies, protozoan and nematode parasites, and bacteria (Edwards and Lofty, 1977). The nature of the coelomic fluid varies between different species of earthworms and depends upon the humidity of the air in which the earthworms reside; hence, it is thicker and more gelatinous in worms in dry conditions than in those from wetter areas.

Intestinal mucus comprises glycoproteins, small glucosidic, and protein molecules (Morris, 1985). This particular mucus is secreted by a proper salivary gland, the pharyngeal bulb of the earthworm. The mucinous salivary secretion accumulates in the pharyngeal cavity and oesophagus. The intestinal mucus is essential for the activity of feeding (Keilin, 1920), where the microbes entering the earthworm guts consume the nitrogenous compounds of mucus (gluco-proteins), which mainly increase their activity, enabling them to contribute enzymes to the digestive processes of the earthworms.

III.1.6. Ecology

At first, Marcel Bouché proposed seven earthworm categories (anecic, endogeic, epigeic, epi-anecic, endo-anecic,epi-endogeic and intermediate) based only on morpho-anatomical characteristics measured on European lumbricids, which many other scientists revised (Bottinelli et al., 2020a; Bottinelli and Capowiez, 2021). Then he simplified the description of these categories into three major ecological categories: epigeic, endogeic, and anecic (Bouché, 1977). Later, these categories were considered functional groups to presume how earthworms influence the soil functioning (Bottinelli et al., 2020b). Overall, these three main categories consider earthworms' essential biological and ecological characteristics such as burrowing abilities (Figure 5), food preferences, body colour, size, and shape (Lavelle, 1988).

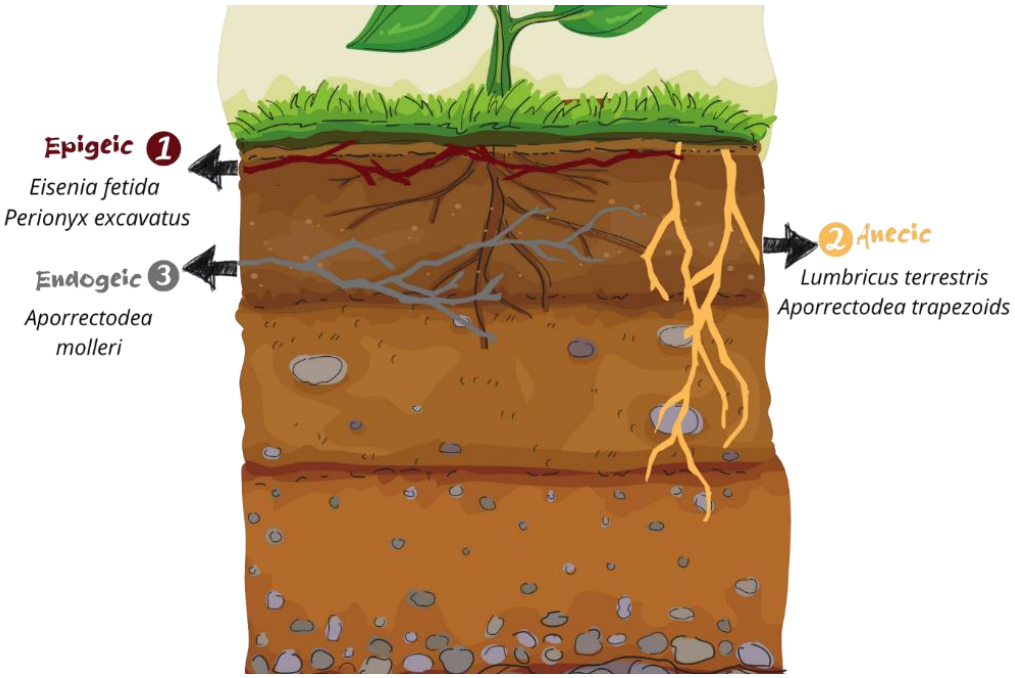


Figure 5. The three main ecological groups of earthworms according to their burrowing and feeding behaviour.

Epigeic species are essentially litter dwellers. They reside in organic horizons in or above the surface litter, feed predominantly on coarse particle organic matter, and absorb enormous volumes of undecomposed litter. These species produce short burrows into the

mineral soil for periods of diapause; therefore, most of their activities and effects are limited primarily to the upper few centimeters of the soil litter interface. They are vital "litter transformers". They are often small (Bouché, 1977), uniformly pigmented species with high metabolic and reproductive rates, demonstrating adaptations to the soil surface's highly different environmental conditions. When the environmental circumstances within heterotrophic decomposition systems are inadequate, or food is insufficient, epigeic species are challenging to find, despite their high potential for fast reproduction. This group of epigeic species includes *Eisenia fetida*, *Perionyx excavatus* (Bouché, 1977) (Figure 6 (a-b)).

Endogeic earthworm species reside more thoroughly in the soil profile and feed mainly on soil and organic matter. They have minimal colouration, and they usually create horizontal, deep-branching tunnel systems that fill with cast material as they travel through the organic-mineral layer of the soil. Earthworms of this type can burrow deep into soils that require a much longer time to achieve their maximum weight and appear to be more tolerant of periods of starvation than are epigeic species (Lakhani and Satchell, 1970). These species are apparently of no significant importance in litter incorporation and decomposition because they feed on subsurface soil material; they are essential in other soil formation processes, including root decomposition, soil mixing, and aeration. This endogeic group includes species such as *Aporrectodea molleri* (Figure 6 (c)).

Anecic earthworm species exist in permanent vertical burrow systems that may reach several meters into the soil profile. The permanent burrows of anecic earthworms generate a microclimatic gradient, and the earthworms can be found at either superficial levels or deep in their burrows, depending on the prevailing soil environmental conditions. They cast at the soil surface and emerge at night to feed mainly on surface litter, manure, and other partially decomposed organic materials, which they draw down into their burrows. Characteristically, these earthworms are as giant as adults and dark in colour anteriorly and dorsally; their reproduction rates are relatively slow. Anecic species of earthworms are essential agents in organic matter decomposition, nutrient cycling, and soil formation, accelerating pedological soil processes worldwide. *Lumbricus terrestris* (Figure 6 (d)) are included in this ecological group of earthworms (Lavelle, 1983).

Figure 6. Example of epigeic earthworms. (a) *Eisenia fetida*, and (b) *Perionyx excavates*. Endogeic earthworm, (c) *Aporrectodea molleri*. Anecic earthworm (d) *Lumbricus terrestris*.

Earthworm populations display huge variety in time and space, with mean population densities and biomass ranging from fewer than ten individuals and 1g/m2, respectively, to more than 1000 individuals and 200 g/m^2, respectively, under favourable conditions. Several environmental factors affect earthworm activity, species richness, abundance, and biomass per m2 (Phillips et al., 2019). Soil organic matter content, soil type, soil moisture content, soil temperature, and soil pH are the most critical factors that frequently regulate the earthworm population (Wood, 1974; Piearce and Lee, 1987; Ulrich et al., 2005). Otherwise, biotic factors such as competition, predation, parasitism, disease and food relationships strongly affect the abundance and distribution of earthworms (Wood, 1974; Piearce and Lee, 1987; Ulrich et al., 2005).

Temperature is a factor of primary importance because it determines individual earthworm metabolic rates. On a global scale, it can have a major role in determining patterns of earthworm distribution, richness and biomass (Phillips et al., 2019). The range of temperatures within which most earthworms can function is narrow but depend on their origin. Then, the optimum temperatures typically in the range 10 to 20°C for cool temperate species and 20 to 30°C for tropical and subtropical species (Edwards and Bohlen 1996).

Faster organic matter degradation rates at higher temperatures result in decreased litter availability. Litter-feeding epigeic and anecic earthworm populations tend to decrease in tropical soils compared with temperate soils (Lavelle et al. 1999). With increasing temperatures, endogeic species utilising resources of increasingly lower quality through more efficient digestive processes, including mutualistic interactions with ingested soil microorganisms, are favoured. Further, than organic matter, the soil qualities that appear to be more critical are texture, pH, and soil moisture. These factors determine the physicochemical parameters of the soil environment, the nature of the vegetation that can be supported, and the quantity and quality of the litter it produces.

Competition for food is generally believed to be important in determining earthworm populations, but there is little information on the nature of this competition and the frequency with which it occurs (Singh et al., 2019). However, the few studies that have formally addressed interspecific interactions in earthworms have shown that competition can occur directly for food or indirectly via some interference mechanism (Abbott, 1980; Hamilton et al., 1988). Earthworms are commonly exposed to high rates of predation and are subject to pathogen and parasite attacks; there may be occasions when predation especially can massively lower population density. However, when populations are not constrained by physicochemical environmental factors, food supply, notably the quality and quantity of the litter input, is the factor that most frequently limits earthworm abundance (Edwards and Lofty, 1977; Edwards and Bohlen, 1996).

Human activities can drastically alter the soil environment and influence earthworm populations directly by physical disturbances and indirectly by altering the physicochemical environment and the food supply. Some of the main ways in which activities such as mining, deforestation and afforestation, grassland management, arable cropping, pesticide use, and water management may influence earthworms. One aspect of human intervention with potentially significant consequences is the accidental or deliberate introduction of exotic species, which could dramatically change abundance and species composition, possibly to the detriment of native species, and influence soils and plant production (Brown et al., 2004).

III.1.7. Impact of earthworms on soil functioning

Earthworms are considered principal terrestrial ecosystem engineers for their ability to modify physical (aggregate structure, porosity), chemical (nutrient supply and cycling), and biological (soil fauna, microbial, and enzyme activities) properties of the soil profile. These beneficial effects have been attributed to improvements in soil properties and structure, increasing the availability of mineral nutrients for plants, and increased microbial populations and biologically active metabolites such as plant growth regulators (Kiyasudeen et al., 2016).

Earthworms are involved in soil formation. Darwin (1881) was among the first to include biota, especially earthworms, in the list of factors responsible for soil formation by accumulating earthworm casts and mixing processes. Surprisingly, little research has considered this role later, while earthworms are considered involved in controlling humification rates through feeding, burrowing, and casting activities and interactions with microorganisms (Bernier, 1998; Dell'agnola and Nardi, 1987; Ponge, 1991). Hence, earthworms bury organic matter from the surface and bring soil particles from deep soil horizons to the surface. The contribution of earthworms to the burial of surface litter at some locations may reach 90–100% of the litter deposited annually on the soil surface by the aboveground vegetation from either 'natural' vegetation or crops (Knollenberg et al., 1985; Raw, 1962). Recent organic matter is buried in the soil, whereas soil from depth is brought to the soil surface by the deposition of casts aboveground, particularly by the anecic species. These surface casts are responsible for an apparent downward migration of stones in the soil profile.

Erosion is also crucial in soil formation, and again, earthworms have a significant role in this process, mainly through the production of casts on soil surfaces. The contribution of casts to erosion appears to occur following their breakdown by the impact of rain rather than the transport of intact cast material (Le Bayon and Binet, 2001, 1999). However, there is debate whether more or less erosion would occur without casts. Some authors suggest that surface-deposited casts of anecic species may resist run-off, thereby reducing erosion. In contrast, others suggest that the erosion of cast material leads to a net increase in erosion (Shipitalo and Protz, 1987).

Earthworms can enhance the soil structure, defined as the arrangement of soil particles and associated pore spaces across various scales. However, physical forces modulate it, the

actions of more extensive soil biota such as plant roots or earthworms, the presence of organic matter and soil tillage in some agricultural systems (Oades, 1993; Milleret et al., 2009a, b). The action of the earthworms can be both compacts and loosen the soil. De-compacting earthworms destroyed macroaggregates formed by compacting ones, whereas compacting earthworms did the same with the casts of de-compacting ones. Such variability dynamically regulates soil structure (Blanchart et al., 1997). In a 20-year study, the experimentally induced absence of earthworms in a grass sward also increased soil bulk density (Clements et al., 1991), which suggested that earthworms can also decrease bulk density. Instead, the absence of earthworms also decreased total soil porosity and affected aggregate size distribution.

Overall, the positive effects of earthworms on soil structure have been widely demonstrated (Lavelle, 1988; Siddiqui, 2020). However, suppose earthworm use is proposed as part of a soil management scheme. There is a need for sufficient and appropriate preliminary soil measurements and then monitoring at relevant time scales. Combining compacting and de-compacting species could also be vital for inoculation to achieve the required objectives in soil structural improvement, given their different behaviors. Recent modeling to simulate the effects of earthworms on soil structure has great merit and is worthy of further development as these activities are an essential ecosystem service (Barot et al., 2007a; Blanchart et al., 2009).

Earthworms are also involved in nutrient cycling. They accelerate organic matter degradation by increasing the available surface area of organic matter through comminution as heterotrophic organisms (Ingham et al., 1985; Seeber et al., 2008). Nitrogen mineralization is consequently increased in the presence of earthworms, either (i) directly through the release of nitrogen by their metabolic products (casts, urine, and mucus, which contains NH_4^+, urea, allantoin, and uric acid) as well as dead tissues, or (ii) indirectly through changes in soil physical properties, fragmentation of organic material, and interactions with other soil organisms (Lee, 1985; Bityutskii et al., 2002).

Earthworms accelerate Nitrogen mineralization from organic matter, but the effect depends on the species and their interactions with other soil biota, soil characteristics, and the location of the organic matter (Butenschoen et al., 2009). Mineral nitrogen released in the casts of earthworms can be necessary concerning crop nitrogen requirements. Otherwise, the casts can potentially have microbial nitrification and denitrification (Palmer et al., 2005; Costello & Lamberti, 2008). Earthworms increase mineral nitrogen in the soil and readily exchangeable

phosphorus, potassium, calcium, and magnesium (Suarez et al., 2004; Adejuyigbe et al., 2006). They can also increase the mineral nitrogen and phosphorus (Dominguez et al., 2004; Suarez et al., 2004; Costello & Lamberti, 2008) because of their effects on soil structure.

Earthworms are also involved in pollution remediation. For both organic and inorganic contaminants, earthworm activity may reduce the amount of sorption on soil particles through the digestive process of organic matter, adjustments of soil chemistry, or both, leading to an increase in the availability of contaminants, and so reducing the timescales required for phytoremediation. A limited number of laboratory studies have been conducted on soil treated with organic compounds and a range of earthworms. These studies have generally used soil amended with polychlorinated biphenyl (PCBs) (Singer et al., 2001; Kelsey et al.,2011), petroleum hydrocarbons (Schaefer et al., 2005; Schaefer et Filser, 2007), or polyaromatic hydrocarbons (Ma et al.,1995, 1998; Eijsackers et al., 2001; Contreras-Ramos et al., 2008,2009). Studies mainly utilize either epigeic or anecic earthworms, with only two authors adopting an endogeic earthworm (Schaefer et al., 2005; Schaefer & Filser, 2007; Kelsey et al., 2011). In general, earthworms expedite the decomposition of organic substances, although the process by which this is achieved is not known. However, it seems likely that this is a combination of increased aeration of the soil, stimulation of the microbial population, which degrades the contaminants, and metabolism of the contaminants by the earthworms themselves. The above studies indicate increases in metal concentration in plant tissues, meaning that earthworm activity always increases plant uptake of metals. Finally, earthworms are also linked to crop growth and health. The positive effect of earthworms on plant growth was noticed earlier by Darwin in 1881. Consequently, the impact of earthworms on primary production has been thoroughly explored in the laboratory and the field (Brown et al. 1999). Several environmental parameters were responsible for variation in biomass production in the presence of earthworms, including soil type, precisely soil texture and carbon content, and the plant functional group (Brown et al. 1999, 2004). Earthworms promote higher biomass production in perennial species, notably trees, than in annual species, although the biomass of legumes is occasionally negatively influenced by the presence of earthworms (Brown et al. 2004). Therefore, aboveground biomass increases typically in the presence of earthworms, but belowground biomass demonstrates a varied response. The positive link between earthworm abundance and crop productivity is not systematic, and differing yields have been found (Baker et al., 1999).

Depending on the earthworm species, it has been described the following changes in physicochemical properties of the soil: (i) modify the soil porosity and aggregation, which changes water and oxygen availability to plants, and (ii) more significant mineralization of SOM, which increases nutrient availability to the plants. The other three mechanisms involve interactions with other organisms: (iii) biocontrol of pests and parasites, (iv) stimulation of symbionts, and (v) production of plant growth regulators via the stimulation of microbial activity.

III.1.8. Interaction earthworms with soil habitat: nematodes, fungi, and bacteria

Earthworm burrowing and casting promote soil mixing and bring microorganisms into contact with inaccessible soil resources, stimulating their populations and activity. The earthworm guts also provide excellent habitat for improved activity levels or multiplication of some microbes; others may be digested, or their activity levels lowered by transit through the earthworm gut (Brown et al. 2000) or the dispersal activity through the soil (Lavelle 1997). The complex effects of earthworms on large populations of soil microorganisms such as saprophytic and mycorrhizal fungi, actinomycetes, bacteria, and microinvertebrates protozoa and microbivore that inhabit the soil depend on the ability of those microorganisms to utilize the earthworm's territory (drilosphere). Furthermore, as earthworms move through the soil matrix, they may disperse microorganisms, both superficially and via ingestion-egestion. The ability of earthworms to distribute microorganisms or stimulate microbial activity and increase microbial populations depends significantly on the earthworm's ecological categories behave.

Bacterial-to-fungal ratios in soils are generally higher in earthworm-worked soils because bioturbation negatively affects fungus populations more than bacteria (Hendrix et al. 1998). In this sense, it has been observed that several species of fungi are ingested preferentially by the earthworm L. terrestris (Moody et al. 1995; Cooke 1983; Bonkowski et al. 2000). In fact, some hyphae and spores may be digested, and many are still infective after passage through the earthworm gut (Reddell and Spain 1991a; Gange 1993).

Plant growth-promoting rhizobacteria (PGPR) may also be dispersed, and their populations or activity increased in the drilosphere (Kozlovskaya and Zdhanni- khova 1961; Loquet et al. 1977; Savalgi and Savalgi 1991; Pederson and Hendriksen 1993). However, these

and other microorganisms such as biocontrol bacteria and fungi cannot spread actively and rapidly through the soil and colonize plant roots extensively so earthworms may act as important dispersal vectors for them (Rouelle 1983; Stephens et al. 1993b; Doube et al. 1994b).

Earthworms may affect the populations and activity of several groups of beneficial soil organisms important in plant litter decomposition and nutrient mineralization processes in soils (Brown 1995). For example, earthworm casts may benefit bacteriophagic nematode populations preferentially over those of other nematode trophic groups (Roessner 1981, 1986; Senapati 1992), but the total numbers of free-living nematodes in earthworm-worked soils may be reduced (Alphei et al. 1996; Dominguez et al. 2003) or increased (Winding et al. 1997), depending on the situation (Edwards, 2004).

III.2. Microorganisms: bacteria, yeasts, and fungi

Soil microbial communities provide the most outstanding source of biodiversity known worldwide (Curtis et al., 2002; Gams, 2007). The soil microbiome consists mainly of bacteria, yeast and fungi occupying the rhizosphere system, defined as a collection of microbial genomes in a given habitat (Bulgarelli et al., 2013). Various microbial communities are used as inoculants and primarily they promote plant growth through nitrogen fixation, phosphate and potassium solubilisation, exopolysaccharide secretion, biocontrol activity, organic matter decomposition, siderophores production, etc.

The rhizosphere, the thin zone of soil surrounding the root, can contain up to 1011 microbial cells per gram root (Egamberdieva et al., 2008) and more than 30,000 prokaryotic species (Mendes et al., 2011). Bacteria are the leading players in biogeochemical cycles in soil, such as carbon, nitrogen, sulphur, and phosphorus (Long et al., 2016) and can affect nutrient absorption by modifying root structure or physiology (Vessey, 2003). The most widespread and abundant soil bacteria are Proteobacteria, Acidobacteria, Actinobacteria, Bacteroidetes, and Verrucomicrobia. Followed by the lesser abundance of Firmicutes, Planctomycetes, Chloroflexi, Gemmatimonadetes, and Cyanobacteria. However, bacteria act within tiny spheres of influence, requiring vectors to broaden their zones of influence across the soil (Kuzyakov and Blagodatskaya, 2015). Those vectors vary from protozoa, nematodes, and earthworms to soil fungi and plant root systems (Daane et al.,1996; Kohlmeier et al., 2005; Warmink and van Elsas, 2009; Glaeser et al., 2016).

Fungi dominate all types of soils and represent the greatest diversity among soil microorganisms. However, the quality and quantity of organic matter have a direct bearing on fungal numbers in soils since fungi are heterotrophic organisms. Fungi are classified into Phycomycetes, Ascomycetes, Basidiomycetes and Fungi imperfecti (Alexander 1977). Members of this class are distinguished by their septate mycelium and a structure called conidiophore from which conidia or spores are continuously produced. Fungi and especially members of the Asco- and Basidimycetes are able to degrade very complex organic compounds such as cellulose or lignin, but many of them also live as root symbionts (mycorrhizas) and obtain simple sugars from their plant partners (Lynch and Hobbie 1988).

The following genera of fungi are most encountered in soils: Acrostalagmus, Aspergillus, Botrytis, Cephalosporium, Gliocladium, Monilia, Penicillium, Scopulariopsis, Spicaria, Trichoderma, Trichothecium, Verticillium, Alternaria, Cladosporium, Pillularia, Cylindrocarpon and Fusarium, Absidia, Cunninghamella, Mortierella, Mucor, Rhizopus, Zygorynchus, Pythium, Chaetomium, and Rhizoctonia (Newman1985; Hawksworth 1991a; Subba Rao 1997). Many yeasts belonging to true Ascomycetes such as Saccharomyces and those belonging to Fungi Imperfecti such as Candida have been isolated from soils. However, their number in soil is relatively low.

Filamentous fungi in soil degrade organic matter and help in soil aggregation. Certain fungi like Alternaria, Aspergillus, Cladosporium, Dematium, Gliocladium, Helminthosporium, Humicola, and Metarhizium produce substances similar to humic substance in soil and, hence, may be important in the maintenance of soil organic matter (Hawksworth 1991b).

As examples, the leading group, “Plant growth-promoting rhizobacteria (PGPR),” increases agronomy efficiency by reducing production costs, and environmental pollution reduces the use of chemical fertilizers (Souza et al., 2015). *Rhizobium sp*. and *Bradyrhizobium sp*. are major microbes in symbiotic N2 fixation (Souza et al., 2015). *Azospirillum* is a free-living nitrogen fixer found to enhance the growth of non-leguminous crops (Lin et al., 2015). *Pseudomonas putida* and *Pseudomonas fluorescens* groups could stimulate plant growth as biocontrol agents, aiding N2 fixation and phosphate solubilization (Lin et al., 2015; Souza et al., 2015). *Azotobacter* and *Azospirillum* effectively enhance production (Rueda et al., 2016). However, bacteria like Bacillus and Pseudomonas and fungi like Aspergillus and Penicillium were considered good phosphate solubilizers (Sharma et al., 2013).

IV. Biological control agents

IV.1. Biological control

The populations of all living organisms are reduced by the natural actions of their predators, parasites, antagonists, and diseases. This process has been referred to as ‘‘*natural control*” but when pests are controlled, this is often called “*biological control*” and the agents that exert the control are frequently called “natural enemies”. Biological control has been defined many times. However, a commonly accepted definition is “*the use of living organisms to suppress the population of a specific pest organism, making it less abundant or less damaging than it would otherwise be*” (Eilenberg et al., 2001). However, different scientists had some disagreement about this biological control definition. Hence, Entomologists started with the concept that biological control was “*the use of living organisms (natural enemies) to manage a pest population*”, while Plant Pathologists suggest that biological control is “*the control of a plant disease by a biological process or the product of a biological process*” (Hajek and Eilenberg, 2004).

In any case, in a broad sense, biological control is the strategy of using of organisms or their functions to combat arthropod pests (Vicente-Díez et al., 2021), weeds, and plant pathogens, as well as vertebrate pests and to protect the crops. It has been used extensively for terrestrial systems, both above and belowground, starting with uses in agriculture and forestry and later being applied to natural ecosystems to control invasive as well as native pests (Hajek and Eilenberg, 2004). In terms of application, biological control can be split into many categories: (i) Classical, (ii) Inundative (or Augmentative), (iii) Inoculative and (iv) Conservation.

Classical biological control can be defined as “the intentional introduction of an exotic biological control agent for the permanent establishment and long-term pest control” (Eilenberg et al., 2001; Lazarovits et al., 2007). Classical biological control is a proven powerful management tool, which can provide significant benefits if practiced cautiously (Lazarovits et al., 2007). Notably, the goal is quite specific: to release an exotic natural enemy into a new environment and establish and regulate a pest population long-term without further intervention. Scientists noted that many introduced pests are not problematic in their areas of origin, where a community of natural enemies often controls them. However, after introducing

a new area, the introduced species increases in number to become a pest in some cases. It is thought that the pest can increase because, in the new area, the natural enemies that would naturally regulate this species are not present. This fundamental assumption of classical biological control has been called the ‘‘*enemy release hypothesis*.” The goal with classical biological control is to re-establish the ‘‘*natural balance*” that controls the pest in its native habitat (Hajek and Eilenberg, 2004).

Inundative biological control is defined as “The use of living organisms to control pests when control is achieved exclusively by the organisms themselves that have been released” (Eilenberg et al., 2001). This technique focuses on the rapid management of pests during the short term. In all circumstances, no reproduction by the natural adversary is envisaged. Because control is only attributable to the released individuals, inundative releases would have to be repeated if pest populations increase after natural enemies are released. The released agents must contact and eliminate a relatively high proportion of the insect pest or decrease the damage level by other methods to provide control. Of course, to achieve sufficient control quickly, it is essential to release a massive number of organisms to inundate the pest population. Inundative management is typically utilized for short-term crops because viable breeding populations of the natural enemies do not arise in the environments offered by temporary monocultures. Inundative Biological Control has also had several accomplishments. For instance, pest management through biological control has become the foundation of integrated pest management programmes in greenhouses (Lazarovits et al., 2007). This system was brought about because there was a desperate need to control pests that rapidly developed resistance to chemical pesticides within closed ecosystems and prompted the search for novel control products, such as entomopathogenic nematodes, which have great potential as a natural enemy of soil pests (Lazarovits et al., 2007).

Inoculative biological control defined as “*The intentional release of a living organism as a biological control agent with the expectation that it will multiply and control the pest for an extended period, but not that it will do so permanently*” (Eilenberg et al., 2001). Inoculative control is due not only to the released organisms themselves but also to their offspring. It provides more long-term and self-sustained control than inundative releases. Suppose an inoculative release is intended for predators, parasitoids, or pathogens. In that case, sufficient pest numbers must be present following the initial release to support a second or third generation of the released agent, and conditions that allow multiplication of the natural enemy

must occur (Hajek and Eilenberg, 2004). Long-term multigenerational survival and recycling through hosts are required for inoculative release programs. As an example of entomopathogenic nematodes (EPN), three conditions are required for an inoculative release program to be successful: (1) moderately susceptible pests need to be present throughout most of the year, (2) pests should have a high economic threshold level, and (3) soil conditions should be favorable for nematode survival (Gaugler and Kaya, 1990).

Conservation Biological Control can be defined as "*Modification of the environment or existing practices to protect and enhance specific natural enemies or other organisms to reduce the effect of pests*" (Eilenberg et al., 2001). This strategy for biological control differs from classical augmentation or inoculation biological control because natural enemies are not released. Instead, the resident populations of natural enemies are conserved or strengthened. Conservation approaches are usually unsuitable for managing pests in high-value crops that can endure moderate damage. A crucial necessity for applying conservation and enhancement is that the biology, behaviour, and ecology of the pest and natural enemies must be understood to some level. To establish effective conservation and enhancement of natural enemies, we need to understand what factors decrease natural enemy numbers or restrict their ability to control pests, and these detractors must be eased. Alternatively, those factors limiting natural enemy populations must be recognized to be adjusted to boost population levels of natural enemies or promote interactions between natural enemies and pests. Conservation and enhancement research has developed to control insects, plant diseases, and plant-parasitic nematodes in more recent years. As a type of conservation and enhancement, Plant Pathologists and Nematologists use communities of organisms that develop within suppressive soils to control pests (Hajek and Eilenberg, 2004; Lazarovits et al., 2007). For example, Duncan et al. (2013) uses the Conservation Biological Control approach by Modifying the orchard planting sites to conserve entomopathogenic nematodes, reduce weevil herbivory, and increase citrus tree growth survival and fruit yield (Duncan et al., 2013).

IV.2. Entomopathogenic nematodes

Entomopathogenic nematodes are a group of soil-dwelling roundworms considered excellent biological control agents. This group of nematodes traditionally includes two phylogenetically distant families, Steinernematidae and Heterorhabditidae, which share similar life histories through convergent evolution (Figure 7) (Stock, 2015; Blaxter et al., 1998; Poinar, 1993). According to Bhat et al., 2020, around 100 valid species of Steinernema and 21 species of Heterorhabditis have been identified from different countries worldwide. This thesis uses two of the newest recently discovered species: *Steinernema khuongi* (Stock et al., 2019) and *Steinernema riojaense* (Půža et al., 2020).

The families Steinernematidae and Heterorhabditidae (Nematoda: Rhabditida) (Figure 7) are a distinct group of nematodes that have evolved the ability to carry and introduce symbiotic bacteria into the body cavity of insects (Campbell and Kaya, 1999; Dillman et al., 2012; Stock, 2015). They are insect pathogens with a host range that includes most insect orders and families, and they can be cultured on a large scale on, or in, artificial solid or liquid media (Shapiro-Ilan and Dolinski, 2015). Steinernematid and heterorhabditid nematodes can kill insects within 48 hours, form a durable, infective stage that can be stored for long periods and applied by conventional methods, and persist in the natural environment. Because the nematodes are adaptable biological organisms, natural populations of insects would not be expected to acquire immunity against them. In addition, plants and mammals are not adversely affected (Gaugler, 2002; Piedra-Buena et al., 2015).

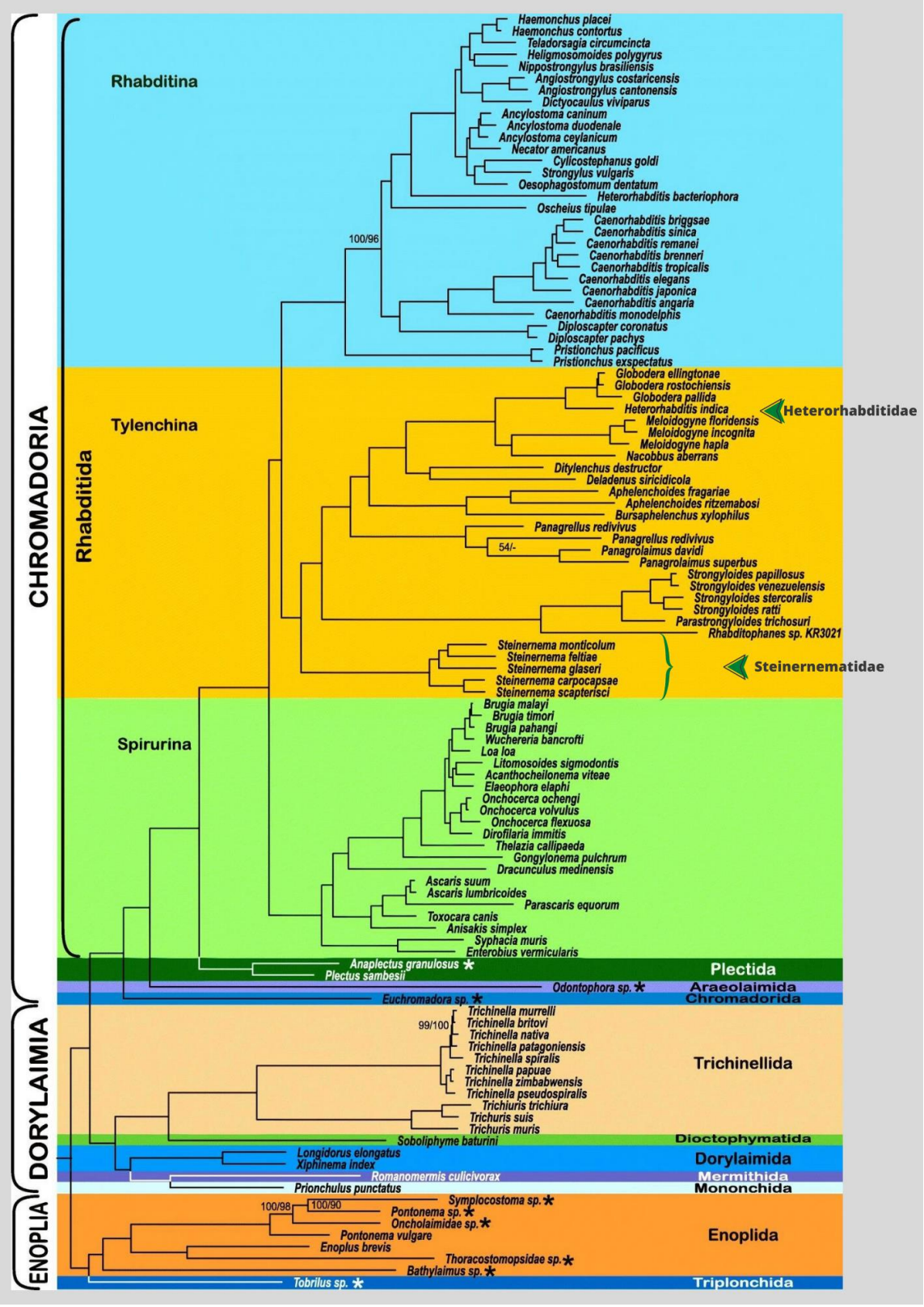


Figure 7. Phylogeny of Nematoda based on the IQ-TREE maximum likelihood analysis of the SCaFoS dataset (modified from Smythe et al., 2019).

IV.2.1. Biology

The invasive stage (infective juveniles, IJs) of EPNs in the genera Steinernema and Heterorhabditis (Nematoda: Rhabditida) carries cells of a symbiotic bacterium Xenorhabdus and Photorhabdus, respectively. The host location is followed by nematode penetration into the hemocoel (Figure 8). Here the symbiotic bacteria are released and quickly kill the host. The nematodes feed upon the bacteria and degrade host tissues, producing more generations. In brief, the bacterial partner transforms the insect into a self-contained microhabitat appropriate for nematode development and reproduction and kill the insect commonly within 48–72 h (Dillman et al., 2012). The EPNs then change to feeding third-stage juveniles, feed on the bacteria, and moult in succession to the fourth stage, where they become adults of the first generation. After reproducing, the females lay eggs that hatch as first-stage juveniles and successfully to the second, third, and fourth stages. In Heterorhabditis, hermaphroditic female replaces the first amphimictic generation in Steinernema. The fourth-stage juveniles develop into adult females and males of the second generation. The adult's mate and the eggs produced by the second-generation females hatch as first-stage juveniles that moult to the second stage. Nematode reproduction continues until resources in the cadaver are depleted, usually allowing for two to three generations. If the food supply is limited, the eggs produced by the first-generation females develop directly into infective juveniles. As resources in the cadaver are extinguished, late second-stage juveniles cease to feed and incorporate a pellet of bacteria. They moult to pre-infective and infective stages, retaining the cuticle of the second stage as a sheath in some of the species. The infective juveniles then typically leave the cadaver searching for a new host. Infective juveniles do not feed and survive in the soil for several months.

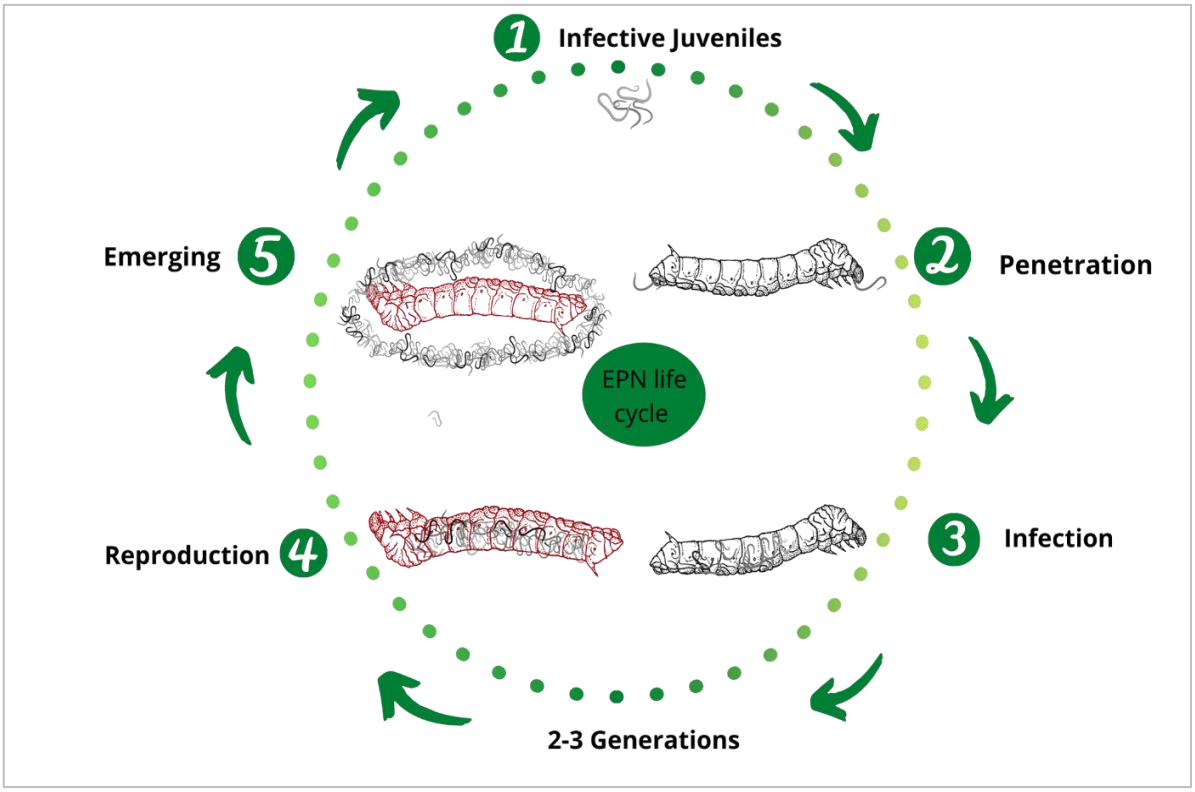


Figure 8. Diagram of the generalized life cycle of entomopathogenic nematodes (1) Infective juvenile (IJs) locate and infect a new host (2) The IJs penetrate and release the symbiont bacteria (3-4) The host is killed in 24-48 h and the IJs develop to adults (5) The IJs emerge from a depleted cadaver and released to the soil searching for a new host.

IV.2.2. Foraging Strategies

The behavior of entomopathogenic nematodes has been studied to make them better biological control agents. However, every aspect of an entomopathogenic nematode's life history is affected by ecological constraints which impact their biological control potential.

The natural reservoir for steinernematid and heterorhabditid nematodes is the soil that parasitizes soil-inhabiting insects. Soil is the only habitat these nematodes can develop and recycle to provide long-term management of insect populations. Host-habitat finding is essential in the infection process because it removes most potentially suitable hosts from becoming parasitized, but it is the least understood.

Once in the proper habitat, predators and parasites initiate host-finding. Two broad categories of foraging strategies have been recognized for entomopathogenic nematodes:

ambush (sit-and wait) and cruise (widely foraging) (Lewis et al., 1992), with a continuum between them. Cruisers allocate more of their foraging time to moving through the environment, scanning for resource-associated cues when moving or during short pauses. Ambushers are more effective than cruise foragers at finding resources with high mobility (Campbell et al., 2003; Lewis et al., 2006), whereas cruisers use a desiccation avoidance strategy and are more active during storage, have a higher basal metabolic rate, are less desiccation-tolerant, and are therefore less stable in the formulations. Cruise foragers, which actively seek hosts, would be less constrained by the type of soil; *H. bacteriophora* is found throughout the soil profile and does not show a diurnal pattern (Campbell et al., 1996). While ambush foragers dedicate more time to prolonged scanning pauses separated by shorter repositioning bouts. Foraging strategy influences the types of resources an organism is likely to encounter. Ambushers tend to use a desiccation tolerance strategy. They are less active during storage, have a lower basal metabolic rate, are more desiccation-tolerant, and are thus more stable in the formulations (Campbell et al., 2003; Lewis et al., 2006). Ambushers would be unable to nictate effectively throughout the soil *S. carpocapsae* is most frequently found in the surface or main 1 cm of soil (Campbell et al., 1996). Nematodes nictating at or near the soil surface would be poorly protected from environmental conditions, which at midday are likely to be unfavourable; *S. carpocapsae* isolations from soil show a diurnal pattern, being lowest at midday.

Foraging strategies of entomopathogenic nematodes are linked directly or indirectly to many biological phenomena such as the relevance of contact (Lewis et al., 1993) and volatile (Figure 9) (Lewis et al., 1993) host cues, sex ratio (Lewis and Gaugler, 1994), mode of movement (Campbell and Kaya, 1999; Campbell et al., 1999), metabolic rate (Lewis et al., 1995), and longevity (Lewis et al., 1995; Lewis et al., 1997) have been studied.

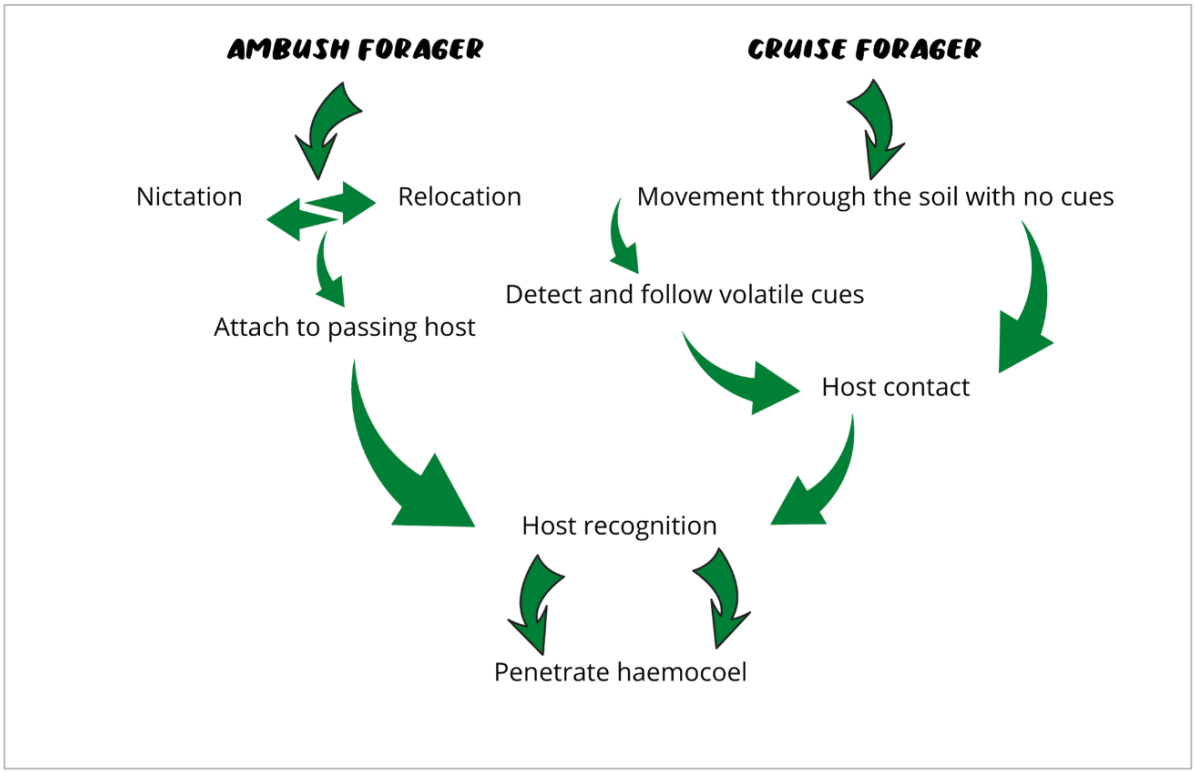


Figure 9. The order of events that occur during the foraging behaviour for ambushing and cruising entomopathogenic nematodes (modified from Gaugler, 2002).

IV.2.3. The Influence of Biotic and Abiotic Factors on EPNs

Many abiotic factors can change the occurrence and persistence of EPNs. These include natural physical or chemical characteristics (e.g., climate, soil pH, soil texture, soil structure) as well as those coming from human actions (e.g., physical or chemical disturbance) (e.g., physical or chemical disturbance). However, most investigations of soil effects on EPNs have concentrated on soil texture (i.e., the composition of soil solids by particle size range) rather than on soil structure (i.e., the arrangement of soil particles into aggregates of various sizes, geometry, and porosity) (Hillel, 1982).

In natural ecosystems, soil type might have more influence on heterorhabditids than steinernematids (Hominick, 2002). However, for *H. bacteriophora* in turfgrass, edaphic factors were relatively constant along transects and only moderately linked with EPN recovery (Campbell et al., 1998). In no-till and conventional–till maize fields in North Carolina, no

significant associations were identified between the occurrence of endemic *S. carpocapsae* or *H. bacteriophora* and soil organic matter, pH, or soil texture (Millar & Barbercheck, 2002).

Moisture is probably the essential abiotic factor influencing soil nematodes (Nickle, 1984). EPNs require appropriate soil moisture for survival and movement, but too much moisture may cause oxygen deprivation and impede mobility (Glazer, 2002). A few EPNs survive once the water has been lost from the substrate, demonstrating the Heterorhabditis limited potential to withstand desiccation (Solomon et al., 1999; Shapiro-Ilan et al., 2005). Otherwise, nematode viability is affected when suddenly exposed to dry soil, but if humidity is decreased progressively, the nematode can adapt and enter anhydrobiosis (Shapiro-Ilan et al., 2005; Koppenhöfer and Fuzy, 2007; Rohde et al., 2010). Moisture also plays a vital role in pest suppression by EPNs in a coastal prairie in California (Preisser and Strong, 2004). It might significantly impact EPN efficacy after applications in peaty versus mineral soils in Ireland (Williams et al., 2013).

Temperature is one of the critical factors affecting both entomopathogenic nematodes and their symbiotic bacteria, with temperatures above 32 °C generally being problematic and above 37 °C generally being fatal (Grewal et al., 1994). Furthermore, optimum temperatures for infection and reproduction vary among nematode species and strains, as well temperatures below 15 °C infections are not suitable, while at temperatures between 15 and 35 °C, infectivity is optimal (Saunders and Webster, 1999; Hazir et al., 2001; Morton and García-del-Pino, 2009).

Entomopathogenic nematodes distribution and abundance could be affected by many biotic factors. Soils contain rich and diverse communities of flora and fauna that could act as predators, parasites, and pathogens or competitors, such as nematophagous fungi, bacteria, protozoa, nematodes, mites, collembolans, and other microarthropods in soil (Ishibashi and Kondo, 1987; Barbercheck and Kaya, 1991a, 1991b; Sayre and Walter, 1991; Barbercheck, 1992; Baur et al., 1998; Strong et al., 1999). These organisms might influence the occurrence and persistence of EPNs in nature (Ishibashi and Kondo, 1987; Sayre and Walter, 1991; Duncan et al., 2007; Pathak et al., 2012).

Competition is defined as any mutually unfavourable relationship that does not directly require predation or parasitism (Wootton, 1994; Pianka, 1999). The competition hypothesis assumes that coexisting species that share limited resources would compete, and that competing species must diverge in resource utilization and reduce niche overlap for competitive exclusion

to be avoided for coexistence to remain. Surveys show that multiple species of EPNs can coexist with as many as five species being reported from a single site (Akhurst & Brooks, 1984; Campos Herrera et al., 2011; Duncan et al., 2003; Stuart & Gaugler, 1994). Coexistence would be expected when behavioural differences and variability in environmental factors enable strong niche separation and avoidance of competition. In laboratory and greenhouse studies, differences in foraging behaviours reduce competition among EPN species and permit coexistence (Koppenhöfer & Kaya, 1996a, 1996b). Specific EPN species might exhibit differential foraging strategies in differing environments and provide further scope for studies of species interactions, competition, and coexistence in diverse habitats (Wilson et al., 2012; Griffin, 2015).

The competition among entomopathogenic nematodes could be interspecific or intraspecific. The dynamics and ramifications of interspecific competition between EPN species are of particular interest because of the potential effects of biological control applications of either exotic or native nematodes on native nematode communities. This competition between EPN species might often be mediated by multiple factors and reflect complex interactions. According to Alatorre-Rosas and Kaya (1991), the result was caused by interference competition between the nematodes within the host cadaver, a direct effect. However, because of the symbiotic bacteria, the outcome for the nematodes might rather be understood as exploitative competition, an indirect impact in which one species indirectly affects a second species by decreasing the abundance of a shared resource.

Intraspecific competition could affect diverse aspects of the biology of EPNs, notably emergence patterns, foraging behaviours, and the dynamics of host invasion, establishment, and reproduction. For example, the emergence of IJs from the host cadaver usually starts unexpectedly, increases over the first few days, and involves tens or hundreds of thousands of IJs (e.g., Stuart et al., 1996; Rolston et al., 2006).

The pattern of IJ emergence and specific characteristics of IJs could be related to the population dynamics of the nematodes and their bacteria within the host cadaver, the accessibility and utilization of available resources, and other conditions and cues that stimulate the formation and release of IJs; and much of this could be associated with the intense intraspecific competition. Moreover, the emerging pattern sets the scene for dispersal, host discovery, and host colonization and could impact possible competition and cooperation among

IJs during the infection process. However, EPNs occur in many habitats and utilize a broad range of hosts (Kaya & Gaugler, 1993), and the temporal and spatial distribution of hosts could vary considerably among habitats or times of the year.

Intraspecific competition may also play a significant role in the commercial production of EPNs by influencing optimal inoculation rates and production settings in vivo and in vitro. Indeed, artificial rearing conditions could have an important influence on the development and reproduction of EPNs, alter the dynamics involved, and select an array of different traits from those that are important. For example, this kind of inadvertent selection has been documented for laboratory cultures of EPNs (Bilgrami et al., 2006; Stuart & Gaugler, 1996; Wang & Grewal, 2002), which could influence the establishment and persistence abilities of mass-reared nematodes when applied in the field.

EPNs and other microbial pest pathogens can compete for insect resources as well. For example, certain virus-infected insects will be infected by EPNs, but the insect cadavers have a brittle integument that can burst open and limit IJ generation (Kaya & Brayton, 1978; Kaya & Burlando, 1989). When EPNs fight for a host insect with other insect pathogens, the host generally dies, but EPN progeny may not be generated from the co-infected hosts (Barbercheck & Kaya, 1990, 1991b). For example, when insects are infected with the fungus *Beauveria bassiana* (Balsamo) Vuillemin and EPNs, the EPNs frequently outcompete the fungus; however, this depends on the target insect (species/developmental stage), exposure methods, concentration, timing, sequence of arrival, and the combination of EPN/EPF species (Shapiro-Ilan et al., 2004; Acevedo et al., 2007; Jabbour et al., 2011;Tarasco et , 2011; Hajek and Meyling, 2018).

IV.3. Entomopathogenic fungi: Biology and ecology

Entomopathogenic fungi (EPF) have been widely investigated as biological control agents of insect pests to improve crop protection sustainability. EPFs are found in the divisions Zygomycota, Ascomycota, and Deuteromycota (Hibbett et al., 2007; Gryganskyi et al., 2012; 2013; Humber, 2012; Barra-Bucarei et al., 2019), and the Chytridiomycota and Oomycota. Many of the genera of entomopathogenic fungi currently under research either belong to the class Deuteromycota and Hyphomycetes (Figure 10), including two of the most widely studied,

Beauveria bassiana (Balsamo) and *Metarhizium anisopliae* (Metchnikoff), both have been used for the biological control of a wide array of foliar and soil-borne invertebrate pests (Roy et al., 2010). In addition, EPF are native inhabitants of most terrestrial habitats, including natural and agricultural zones (Bidochka et al. 1998, McGuire and Northfield 2020). Consequently, EPFs can interact with arthropod hosts as parasites or saprophytes (Charnley and Collins, 2007).

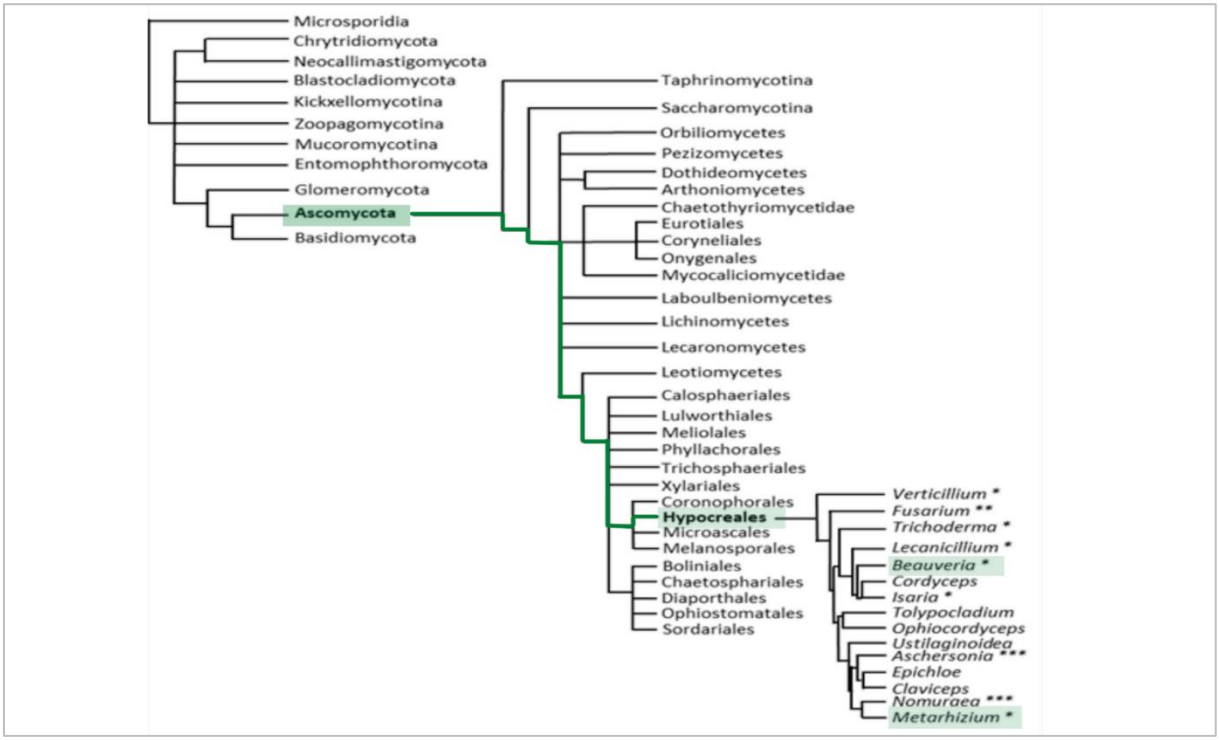


Figure 10. Taxonomy of the entomopathogenic fungi, following Hibbet et al. (2007), Humber (2012), Gryganskyi et al. (2012; 2013), Wang et al. (2016), and adapted from Fernández-Bravo (2017). From each group, a representative species with commercial use is presented *Commercialized in the conidial formula; **Micotoxins formula commercialized; ***No longer commercially available, no data (Lacey et al., 2015; Hernández-Rosas et al., 2020). The species used in this thesis are highlighted in green.

Asexually generated fungal spores or conidia are generally responsible for infection and are spread throughout the insect hosts' surroundings. After the conidia infect the host; the fungus produces various compounds responsible for host death (Oreste et al. 2012, Altinok et al., 2019) and other secondary metabolites with an insecticidal activity, such as destruxins, oosporein, beauvericin, bassianolide, and beauveriolides (Quesada-Moraga et al., 2006) to defend the cadaver from opportunistic (Strasser et al., 2000). Mycelia cover the entire body cavity during

the saprophytic phase, eventually producing emergent mycelia and conidiophores for passive spore dispersion (Figure 11) (Roy et al., 2006; Vega et al., 2008).

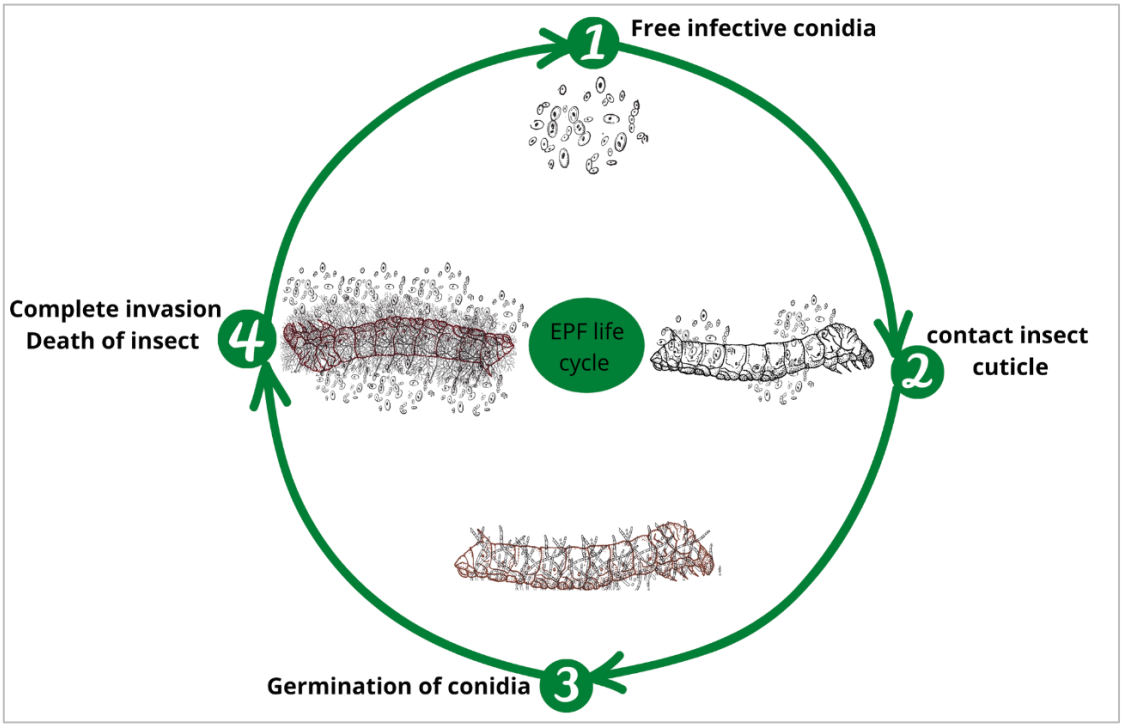


Figure 11. Schematic life cycle of an entomopathogenic fungi. (1) EPF conidia, (2) conidia contact with insect cuticle (parasitic phase), (3) the germination tube penetrates the arthropod hemocoel, leading to cell proliferation inside of it until the insect dies. (4) Hyphae go through corpse insect cuticle and produce conidiophores in the cadaver exterior, to help conidia dispersion (saprophytic phase).

A comprehensive and in-depth understanding of the influence of the soil environment (abiotic factors) on EPF efficacy and persistence is crucial to the fungi's long-term utility. Hence, the temperature is a significant factor in EPF efficacy, whether in the soil or on the phylloplane. For *B. bassiana* and *M. anisopliae* conidial germination and mycelial growth, which are critical events for efficacy, the optimal temperature is 25-28°C, with a rapid decrease as temperatures exceed 30°C, a slight decrease as temperatures decreases toward 15-18°C, and a more rapid subsequent decline, with little or no growth, below 8-9°C (Fargues et al., 1997, 1992).

Similarly, the influence of soil texture on infectivity of EPF has been examined experimentally in only a very few studies. For example, Studdert and Kaya (1990) reported

considerable differences in *Spodoptera exigua* pupal mortality resulting from exposure to *B. bassiana* conidia between peat and fine sandy loam and soils with significantly different pore structures. However, the difference between the two soils was significant only at higher moisture content. Infection rates among the pupae and adults differed between the two soils at higher moisture levels. It may be that interaction between soil moisture and the very different pore structures of the two soils affected fungal efficacy (Studdert and Kaya, 1990).

Agricultural inputs and practices considerably impact soil microorganisms, impacting size and dynamics, either directly through the nutrient provision or acidity or indirectly through plant growth stimulation and subsequent carbon flow from roots (Barron and Hawksworth, 1992). (Barron and Hawksworth, 1992). Crop cover, soil pH, fertilizers, rotations, pesticides, and tillage were recognized as significant human inputs that influence microbial diversity in soil (Kennedy and Gewin, 1997). For example, conventional tillage consistently and significantly lowered *B. bassiana*, *M. anisopliae*, and *Paecilomyces spp*. (Sosa-Gomez and Moscardi, 1994).

Biotic factors are essential in EPF ecology, particularly regarding the persistence of fungi. It is well-known that many soil organisms interact with EPF differently (Jaronski, 2007). However, relatively few studies have examined the direct interaction between EPF and specific members of the soil microbiota. *B. bassiana* and *M. anisopliae*, on the other hand, did not germinate on unsterilized sandy loam soil, duff (decaying organic matter layer), or fresh pine needles but did so when the conidia were transferred to the sterile medium or when these substrates had previously been sterilized (Walstad et al., 1970). According to Groden and Lockwood (1991), a reversible fungistasis of *B. bassiana* conidia in four soils was observed. Moreover, indirect interaction with microorganisms alters EPF growth and germination by specific bacterial toxic or secondary metabolites (Jaronski, 2007).

Also, various soil organisms from the mesofauna (collembolan, mites) and macrofauna (earthworms) can predate on them (Broza et al., 2001; Dromph, 2003; Brownbridge and Glare, 2007; Jaronski, 2007; Shapiro-Ilan and Brown, 2013; Chelkha et al., 2021; Zhou et al., 2021). Also, it is known that several common soil arthropods, certain nematodes, oribatid mites (Mitchell and Parkinson, 1976), prostigmatid mites (Lussenhop, 1992), pauropods (Scheller, 1990), and enchytrids (Didden, 1990) are fungivores, and presumably can target on EPF conidia and mycelium in the soil. In particular, there is evidence that the earthworms *Lumbricus*

terrestris, *L. rubellus*, and *Approrrectodea caliginosa* can feed extensively on fungi (Lee, 1985).

Finally, some of these partnerships can also be beneficial, such as the phoretic dissemination of the EPF by collembola or earthworms that can improve their distribution in the soil and, consequently, the capacity to be in contact with arthropod host (Dromph, 2003; Shapiro-Ilan and Brown, 2013).

IV.4. Nematophagus fungi: Biology and ecology

The NF are widespread in natural and agricultural soils globally and can be regarded as beneficial soil organisms for their potential to suppress pathogenic nematodes of crops and cattle (Nordbring-Hertz et al., 2006; Braga and Araújo, 2014; Li et al., 2015). There are more than 700 described species of Nematophagous fungi found in all major groups of fungi. However, most nematophagous asexual fungi, including nematode-trapping and endoparasitic species, are deuteromycetes. Moreover, regarding the sexual stages of several *Arthrobotrys*, *Monacrosporium*, and *Dactylella* species, they belong to the discomycetes (Ascomycetes) (Hsueh et al., 2013, 2017, Soares et al., 2018).

Each NF species is carnivorous and obtains its nutrients by predating or parasitizing nematodes and demonstrating a specific technique to trap the nematodes, such as adhesive nets, branches, knobs, or hyphae constricting rings, zoospores, or appressoria (Figure 12) (Nordbring-Hertz et al., 2006). NF are classified into three main groups:

1) **Endoparasites**: They are primarily obligate parasites and, in most cases, have a restricted host range. They infect nematodes by their spores, either through ingestion or attachment to the cuticle (Morton et al., 2004). Certain endoparasites create zoospores attracted to the nematodes before adherence and encystment on the cuticle surface.

2) **Predators or trap-forming fungi**: this group is the most studied, including the genera *Arthrobotrys*, *Duddingtonia*, and *Monacrosporium*. Nematode-trapping fungi are generalists, and they can infect many different nematode species (Nordbring-Hertz et al., 2006). To catch nematodes, they produce unique extracellular structures of adhesive networks and tridimensional traps, which can be differentiated into six types: (a) non-differentiated adhesive hyphae, (b) ramifications of hyphae that undergoes anastomosis, forming three-dimensional

adhesive networks, (c) adhesive ramifications, which, at times, can join together forming simple two-dimensional adhesive networks, (d) adhesive nodules, (e) constricting rings, and (f) non-constricting rings (Yang et al. 2007a, b). The constricting ring is the only one that actively catches nematodes.

3) **Opportunistic fungi that parasitize eggs, cysts, and female nematodes**: this group is used to decrease nematode and helminth populations because they reduce levels of viable eggs in the soil (Frassy et al. 2010; de Mello et al., 2014; Braga and de Araújo 2014). Generally, include several Trichoderma spp., *Purpureocillium lilacinus*, and *Dactyella ovoparasitica*. Their hyphae penetrate the eggshell through the tiny pores in the vitelline layer, causing changes in the permeability of the shell and expanding its volume. The hyphae then penetrate through the adjacent layer of chitin and lipid. They thus colonize the egg's inside and the developing larvae (Frassy et al. 2010; Dallemole-Giaretta et al. 2012; Araujo et al., 2013).

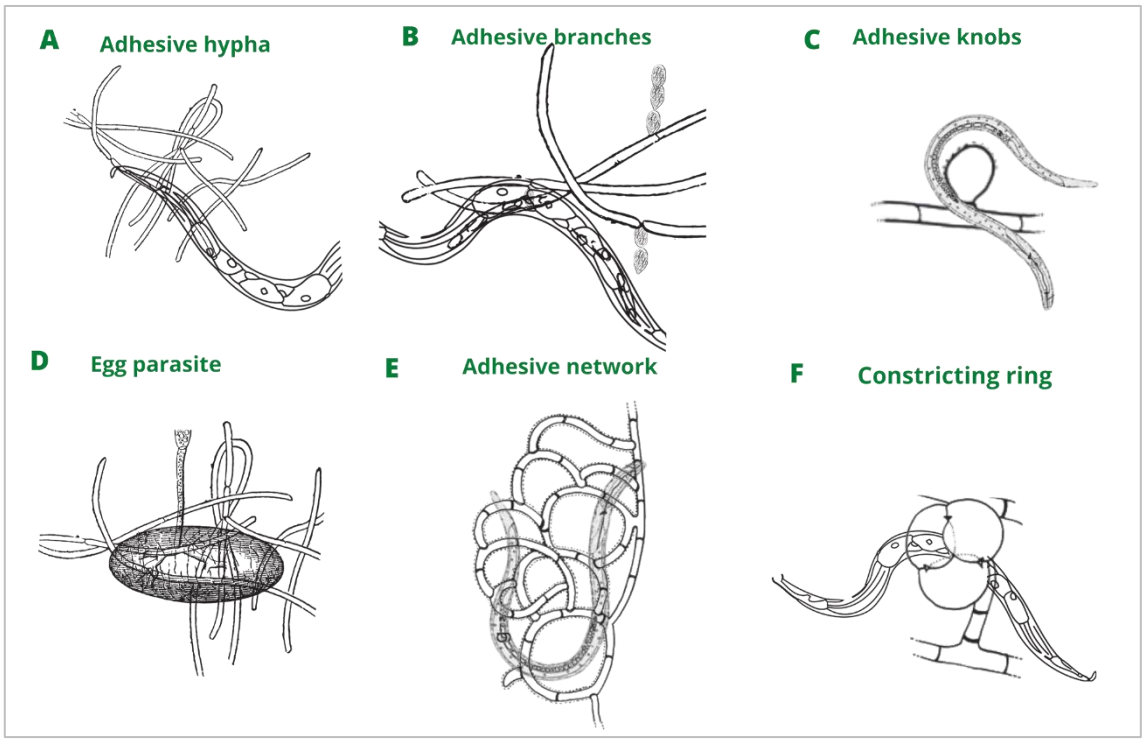


Figure 12. The trapping structures of nematophagous fungi with (a) Nematode attached to the adhesive hypha of *Stylopage hadra* by means of globular protuberances and the body filled with trophic hyphae. (b) Adhesive branches fused to form scalariform network in *Monacrosporium gephyrophagum*. Nematode filled with assimilative hyphae attached to the adhesive branch is seen. (c) Nematode captured by adhesive knobs of *Monacrosporium parvicollis*. (d) Root-knot

nematode egg parasitized by *Purpureocillium lilacinum*. (e) Freshly captured nematode in 3-D adhesive network of *Arthrobotrys conoides*. (f) Nematode captured in constricting rings of *Arthrobotrys brochopaga* at its head and tail region. (Modified from Saxena, 2018).

In soil habitats, various abiotic factors affect the growth rate and competitive saprobic capacities of NF, such as organic materials, temperature, pH, potassium, electrical conductivity, accessible water content, vitamins, biotin, abscisic acid, sodium nitroprusside, and metals (Pathak et al., 2017). Therefore, the trapping fungus can respond positively to some organic substrates, such as lupine leaves, grass leaves, dead isopods, dead moth larvae, isopod faeces, deer faeces, shrimp shells, and powdered chitin (Nguyen et al. 2007). Therefore, low C: N ratios usually induced considerable increases in *Arthrobotrys oligospora* populations, whereas those with higher C:N ratios did not. *Arthrobotrys oligospora* and *Arthrobotrys thaumasia*, which build adhesive networks, *Drechslerella anchonia*, *D. dactyloides*, *D. bembicodes* and *D. doedycoides*, which form constricting rings, require thiamine for growth (Satchuthananthavale and Cooke 1967b).

Temperature and pH can also alter the radial growth and predatory activity of various isolates of nematode-trapping fungi. They may grow in a medium with a pH of 5.0 to 8.0 at 10–30°C. The ideal conditions for fungal development were pH 6.0–6.5 and 20–25°C. Fernandez et al. (1999) discovered that all nematode-trapping fungal isolates examined showed a greater growth rate at 20°C. At 10 and 15°C, all *Arthrobotrys flagrans* isolates displayed similar radial development patterns at both constant and fluctuating temperatures. However, at 20°C, they grew significantly faster at constant than at variable temperatures.

Nematophagous fungi occupy the rhizosphere upper 20 cm of the soil and appear to be almost absent below 40 cm, and so do most of the fungus soil population (Persmark et al., 1996). The rhizosphere could be an aria of biotic interaction that affects the NFs. Many nematode-trapping fungi have been found to occur more frequently in the rhizospheres of several plants, especially leguminous plants, than in root-free soil. The ability of the nematophagous fungi to grow in the rhizosphere is of great importance for its capacity to control plant-parasitic nematodes. However, a correlation exists between nematophagous fungi and the number of nematodes in some soils.

Experiments in soil microcosms utilizing the endoparasitic fungus *H.rhossoliensis* and plant-parasitic nematodes have demonstrated that fungal parasitism depends on the nematode density. However, there is a relatively long-time lag in the response of the fungal population to changes in the number of nematodes (Jaffee et al., 1992). Otherwise, the NFs interact also with entomopathogenic nematodes; Bueno-Pallero et al. (2018) showed that the combined applications of the EPN, *Steinernema feltiae*, and NFs, *Arthrobotrys musiformis* and *Purpureocilium lilacinum*, or EPN, *S. feltiae*, + EPF, *Beauveria bassiana*, +NFs were additive or even synergistic, respectively and IJs of some EPN species can protect themselves against NFs.

Apart from attacking nematodes, nematophagous fungi also can infect other fungi. Nematode-trapping fungus such as *A. oligospora* attacks their host fungi by coiling the hyphae of the nematode-trapping fungi around the host hyphae, which disintegrates the host cell cytoplasm without penetration of the host. It was demonstrated that nutrient transfer occurs between the nematode-trapping fungus *A. oligospora* and its host *R. solani* using radioactive phosphorous tracing (Olsson and Persson, 1994). *A. oligospora*, *P. chlamydosporia*, and other nematophagous fungi can colonize plant roots (Bordallo et al., 2002). The fungus grows inter- and intracellularly and generates appressoria when entering plant cell walls of epidermis and cortex cells but never reaches vascular tissues (endophytic) (Monfort et al., 2005).

PART 2: PRACTICAL PART

Chapter 1: MATERIALS AND METHODS

I. Cutaneous excreta of the earthworm *Eisenia fetida* (haplotaxida: lumbricidae) might hinder the biological control performance of entomopathogenic nematodes.

I.1. Entomopathogens nematode, earthworms, cutaneous excreta, insect, and substrates

Six EPN species were tested: two heterorhabditids (*Heterorhabditis zealandica* Btw population, and *H. bacteriophora* AM-203 population) and four steinernematids (*Steinernema feltiae* AM-25 population, *S. khuongi* Arc population, *S. glaseri* NC population, and *S. carpocapsae* ALL population) (Figure 13). EPNs were cultured in last instar larvae of *Galleria mellonella* (Lepidoptera: Pyralidae) reared at University of Algarve (Portugal). The IJs were harvested in mineral water and stored at 12-14°C (Figure 13a_b). All nematodes were used within two-week after harvest. Experiments were performed with the last instar larvae of *G. mellonella* (250-300 mg) (Figure 14).

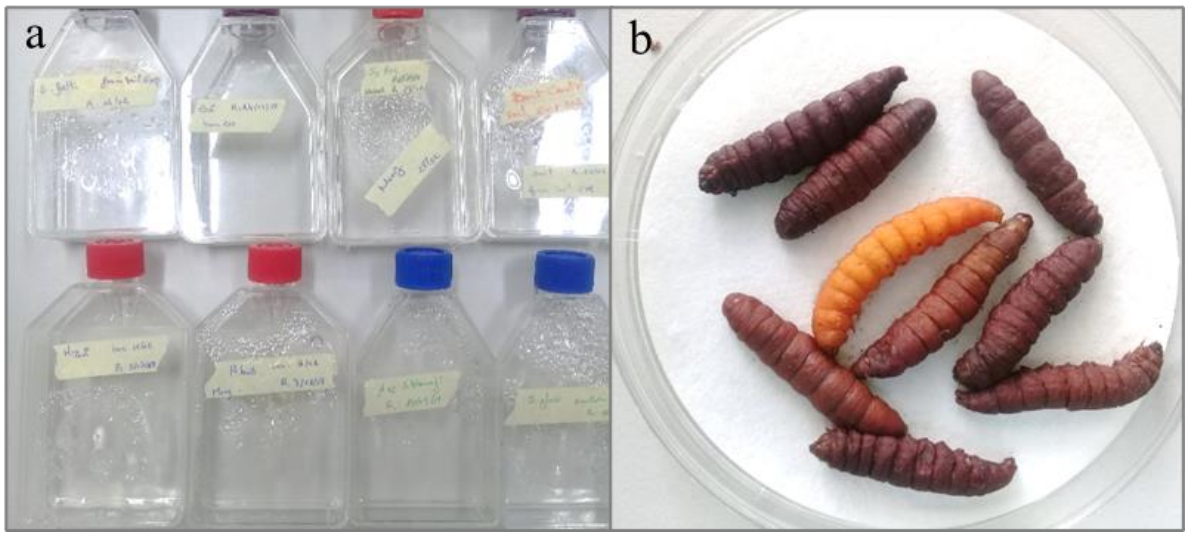


Figure 13: (a) cell culture flask for entomopathogenic nematodes, (b) larva infected by *H. bacteriophora*.

Figure 14: *Galleria mellonella.*

The earthworm *E. fetida* (Figure 15) was obtained from the commercial stock of natural worms' nurseries for vermicomposting at "O Minhocario" (Pedro José Lanza, Lisbon, Portugal). All the earthworms were adults of similar size (0.3-0.5 g and length 5.0-5.5 cm). Earthworms were maintained in the shipping substrate supplemented with fresh vegetables and autoclaved soil (see below) in a moistened environment without a photoperiod. They were used within 2 weeks after their arrival after being starved for 24 hours in moistened autoclaved soil to avoid cross-contamination with their casts. Fresh CEx was obtained under the effect of a petroleum ether saturated atmosphere (Figure 16) (El Harti et al., 2001), recovered in distilled water and maintained on ice for immediate use. The earthworms from which CEx was obtained were discarded. Fresh CEx and new earthworm were employed in every experiment/trial.

Figure 15: *E. fetida.*

Two different media were used in the experiments described below: (i) pure mineral sand (Vale do Lobo, Loulé, Portugal) and (ii) commercial soil (MaxMat", Faro, Portugal; 92% sand, 7% silt, 1% clay; OM = 46% (v:v), pH = 5.8) (Figure 17). Both substrates were first autoclaved for 1 hour (two times) and thereafter oven-dried in an oven at 40ºC with ventilation. The substrates were maintained in the laboratory for more than a week under laboratory conditions before being used (Chiriboga et al., 2017).

Figure 16: *E.fetida*'s cutaneous excreta obtained under the effect of a petroleum ether saturated atmosphere.

I.2. Impact of the presence of earthworms or their cutaneous excretion on the potential biocontrol activity of entomopathogenic nematodes

Following Campos-Herrera et al. (2006), we employ a laboratory proxy for the proof of concept of the interaction between the earthworm or their CEx and the EPN. The experimental unit was a plastic Petri dishes (9 cm diam.) filled with 45 g of autoclaved soil moistened to 24% (w/v) with distilled water and each treatment had 6 replicates (Figure 17). The treatments were: (i) control (distilled water), (ii) each of the EPN species, (iii) earthworms, (iv) CEx, (v) EPN+earthworms, and (vi) EPN+CEx. A preliminary experiment with serial concentrations of IJs in the same conditions were performed to establish the best range to perform the experiment, finally adjusted to 48 IJs per Petri dish. Two earthworms or the CEx corresponding to 2 earthworms (in 1 mL) were added per Petri dish in the corresponding treatments. The Petri dishes were sealed with parafilm and incubated at 24ºC in the dark for 3 days. Thereafter, each

Petri dish was open to place 5 *G. mellonella* larvae. Larval mortality was recorded every two days during 8 days, removing the cadavers and placing them individually in new dishes. This first part of the experiment assessed the impact on the EPN performance when combined with earthworms or their CEx, measured as larval mortality and time to kill. In addition, we evaluated whether the earthworm's cast could contain IJs, following Shapiro-Ilan and Brown (2013). For this part, at the end of the soil exposure, earthworms were cleaned, rinsed with tap water, surface sterilized in 70% ethanol, and followed by another bath with distilled water (see Shapiro-Ilan and Brown, 2013). Subsequently, each of the 2 earthworms included in the plates with soil (with or without nematodes) were then placed in 90 mm Petri dishes lined with moistened filter paper, sealed with parafilm and allowed to cast for 48 h at 24ºC in the dark (Figure 17) (Shapiro-Ilan and Brown, 2013). Thereafter, 3 *G. mellonella* larvae were added per dish, and the mortality was checked for another 6 days. The whole experiment was performed for each of the 6 EPN species at 2 different times, with new earthworms, freshly produced nematodes and hosts as well as soil preparation.

Figure 17: Commercial soil (MaxMat", Faro, Portugal; 92% sand, 7% silt, 1% clay; OM = 46%, pH = 5.8) inoculated with the experiment treatments.

I.3. Impact of the cutaneous excreta in the infectivity and reproductive success of the entomopathogenic nematodes

In this experiment, we used two 24-well plates (Falcon Multiwell, 24 well Polystyrene, Corning Incorporated-Life Sciences, Duham, USA) per treatment, and 10 wells per 24-wells plate (n = 20) (Figure 18). Fresh CEx equivalent to 5 earthworms for each treatment was prepared (El Harti et al., 2001), splitting in aliquots of 100 µL per well (equivalent to have the amount of CEx produced by ¼ of earthworm per well). Each well was filled with 1 g of sterile sand, and moistened to account for a final volume of 200 µL, being water, CEx, IJ suspension or their combination depending on the treatment: (i) control (distilled water), (ii) EPNs (suspension of IJs in water), (iii) CEx (100 µL CEx suspension and 100 µL distilled water), and (iv) EPNs+CEx (suspension of IJs, 100 µL of CEx, and remaining volume as distilled water, if necessary). For each of the six EPN species, two different IJ inocula were tested (Campos-Herrera et al., 2015): low EPN concentration (3 IJs per well, equivalent to 1.5 IJs/cm2) and high EPN concentration (20 IJs per well, equivalent to 10 IJs/cm2). For low EPN concentration inoculum, the IJs were first hand fished (using insect pins No. 000) and placed them in a 50 µL drop of distilled water. In the case of the 20 IJs per well, a nematode suspension was prepared and adjusted to 100 µL per well. Then, in each well, 1 g of sterilized sand was added, including one larva of *G. mellonella* after 24 hours' incubation in a dark room at 22°C. Thereafter, larval mortality was recorded for a six days period, removing the cadavers, cleaning them with tap water and placing them individually in White traps (White, 1927). The nematode emergence from the cadavers was observed every 2–3 days over a period of 30 days. Once the first nematode emergence was observed, the final production of IJs per cadaver was counted 9–10 days after the onset of emergence (Blanco-Pérez et al. 2019). For each of the nematode species and concentrations, the experiment was conducted at 2 different times, with new earthworms, freshly produced nematodes and hosts as well as arena preparation.

Figure 18: 24-wells plates (Falcon Multiwell, 24 well Polystyrene, Corning Incorporated-Life Sciences, Duham, USA) inoculate with treatments.

I.4. Statistical analysis

All variables expressed as frequencies were arcsine transformed before statistical analysis, while quantitative variables were log (x +1) transformed. Thereafter, we confirmed that the data of the two independent trials per experiment and species could be pooled by two way ANOVA (data not shown). Then, to test the impact of the presence of earthworms or their CEx on the potential biocontrol activity of EPNs, we analyzed the effect of each treatment (three levels: IJs applied alone, IJs in the presence of earthworms, and IJs in the presence of CEx) on the cumulative *G. mellonella* larval mortality (2 to 8 days' post-exposure), by using one-way ANOVA and Tukey's HSD test. Similarly, for the study of the impact of the CEx on EPN infectivity and reproduction, we analyzed the effect of the presence or the absence of CEx, and the IJ inoculum (3 or 20), on the following variables: (i) cumulative larval mortality (for 2 to 6 days' post-exposure), (ii) frequency of larvae producing IJs, (iii) days needed for IJs to emerge from cadavers, and (iv) number of IJs produced per larva, by using two-way ANOVA test. We performed all the analyses with SPSS 24.0 (SPSS Statistics, SPSS Inc., Chicago, IL, USA). Statistical differences were assessed for $P < 0.05$. We used least square means ± S. E. as descriptive statistics.

II. Earthworms and their cutaneous excreta can modify the virulence and reproductive capability of entomopathogenic nematodes and fungi

II.1. Organisms and substrates

We employed three earthworm species: the redworm *Eisenia fetida* (Haplotaxida: Lumbricidae) (0.3–0.5 g and 5.0–5.5 cm length), the common earthworm *Lumbricus terrestris* (Haplotaxida: Lumbricidae) (0.2-0.3 g and 4.2–5.5 cm length), and the Indian blues *Perionyx excavatus* (Haplotaxida: Megascolecidae) (0.9–1.3 g and 12–16 cm length) (Figure 19). All earthworms were adults of similar size, regularly provided from a commercial stock of natural worms' nursery for vermicomposting at O Minhocario (Pedro José Lanza, Lisbon, Portugal). The specimens were kept under laboratory and dark conditions (22-24ºC) and reared in the original shipping soil with fresh vegetables. Earthworms were used within two weeks after their arrival after being starved over 24 h in a moistened autoclaved soil (see below) to avoid cross-contamination with their casts. We obtained the fresh CEx by exposing the earthworms to the effect of a petroleum ether saturated atmosphere (El Harti et al., 2001), recovered it in distilled water, and maintained it on ice for immediate use. The final volume of CEx and distilled water combination was the same for all the earthworm species. We employed fresh CEx and new earthworm for each experiment and trial.

Figure 19: *(a) Eisenia fetida (b) Lumbricus terrestris* (c)*Perionyx excavatus.*

The EPN populations used for this study were native to vineyards from La Rioja, Spain (Blanco-Pérez et al., 2020): two steinernematid species *Steinernema feltiae* RM107 (ITS region GenBank accession number: MW480131) and *Steinernema riojaense* RM30 (ITS region GenBank accession number: MK503133), and the heterorhabditids species *Heterorhabditis*

bacteriophora RM102 (ITS region GenBank accession number MW480132). EPNs were cultured in the last instar larvae of *Galleria mellonella* (Lepidoptera: Pyralidae), reared at the Instituto de Ciencias de la Vid y del Vino (ICVV), Logroño (Spain). The infective juveniles (IJs) that emerged from the insect cadavers were harvested in tap water and stored at 14ºC and dark conditions until use. We employed two-week harvest nematodes for each experiment and trial.

The populations of the two EPF species investigated, *B. bassiana* DF83 (ITS region GenBank accession number: MG515530) and *M. anisopliae* DF89 (ITS region GenBank accession number: MN808333), were native from Algarve, Portugal (Bueno-Pallero et al., 2018, 2020). Both EPF species were cultured on 90 mm diam. Petri dishes with Potato Dextrose Agar (PDA, Biokar) at 25±1 °C, not sub-cultured more than once during the study (Shapiro-Ilan et al., 2004). The fungal material was stored at 4 °C and dark conditions until use (Goettel et al., 2000). The conidia suspensions were prepared using PDA culture (3-4week old), suspended in Ringer's solution and 0.05% Tween 80° (PanReac ApplChem) (Doberski and Tribe, 1980), and adjusted by hemocytometer (Neubauer improved) (Bueno-Pallero et al., 2018). We used the last instar larvae of *G. mellonella* (300-350 mg size) to test the virulence and reproductive potential of EPNs and EPF.

We employed two different substrates as arenas for the experiments performed (as described below): (i) pure mineral sand (Vale do Lobo, Loulé, Portugal) and (ii) commercial soil (MaxMat", Faro, Portugal; 92% sand, 7% silt, 1% clay; 46% SOM and pH 5.8). Both substrates were autoclaved twice, extended in trays, oven-dried in an oven at 40ºC with ventilation, and stored under laboratory conditions for over a week before being used (Chiriboga et al., 2017).

II.2. Interaction between earthworms, their cutaneous excreta and entomopathogenic nematodes or fungi in sterilized soil

We evaluated the impact of the presence of earthworms or their CEx on EPN and EPF activity using Petri dishes (9 cm diam.) filled with 45 g sterilized soil moistened to 24% (w/v) with distilled water as experimental units (Campos-Herrera et al., 2006; Chelkha et al., 2020). Following El Harti et al. (2001) and Chelkha et al. (2020) experimental procedures, 1 mL of

fresh CEx secreted from two earthworms was prepared per sample. For each earthworm species, the treatments (n = 6) were: (i) a control (distilled water), (ii) two live earthworms, (iii) the CEx secreted from two earthworms, (iv) a single EPN or EPF species, and the combinations (v) two live earthworms + a single EPN or EPF species, and (vi) the CEx secreted from two earthworms + a single EPN or EPF species. We established the EPN inoculum at 48 Ijs per Petri dish (0.8 Ijs/cm^2) (see Chelkha et al., 2020) and the EPF inoculum at 2-4 x 10^7 conidia per Petri dish (3.2-6.3 x 10^5 conidia/cm^2) after running a preliminary experiment with serial concentrations for the two EPF species investigated. The experimental setup was incubated at 24°C in the dark for three days. Thereafter, we added six last instar larvae of *G. mellonella* to every Petri dish. The larval mortality was checked daily for a week for EPNs, and every two days for two weeks, starting after day four post-application, for EPF. At the end of the experiment, all the earthworms were removed and verified that all the specimens survived and were in good conditions. All insect cadavers were cleaned with distilled water and placed in White traps to recover EPN emergence (Figure 20a-b)or EPF growth (Figure 21a-b) (Campos-Herrera and Lacey, 2018). The experiment was conducted twice with freshly produced organisms, CEx, and soil preparations.

To test the effect of earthworms and their CEx on EPN and EPF virulence and reproductive rates, we ran generalized linear models (GLM) with a binomial distribution (logit-link function). We ran two independent analyses. First, we tested for the effect of the earthworm species (two or three levels for EPF and EPNs, respectively), the earthworm treatment (two levels: two individual earthworms versus the CEx extracted from two earthworms), and their interaction (all fixed factors). Consecutively, we tested for each earthworm species combination (two earthworms or their CEx) versus single applications of entomopathogens (control treatments). We performed all analyses with SPSS 25.0 (SPSS Statistics, SPSS Inc., Chicago, IL, USA), using $P < 0.05$ for assessing statistical differences. We used least-square means ± SE as descriptive statistics.

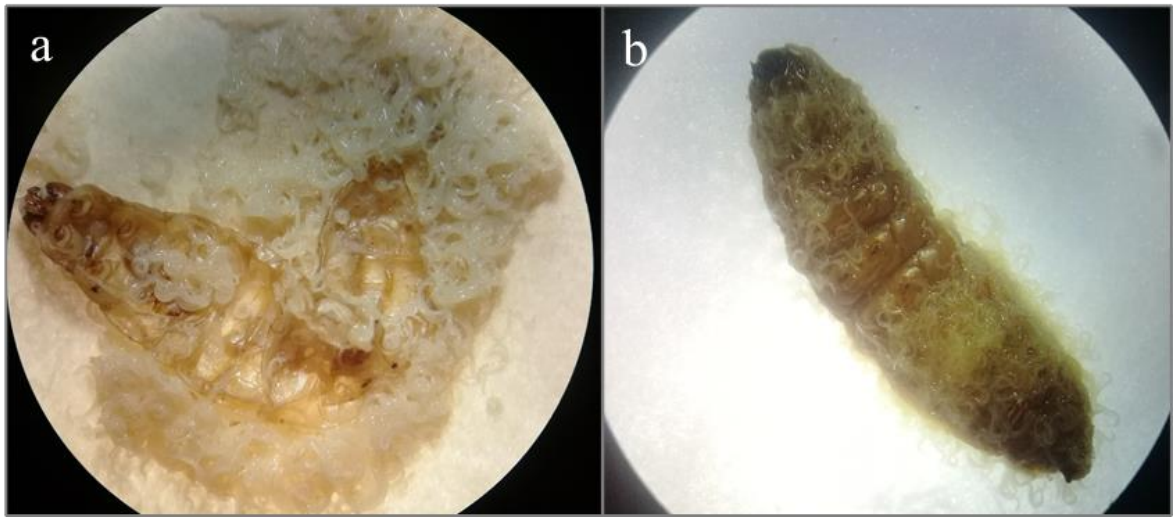


Figure 20: (a) IJs of *Steinernema riojaense* emerged from *G.mellonela* larva, (b) IJs of *Steinernema feltiae* emerged from larva.

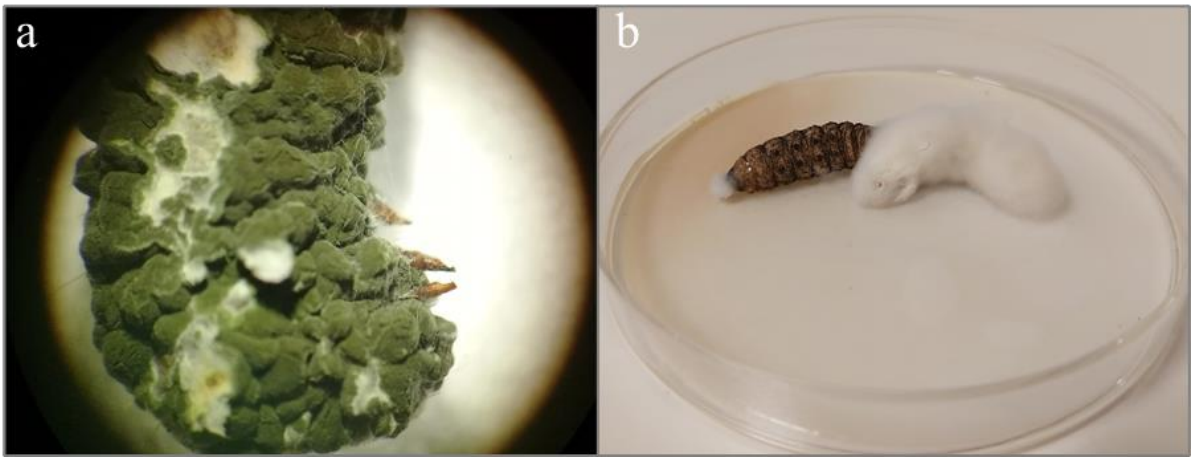


Figure 21: (a) *M.anisopliae* (b) *B.bassiana* emerged from *G.mellonela* larva.

II.3. Impact of the cutaneous excreta from earthworms on entomopathogenic nematodes and fungi applied at different concentrations

We employed 24-well plates (Falcon Multiwell, 24 well Polystyrene, Corning Incorporated-Life Sciences, Duham, USA) to evaluate the impact of CEx on EPN and EPF virulence and reproductiveness. New fresh CEx secreted from five earthworms (see Chelkha et al., 2020) was prepared per treatment, splitting in aliquots of 100 µl per well. For each

earthworm species, the treatments (n=24) were: (i) a control (distilled water), (ii) CEx suspension of each earthworm species, (iii) a single EPN or EPF species (IJs or conidia suspension), and (iv) the CEx of each earthworm species + only EPN or EPF species. We added 200 µl final volume per well, using two 24-well plates per treatment and selecting 12 alternated wells in each one (Klingen et al., 2002). Following Campos-Herrera et al. (2015) and Blanco-Pérez et al. (2019) procedures, we tested for two EPN concentrations: 3 IJs per well (1.5 IJs/cm²) and 20 IJs per well (10 IJs/cm²); and two EPF concentrations (determined after a preliminary test): 1-2 x 10^7 conidia/mL (1-2 x 10^6 conidia per well) and 1-2 x 10^8 conidia/mL (1-2 x 10^7 conidia per well). Plates were incubated at 24°C in the dark for 24 h. Then, we added 1g of sterile sand and a *G. mellonella* larva per well. Then, all plates were incubated again for 48 h at 24°C in the dark. The larval mortality was checked every two days, for six days for EPNs, and two weeks, starting after day six post-application, for EPF. All insect cadavers were cleaned with distilled water, placed in White traps, and incubated at 22-24°C in the dark. The EPN emergence and EPF growth were checked three days a week over 30 days (Chelkha et al., 2020). The experiment was conducted twice with freshly produced CEx, organisms, and substrate preparations.

In addition, we evaluated the viability of EPF conidia for the low concentration application after 48h exposition to earthworms' CEx. In a subsequent experiment conducted as described above, we added the 200 µl final volume of each treatment into six Eppendorf tubes instead of 24-well plates. After a 48h incubation at 24°C in the dark, we made three serial dilutions from each Eppendorf tube to reach a final dilution of 1-2 x 104 conidia/mL that we subsequently plated in 9 cm diam. Petri dishes with selective media were prepared as described by Doberski and Tribe (1980) with some modifications. Specifically, we dissolved 40 g glucose, 40 g nutrient agar (DIN, VWR Chemicals), 0.01 g crystal violet (Merck), and 1 g chloramphenicol (VWR Life Sciences) in 1L distilled water and autoclaved it. The cycloheximide dilution (PanReac ApplChem) at 10 g/L concentration was prepared and autoclaved separately. Once they cooled down, and right before we poured on the Petri dishes, we added 25 mL cycloheximide dilution per 1L of media. We spread 20 µL of each last serial dilution tubes into two selective media Petri dishes (12 Petri dishes per treatment). Finally, after ten days of incubation at darkness at 22-24ºC for the growth of EPF colonies, we counted for the germination of conidia (Figure 22). The experiment was conducted twice with freshly produced CEx and new culture/suspension for the two EPF species.

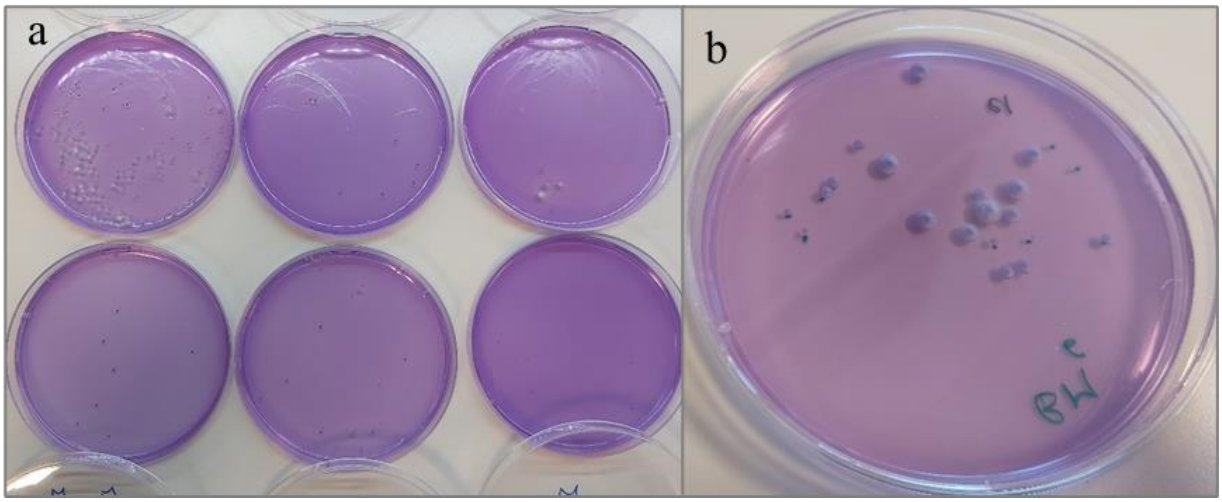


Figure 22: (a) *B.bassiana* (b) *M.anisopliae* growth in selective media after treatments.

To test the effect of the CEx from earthworms on EPN and EPF virulence and reproductive rates, we ran generalized linear models (GLM) with a binomial distribution (logit-link function). We ran two independent analyses to test for differences among earthworm species (two or three levels for EPF and EPNs, respectively) and between only applications of entomopathogens (control treatments) and their combination with earthworm CEx. Finally, after log (x+1) transformations, we performed two-way ANOVA and Tukey's HSD tests on numbers of conidial germination in selective media. We performed all analyses with SPSS 25.0, using $P < 0.05$ for assessing statistical differences. We used least-square means ± SE as descriptive statistics.

III. New insights on the impact of earthworm extract on the growth of beneficial soil fungi: species-specific alteration of the nematophagous fungal growth and limitation of an entomopathogenic fungus

III.1. Earthworms and fungi

Earthworms were obtained directly from the soil by digging and manual labour in the Akrach-Rabat region of Morocco (latitude 33° 56′ 15 N, longitude 6° 46′ 43 W). Sampling was conducted between 8:00 and 10:00 AM, when earthworms are most active near the surface (0.25 m and 0.5 m depth). The species *Aporrectodea molleri* was identified morphologically (determination key) and genetically based on the cytochrome c oxidase subunit I gene (COX1, GenBank accession number MT878074) in collaboration with the "Grupo de Ecologia Animal

(GEA)" at the University of Vigo-Spain (Yakkou et al., 2021). Individuals of *A. molleri* were kept in good condition in a plastic pot with soil and high humidity before being transferred to the laboratory.

We evaluated two species of NFs: a trapping fungus *Arthrobotrys musiformis* Drechsler (Orbiliales: Orbiliaceae; strain 11, GenBank accession number KJ938572) provided by L.W. Duncan (University of Florida, USA), and an egg- or cyst-parasitic fungus *Purpureocillium lilacinum* (Thom) (Hypocreales: Ophiocordycipitaceae; ARSEF 9357, GenBank accession number KJ938575), provided by ARSEF culture collection in Ithaca, New York (Luangsa-Ard et al., 2011). Also, we evaluated a species of entomopathogenic fungus (EPF) *Beauveria bassiana* (Balsamo) Hypocreales: Clavicipitaceae, native to the Algarve; GenBank accession number MG515530) supplied by F.A. Bueno-Pallero (Universidade do Algarve, Portugal). Fungal species were first grown in 90 mm diameter Petri dishes with potato dextrose agar (PDA, Biokar) at 25 ± 1 °C. The 3- to 4-week-old fungal material was stored at 4 °C and in the dark until use no longer than 6 weeks.

III.2. Preparation of earthworm extract and culture media

Freshly recovered earthworms were carefully cleaned with tap water, passed through absorbent paper to eliminate the excess of water and weighed. Individuals providing a total of 100 g were directly oven-dried at 60 °C in a glass Petri dish (fresh earthworm, FE). Another 100 g was kept in the dark in a Petri dish with filter paper with high humidity for fasting to ensure cleaning of the intestinal contents. After 8 to 10 days, we washed the worms and put them in a 60 °C oven (worms without intestinal contents, EDG). After seven days in the oven, the earthworm tissue was completely dried. We ground it to a powder with a mortar and pestle and dissolved it by shaking it with distilled water in an Erlenmeyer flask. The solution was placed in the oven at 70 °C for maceration in distilled water baths. At each maceration, the supernatant was collected, then the maceration baths were continued by adding distilled water in the same way until there was no more substance to dissolve, and the water became clear. The successive macerates were accumulated and filtered through cotton. The filtrate was dried in an oven (70 °C) and weighed to obtain the dry weight. The whole process takes about 15 days. From the 100 g fresh weight of earthworm (EDG), 11% of the extract is recovered on a dry weight basis, and from the 100 g fresh weight of FE, 13% of the earthworm extract is collected on a dry weight basis (Yakkou et al., 2021b).

Six concentrations were prepared for each medium: C1 = 40 g/L C2 = 20 g/L, C3 = 10 g/L C4 = 5 g/L, C5 = 2.5 g/L, and C6 = 1.25 g/L of the extracts and added 14 g of bacteriological agar (Agar No.1, 500g, Oxoid) to solidify the media (Figure 23). The solution was autoclaved at 121 °C for 15 min. Then, 16 mL of each culture medium was poured into a 9 cm diameter plastic Petri dish (Figure 23). In addition, potato dextrose agar (PDA, C = 24 g/L; the composition 4 g (from 200 g infused potato) potatoes, 20 g dextrose, 20 g agar powder) and heart-brain agar (BHI Brain Heart Infusion Agar, dehydrated, Oxoid, C = 3.7 g/l; the composition per 1L: 10g of peptone, 10g of beef heart infusion; 7.5g calf brain infusion, 2g dextrose; 2.5g of Na2HPO4, 5g of NaCl, 15g of bacteriological agar) were prepared as the additional media proved suitable for the three fungi studied. A liquid medium was prepared using the same concentration as the previous one, and for the control, we prepared potato dextrose (PD) and Brain Heart Infusion (BHI).

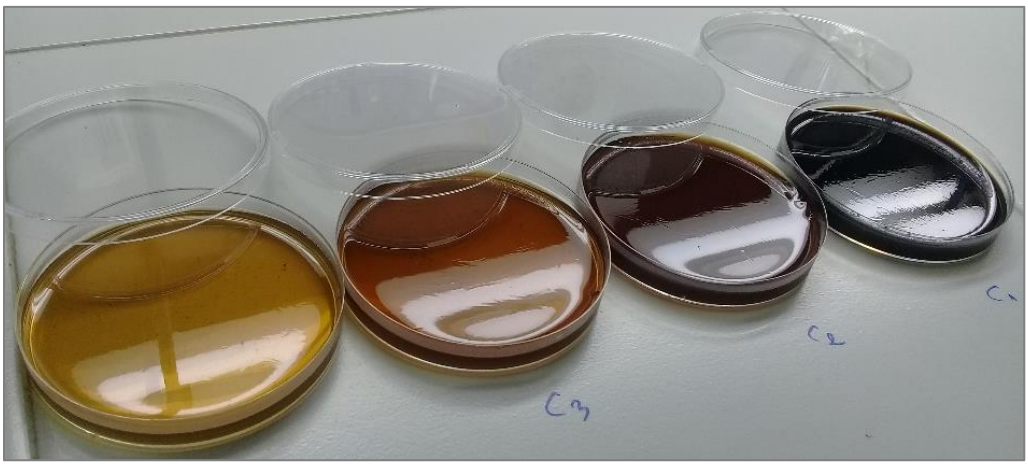


Figure 23: Earthworm extract media in four different concentrations.

III.3. Evaluation of mycelial growth, reproduction, and germination of conidia for *Arthrobotrys musiformis*, *Purpureocillium lilacinum* and *Beauveria bassiana*

Fungi from 15-day-old PDA media were used as inoculate in this study. A 5 mm disc of the corresponding fungus was taken with a sterilized cylinder from the peripheric (the active growing zone) and placed in the middle of the dish (n = 3 per treatment). The dishes were closed with parafilm and incubated at 28 °C in the dark. The treatments were: (i) PDA, (ii) BHI, (iii) Fresh Earthworms (FE) (six concentrations C1, C2, C3, C4, C5, C6), and (iv) Earthworms

without gut contents (EDG) (six concentrations C1, C2, C3, C4, C5, C6). Diameter measurement was performed using a digital calliper every 24 h starting on day 2 for 18 days. After 18 days, a measurement was made to quantify the conidia produced by the fungus under each growth condition. A 5 mm disc of fungus was suspended in 1 mL of tween solution (distilled water and 0.05% Tween 80) and counted using a Malassez chamber (n = 3). The experiment was performed twice.

We prepared a liquid medium using the same six concentrations of 40 g/L, 20 g/L, 10 g/L, 5 g/L 2.5 g/L, and 1.25 g/L of the two earthworm extracts (FE and EDG), and for the control, we prepared potato dextrose (PD), Brain Heart Infusion (BHI), and distilled water (NC), The solution was autoclaved for 15 min at 121 °C. The treatments were: (i) PD, (ii) BHI, (iii) NC, (iv) fresh earthworms, (FE, six concentrations C1, C2, C3, C4, C5, C6), and (v) earthworms devoid of intestinal contents (EDG, six concentrations C1, C2, C3, C4, C5, C6). A conidial suspension was prepared using a 2-weeks-old PDA culture and adjusted by Malassez counting chamber to 5-6 x 10^6. We added 100 µL of our suspension to a 1 mL Eppendorf tube containing 400 µL of sterilized medium and incubated for 48 h at 28 °C and kept in the dark. Germinated and un-germinated conidia were counted microscopically by Malassez chamber (n = 5), and we calculated the germination percentage. The experiment was performed twice.

III.4. Statistical analysis

After checking the homogeneity of the results (data not shown), the two independent trials per experiment and species were combined for further analysis. We performed all analyses individually for each of the three fungal species. First, we used one-way ANOVA and Tukey's HSD to compare FE and EDG media growth at maximum concentrations (C1) with additional media (PDA and BHI). We analysed differential growth on days 3, 6, 9, 12, 15, and 18. Next, we elucidated the effect of intestinal content of four concentrations (C1 to C4). Finally, we used a two-way ANOVA using the following factors: (i) type of medium-with or without intestinal content, (ii) concentration, and (iii) interaction. We performed this analysis on days 3, 6, 9, 12, 15, and 18. Similarly, we evaluated statistical differences between conidial reproduction and germination. First, we compared conidial production and germination percentage observed in additional media (PDA and BHI) with those prepared from earthworms (FE and EDG) by one-way ANOVA. Subsequently, we compared the effect of concentration and media on conidia production by a two-way ANOVA analysis (factors: (i) type of media with or without gut

content, (ii) concentration, and (iii) interaction). All statistical differences were evaluated for $p < 0.05$ (SPSS 25.0, SPSS Statistics, SPSS Inc., Chicago, IL, USA), and data are presented as least squares means ± S. E. as descriptive data.

Chapter 2: RESULTS AND DISCUSSIONS

I. Cutaneous excreta of the earthworm *Eisenia fetida* (haplotaxida: lumbricidae) might hinder the biological control performance of entomopathogenic nematodes.

I.1. Impact of the presence of earthworms or their cutaneous excreta on the entomopathogenic nematode virulence

For some steinernematid species, the larval mortality achieved when the IJs were combined with earthworms or CEx was reduced when compared with EPN-only treatments in a species-specific manner (Figure 24A-D; Table II). Virulence (number of killed larvae) of *S. feltiae* in the presence of CEx was significantly reduced from day 4 to 8, while for *S. glaseri* virulence was lower only on day 4, and no differences were observed for *S. carpocapsae* neither for *S. khuongi*. Similarly, the presence of earthworms also resulted in significantly less larval mortality for *S. feltiae* (for day 6), *S. carpocapsae* (for day 4), and *S. khuongi* (for day 2). No differences were observed between the larval mortality caused when earthworms or CEx were inoculated with steinernematids (Table II). In contrast, neither earthworm or CEx had detectable deleterious effects on the virulence of either heterorhabditid species (Figure 24 E-F). Indeed, the only measurable treatment effect on heterorhabditids was higher larval mortality at 2 days after application when *H. zealandica* IJs were combined with earthworms rather than CEx (Figure 24F). No larval mortality was recorded when *G. mellonella* larvae were exposed to the earthworm cast (data not shown).

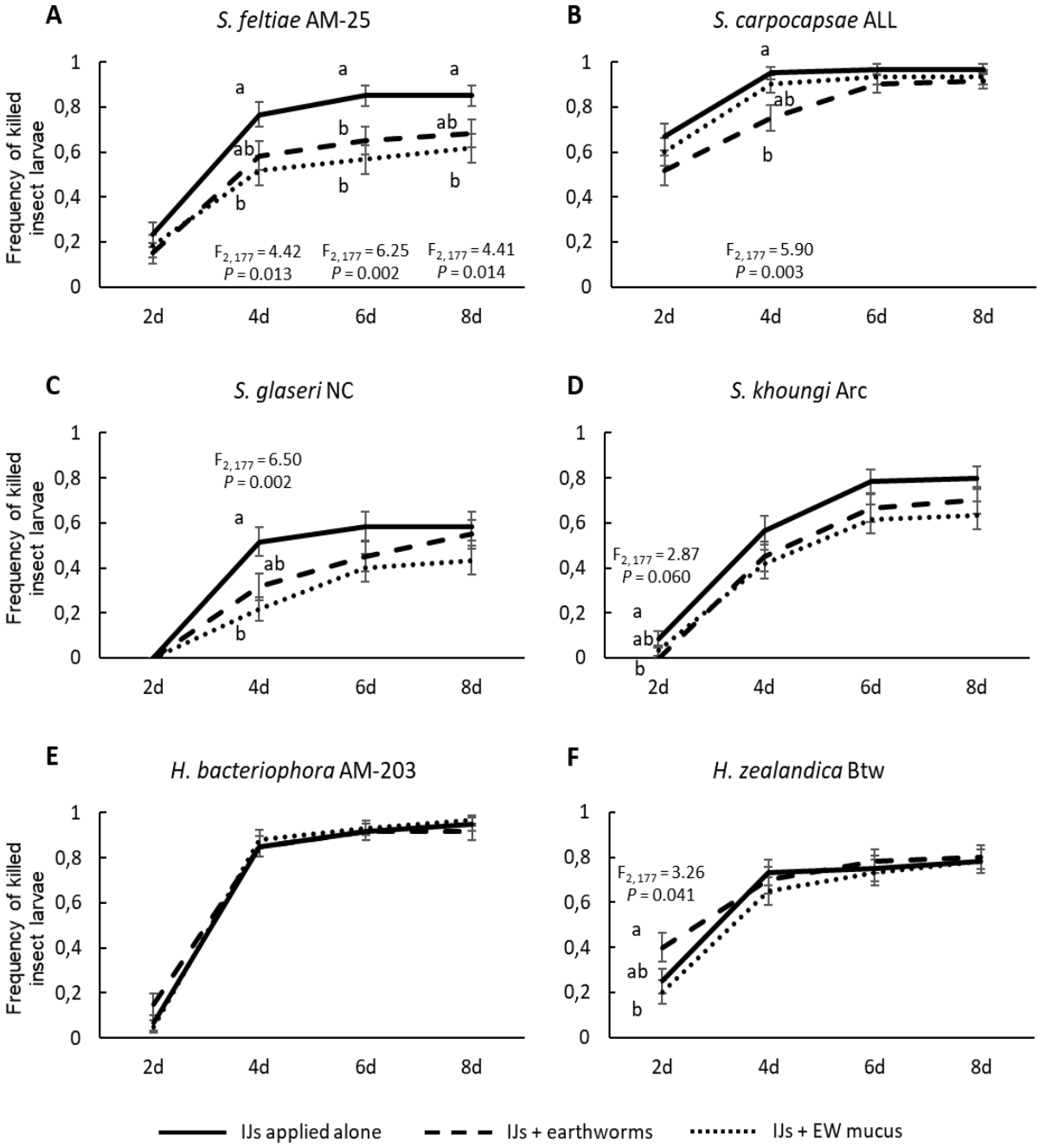


Figure 24: Cumulative *Galleria mellonella* larval mortality (2 to 8 days) after exposure to 48 infective juveniles (IJs) of the entomopathogenic nematode species (A) *Steinernema feltiae*, (B) *S. carpocapsae*, (C) *S. glaseri*, (D) *S. khoungi*, (E) *Heterorhabditis bacteriophora*, and (F) *H. zealandica*, applied alone or with 2 two earthworms, or the cutaneous excreta (CEx) of 2 earthworms. F and P values are showed when treatments are segregated into different Tukey's test (HSD) subsets (each group "a", "ab" or "b"). Values are least-square means ± SE.

Table II. Statistics (One-way ANOVA) of the cumulative *Galleria mellonella* larval mortality (2 to 8 days) after exposure to 48 infective juveniles (IJs) of the entomopathogenic nematode species: *Steinernema feltiae, S. carpocapsae, S. glaseri, S. khoungi, Heterorhabditis bacteriophora, and H. zealandica,* applied alone or with 2 two earthworms (EW), or with the mucus secreted by 2 EW. Significant differences are highlighted in bold.

		Day after exposure			
		2	4	6	8
Steinernema feltiae	$F_{2,177}$	0.68	4.42	6.25	4.41
	P	0.507	**0.013**	**0.002**	**0.014**
Steinernema carpocapsae	$F_{2,177}$	1.40	5.90	1.07	0.67
	P	0.248	**0.003**	0.346	0.512
Steinernema glaseri	$F_{2,177}$	-	6.50	2.18	1.49
	P	-	**0.002**	0.117	0.228
Steinernema khoungi	$F_{2,177}$	2.87	1.49	2.06	2.07
	P	0.060	0.228	0.130	0.129
Heterorhabditis bacteriophora	$F_{2,177}$	2.14	0.18	0.08	0.74
	P	0.120	0.833	0.927	0.481
Heterorhabditis zealandica	$F_{2,177}$	3.26	0.49	0.21	0.03
	P	**0.041**	0.612	0.813	0.968

I.2. Impact of the earthworm cutaneous excreta on the entomopathogenic nematode infectivity and reproductive success

The IJ inoculum mainly drove differences in larval mortality so that higher initial IJ concentrations (20 IJs) achieved higher larval mortality values than the initial 3 IJs concentration for both steinernematid and heterorhabditid species (Figure 25; **Table** III). Larval mortality was lower when steinernematid IJs were combined with CEx than the mortality recorded in the corresponding IJs alone treatment (except in the case of *S. carpocapsae*) in a species-specific manner (Figure 25A-D, **Table** III). For example, the combined inoculation of CEx and *S. feltiae* resulted in lower larval mortality rates than the corresponding IJs applied alone treatment in an early time post-exposure (2 days), for *S. glaseri* at most days, and for *S. khoungi* only after more than 5 days of exposure. The presence of CEx did not interfere with larval mortality caused by heterorhabditids (Figure 25E-F, **Table** III). Similarly, larger IJ

inocula resulted in higher frequencies of larvae producing EPN progeny for all EPN species, while no impact was observed due to CEx exposure (Figure 26). The number of days required for IJs to emerge was higher for lower IJ inocula (3 IJs) than higher (20 IJs) for the EPN species *S. feltiae* and *S. carpocapsae*. CEx delayed emergence only when combined with the low *S. feltiae* IJ inoculum (Figure 27 A-B). Lower IJ inoculum also resulted in fewer emerged IJs per larva only for *S. feltiae* offspring (Figure 28A), while the presence of CEx reduced nematode progeny when applied with *S. feltiae* and *H. zealandica* (Figure 28A, F). This last case was the only record in which the presence of CEx negatively affected the virulence or reproductive success of any heterorhabditid species.

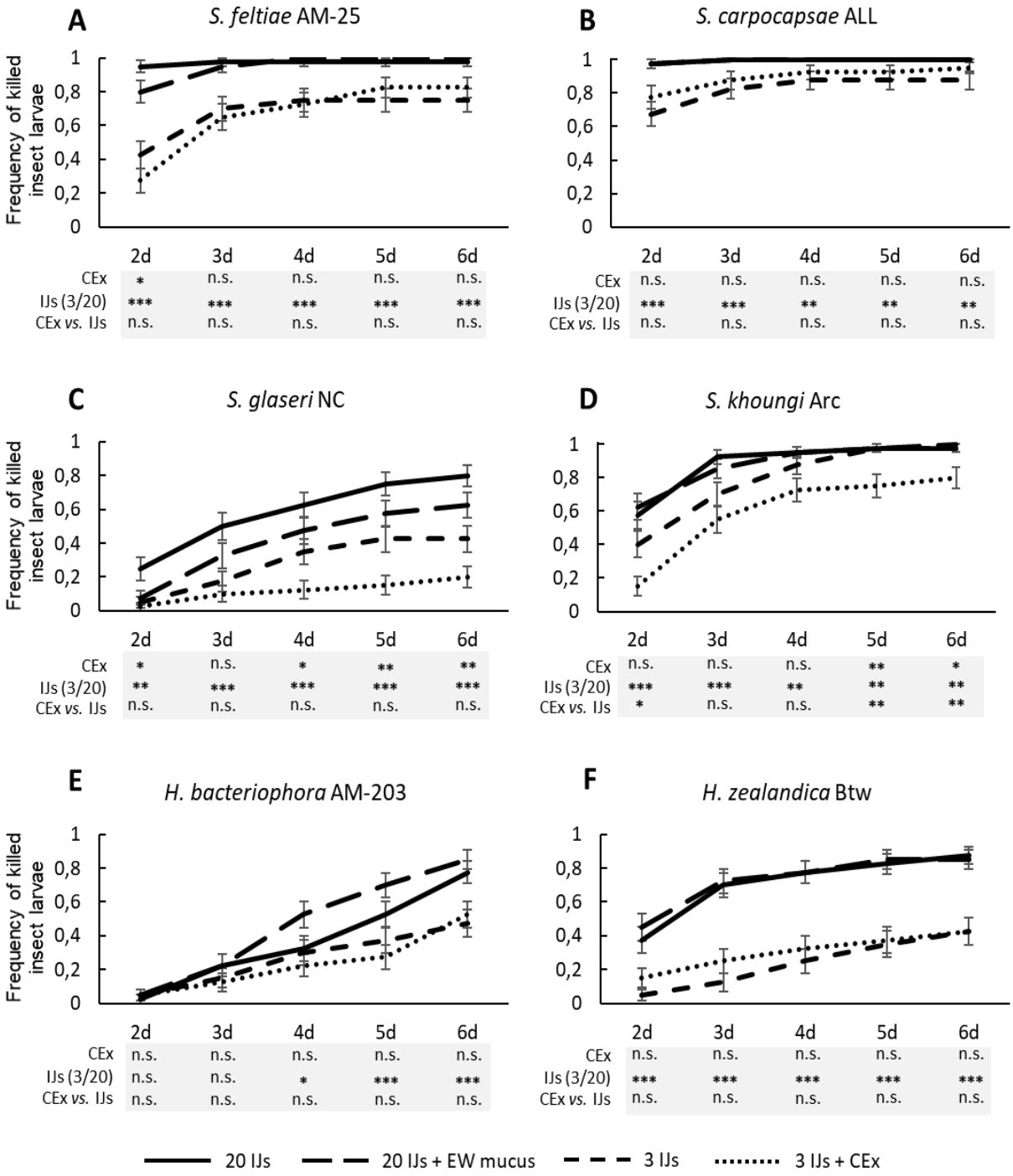


Figure 25: Cumulative *Galleria mellonella* larval mortality (2 to 6 days) after exposure to 3 or 20 infective juveniles (IJs) of the entomopathogenic nematode species (A) *Steinernema feltiae*, (B) *S. carpocapsae*, (C) *S. glaseri*, (D) *S. khoungi*, (E) *Heterorhabditis bacteriophora*, and (F) *H. zealandica*, applied alone or with 100 µL of the cutaneous excreta (CEx) of corresponding with 0.25 earthworms per well. Asterisks indicate significant differences within treatment comparisons at * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and n.s., not significant. Values are least-square means ± SE.

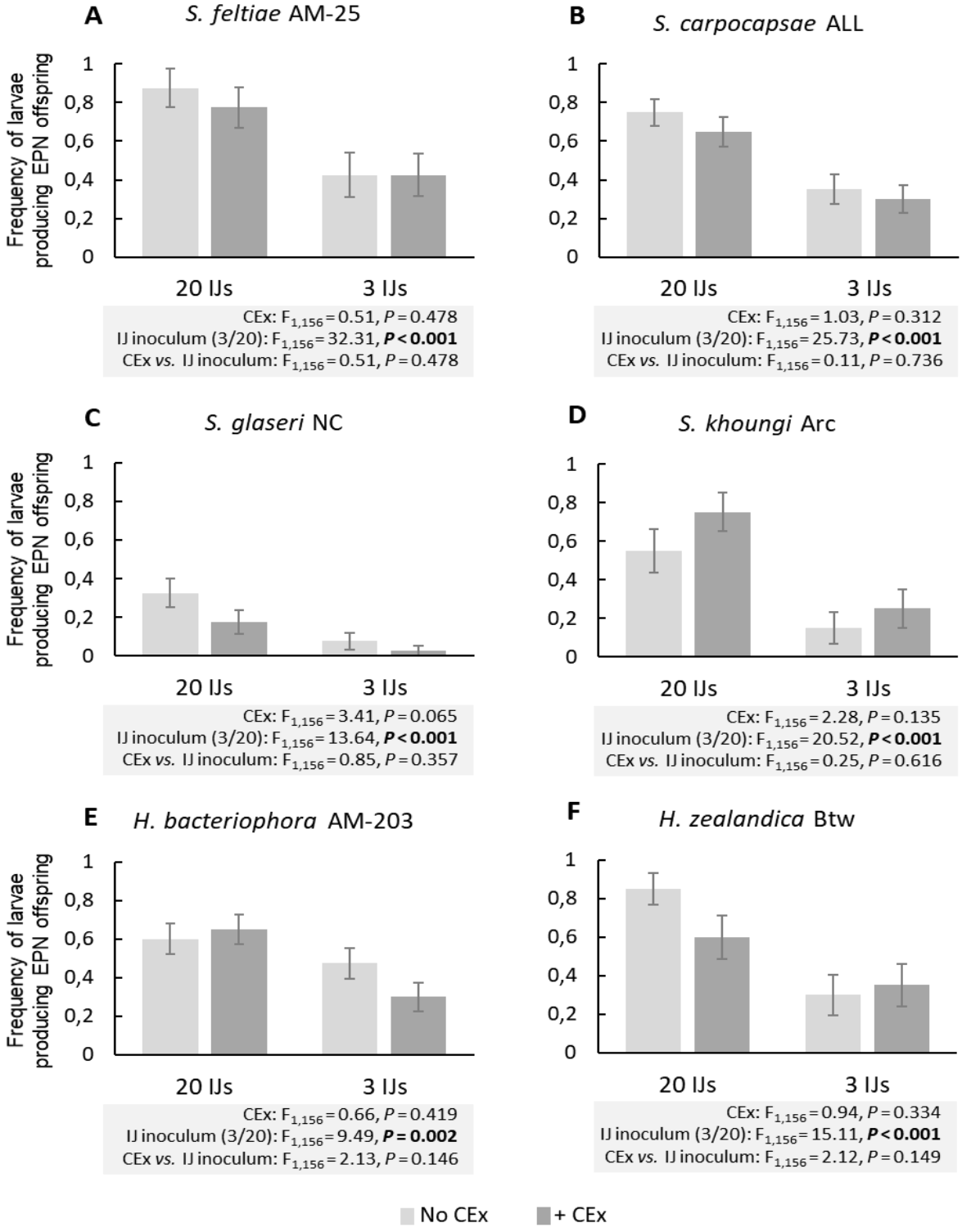


Figure 26: Effect of the presence of cutaneous excreta (CEx) of earthworms on the frequency of *Galleria mellonella* larvae producing entomopathogenic nematode (EPN) offspring when exposed to 3 or 20 infective juveniles (IJs) of the EPN species (A) *Steinernema feltiae*, (B) *S. carpocapsae*, (C) *S. glaseri*, (D) *S. khoungi*, (E) *Heterorhabditis bacteriophora*, and (F) H. zealandica. Significant differences are highlighted in bold. Values are least-square means ± SE.

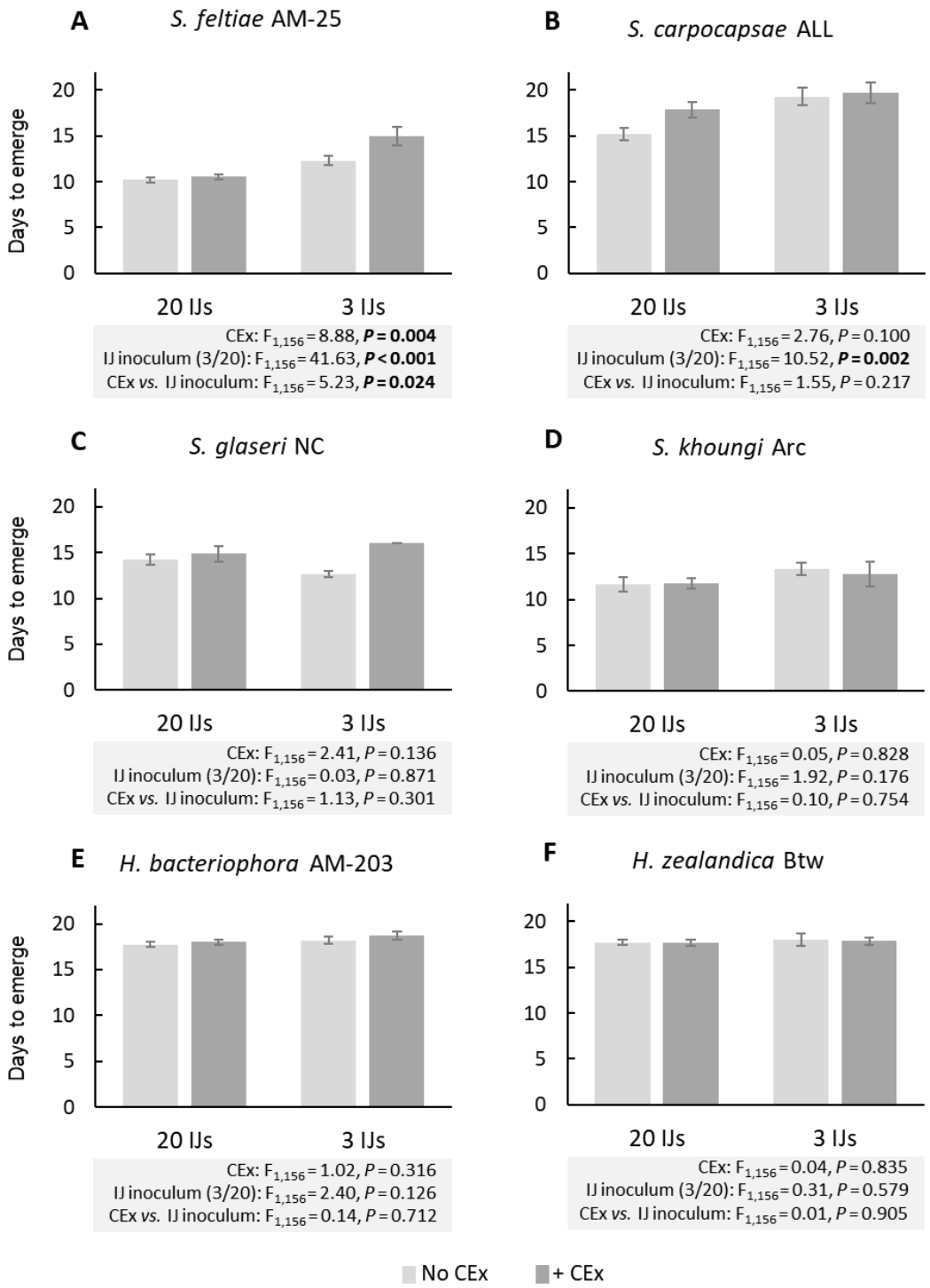


Figure 27: Effect of the presence of cutaneous excreta (CEx) of earthworms on the number of days needed for retrieving the progeny of infective juveniles (IJs) emerging from *Galleria mellonella* cadavers when exposed to 3 or 20 IJs of the entomopathogenic nematode species (A) *Steinernema feltiae*, (B) *S. carpocapsae*, (C) *S. glaseri*, (D) *S. khoungi*, (E) *Heterorhabditis bacteriophora*, and (F) *H. zealandica*. Significant differences are highlighted in bold. Values are least-square means ± SE.

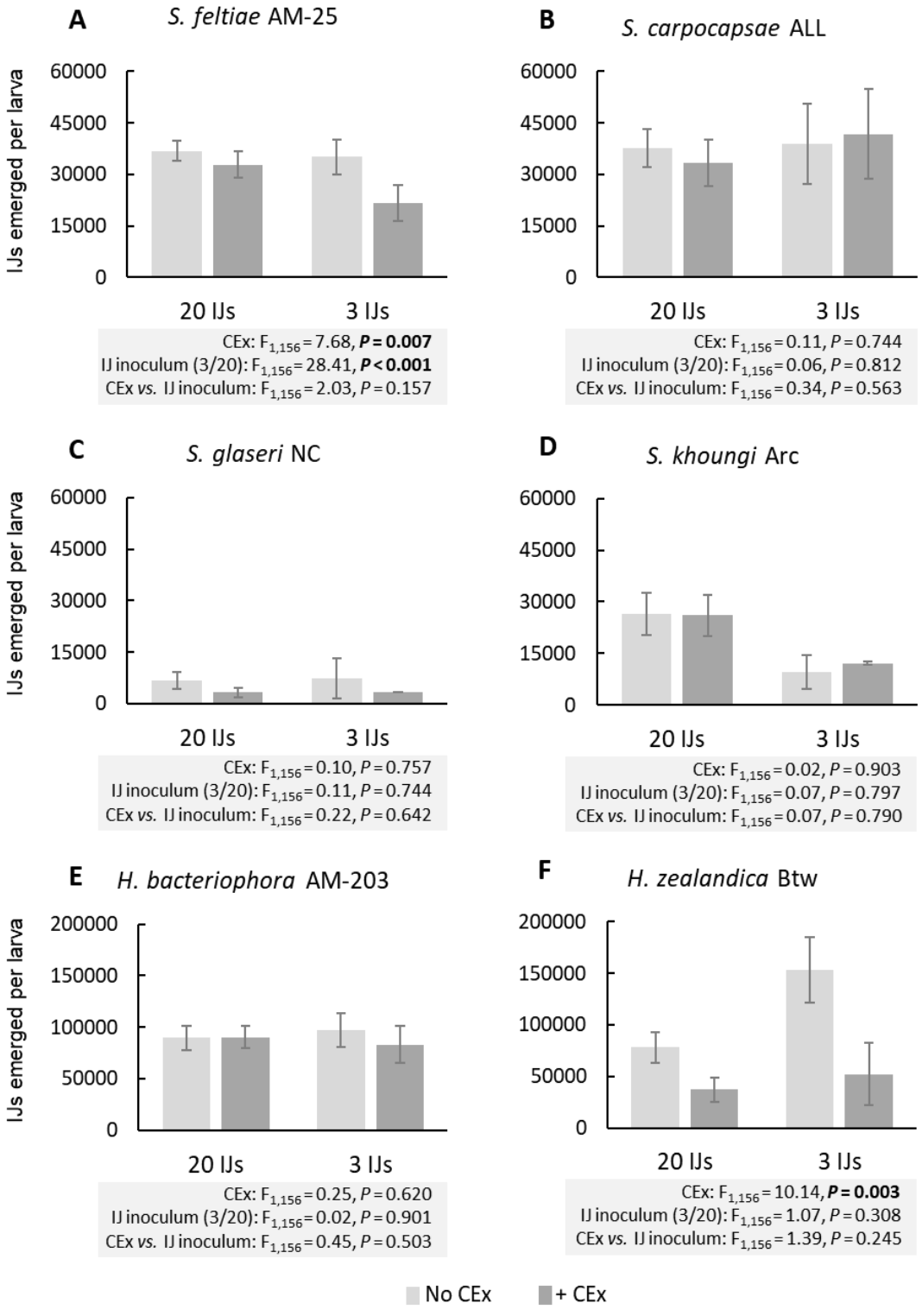


Figure 28: Effect of the presence of cutaneous excreta (CEx) of earthworms on the production of infective juveniles (IJs) emerged per *Galleria mellonella* cadaver when exposed to 3 or 20 IJs of the entomopathogenic nematode species (A) *Steinernema feltiae*, (B) *S. carpocapsae*, (C) *S. glaseri*, (D) *S. khoungi*, (E) *Heterorhabditis bacteriophora*, and (F) *H. zealandica*. Significant differences are highlighted in bold. Values are least-square means ± SE.

Table III. Statistics (two-way ANOVA) of the cumulative *Galleria mellonella* larval mortality (2 to 6 days) after exposure to 3 or 20 infective juveniles (IJs) of the entomopathogenic nematode species: *Steinernema feltiae*, *S. carpocapsae*, *S. glaseri*, *S. khoungi, Heterorhabditis bacteriophora*, and *H. zealandica*, applied alone or with 100 µL of earthworm (EW) mucus. Significant differences are highlighted in bold.

			Day after exposure				
			2	3	4	5	6
Steinernema feltiae	EW mucus	$F_{1,156}$	5.39	0.43	0.00	1.10	1.10
		P	**0.022**	0.513	1.000	0.297	0.297
	IJ inoculum	$F_{1,156}$	66.02	25.31	23.71	17.52	17.52
		P	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
	EW mucus vs. IJ inoculum	$F_{1,156}$	0.00	0.05	0.24	0.27	0.27
		P	1.000	0.827	0.627	0.602	0.602
Steinernema carpocapsae	EW mucus	$F_{1,156}$	0.88	0.38	0.55	0.55	1.40
		P	0.349	0.536	0.461	0.461	0.239
	IJ inoculum	$F_{1,156}$	22.03	13.83	8.73	8.73	7.61
		P	**<0.001**	**<0.001**	**0.004**	**0.004**	**0.006**
	EW mucus vs. IJ inoculum	$F_{1,156}$	0.88	0.38	0.55	0.55	1.40
		P	0.349	0.536	0.461	0.461	0.239
Steinernema glaseri	EW mucus	$F_{1,156}$	4.75	3.46	6.68	9.83	7.81
		P	**0.031**	0.065	**0.011**	**0.002**	**0.006**
	IJ inoculum	$F_{1,156}$	7.41	16.76	18.56	27.29	31.25
		P	**0.007**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
	EW mucus vs. IJ inoculum	$F_{1,156}$	2.67	0.55	0.27	0.49	0.12
		P	0.104	0.458	0.606	0.487	0.727
Steinernema khoungi	EW mucus	$F_{1,156}$	1.84	3.02	2.17	7.58	4.20
		P	0.177	0.084	0.142	**0.007**	**0.042**
	IJ inoculum	$F_{1,156}$	19.47	16.43	8.69	7.58	7.47
		P	**<0.001**	**<0.001**	**0.004**	**0.007**	**0.007**
	EW mucus vs. IJ inoculum	$F_{1,156}$	4.148	0.34	2.17	7.58	7.47
		P	0.043	0.563	0.142	**0.007**	**0.007**
Heterorhabditis bacteriophora	EW mucus	$F_{1,156}$	0.15	0.04	0.71	0.25	0.76
		P	0.703	0.839	0.399	0.621	0.384
	IJ inoculum	$F_{1,156}$	0.15	2.04	4.83	14.44	19.03
		P	0.703	0.155	**0.029**	**<0.001**	**<0.001**
	EW mucus vs. IJ inoculum	$F_{1,156}$	0.15	0.04	3.46	3.30	0.03
		P	0.703	0.839	0.065	0.071	0.862
Heterorhabditis zealandica	EW mucus	$F_{1,156}$	1.82	1.24	0.29	0.13	0.003
		P	0.179	0.267	0.591	0.716	0.855
	IJ inoculum	$F_{1,156}$	62.38	60.88	49.07	47.97	41.15
		P	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
	EW mucus vs. IJ inoculum	$F_{1,156}$	0.04	0.55	0.29	0.00	0.003
		P	0.847	0.459	0.591	1.00	0.855

I.3. Discussion

This study showed, as we hypothesized, that CEx of earthworms had a potential sublethal impact on the EPN, observed in detrimental effects on larval mortality and EPN fitness, but only in certain species and conditions. Furthermore, as shown previously for pathogenic fungi (Plavšin et al., 2017), our results suggest that the CEx itself might have a deleterious effect on the biological control provided by certain steinernematids (some variables for *S. feltiae*, and in more limited occasions on *S. glaseri* and *S. khoungi)*, although the long-term impacts remained unknown.

First, the earthworms and/or the presence of CEx might affect the IJs' survival and hence, the activity as biocontrol. Campos-Herrera et al. (2006) observed that the EPN *S. feltiae* did not survive the gut passage of the earthworms *E. fetida*, considering that the feeding behavior will have a deleterious effect on the presence of EPN. However, in the current study, we did not observe the survival of the EPN exposed in the soil nor those exposed directly to the CEx, and hence, we cannot determine if the decrease in the activity is due to low survival or changes in the infectivity phase of the IJs. This phenomenon implies that often, the IJs can be in-active. Hence, the traditional insect bait technique cannot register their presence. This infectivity phase is known to occur to the EPN in the soil, and although starvation might be one key factor of this inactivation, still, the causes are not well known (Hominick, 2002; Griffin, 2015). Both laboratory and field studies have shown that the number of IJs and the ability to kill might not always be correlated and will depend on the method of nematode extraction selected. For example, Hass et al. (1999), recovered only 20% of *Heterorhabditis megidis* from the soil immediately after application. However, these authors retrieved values above 50% when the Baermann funnel method was employed based on nematode mobility. Similarly, Jaffuel et al. (2017) showed that the high number of *H. bacteriophora* quantified using real-time qPCR were not translated into high activity records in the baiting test. It could be possible that the CEx produces an effect that reduces the mobility and ability to kill the EPN or even have the potential to kill the nematodes, but further studies are needed to unravel the potential mechanisms involved in these interactions.

The differences in the impact of the CEx of earthworms on the fitness of different EPN species might be related to the nematode cuticle, the first barrier for IJs to interact with the external environment. This exoskeleton is critical for maintaining nematode body shape and

permitting mobility (Bird, 1971). The composition and properties of the cuticle might depend on the nematode stage and species, and cuticular differences can even occur between genera of the same family, as described for trichodorid nematodes (Decraemer et al., 2001). Slight differences in the composition or structure of EPN cuticles could be related to other differences between these two genera. For example, the third stage (IJ) heterorhabditids often maintain the second stage juvenile cuticle, whereas steinernematids usually discard it quickly. The presence of this extra cuticle is not a limiting factor for heterorhabditid infectivity (Campbell and Gaugler, 1992), and has been proposed to defend against some perturbations, including nematophagous fungi (Timper and Kaya, 1989, 1992; Timper et al., 1991), although also in a species-specific manner (El Borai et al., 2009).

The IJs of different EPN species also differ in mobility and host searching behavior, broadly described as a continuum between "ambusher" (sit and wait) and "cruiser" (active search) nematodes. Thus, *S. carpocapsae* is considered an ambusher, whereas larger steinernematids (*S. glaseri* and *S. khuongi* probably) and heterorhabditids are ranked as cruisers. Other EPN species, such as *S. feltiae*, display an "intermediate" searching behavior (Lewis et al., 1992; Campbell and Gaugler, 1997; Campbell et al., 2003). Interestingly, Campbell and Gaugler (1992) observed that exsheathed *S. carpocapsae* IJs increased their mobility compared to those that retained the second stage cuticle, whereas *H. bacteriophora* IJs were more mobile when the sheath was retained. Certain ambusher steinernematid might be expected to encounter CEx less frequently due to their reduced mobility than cruiser steinernematids that are more likely to contact this material while searching for hosts. However, the own earthworm mobility could compensate the effect of restricted movement of the typical ambusher nematodes. In any case, it may explain why *S. carpocapsae* pathogenicity and reproductive success were not affected by the presence of CEx, only by the presence of motile earthworms. Even if true, the importance of CEx to individual species in nature requires further study, given that ambush-cruise foraging strategy is strongly dependent on environmental conditions that can promote cruising behavior in such a typical ambusher as *S. carpocapsae* (Wilson et al., 2012; Griffin, 2015).

The IJ size could also explain the species-specific differences observed, due to the differences in the overall surface that would be in contact with CEx. In our study, the presence of CEx did not affect the fitness of the EPN species whose IJs are described as below 600 μm length on average (*S. carpocapsae*, and *H. bacteriophora*) (Nguyen and Hunt, 2007; Nguyen et al., 2007). By contrast, virulence of the slightly larger *H. zealandica* (IJs≈685 μm) was not

affected, although the species suffered sub-lethal effects (lower offspring), and the virulence of the largest species (IJ *S. feltiae* ≈879 μm, *S. glaseri* ≈1130 μm, and *S. khoungi* ≈1066 μm (Nguyen and Hunt, 2007; Nguyen et al., 2007; Stock et al., 2018) was seriously affected. Additional studies considering other nematode species and a broad range of size could provide some insight to the impact of CEx depending on the EPN size.

In conclusion, we have observed that the cutaneous excreta produced by the earthworm *E. fetida* might be deleterious to EPN IJs, altering their ability to kill insect larvae and, in some cases, even decreasing their reproductive success. Because this earthworm is mainly found associated with composting soil, our observations might limit the described potential beneficial effect of the application of EPN in mature compost produced by vermicomposting (Herren et al. 2018). Still is in unknown whether this effect is also induced by other earthworm species with more generalist activity and whether other soil entomopathogens are affected (e.g., entomopathogenic fungi). In any case, this study highlights that in addition to the known positive impacts on EPN behavior (Shapiro et al., 1993; 1995; Shapiro-Ilan and Brown, 2013), earthworms and their cutaneous excreta can adversely affect IJs.

II. Earthworms and their cutaneous excreta can modify the virulence and reproductive capability of entomopathogenic nematodes and fungi

II.1. Impact of earthworms, their cutaneous excreta on the virulence and reproductive capability of entomopathogenic nematodes and fungi in sterilized soil

Overall, we found that the impact of earthworms and their CEx on EPN virulence was species-specific, particularly for *S. feltiae* and *H. bacteriophora* (Figure 29), but not on EPN reproductive capability (Figure 30). Compared to individual earthworm applications, the addition of CEx only resulted in significantly higher larval mortality rates of *S. feltiae* and reproductive rates of *S. feltiae* and *H. bacteriophora* (Figure 29 and 30). Regarding *S. feltiae*, both *P. excavatus* treatments (earthworms or their CEx) caused significantly higher larval mortality rates than the control, while significantly lower when combined with *E. fetida* earthworms (Figure 29A). We observed opposite results for *H. bacteriophora*: its virulence was significantly higher for *E. fetida* treatments and lower when combined with *P. excavatus* CEx (Figure 29C). Lastly, *S. riojaense* virulence only decreased with *E. fetida* CEx (Figure 29B). The earthworm species *L. terrestris* did not affect the virulence nor the reproductive capability of EPNs except for the significantly higher mortality rates recorded for

H. bacteriophora three days after CEx inoculation (Figure 29C). Indeed, earthworms did not affect EPN reproductive rates except by increasing it for *S. feltiae* when combined with *E. fetida* CEx (Figure 30A).

The two earthworm species and their CEx differently affected EPF virulence and growth (Figure 31 and 32). First, individuals of *E. fetida* only reduced *B. bassiana* virulence after eight days of exposure (Figure 31A), while *M. anisoliae* showed an overall negative impact from day 8 until 14 (Figure 31B), which translated in a reduction of the reproductive potential (Figure 32B). Exposure to *L. terrestris* reduced *B. bassiana* virulence (Figure 31A), while *M. anisopliae* was unaffected (Figure 31B). Concerning CEx exposure, *E. fetida* CEx reduced *B. bassiana* virulence (Figure 31A) and reproductive potential (Figure 32A), whereas *M. anisopliae* exhibited an increase in its virulence (Figure 31B). On the other hand, *L. terrestris* treatments only affected *B. bassiana* by decreasing its virulence (Figure 31A).

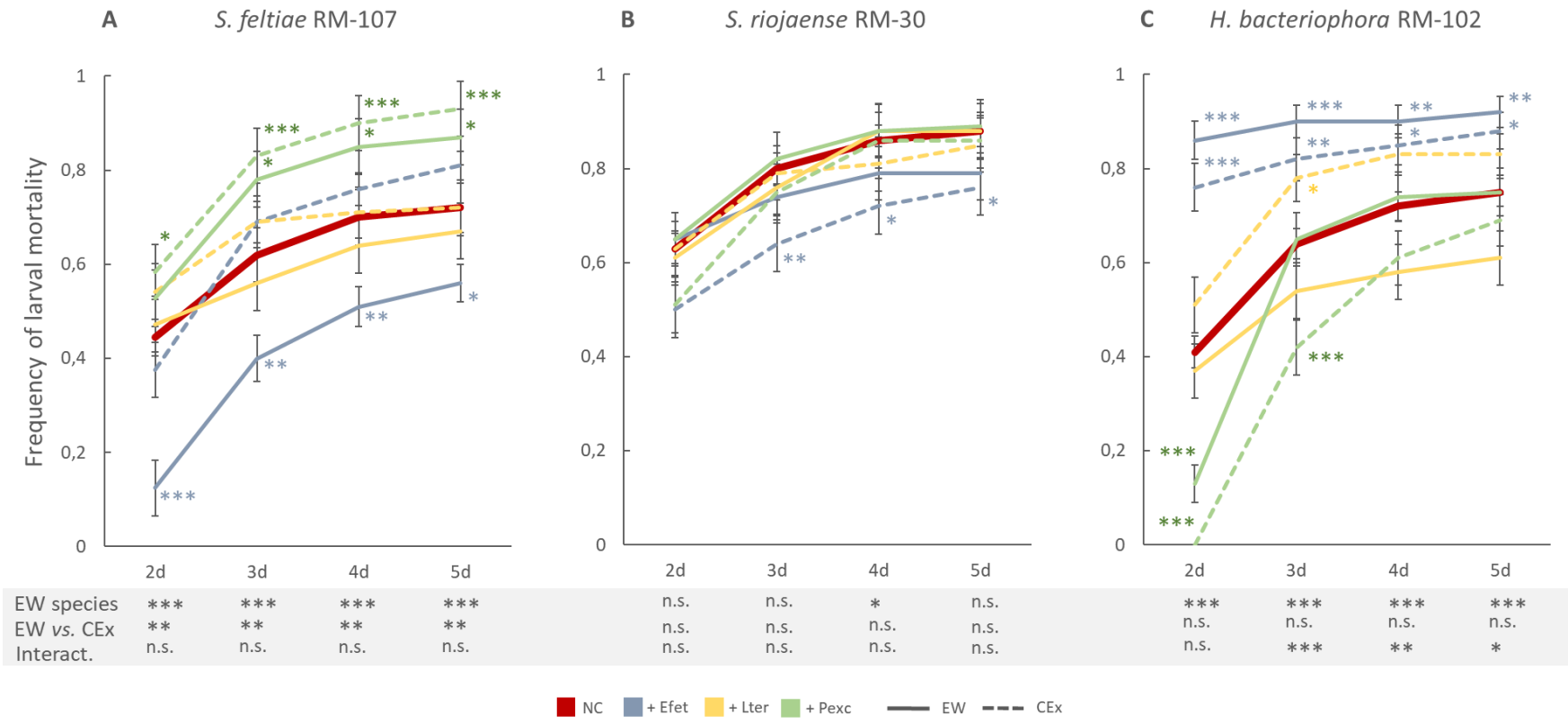


Figure 29. Cumulative *Galleria mellonella* larval mortality (2–5 days) after exposure to 48 infective juveniles (IJs) of the entomopathogenic nematode species (A) *Steinernema feltiae*, (B) *S. riojaense*, and (C) *Heterorhabditis bacteriophora*, IJs only applied or combined with two earthworms (EW) or their cutaneous excreta (CEx) of the species *Eisenia fetida* (Efet), *Lumbricus terrestris* (Lter), and *Perionyx excavatus* (Pexc). Asterisks indicate significant differences within treatment comparisons at * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and n.s., not significant, for two independent generalized linear models testing for the effect of (i) the earthworm species, the earthworm treatment (EW *vs.* CEx), and their interaction (detailed under graphs), and (ii) each EW treatment (EW or CEx) *versus* IJ only applications (NC) (pictured on graphs). Values are least-square means ± SE.

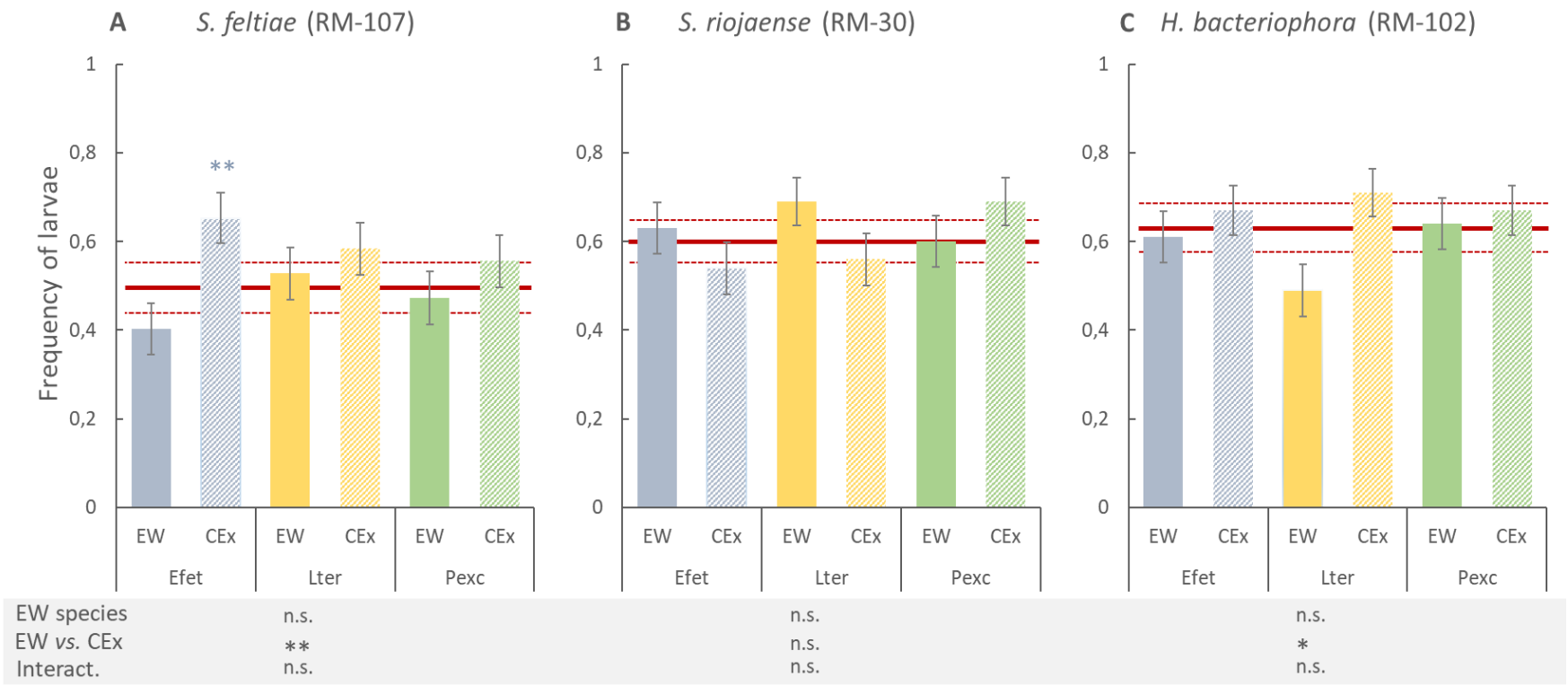


Figure 30. Frequency of *Galleria mellonella* larvae producing nematode offspring after exposure to 48 infective juveniles (IJs) of the entomopathogenic nematode species (A) *Steinernema feltiae*, (B) *S. riojaense*, and (C) *Heterorhabditis bacteriophora*, IJs only applied or combined with two earthworms (EW) or their cutaneous excreta (CEx) of the species *Eisenia fetida* (Efet), *Lumbricus terrestris* (Lter), and *Perionyx excavatus* (Pexc). Asterisks indicate significant differences within treatment comparisons at * $P < 0.05$, ** $P < 0.01$, and n.s., not significant, for two independent generalized linear models testing for the effect of (i) the earthworm species, the earthworm treatment (EW *vs*. CEx), and their interaction (detailed under graphs), and (ii) each EW treatment (EW or CEx) *versus* IJ only applications. The negative control is presented as horizontal red bar for the mean values and dashed lines for standard errors. Values are least-square means ± SE.

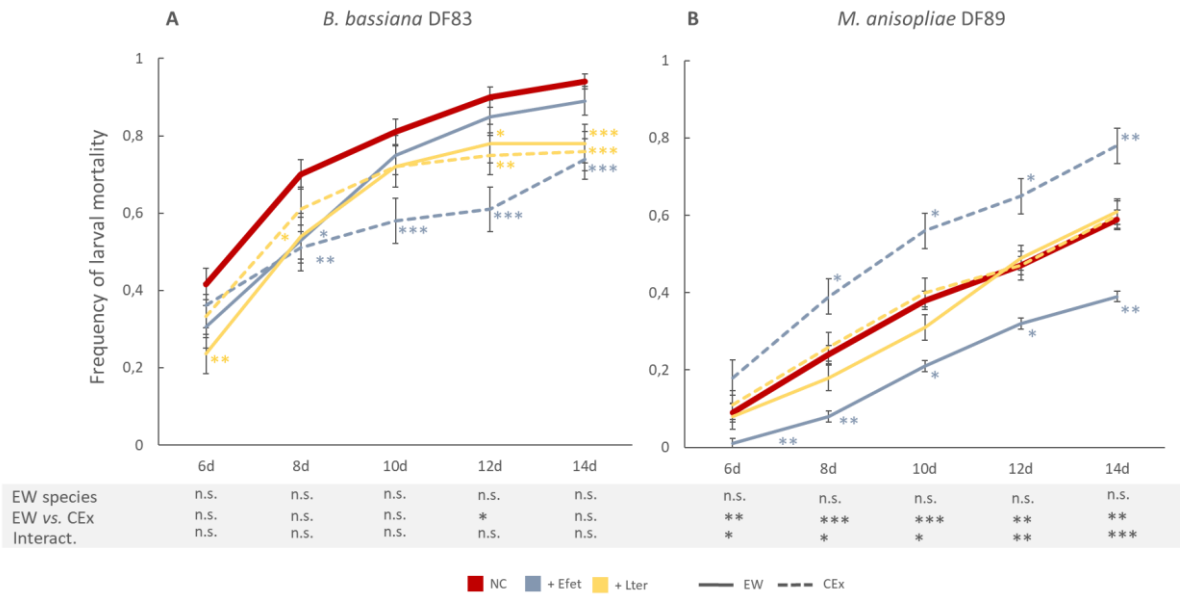


Figure 31. Cumulative *Galleria mellonella* larval mortality (6–14 days) after exposure to 10^7 conidia per Petri dish of the entomopathogenic fungi species (A) *Beauberia bassiana* and (B) *Metarhizium anisopliae*, conidia only applied or combined with two earthworms (EW) or their cutaneous excreta (CEx) of the species *Eisenia fetida* (Efet) and *Lumbricus terrestris* (Lter). Asterisks indicate significant differences within treatment comparisons at * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and n.s., not significant, for two independent generalized linear models testing for the effect of (i) the earthworm species, the earthworm treatment (EW *vs*. CEx), and their interaction (detailed under graphs), and (ii) each EW treatment (EW or CEx) *versus* conidia only applications (NC) (pictured on graphs). Values are least-square means ± SE.

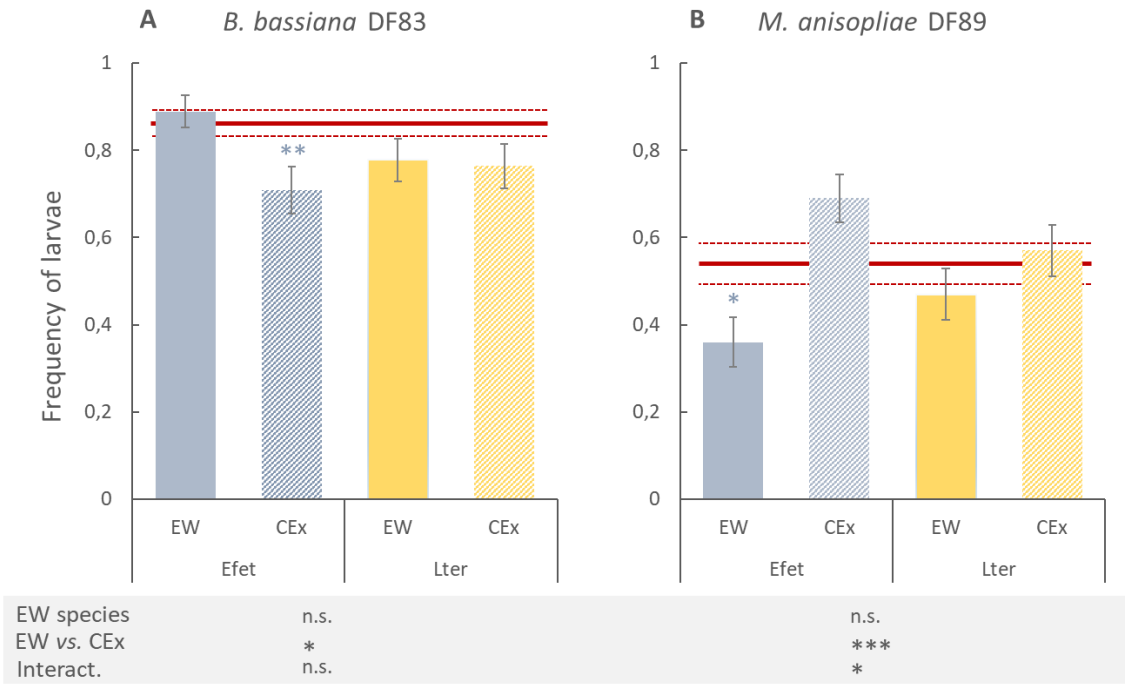


Figure 32. Frequency of *Galleria mellonella* larvae producing mycelia after exposure to 10^7 conidia per Petri dish of the entomopathogenic fungi species (A) *Beauberia bassiana* and (B) *Metarhizium anisopliae*, conidia only applied or combined with two earthworms (EW) or their cutaneous excreta (CEx) of the species *Eisenia fetida* (Efet) and *Lumbricus terrestris* (Lter). Asterisks indicate significant differences within treatment comparisons at * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and n.s., not significant, for two independent generalized linear models testing for the effect of (i) the earthworm species, the earthworm treatment (EW *vs.* CEx), and their interaction (detailed under graphs), and (ii) each EW treatment (EW or CEx) *versus* conidia only applications. The negative control is presented as horizontal red bar for the mean values and dashed lines for standard errors. Values are least-square means ± SE.

II.2. Impact of the cutaneous excreta from earthworms on the virulence and reproductive capability of entomopathogenic nematodes and fungi applied at different concentrations

Overall, higher IJ inocula resulted in higher larval mortality and EPN reproductive rates (Fig. 33 and 34). We observed that, in particular cases, the species-specific nature of the impact of earthworms' CEx on EPN virulence and reproductive capability differs between initial IJ inocula. Thus, we found differences among earthworm species for the mortality rates reported for the high IJ inoculum of *H. bacteriophora* but not for the low IJ inoculum (Figure 33E and F), and the opposite for the frequency of larvae producing *S. feltiae* offspring (Figure 34 A and B). However, independently of the initial IJ inocula, we also found differences among

earthworm species for the infectivity and reproductive rates of *S. feltiae* (Figure 33A and B) and *S. riojaense* (Figure 34C and D), respectively. In contrast, the infectivity of *S. riojaense* (Figure 33C and D) and reproductive rates of *H. bacteriophora* (Figure 34E and F) were not affected. Specifically, *E. fetida* CEx negatively affected the virulence and reproductive capability of low *S. feltiae* inoculum, while we observed opposite results for *P. excavatus* CEx (Figure 33A and 34A). Besides, for the high *S. feltiae* inoculum, *P. excavatus* CEx negatively affected EPN virulence (Figure 33B) while *L. terrestris* CEx positively affected EPN reproductive capability (Figure 34B). Regarding *S. riojaense*, *E. fetida* and *L. terrestris* CEx reduced mortality rates for low and high IJ inoculum, respectively (Figure 33C and D). Moreover, *S. riojaense* reproductive rates decreased when exposed to *E. fetida* CEx at low IJ inoculum (Figure 34C) but increased in the presence of the *L. terrestris* and *P. excavatus* CEx at high IJ inoculum (Figure 34D). Finally, CEx from earthworms only affected *H. bacteriophora* by reducing its virulence when exposed to *L. terrestris* CEx at high IJ inoculum (Figure 33F).

As observed for EPNs, higher conidial inoculum resulted in higher larval mortality and fungal growth rates (Figure 35 and 36). Overall, the CEx from earthworms reduced the EPF virulence and growth, although not significantly for the combination of *L. terrestris* and *M. anisopliae* (Figure 35 and 36). However, except for the low conidial inoculum of *M. anisopliae* (Figure 36C), *E. fetida* CEx decreased the EPF virulence and growth significantly more than *L. terrestris* CEx (Figure 35 and 36). Finally, the EPF conidial viability was significantly higher for *B. bassiana* combined with *L. terrestris* CEx (Figure 37A) and lower for *M. anisopliae* combined with *E. fetida* CEx (Figure 37B)

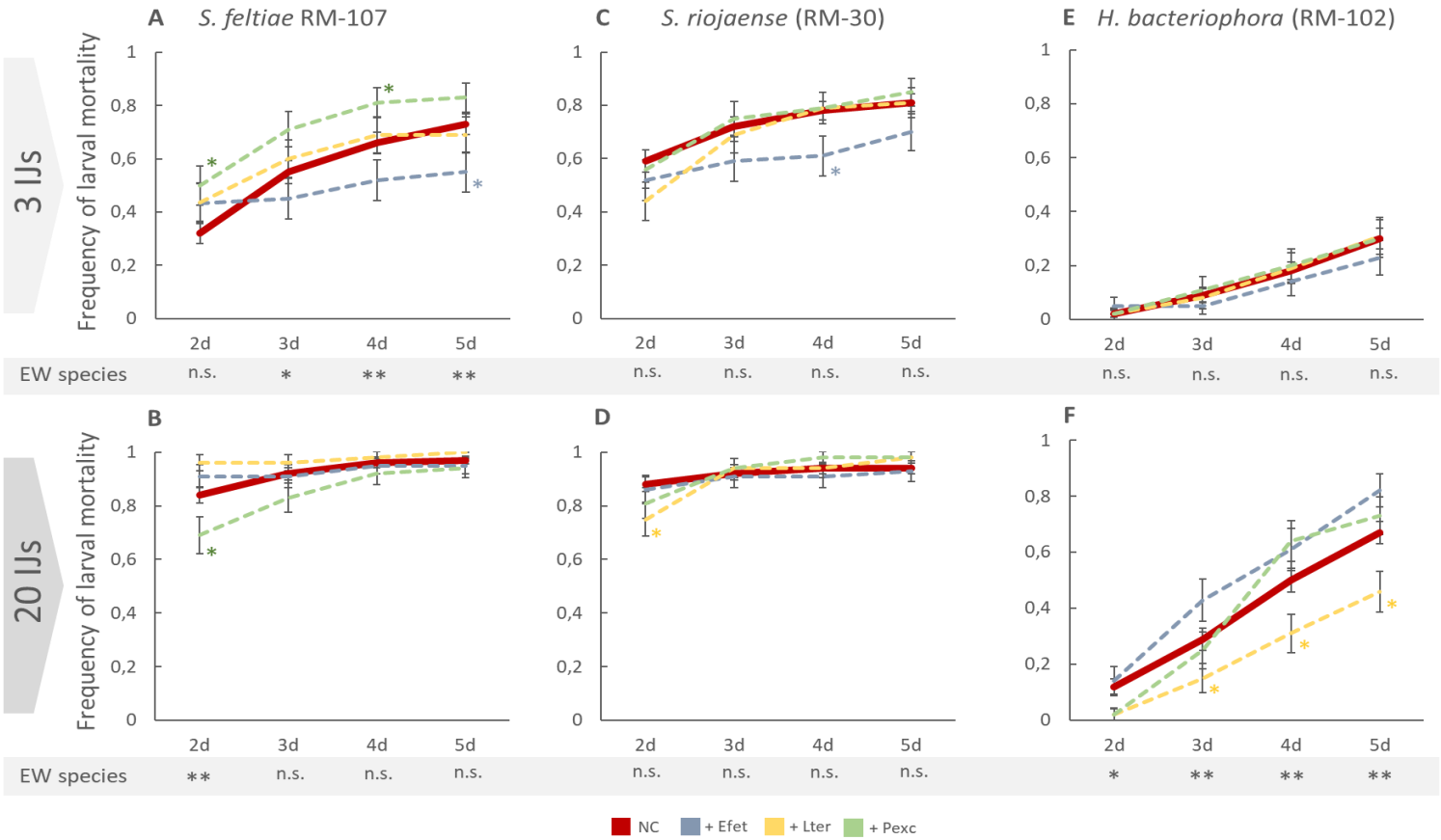


Figure 33. Cumulative *Galleria mellonella* larval mortality (2–5 days) after exposure to 3 or 20 infective juveniles (IJs) of the entomopathogenic nematode species: (A-B) *Steinernema feltiae*, (C-D) *S. riojaense*, and (E-F) *Heterorhabditis bacteriophora*, IJs only applied or combined with the cutaneous excreta (CEx) of the earthworm species *Eisenia fetida* (Efet), *Lumbricus terrestris* (Lter), and *Perionyx excavatus* (Pexc). Asterisks indicate significant differences within treatment comparisons at * $P < 0.05$, ** $P < 0.01$, and n.s., not significant, for two independent generalized linear models testing for the effect of (i) the earthworm species (detailed under graphs), and (ii) each CEx treatment *versus* IJ only applications (NC) (pictured on graphs). Values are least-square means ± SE.

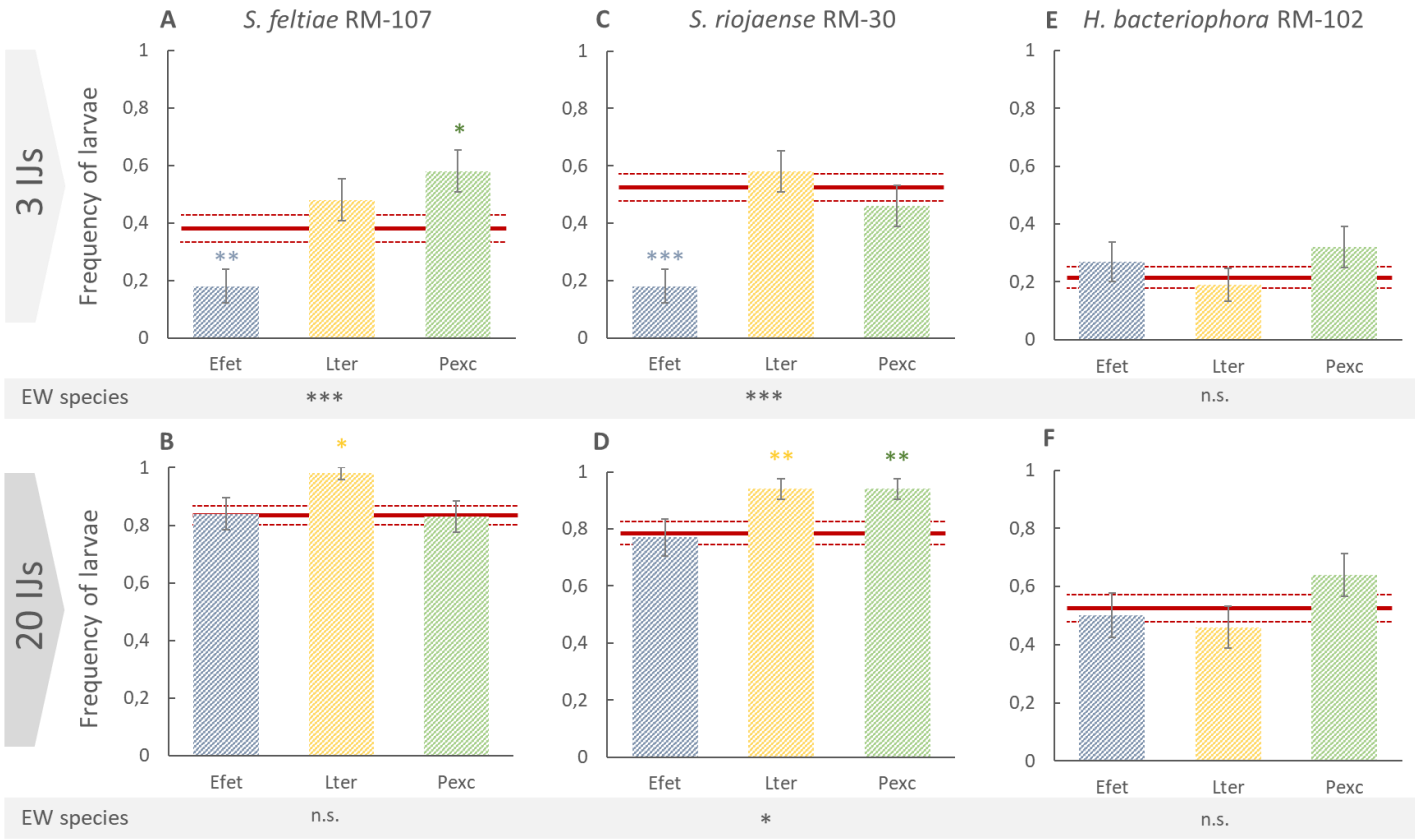


Figure 34. Frequency of *Galleria mellonella* larvae producing nematode offspring after exposure to 3 or 20 infective juveniles (IJs) of the entomopathogenic nematode species (A-B) *Steinernema feltiae*, (C-D) *S. riojaense*, and (E-F) *Heterorhabditis bacteriophora*, IJs only applied or combined with the cutaneous excreta (CEx) of the earthworm species *Eisenia fetida* (Efet), *Lumbricus terrestris* (Lter), and *Perionyx excavatus* (Pexc). Asterisks indicate significant differences within treatment comparisons at * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and n.s., not significant, for two independent generalized linear models testing for the effect of (i) the earthworm species (detailed under graphs), and (ii) each CEx treatment *versus* IJ only applications. The negative control is presented as horizontal red for the mean values and dashed lines for standard errors. Values are least-square means ± SE.

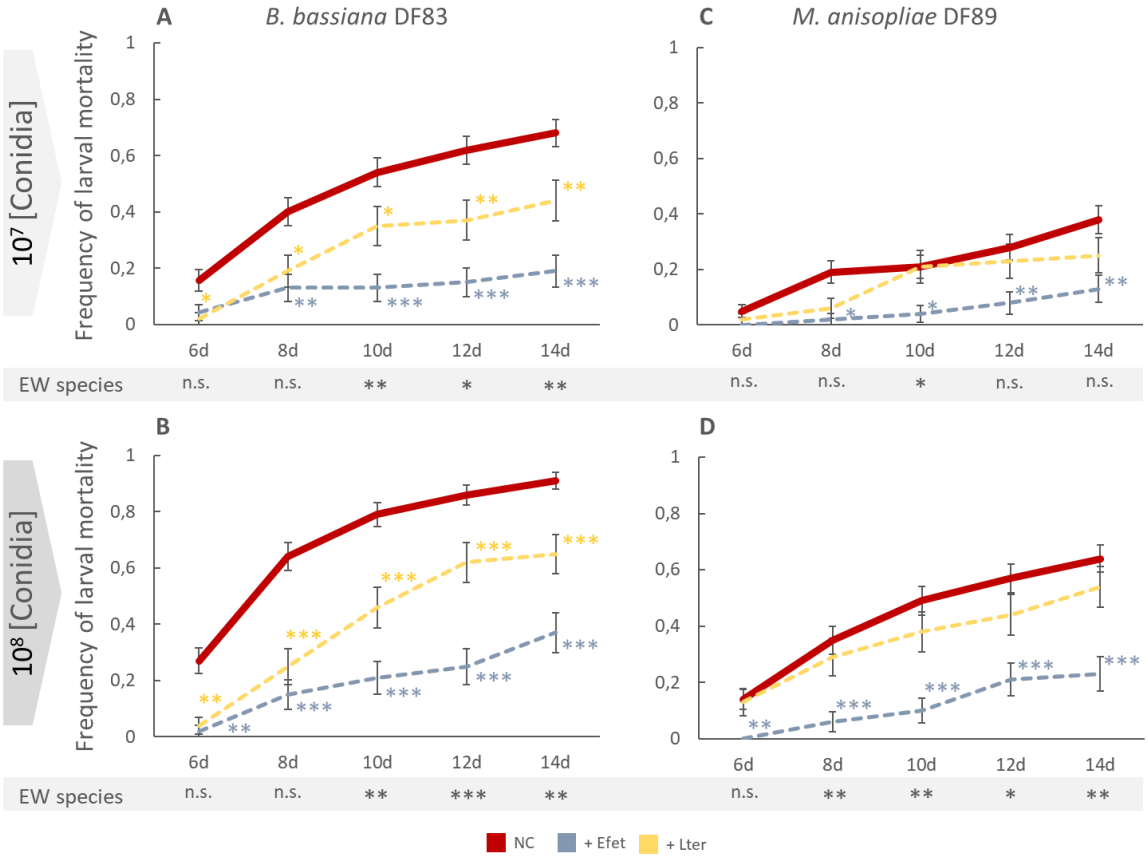


Figure 35. Cumulative *Galleria mellonella* larval mortality (6–14 days) after exposure to 10^7 or 10^8 conidia per well of the entomopathogenic fungi species (A-B) *Beauveria bassiana* and (C-D) *Metarhizium anisopliae*, conidia only applied or combined with the cutaneous excreta (CEx) of the earthworm species *Eisenia fetida* (Efet) and *Lumbricus terrestris* (Lter). Asterisks indicate significant differences within treatment comparisons at * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and n.s., not significant, for two independent generalized linear models testing for the effect of (i) the earthworm species (detailed under graphs), and (ii) each CEx treatment *versus* conidia only applications (NC) (pictured on graphs). Values are least-square means ± SE.

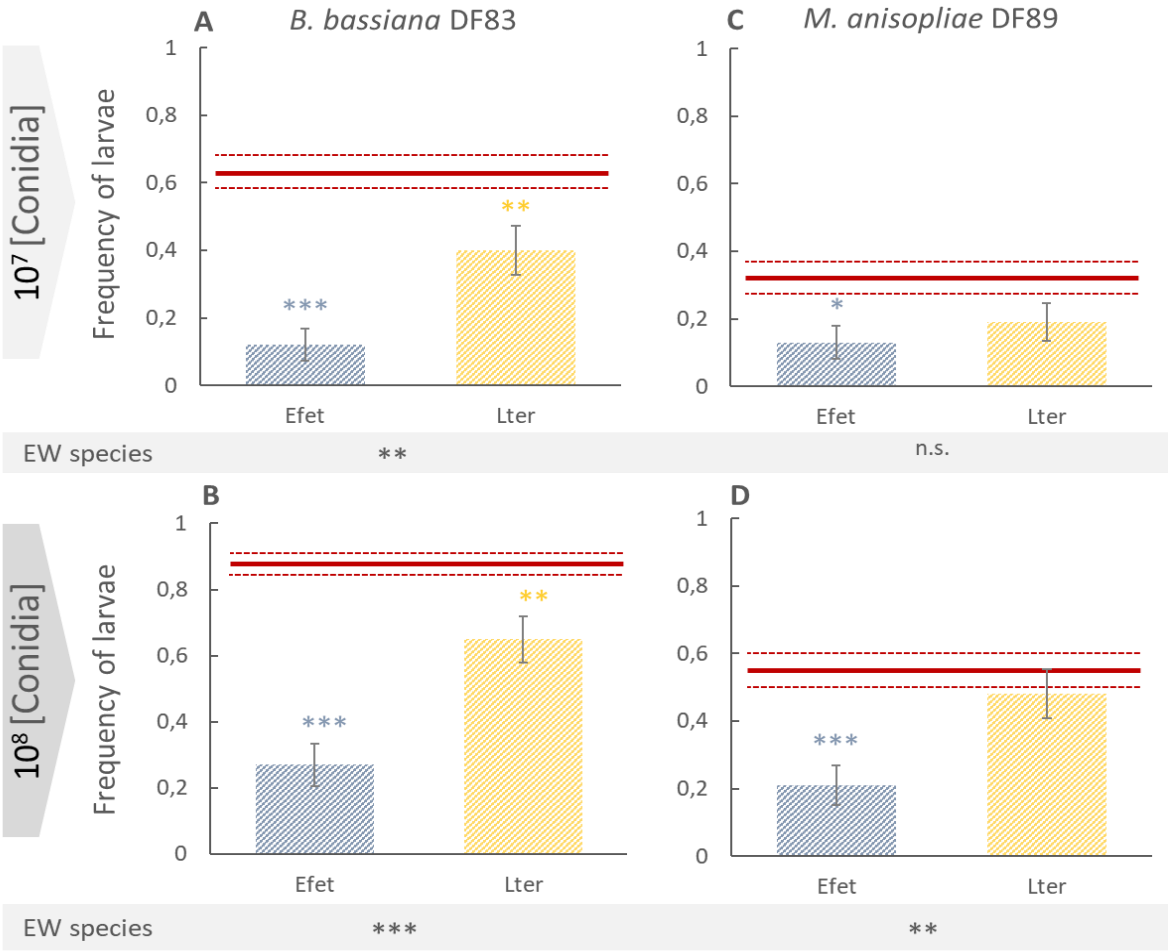


Figure 36. Frequency of *Galleria mellonella* larvae producing mycelia after exposure to 10^7 or 10^8 conidia per well of the entomopathogenic fungi species (A-B) *Beauberia bassiana* and (C-D) *Metarhizium anisopliae*, conidia only applied or combined with the cutaneous excreta (CEx) of the earthworm species *Eisenia fetida* (Efet) and *Lumbricus terrestris* (Lter). Asterisks indicate significant differences within treatment comparisons at * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and n.s., not significant, for two independent generalized linear models testing for the effect of (i) the earthworm species (detailed under graphs), and (ii) each CEx treatment *versus* conidia only applications. The negative control is presented as horizontal red for the mean values and dashed lines for standard errors. Values are least-square means ± SE.

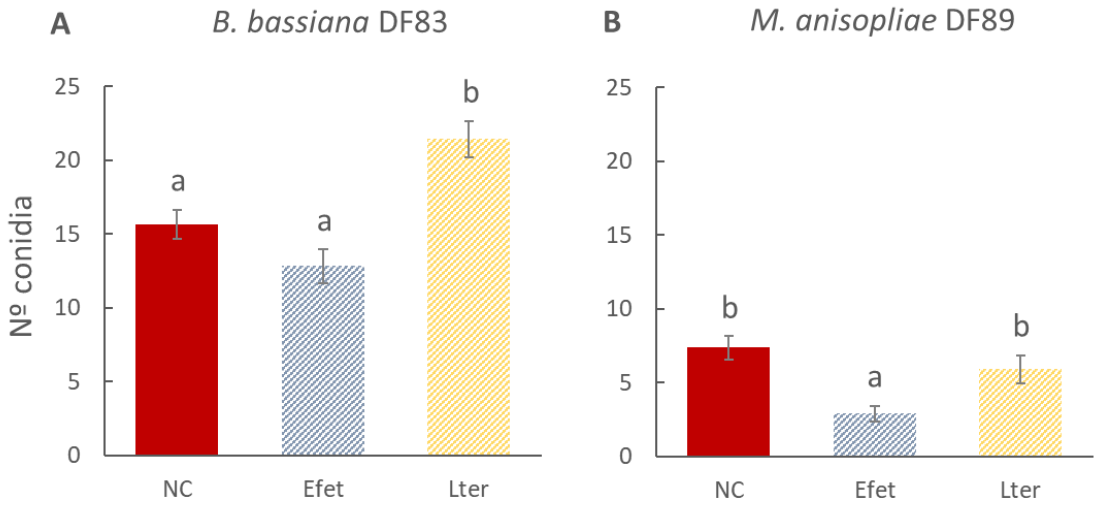


Figure 37. Number of conidia germinated in selective media after exposure to 10^4 conidia per well of the entomopathogenic fungi species (A) *Beauberia bassiana* and (B) *Metarhizium anisopliae*, single applied (NC) or combined with the cutaneous excreta (CEx) of the earthworm species *Eisenia fetida* (Efet) and *Lumbricus terrestris* (Lter). Letter indicates different Tukey's test (HSD) subsets ($P < 0.05$). Values are least-square means ± SE.

II.3. Discussion

In agreement with our first hypothesis, we found that the impact of earthworms (individuals or CEx) on EPNs was species-specific. Overall, the earthworms themselves caused mostly neutral effects on EPN virulence and reproduction, but some positive and negative outcomes deserve some attention. Chelkha et al. (2020) showed that the CEx produced by *E. fetida* exhibited a deleterious effect on certain steinernematids at specific timings and conditions such as exposure to low IJ concentrations. However, our results showed some discrepancies with Chelkha et al. (2020). For example, we observed two divergent outcomes for its impact on the *S. feltiae* RM-107 population, the inhibition of its virulence in the presence of the earthworm *E. fetida* but not by its CEx. Moreover, we reported that the earthworm species *E. fetida* and its CEx increased the virulence of *H. bacteriophora* RM-102, while Chelkha et al. (2020) did not observe any effect on *H. bacteriophora* AM-203. These results suggest that the impact of earthworms on EPN virulence maybe not just species-specific but intraspecific. We studied EPN populations isolated from vineyards in La Rioja (Spain) (Blanco-Pérez et al., 2020) and those reported by Chelkha et al. (2020) from natural areas and citrus orchards in Algarve (Portugal) (Campos-Herrera et al., 2019). Diverse origins can explain significant physiological and ecological differences among EPN traits (Poinar, 1992). For

example, populations of *S. feltiae* isolated in La Rioja from agricultural areas or the edges of cultivated fields differed for relevant variables related to infectious dynamics and capability to complete their life cycles (Campos-Herrera and Gutiérrez, 2014). Similarly, different populations of *S. feltiae* showing intraspecific divergences differed in their virulence against various insect pests (Campos-Herrera and Gutiérrez, 2009). Another cause might be related to the earthworms. It is plausible that unnoticed changes in the culturing procedures or variable food supplied during the rearing in the commercial installation might alter the chemical composition of the CEx, which can be translated into a different impact on EPNs. Further studies are required to unravel the effect of multi-organism interactions on EPN populations.

The two earthworm species added in this study highlighted the complexity of the interactions between earthworms and EPNs, detecting positive, neutral, or negative impact depending on the EPN species, concentration applied, and the presence of earthworms or its CEx. Contrary to the results obtained for *E. fetida*, the earthworm species *L. terrestis* or CEx reduced *H. bacteriophora virulence* while, saving for a minor exception, did not affect steinernematids. Also, opposite to the pattern observed for *E. fetida*, we reported positive results for the EPN reproductive capability, often recording higher frequencies of insect cadavers producing progeny for steinernematids applied at high IJ concentrations when exposed to *L. terrestis* CEx. The impact of *P. excavatus on EPNs* also differed for the two other earthworm species evaluated.

Only *S. riojaense,* characterized by a larger IJ size (Půža et al., 2020), was unaffected by *P. excavatus* or its CEx at any IJ concentrations. On the other hand, *S. feltiae* virulence and reproductiveness were favored in the presence of this earthworm species, while its CEx exposed in the soil resulted detrimental for *H. bacteriophora.* Accordingly, the species-specific nature of these interactions might be based not only on EPNs but also on earthworm species. The specific behavior of each earthworm species evaluated could explain the differential impact on EPNs observed. Indeed, *E. fetida* and *P. excavatus* are epigeic (litter inhabitant) species, while *L. terrestris* is epi-anecic (mineral dweller) species (Römbke et al., 2005; Bottinelli et al., 2020). It seems plausible that minor changes in feeding and drilling behaviors could generate diverse ecological niches in the soil that affect the IJ survival and capacity to search for hosts differently. Besides, even if the chemical CEx composition could be similar for different earthworm species (Guhra et al., 2020), the presence of particular immune cells and antibiotics (Dales and Kalaç, 1992; Bilej et al., 1995, 1991; Kasschau et al., 2007; Fiołka et al., 2012) confer species specificity. Moreover, internal gut microbiota and environmental conditions (soil

type or organic matter content, for example) can also modulate the final CEx composition (Guhra et al., 2020). Hence, we suggest that specific compounds of each of the CEx investigated contribute to explain the differential impact observed on EPNs. Another possible explanation can be linked to the amount of CEx excreted per earthworm species. Although we applied the same final volume for all earthworm species, differences in size and weight could alter the concentration of the CEx excreted. In any case, further studies are required to compare the chemical and biological composition of the CEx evaluated to support our hypothesis and link them to our observations. In addition, the production of the CEx by the earthworm is another relevant factor to establish the real impact in this interaction. Despite the present and Chelhka et al. (2020) studies involved CEx produced under stress, recent experimentation performed with mucus derived from no-stressed earthworms has shown that in a two-arms olfactometer, the EPN species *H. megidis* preferred to move to maize-roots not treated with earthworm CEx, even if a high production of the EPN-attractant Eβc was reported (Fattore et al., 2020). Hence, independently of the form of extraction, there is evidence that the CEx produced by certain earthworms are negatively related to EPNs. The results by Fattore et al. (2020) and those due to the infectivity of EPN exposed to CEx presented here and by Chelkha et al. (2020) suggest that one possible explanation to the impact on EPNs is that IJs can sense the presence of the CEx and react to them by stimulating activity, or by blocking perception of host stimuli. Also, Chelkha et al. (2020) mentioned that intraspecific differences in the IJ cuticle might also be related to these results. For example, different species and stages of trichodorid nematodes differ in their cuticle composition and physical properties (Karanastasi et al., 2001). Since the two genera traditionally considered EPNs belong to phylogenetically distant families (Steinernematidae and Heterorhabditidae) that become entomopathogens by convergent evolution (Blaxter et al., 1998), it would be plausible that this assumption would also apply to EPNs. In summary, we cannot point to clear evidence that explains the differential impact of earthworms or their CEx on EPNs but, according to our results, it would be necessary to select the most compatible EPN species to apply in mature compost produced by vermicomposting (Herren et al., 2018) or other approaches. That is of primary relevance in the cases that require the use of *E. fetida* or other earthworm species for which detrimental effects have been proved (Chelkha et al., 2020). In any case, additional studies are required to establish the significance of these interactions in more naturalized conditions.

Here, we evaluated for the first time the impact of earthworms and their CEx on the virulence and reproductive capability of EPF. We observed a general reduction of *B. bassiana*

virulence in the presence of any of the CEx tested. In addition, the earthworm *L. terrestris* reduced the virulence at various timings, while *E. fetida* specimens only significantly decreased *B. bassiana* virulence at day eight after exposure. Taking all together, the overall trend for this EPF was a negative impact. However, we observed contrasting results for *M. anisopliae*, particularly for the soil application context. While no effect was reported for the interaction between *L. terrestris* and *M. anisopliae, E. fetida* CEx increased its virulence, but the specimens significantly reduced it. Plavšin et al. (2017) also reported a reduction in the growth of the phytopathogenic fungi *Fusarium oxysporum* (Hypocrealeas: Nectriaceae) when exposed to the coelomic fluids of the earthworm species *Dendrobaena vineta* and *E. fetida*. As mentioned for EPNs, specific immune cells and antibiotic and antifungal metabolites contained in the earthworms' CEx (Dales and Kalaç, 1992; Bilej et al., 1995, 1991; Kasschau et al., 2007; Fiołka et al., 2012) might contribute to inhibit EPF virulence and growth. However, whether the general detrimental effects are the natural trend, depend on the ecological scenarios evaluated, the species or strains tested, or a combination of these is unknown. Thus, further research is required to unravel the nature of these interactions and expand the possible impact of their co-occurrence in a natural environment.

On the other hand, our data provide some evidence on the possible consumption of EPF by the two earthworm species tested. Indeed, some studies suggest that earthworms commonly feed on fungi (Bonkowski et al., 2000; Maraun et al., 2003).

Thus, a decrease in the number or viability of conidia per Petri dish after earthworm feeding and movement activity could explain our results. *However*, Shapiro-Ilan and Brown (2013) observed that the earthworm species *L. terrestris* enhanced the dispersal of the EPF species *B. bassiana*, and the conidia recovered from the earthworm casts remained active against *G. mellonella* larva. Perhaps the 1.17 x 10^7 conidia/cm^2 concentration employed by Shapiro-Ilan and Brown (2013), two orders of magnitude higher than tested in our study (3.1-6.3 x 10^5 conidia/cm^2), masked the impact of the earthworms on the viability of EPF conidia while feeding and moving. However, we could not evaluate the effect on the conidia dispersion in experiments designed in Petri dishes. Probably, under other experimental conditions that allow the free movement of earthworms, it could be shown if the conidia dissemination would compensate for the negative impact observed on EPF infectivity and growth.

The use of beneficial soil organisms arises as an essential tool for pest and disease control in sustainable agriculture. Despite the absence of conclusive profits for crops, previous studies highlight the compatibility of diverse beneficial soil organism applications, including

EPNs, pseudomonads bacteria, and arbuscular mycorrhizal fungi (Imperiali et al., 2017; Jaffuel et al., 2019). However, the lack of information on the multitrophic interactions of the large variety of organisms existing in soils is critical to understand their impact on target crops. Laboratory studies provided evidence of the plasticity of EPN and EPF activity depending on the fine-tuning (application time or selection of populations and concentrations to apply) of their co-occurrence or co-application among other soil organisms such as nematophagous fungi (Bueno-Pallero et al., 2018). Also, olfactometer-based bioassays revealed that EPNs' response to the attractant Eβc alone was double in earthworm-worked soil than in earthworm-free soil (Fattore et al., 2020). However, the EPN movement towards plants treated with mucus was strongly limited despite high Eβc production, highlighting the complex interactions occurring in the soil among entomopathogens, earthworms, insects, and plants. Here we have shown that the impact of the earthworms or their CEx on the entomopathogenic activity was, despite some exceptions, species-specific, particularly for EPNs. Additional experiments based on more natural conditions, such as macrocosm composed of plants and natural (not autoclaved) soil, will allow us to understand complex interactions mediated by earthworms and entomopathogens that commonly occur in soils. Expanding our knowledge on the impact of co-occurrence of selected soil inhabitants must prevent inconsistencies when applying them. In the current context of social demands towards sustainable agriculture, this information is critical to providing effective crop management (for both implementation and conservation programs) based on beneficial soil organisms as entomopathogens.

III. New insights on the impact of earthworm extract on the growth of beneficial soil fungi: species-specific alteration of the nematophagous fungal growth and limitation of an entomopathogenic fungus

III.1. The effect of earthworm extract on growth, production, and germination of *Arthrobotrys musiformis* conidia

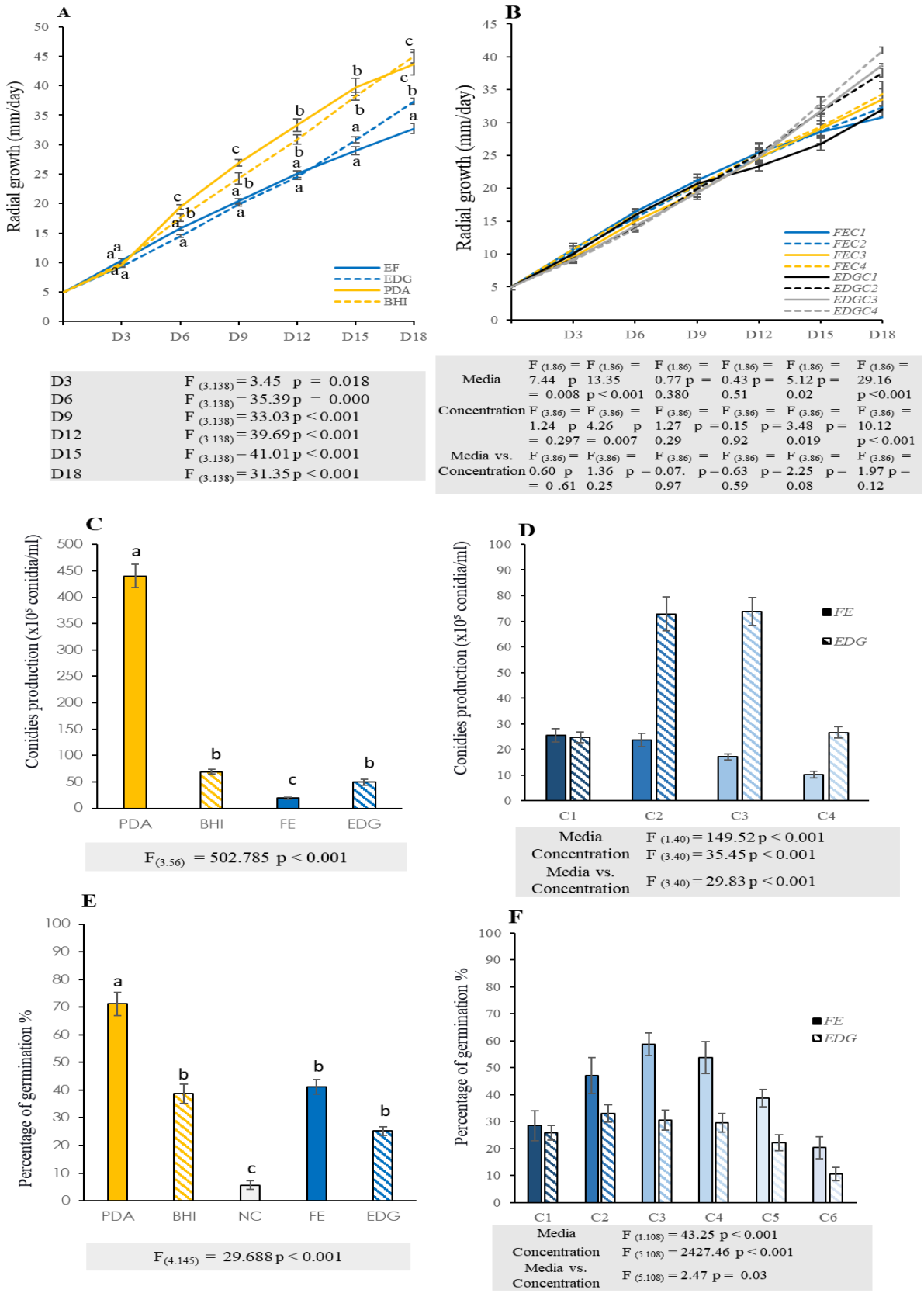
The type of earthworm medium and concentration affected the growth of *A. musiformis* (**Table IV**). Specifically, the differences on the growth in each media were expanding the differences while advancing in time (Figure 38a). Differences with additional media regarding worm extract-based media (FE and EDG) were observed after 6 days of growth. However, growth in the EDG medium was significantly higher than in FE after 18 days of development (Figure 38a). In addition, both worm extract-based media dilutions promoted higher fungal growth (Figure 38b). A significant impact of concentration and media was observed again

without substantial interaction on days 6 and 18. At the end of the experiment, the lower concentrations of both media favoured fungal growth, with EDG media (C4, C3, C2) having higher growth than FE media (Figure 38b).

Concerning sporulation, *A. musiformis* produced 4 to 10 times more conidia on PDA than on any other medium (Figure 38c). Conidial production in FE media was significantly lower than in EDG medium and the BHI positive control. Sporulation was affected by medium and concentration and their interaction (Figure 38d). In the FE medium, the lower the concentration, the fewer conidia were produced; however, optimal conidial production was achieved at intermediate concentrations (C2 and C3) in the EDG medium.

Evaluation of the germination capacity of these conidia showed that PDA was the best performing medium, recording ~ 70% germination (Figure 38e). In contrast to conidia formation, the EDG medium generated a lower germination percentage, but it was not significantly different between FE and BHI media (Figure 38e). When comparing the effect of earthworm extract media concentrations in detail, we observed an opposite trend in conidial production (Figure 38f). Regarding the germination percentage, the EF recorded the maximum values in the intermediate concentrations (C2-C3 and C4) with maximum germination for C3 (58.7 ± 4.25) (Figure 38f), while in EDG media, the germination percentage decreased with the dilution of the medium (Figure 38f).

Finally, overall, mycelial density was low at the low concentrations (C3 and C4) and almost zero for C5 and C6 (data not shown) (Figure 39).



D3	$F_{(3.138)} = 3.45$ p = 0.018
D6	$F_{(3.138)} = 35.39$ p = 0.000
D9	$F_{(3.138)} = 33.03$ p < 0.001
D12	$F_{(3.138)} = 39.69$ p < 0.001
D15	$F_{(3.138)} = 41.01$ p < 0.001
D18	$F_{(3.138)} = 31.35$ p < 0.001

Media	$F_{(1.86)}$ = 7.44 p = 0.008	$F_{(1.86)}$ = 13.35 p < 0.001	$F_{(1.86)}$ = 0.77 p = 0.380	$F_{(1.86)}$ = 0.43 p = 0.51	$F_{(1.86)}$ = 5.12 p = 0.02	$F_{(1.86)}$ = 29.16 p <0.001
Concentration	$F_{(3.86)}$ = 1.24 p = 0.297	$F_{(3.86)}$ = 4.26 p = 0.007	$F_{(3.86)}$ = 1.27 p = 0.29	$F_{(3.86)}$ = 0.15 p = 0.92	$F_{(3.86)}$ = 3.48 p = 0.019	$F_{(3.86)}$ = 10.12 p < 0.001
Media vs. Concentration	$F_{(3.86)}$ = 0.60 p = 0 .61	$F_{(3.86)}$ = 1.36 p = 0.25	$F_{(3.86)}$ = 0.07. p = 0.97	$F_{(3.86)}$ = 0.63 p = 0.59	$F_{(3.86)}$ = 2.25 p = 0.08	$F_{(3.86)}$ = 1.97 p = 0.12

$F_{(3.56)} = 502.785$ p < 0.001

Media	$F_{(1.40)} = 149.52$ p < 0.001
Concentration	$F_{(3.40)} = 35.45$ p < 0.001
Media vs. Concentration	$F_{(3.40)} = 29.83$ p < 0.001

$F_{(4.145)} = 29.688$ p < 0.001

Media	$F_{(1.108)} = 43.25$ p < 0.001
Concentration	$F_{(5.108)} = 2427.46$ p < 0.001
Media vs. Concentration	$F_{(5.108)} = 2.47$ p = 0.03

Figure 38. Evaluation of vegetative growth, conidial production and germination in the fungus *Arthrobotris musiformis* exposed to two earthworms' extracts: fresh earthworm (FE),

earthworms devoid of intestinal contents (EDG) and two conventional media: potato dextrose agar (PDA), and brain heart infusion agar (BHI). A. Accumulated growth from 3 to 18 days according to conventional and earthworm-based media. B. Accumulated growth as a function of concentration and earthworm-based medium. C. Conidia production (x10^5 conidia/ml) according to conventional and earthworm-based media. D. Conidia production (x10^5 conidia/ml) according to concentration and earthworm-based medium. E. Percent germination on conventional and earthworm-based media. F. Percent germination as a function of concentration and earthworm-based medium. Concentrations are equivalent to C1 = 40 g/l, C2 = 20 g/l, C3 = 10g/l, and C4 = 5g/l. Results of one-way ANOVA (A, C, E) or two-way ANOVA (B, D, F), and differences are significant at Tukey's test (HSD) and groups "a", "b" and "c".

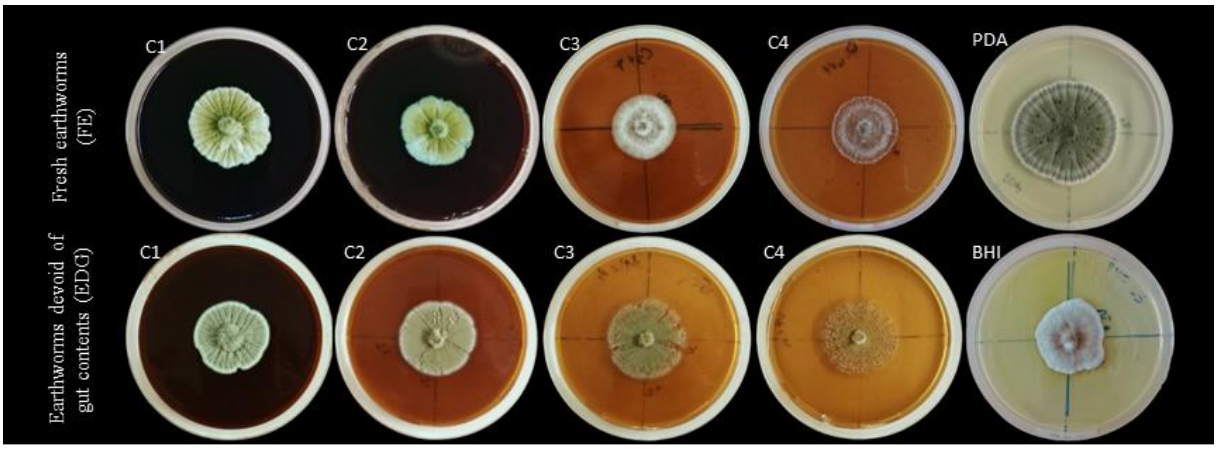


Figure 39. Growth of *Arthrobotrys musiformis* after 20 days post-exposer to two different earthworm based media: fresh earthworms (FE) (four concentration C1, C2, C3, and C4), and earthworms devoid of gut contents (EDG) (four concentration C1, C2, C3, and C4), C1 = 40g/l, C2 = 20g/l, C3 = 10g/l, C4 = 5g/l , and two rich media: Potato Dextrose Agar (PDA), and Brain heart infusion (BHI).

Table IV : Statistics (one-way ANOVA) of accumulative vegetative growth of the fungus *Arthrobotris musiformis* (3 to 18 days) and conidia production (x10^5 conidia/ml) after cultivation on two earthworm extract-based media (EF and EDG) at six concentrations (C1... C6) compared to two conventional media (PDA and BHI), and the percentage of germination on liquid earthworm extract media (EF and EDG) compared to two conventional media (PDA and BHI) and distilled water as negative control (NC). Groups are indicated when treatments are separated into different subsets according to Tukey's test (HSD). Values are least squares means ± SE. Significant differences are highlighted in bold.

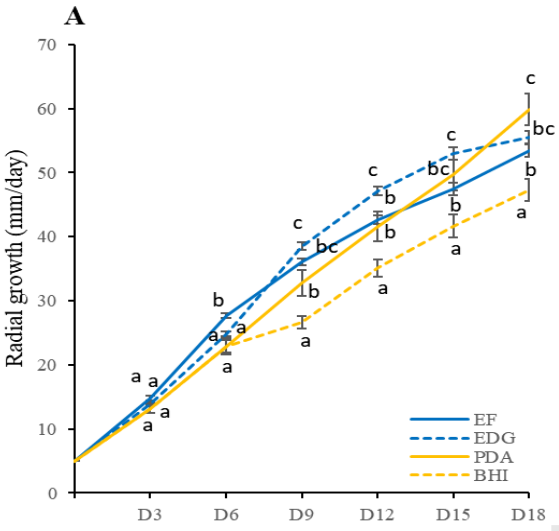
			C1=40 g/l	C2=20g/l	C3=10 g/l	C4=5 g/l	C5=2,5g/l	C6=1,25g/l	PDA=24g/l	BHI=37g/l	NC
Growth mm/Day	D3	FE	10,77±0,48a	10,38±0,56a	9,59±0,31a	10,71±0,87a	_	_	9,62±0,30a	9,94±0,16a	_
		EDG	10,01±0,27a	9,31±0,38a	9,22±0,40a	9,01±0,41a	_	_			
	D6	FE	16,46±0,45bc	15,52±0,41abc	15,00±0,50ab	16,04±0,78abc	_	_	**19,44±0,39d**	17,58±0,60cd	_
		EDG	15,89±0,60abc	13,94±0,26ab	14,34±0,23ab	13,76±0,42a	_	_			
	D9	FE	21,21±0,90ab	20,37±0,63ab	19,40±1,08a	21,49±1,24ab	_	_	**26,91±0,60c**	24,27±1,00bc	_
		EDG	20,63±0,54ab	19,92±0,40a	19,27±0,512a	19,50±0,84a	_	_			
	D12	FE	25,44±1,25a	24,62±0,78a	24,63±0,84a	26,8±1,49a	_	_	**33,30±1,03b**	**30,845±0,79b**	_
		EDG	23,33±0,63a	25,21±0,91a	24,69±0,74a	24,78±1,11a	_	_			
	D15	FE	28,58±1,65a	28,68±1,02ab	29,14±1,10ab	30,98±1,82ab	_	_	**39,77±1,44d**	38,23±0,67cd	_
		EDG	26,80±0,99ab	31,76±0,78ab	31,80±0,37ab	32,99±0,89bc	_	_			
	D18	FE	30,83±1,76a	32,31±1,30ab	33,59±1,53ab	35,24±2,16ab	_	_	43,71±1,92de	**45,00±1,10e**	_
		EDG	32,02±0,46ab	37,59±0,62abcd	38,77±0,21bcde	40,98±0,53cde	_	_			
Production des conidies		FE	25,55±2,61a	23,75±2,63a	17,08±1,2a	10,13±1,31a		_	**440,00±22,26c**	**69,30±4,88b**	_
		EDG	24,72±2,18a	72,77±6,56b	73,75±5,33b	26,66±2,25a	_	_			
Pourcentage of Germination %		FE	28,46±5,52bcd	47,12±6,63def	**58,69±4,25fg**	53,75±5,84efg	38,7±3,16cde	20,36±4,08abc	**71,13±2,16g**	38,67±2,43cde	5,63±1,77a
		EDG	25,76±2,74bc	32,97±3,31cd	30,48±3,63cd	29,47±3,51bcd	22,13±2,85abc	10,55±2,44ab			

III.2. The effect of earthworm extract on the growth, production, and germination of *Purpureocillium lilacinum* conidia

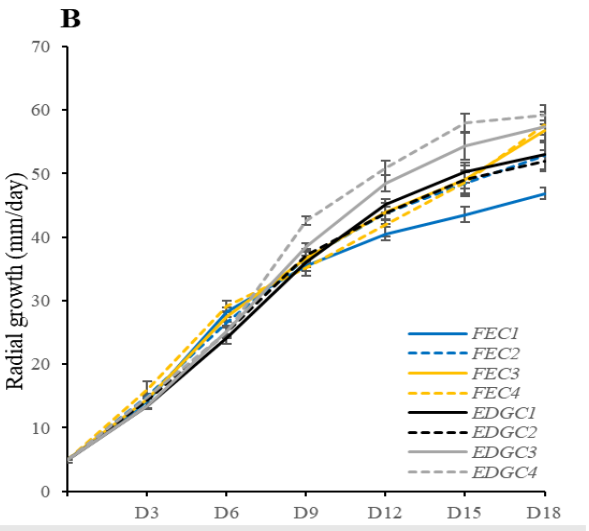
The type of earthworm medium and concentration affected the growth of *P. lilacinum*, contrary to that of *A. musiformis* (**Table V**). Specifically, the earthworm medium produced such differences since day 6 (Figure 40a). In detail, from day 9 to day 12, EDG media supported the highest growth of the fungus (Figure 40a), although, by day 18, PDA, FE, and EDG minimized the differences. In the concentration-effect analysis, media and concentration were statistically significant from day 9 to day 15, and the interaction was also significant on days 9 and 12. Overall, C3 and C4 in EDG media provided the highest fungal growth. At the end of the experiment (day 18), the lowest concentrations (C3 and C4) of the FE medium also supported *P. lilacinum* growth (Figure 40b).

Conidial production in earthworm media (FE and EDG) was lower than that observed in conventional PDA and BHI media (Figure 40c). Conidial production was affected by both factors (concentration/medium) and interaction (Figure 3d), with an opposite pattern observed for *A. musiformis*. Second, the FE medium showed an optimum at C3, whereas conidia production in the EDG medium decreased when the medium was diluted (Figure 40d). Finally, the germination percentage was more than twice as high in the conventional PDA medium as in BHI or any other earthworm medium (Figure 40e). The percentage of germination in both media (FE and EDG) decreased when the concentration was lower, with maximum germination for C1 = 45.9 ± 1.17 for FE medium (Figure 40f).

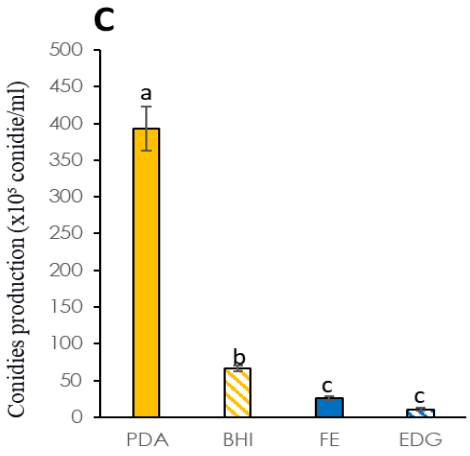
Finally, overall, the mycelial morphology was different depending on the treatment and invisible for concentrations C5 and C6 (data not shown) (Figure 41).



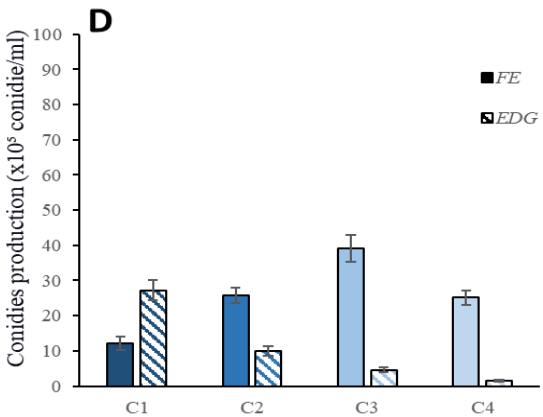
D3	$F_{(3.128)} = 3.113$ p = 0.029
D6	$F_{(3.124)} = 12.744$ p < 0.001
D9	$F_{(3.120)} = 30.577$ p < 0.001
D12	$F_{(3.120)} = 18.701$ p < 0.001
D15	$F_{(3.120)} = 11.483$ p < 0.001
D18	$F_{(3.120)} = 10.386$ p < 0.001



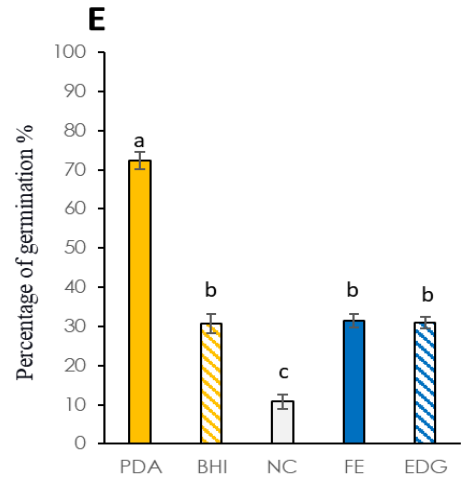
Media	$F_{(1.80)}$ = 1.70 p = 0.19	$F_{(1.76)}$ = 38.44 p < 0.001	$F_{(1.76)}$ = 15.49 p < 0.001	$F_{(1.72)}$ = 23.96 p < 0.001	$F_{(1.72)}$ = 19.08 p < 0.001	$F_{(1.72)}$ = 2.16 p = 0.14
Concentration	$F_{(3.80)}$ = 5.23 p = 0.002	$F_{(3.76)}$ = 1.71 p = 0.17	$F_{(3.76)}$ = 3.82 p = 0.013	$F_{(3.72)}$ = 3.94 p = 0.012	$F_{(3.72)}$ = 5.22 p = 0.003	$F_{(3.72)}$ = 9.95 p < 0.001
Media vs. Concentration	$F_{(3.80)}$ = 0.06 p = 0.97	$F_{(3.76)}$ = 0.80 p = 0.49	$F_{(3.76)}$ = 5.92 p < 0.001	$F_{(3.72)}$ = 3.47 p = 0.02	$F_{(3.72)}$ = 1.97 p = 0.12	$F_{(3.72)}$ = 1.53 p = 0.21



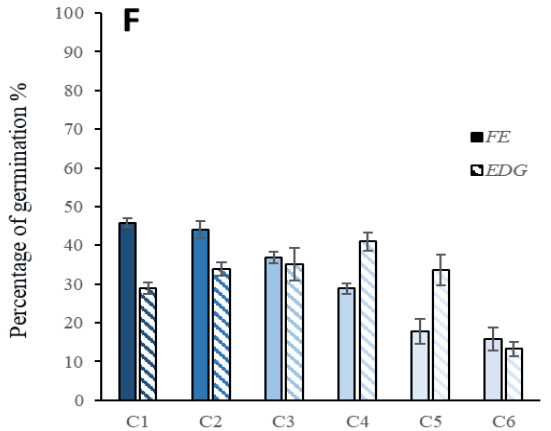
$F_{(3.56)} = 435.355$ p < 0.001



Media	$F_{(1.40)} = 93.12$ p < 0.001
Concentration	$F_{(3.40)} = 5.59$ p = 0.003
Media vs. Concentration	$F_{(3.40)} = 48.43$ p < 0.001



$F_{(4.145)} = 35.698$ p < 0.001



Media	$F_{(1.108)} = 0.148$ p = 0.70
Concentration	$F_{(5.108)} = 27.32$ p < 0.001
Media vs. Concentration	$F_{(5.108)} = 12.27$ p < 0.001

Figure 40. Evaluation of vegetative growth, conidial production and germination in the fungus *Purpureocillium lilacinum* exposed to two earthworm extracts: fresh earthworm (FE), earthworms devoid of intestinal contents (EDG) and two conventional media: potato dextrose agar (PDA), and brain heart infusion agar (BHI). A. Accumulated growth from 3 to 18 days according to conventional and earthworm-based media. B. Accumulated growth as a function of concentration and earthworm-based medium. C. Conidia production (x10^5 conidia/ml) according to conventional and earthworm-based media. D. Conidia production (x10^5 conidia/ml) according to concentration and earthworm-based medium. E. Percent germination on conventional and earthworm-based media. F. Percent germination as a function of concentration and earthworm-based medium. Concentrations are equivalent to C1 = 40 g/l, C2 = 20 g/l, C3 = 10g/l, and C4 = 5g/l. Results of one-way ANOVA (A, C, E) or two-way ANOVA (B, D, F), and differences are significant at Tukey's test (HSD) and groups "a", "b" and "c".

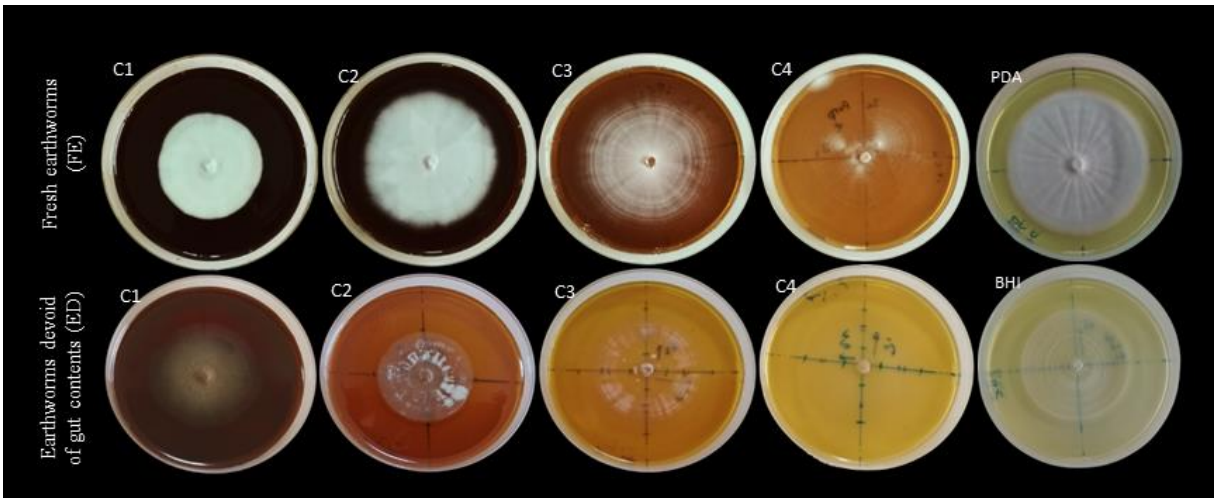


Figure 41. Growth of *Purpureocillium lilacinum* after 20 days post-exposer to two different earthworm based media: fresh earthworms (FE) (four concentration C1, C2, C3, and C4), and earthworms devoid of gut contents (EDG) (four concentration C1, C2, C3, and C4), C1 = 40g/l, C2 = 20g/l, C3 = 10g/l, C4 = 5g/l , and two rich media: Potato Dextrose Agar (PDA), and Brain heart infusion (BHI).

Table V: Statistics (one-way ANOVA) of accumulative vegetative growth of *Purpureocillium lilacinum* fungus (3 to 18 days) and conidia production (x10^5 conidia/ml) after cultivation to two earthworm extract-based media (EF and EDG) at six concentrations (C1... C6) compared to two conventional media (PDA and BHI), and the percentage of germination on liquid earthworm extract media (EF and EDG) compared to two conventional media (PDA and BHI) and distilled water as negative control (NC). Groups are indicated when treatments are separated into different subsets according to Tukey's test (HSD). Values are least squares means ± SE. Significant differences are highlighted in bold.

			C1=40 g/l	C2=20g/l	C3=10 g/l	C4=5 g/l	C5=2,5g/l	C6=1,25g/l	PDA=24g/l	BHI=37g/l	NC
Growth mm/day	D3	FE	13,91±0,40a	14,95±0,37a	14,46±0,50a	15,99±1,37a	_	_	13,10±0,63a	13,21±0,69a	_
		EDG	13,43±0,50a	14,43±0,35a	13,66±0,37a	15,67±0,58a	_	_			
	D6	FE	28,17±0,71bc	26,57±0,78abc	27,44±0,70abc	**29,14±0,81c**	_	_	22,89±1,28a	22,93±0,97a	_
		EDG	24,09±0,49ab	24,20±0,96ab	24,94±0,46abc	25,13±0,93abc	_	_			
	D9	FE	35,44±0,75b	36,69±1,15bc	36,75±0,97bc	35,11±1,15b	_	_	32,76±2,04ab	26,65±0,98a	_
		EDG	36,16±0,85bc	37,07±1,04bc	38,50±0,54bc	**42,55±0,72c**	_	_			
	D12	FE	40,52±1,02ab	43,73±1,64bc	44,00±1,33bc	41,95±1,86ab	_	_	41,65±2,35ab	35,11±1,33a	_
		EDG	45,07±0,89bc	43,83±0,98bc	48,44±1,31bc	**50,86±1,10c**	_	_			
	D15	FE	43,53±1,21a	48,44±2,07ab	49,01±2,20ab	48,55±1,67a	_	_	49,63±2,4ab	41,66±1,8a	_
		EDG	50,26±1,4ab	49,03±1,35ab	**54,30±2,09b**	**58,04±1,42b**	_	_			
	D18	FE	46,84±0,93a	52,91±2,29abc	56,73±1,56abc	**57,69±1,57c**	_	_	**59,90±2,43c**	47,31±1,69ab	
		EDG	53,06±1,77abc	51,99±1,63abc	57,39±2,33bc	**59,21±1,52c**	_	_			
Reproduction		FE	12,22±1,8a	25,83±2,16ab	39,02±3,82ab	25,13±1,98ab		_	**392,91±29,54c**	66,11±3,64b	_
		EDG	27,22±2,84ab	9,99±1,37a	4,58±0,63a	1,52±0,25a	_	_			
Pourcentage of Germination %		FE	**45,89±1,17f**	44,00±2,30ef	36,88±1,28cdef	28,85±1,40bc	17,72±3,19ab	15,79±3,017a	**72,39±4,30g**	30,77±3,40cd	10,81±1,63a
		EDG	28,92±1,45bc	33,88±1,74cdef	35,09±4,26cdef	41,03±2,38def	33,62±3,90cde	13,20±1,96a			

III.3. The effect of earthworm extract on the growth, production, and germination of *Beauveria bassiana* conidia

The *B. bassiana* fungus did not grow in either earthworm medium. However, *B. bassiana* conidia germinated on both earthworm-based media at a rate similar to that of the conventional BHI medium (~ 20-30%), whereas the PDA supported > 60% conidial germination (Figure 5a). Otherwise, the medium did not affect germination, while the concentration affected their germination, with the lower concentrations of C5 and C6 reducing about half of the germination capacity (Figure 42b), with maximum germination for C4 = 45.2 ± 3.55 for EDG medium (**Table VI**).

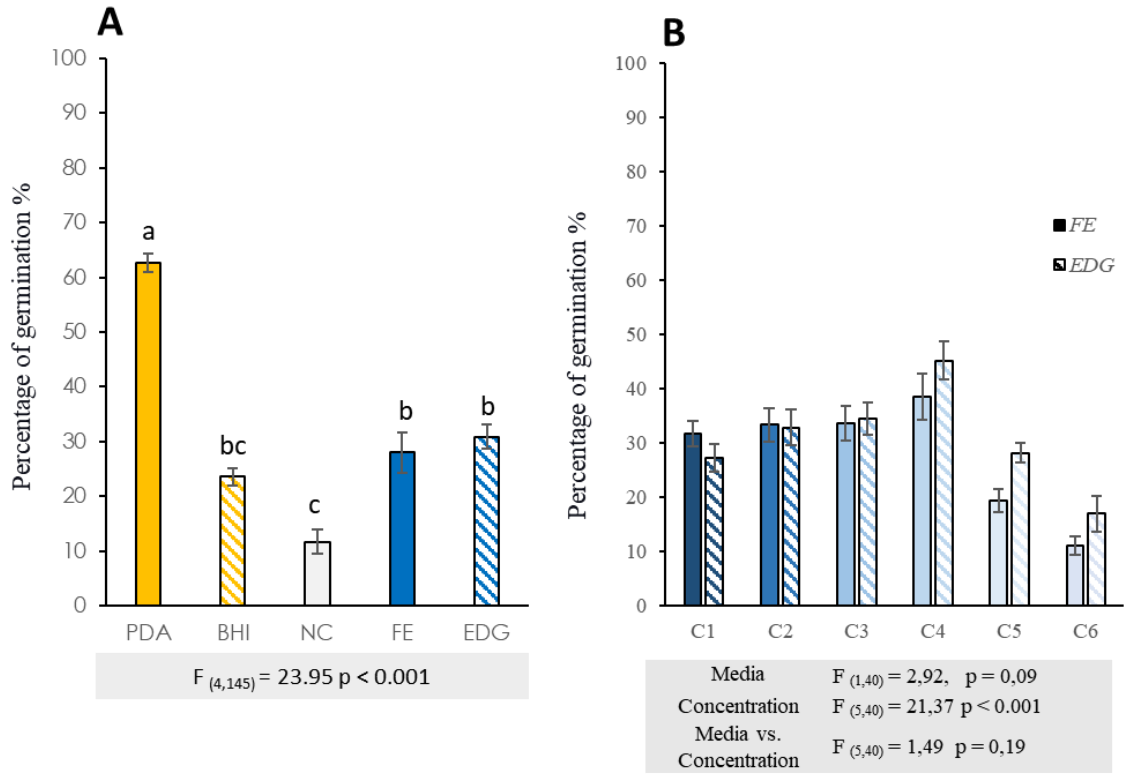


Figure 42. Evaluation of conidial germination of the fungus *Beauveria bassiana* exposed to two different earthworm extracts: fresh earthworms (FE) and earthworms without gut contents, EDG, and two conventional media: potato dextrose agar (PDA), and brain heart infusion agar (BHI). A. Percentage germination on conventional and earthworm-based media. B. Percent germination as a function of concentration and earthworm-based medium. Concentrations are equivalent to C1 = 40 g/l, C2 = 20 g/l, C3 = 10g/l, and C4 = 5g/l. Results of one-way ANOVA (A) or two-way ANOVA (B), and differences are significant according to Tukey's test (HSD) and groups "a", "b" and "c".

Table VI: Statistics (one-way ANOVA) of the percentage of germination of *Beauveria bassiana* fungus (3 to 18 days) on liquid earthworm extract media (EF and EDG) at six concentrations (C1...C6) compared to two conventional media (PDA and BHI) and distilled water as negative control (NC). Groups are indicated when treatments are separated into different subsets according to Tukey's test (HSD). Values are least squares means ± SE. Significant differences are highlighted in bold.

		C1=40 g/l	C2=20g/l	C3=10 g/l	C4=5 g/l	C5=2,5g/l	C6=1,25g/l	PDA=24g/l	BHI=37g/l	NC
Percentage of Germination %	FE	31,79±2,40bcde	33,38±3,07cdef	33,64±3,13cdef	38,57±4,22ef	19,39±2,15abc	11,07±1,74a	**62,63±4,09g**	23,62±3,65abc	11,67±2,18a
	EDG	27,29±2,52bcde	32,84±3,29cdef	34,61±2,96def	**45,22±3,55f**	28,21±1,79bcd	17,02±3,26ab			

III.4. Discussion

In agreement with our hypothesis, the results showed that different media based on earthworm products could allow the growth and generation of reproductive structures for certain fungi in a species-specific manner. This can be translated into the possible impact of decaying earthworms in nature, which could stimulate the growth and reproduction of specific biological control agents naturally present in the soil. However, their support is species-specific and depends on specialization. Thus, the NF *P. lilacinum*, which could have saprophytic activity as a parasite of eggs and organic matter, showed higher growth in earthworm environments than in conventional environments. At the same time, the NF *A. musiformis*, could grow in earthworm environments but more slowly than in conventional environments. Finally, the entomopathogenic fungus *B. bassiana* could not grow in any of the earthworm-based media.

The fact that dilution of the earthworm-based media contributed to the overall improvement in fungal growth prompts the consideration that the high concentration of FE and EDG media may have an overabundance of nutrients and certain proteins; thus, the presence of certain compounds derived from earthworm cutaneous excreta and coelomic fluid or a combination of all these factors may alter their activity (Zhou et al., 2021; Chelkha et al., 2021). Indeed, inhibitory growth activity was observed when the phytopathogenic fungi *Berkeleyomyces basicola, Fusarium culmorum, Fusarium oxysporum, Globisporangium irregulare, Rhizoctonia solani, Macrophomina phaseolina*, and *Sclerotinia sclerotiorum* were exposed to the coelomic fluid extract of some earthworm species (Ečimović et al., 2021; Plavšin et al., 2017). In addition, the paste or powder of *Perionyx excavatus* and *Eudrilus eugeniae* were found to be antifungal against *Aspergillus flavus, Aspergillus niger*, and *Candida albicans* (Punu et al., 2016; Sethulakshmi et al., 2018).

Overall, when the two earthworm-based media were compared, the EDG allowed for greater growth of NF mycelia than the FE extract, suggesting that certain components of the earthworm gut present in the FE medium may help inhibit or limit the growth of mycelia of both fungi (Shobha and Kale, 2008; Bhorgin and Uma, 2014; Chauhan, 2014). The earthworm gut is well known to mineralize organic matter into finer particles through microbial decomposition (Brown et al., 2000). This activity results in a decisive release of nutrients, which has been shown to promote plant growth (Scheu, 1987; Whalen and Parmelee, 2000; Brown et al., 2004; Amador et al., 2006). However, as our results suggest, this mineralization may not

promote NF growth. Also, it is also plausible that the high FE concentration included an overabundance of nutrients because the negative effect observed in the high concentration treatments was reduced in the treatments with the dilution of the FE.

In addition to the impact on mycelial growth, the earthworm-based media also influenced conidial production and germination. Indeed, conidial germination was the only plausible activity of *B. bassiana* when grown in one of the two earthworm-based media. In this case, the germination did not depend on the presence or absence of gut contents (FE or EDG, respectively) but their concentration, suggesting that the inhibitory effect originates from the earthworm itself and not from the gut-associated microbiota. For NF, the lowest conidial production was recorded for FE media for both NF species, but this effect was not as strong when its germination was studied.

Overall, the dilutions (C2-C4) improved the potential reproductive values of both NF (conidial production and germination percentage), with some exceptions. This result agrees with the impact of earthworm-based media and the dilutions studied on mycelial growth, which can also be attributed to nutrient overabundance, the presence of certain antibiotics and antifungal derivatives, or a combination of all these factors (Zhou et al., 2021; Chelkha et al., 2021). In conclusion, the presence of earthworm cadavers in the soil can modulate the activity of some soil inhabitants and provides nutrients for the vegetative and reproductive actions of NF and EPFs in a species-specific manner.

Chapter 3: GENERAL CONCLUSION AND PERSPECTIVES

I. GENERAL DISCUSSION

Agricultural intensification can deliver high crop yields but typically comes at the cost of significant land and biodiversity degradation, ultimately causing significant issues for long-term food supplies (Pimentel, 2000). Various sustainable approaches are being applied to counterbalance the unfavorable consequences of intensive crop farming, such as organic agriculture and intercropping cultivation, which allow minimum disturbance to agroecosystems and maximal preservation of soil biodiversity, functions, and services (Altieri, 2004). Sustainable farming supports complementary approaches, such as integrated pest management (IPM), which emphasizes using biological control agents to reduce insect populations. Entomopathogenic nematodes (EPNs), entomopathogenic fungi (EPFs), and nematophagous fungi (NFs) are among the most promising biocontrol agents. Generally, they can actively locate a host (pest or nematode), kill them in a matter of days (depending on the agents), and are easily mass-produced, allowing commercial use.

Likewise, earthworms favourably affect plant performance through feeding, burrowing, and casting behaviours. Overall, earthworms can improve soil aeration and soil water retention capacity, promote nutrient turnover, and stimulate beneficial microbial biomass and activity, boosting plant growth (le Bayon et al., 2017). Earthworms come into intimate contact with many soil organisms, and hence, they can modify their activity directly by affecting their virulence or displacement or indirectly by modifying the Physico-chemical properties of the soil (Edwards, 2020). The combination of various beneficial soil organisms such as earthworms with any biocontrol agent such as nematode or fungi needs a deep understanding of their interaction to optimize their use.

This thesis widens our knowledge of the multitrophic interaction between earthworms as an abundant invertebrate in soil and various biological control agents. We performed a comprehensive study of the impact of BCs agents' efficiency in the presence of an earthworm, their cutaneous excreta, or their main cadavers. An extensive range of biological control agents was used in this thesis, comprising entomopathogenic nematodes and fungi as well as

nematophagous fungi. In detail, we investigated entomopathogenic nematodes from the family steinernematids: *S. feltiae* AM-25 and RM107 population, *S. khuongi* Arc population (Stock et al., 2019), *S. glaseri* NC population, and *S. carpocapsae* ALL population, and the new species *S. riojaense* RM30 (Půža et al., 2020), and members of the family Heterorhanditidae: *H. zealandica* Btw population and *H. bacteriophora* AM-203 and RM102 populations). We also evaluated several fungi employed as biological control agentes. Two entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae*, and two nematophagous fungi: *Arthrobotrys musiformis* and *Purpureocilium lilacinus*. Otherwise, four we used earthworm species: *Eisenia fetida, Lumbricus terrestris*, *Perionyx excavatus* (Haplotaxida) from commercial sources, and one native species from Morocco *Aporrectodea molleri* MT878074.1 (Table VII) (Yakkou et al., 2021).

In this thesis, and for the first time, we explored (i) whether the presence of earthworms or their CEx might have a sub-lethal effect on the EPN and EPF, (ii) if the EPN/EPF biocontrol activity can be altered in the presence of earthworms by the feeding or the CEx, and (iii) whether the earthworm body tissue can support the growth of NF and EPF. Hence, altogether, our data should be considered for the optimization of the use of biological control agents in soil with a high abundance of earthworms or the combination of these agents (EPN/EPF and NF) with the vermicompost that includes a high concentration of CEx or the earthworm body tissue associated with the earthworm's mortality. Additionally, extending our knowledge on the influence of co-occurrence of selected soil inhabitants must prevent discrepancies when utilizing them. In the modern context of social pressures toward sustainable agriculture, this information is crucial to provide proper crop management based on beneficial soil organisms as entomopathogens.

Table VII: The biological material used during this thesis.

Species name	Isolate name	Origin	GenBank n°	References
Entomopathogenic nematodes				
S. feltiae	AM-25	Algarve	MG551674	(Bueno-Pallero et al., 2018)
S. khuongi	Arc	Florida, USA	GU177835	(Stock et al., 2019)
S. glaseri	NC	USA	AF331908	(Stock et al., 2019)
S. carpocapsae	ALL	USA	AF331900	(Mráček et al., 2014)
S. feltiae	RM107	La Rioja, Spain	MW480131	(Blanco-Pérez et al., 2020)
S. riojaense	RM30	La Rioja, Spain	MK503133	(Půža et al., 2020)
H. bacteriophora	AM-203	Algarve, Portugal	MG551677	(Blanco-Pérez et al., 2019)
H. bacteriophora	RM102	La Rioja, Spain	MW480132	(Blanco-Pérez et al., 2020)
H. zealandica	Btw	USA	GU174009	
Entomopathogenic fungus				
B. bassiana	Balsamo DF83	Algarve, Portugal	MG515530	(Bueno-Pallero et al., 2018)
M.anisopliae	DF89	Algarve, Portugal	MN808333	(Bueno-Pallero et al., 2020)
Nematophagous fungi				
Arthrobotrys musiformis	Drechsler 11	Florida, USA	KJ938572	(Bueno-Pallero et al., 2018)
Purpureocillium lilacinum	Thom 9357	Florida, USA	KJ938575	(Campos-Herrera et al., 2015)
Earthworms				
Eisenia fetida		The commercial stock of natural worms' nurseries for vermicomposting at "O Minhocario" (Pedro José Lanza, Lisbon, Portugal)		
Lumbricus terrestris				
***Perionyx excavatus* (Haplotaxida)**				
Aporrectodea molleri		Rabat, Morocco	MT878074.1	(Yakkou et al., 2021)

Little is known about the complex interplay between earthworms and EPNs in the soil. For the first time, we evaluated the impact of the earthworm *E.fetida* or their CEx on the potential biocontrol activity of EPN in the soil as a first approach (proof of concept) (Chelkha et al., 2020) . The aim was to elucidate whether CEx can affect the infectivity and reproductive success of EPNs. As a result, we observed that the CEx produced by the earthworm *E. fetida* might be deleterious to EPN infective juvenile (IJs), altering their ability to kill insect larvae and even decreasing their reproductive success, and hence, the biocontrol activity, but only in certain species and under certain circumstances, suggesting a possible species-specific effect.

Later, Chelkha et al. (2021) unravelled how the patterns observed with the earthworm *E. fetida* could be extended to other earthworm and EPN species. This study evaluated how the presence of three earthworm species (*Eisenia fetida, Lumbricus terrestris*, and *Perionyx excavatus*) or their CEx can affect the virulence and reproduction capability of different species and populations of EPNs. We also aimed to investigate whether the CEx affects the infectivity and reproductive success of EPNs applied at high or low concentrations. Our findings confirmed that the impact of earthworms on EPN virulence might be species-specific as well as intraspecific. The intricacy of interactions between earthworms and EPNs, detecting positive, neutral, or negative impact depending on EPN species, the concentration used, and the presence of earthworms or its CEx, illustrated the complexity of these interactions and determined the relevance of the study to ensure a successful combination of various beneficial soil organisms.

Few studies investigated the interaction between earthworms and entomopathogenic nematodes. Shapiro et al. (1993, 1995) and Shapiro-Ilan and Brown (2013) demonstrated that earthworms could indirectly affect root feeders by increasing EPN's dispersal and biocontrol capacity. In addition, Campos-Herrera et al. (2006) observed that the EPN *S. feltiae* did not survive throughout the gut passage of the earthworms *E. fetida*, considering that the feeding behaviour might have a deleterious effect on the presence of EPN. Furthermore, a recent study by Fattore et al. (2020) found that EPNs moved away from mucus-watered plants (which also included CEx) by a factor of three when compared to controls. Those studies reinforced our research results and proved that the interaction could be positive, neutral, or negative depending first on the EPN species, population, origins, size, and behaviour (infectious dynamics, mobility) and the design of the experiments, the nematodes rearing conditions, and the host used. From the earthworm point of view, it is also essential to consider the species, the

ecological category, the rearing conditions, and the food served, besides the CEx extraction methods, concentration, and compositions.

We evaluate the interaction of earthworms with EPF, and for the first time, with their CEx. We hypothesized that diverse earthworm species could differently affect EPF infectivity and reproductive capacity. Our study assessed the interaction of different earthworm species (*Eisenia fetida, Lumbricus terrestris*) with two EPF species, *Beauveria bassiana* and *Metarhizium anisopliae,* in two independent approaches. Overall, our results suggest a general reduction of *B. bassiana* virulence in the presence of any of the CEx examined, whereas no effect was recorded for the interaction between *L. terrestris* and *M. anisopliae*. In general, earthworm CEx affected EPF, but still, our hypotheses were supported in species-specific ways. Additionally, we explored the impact of earthworm body tissue on the growth of EPF. However, we observed that the EPF *B. bassiana* did not grow in any media (FE, EDG), but it showed germination in the liquid media.

Few investigations have demonstrated the link between earthworms and EPF. Shapiro-Ilan and Brown (2013) discovered that the earthworm specie *L. terrestris* aided in spreading the EPF species *B. bassiana*, and that the conidia obtained from earthworm castings remained active against *G. mellonella* larva. This study is interested in the phoretic relationship, which is the ability of earthworms to distribute from outside by movement and through the gut, so earthworms could be utilized as vectors to introduce or disperse entomopathogenic fungi. However, the recent study by Zhou et al. (2021) revealed that inoculating A. hetaohei larvae with a mixture of *B. bassiana* and epidermal mucus of *E. fetida* could reduce *B. bassiana* vitality and pathogenicity to the insect, resulting in a slower disease course and lower mortality which reinforces our results. Thus, further research is required to unravel the nature of these interactions and expand the possible impact of their co-occurrence in a natural environment. The effect of earthworms' body tissue on the growth of nematophagous fungi (*Arthrobotrys musiformis,* and *Purpureocillium lilacinum*).

Few investigations explored the impact of earthworm body tissue on fungus naturally occurring in the soil. However, our study also considers this possible interaction. We hypothesize that earthworms extract (body tissues) could boost the growth of NF and EPF in a species-specific way. However, only the NF could grow and multiply in earthworm-based media (with or without the gut content), except when those media were highly diluted,

following a species-specific pattern. In any case, the earthworm-based medium lowered the fungal growth compared with the PDA-rich medium. Our data imply that the use of earthworms as a resource depends on the predicted specialization the NF *A. musiformis* slightly influenced, and *P. lilacinum* as unaffected. The only studies showing a similar approach were made by Punu et al. (2016), and Sethulakshmi et al. (2018) using *Perionyx excavatus* and *Eudrilus eugeniae* paste or powder that resulted in being antifungal against *Aspergillus flavus*, *Aspergillus niger*, and Candida albicans. However, still, they are not testing any biological control agents. So, our result remains unique and can be comparable to any similar studies.

II. PERSPECTIVES

For the first time, we investigated how earthworm CEx can interact with EPN. One of the limitations of our experimental procedure was that we could not determine if the decrease in the activity was due to low IJ survival or changes in their infectivity phase of them because the traditional insect bait technique cannot register their presence. Furthermore, earthworms secrete various liquids such as the mucus, the epidermal mucus, and coelomic fluid discharged over their body surface that named the "cutaneous excreta" which is a water-soluble mixture of low molecular weight carbohydrates, aminoacids, glycosides and glycoproteins, and mucus proteins from the immunity system (Zhang et al., 2016). This complex liquid has been shown to suppress some bacteria and impact the activity and abundance of bacteria (Wang et al., 2007). In our studies, we did not search for the component of the CEx responsible for lowering the infectivity of EPNs. Further studies are needed to determine the chemical and biological composition of the CEx of various earthworms' species to understand which could be responsible for that deleterious effect.

Nevertheless, our results agree with the relevance of selecting the most compatible EPN species to apply in mature compost produced by vermicomposting (Herren et al., 2018) or other eco-friendly approaches (Singh et al., 2020; Tóthné Bogdányi et al., 2021). Especially in the cases that require the use of earthworm species for which detrimental effects have been already proved (Chelkha et al., 2020, 2021). In any case, additional studies are required to establish the significance of these interactions. Plus, it would be interesting to investigate the interaction between other non-commercial earthworms to determine the possible impact on natural conditions. For example, it can be investigated the interaction between the earthworm *Aporrectodea molleri* (GenBank Accession number MT878074) isolated from

Morocco (Yakkou et al., 2021), and some native EPN Moroccan populations such as *H. bacteriophora* (GenBank Accession number MN420270) or *S. feltiae* (GenBank Accession number MN749619) isolate from different regions (Meknès-El Hajeb, Ifrane and Tafilalet) (Benseddik et al., 2020)

Additional to the laboratory studies, greenhouse and field trials are required to unravel the impact on the biocontrol activity of EPN when we combine them with earthworms in more commercial applications. Finally, in the context of global warming, exploring how the principal abiotic factors (atmospheric CO2, temperature, and soil moisture) can affect those interactions will support predictive models of their efficacy in combined scenarios (Guyer et al., 2021). In any case, this thesis highlighted that the beneficial potential of the earthworms and EPN is species-specific and depends on a multifactorial environment, and further research is warranted to use their beneficial actions better.

Expanding the knowledge on the interaction of earthworms with EPFs is crucial to understanding their influence on the target insect pest, the environment, and the management attributes. Under laboratory circumstances, the present study's limitations can modify EPF activity, such as the common use of sterile conditions, selected populations, and concentrations. Nevertheless, the controlled conditions are a suitable system to simplify complex scenarios that can help understand the main drivers of the interactions. At the same time, the controlled conditions solve other critical factors and multitrophic interactions that exist in natural conditions and create a drastic alteration to the efficiency of EPF. Further studies using more natural settings, such as field experiments or macrocosms composed of plants and natural soil, can help us to unravel the intricate interactions mediated by earthworms and EPF, which typically occur in natural and cultivated soils. Also, we are aware that our study was limited to only two EPFs, *M. anisopliae* and *B. bassiana, two of the most extensively used and encountered EPF (Lacey et al., 2015). However,* more than 300 entomopathogenic species are described, some of the most common belonging to the genera *Aspergillus, Metarhizium, Hirsutella, Beauveria, Aschersonia Culicinomyces, Lecanicillium, Paecilomyces, and Tolypocladium (Sinha et al., 2016).* In addition, the Phyla Ascomycota and Deuteromycota, other phyla such as Chytridiomycota, Oomycota, Zygomycota, and Basidiomycota, include some genera and hundreds of species described as entomopathogens (Sinha et al., 2016). Hence, extending the research with other species is very recommended. Also, studying other EPF is critical to understanding the pattern's extent.

There is a general ambition for searching for new strategies to minimize fertilization and pesticides, and earthworms as biofertilizers is a new concept that is attracting attention exponentially. Indeed, earthworms have been introduced into the industry nowadays, but their formulation is still poorly used. This study suggests the use of earthworms as an alternative source of protein (Sun et al., 1997; Parolini et al., 2020; Chakraborty et al., 2021) and as a rich media to grow biological control agents and preserve their pathogenicity and efficiency in a low cost and sustainable strategy. Additionally, earthworm mortality in the natural territory could be exposed to numerous environmental factors that enhance the degradation of the body tissue. In controlled conditions, we limit the degradation, but this fact does not reproduce the natural conditions. Also, the soil organisms enhance the degradation, and the body vanishes in a few days. A naturalized approach should be investigated to clarify the real impact of earthworms' tissue on NF behavior. Adding plants to the mesocosm containing NF, earthworm tissue, and the soil nematode community could give real meaning to the study and extend the knowledge of NF behavior (Fattore et al., 2020).

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PUBLICATION, GRANTS, AWARDS, AND MEETING

ARTICLES

- **New insights on the impact of earthworm extract on the growth of beneficial soil fungi: species-specific alteration of the nematophagous fungal growth and limitation of an entomopathogenic fungus. Chelkha, M**., Yakkou, L., Houida, S., Raouane, M., Amghar, S., El Harti, A. Campos-Herrera, R (2022). Turkish journal of zoology.
- **Cutaneous excreta of the earthworm *Eisenia fetida* (Haplotaxida: Lumbricidae) might hinder the biological control performance of entomopathogenic nematodes. Chelkha, M**., Blanco-Pérez, R., Bueno-Pallero, F. Á., Amghar, S., El Harti, A., & Campos-Herrera, R. (2020).Soil Biology and Biochemistry, 141, 107691. https://doi:10.1016/j.soilbio.2019.107691
- **Earthworms and their cutaneous excreta can modify the virulence and reproductive capability of entomopathogenic nematodes and fungi. Chelkha, M**., Blanco-Pérez, R., Vicente-Díez, I., Bueno-Pallero, F. Á., Amghar, S., El Harti, A., & Campos-Herrera, R. (2021). Journal of Invertebrate Pathology, 184, 107620. https://doi:10.1016/j.jip.2021.107620
- **Intraspecific virulence of entomopathogenic nematodes against the pests Frankliniella occidentalis (Thysanoptera: Thripidae) and Tuta absoluta (Lepidoptera: Gelechiidae).** Campos-Herrera, R., Vicente-Díez, I., Galeano, M., **Chelkha, M.,** del Mar González-Trujillo, M., Puelles, M., ... & Belda, J. E. (2021). Journal of Nematology, 53. https://doi.org/10.21307/jofnem-2021-102
- **Exploring the use of entomopathogenic nematodes and the natural products derived from their symbiotic bacteria to control the grapevine moth, *Lobesia botrana* (Lepidoptera: Tortricidae)**. Vicente-Díez, I., Blanco-Pérez, R., **Chelkha, M.**, Puelles, M., Pou, A., & Campos-Herrera, R. (2021). Insects, 12(11), 1033. https://doi.org/10.3390/insects12111033
- **Positioning entomopathogenic nematodes for the future viticulture: Exploring their use against biotic threats and as bioindicators of soil health.** Campos-Herrera, R., Vicente-Diez, I., Blanco-Perez, R., **Chelkha, M.**, González-Trujillo, M., Puelles,

M., Cepulite, R. Pou, A. (2021). Turkish Journal of Zoology 45:335–346. https://doi:10.3906/zoo-2106-40

- **Coexistencia de dos organismos beneficiosos del suelo:¿ Pueden las lombrices de tierra alterar la actividad beneficiosa de los nematodos entomopatógenos como agentes de control biológico?. Chelkha, M.**, Pérez, R. B., Pallero, F. B., Díez, I. V., Amghar, S., El Harti, A., & Herrera, R. C. (2020). AE. Revista Agroecológica de Divulgación, (39), 4.

INTERNATIONAL MEETING

- Poster: **Plasticity in the use *Xenorhabdus nematophila* and *Photorhabdus laumondii* against *Botrytis cinerea*.** Ignacio Vicente-Díez, Rubén Blanco-Pérez, **Maryam Chelkha**, Alicia Pou, Raquel Campos-Herrera. ICN 2022 - 7th International Congress of Nematology which took place in Antibes Juan-Les-Pins from 1 to 6 May 2022.

- Poster: ***Steinernema carpocapsae* and *Xenorhabdus nematophila* based products for the control of the grapevine moth and the grey mold in vineyards.** Ignacio Vicente-Díez, Rubén Blanco-Pérez, **Maryam Chelkha**, Miguel Puelles, Alicia Pou, Raquel Campos-Herrera. International Congress on Invertebrate Pathology and Microbial Control & 53rd Annual Meeting of the Society for Invertebrate Pathology. 28th June - 2nd July 2021.
- Poster: **Screening of adjuvants to enhance the entomopathogenic nematode survival and adherence after aerial application on grapevine leaves.** María del Mar González-Trujillo, Rasa Čepulyte, Ignacio Vicente-Díez, Rubén Blanco-Pérez, **Maryam Chelkha**, Miguel Puelles, Anna Gámez, José Luis Ramos-Sáez de Ojer, Raquel Campos-Herrera. International Congress on Invertebrate Pathology and Microbial Control & 53rd Annual Meeting of the Society for Invertebrate Pathology. 28th June - 2nd July 2021.
- Poster: **Unravelling the effect of the presence of earthworms or their cutaneous excreta and entomopathogenic nematodes in the soil bacterial community, biocontrol capacity, and plant traits**. **Maryam Chelkha**, María del Toro Hernández, Ignacio Vicente-Díez, Rubén Blanco-Pérez, Souad Amghar, Abdellatif El Harti, Alicia Pou, Raquel Campos-Herrera. International Congress on Invertebrate Pathology and

Microbial Control & 53rd Annual Meeting of the Society for Invertebrate Pathology. 28th June - 2nd July 2021.

- Poster: **Unravelling earthworms' impact over entomopathogenic nematode infectivity: general trend or species-specific dependent?**. **Maryam Chelkha**, Rubén Blanco-Pérez, Ignacio Vicente-Díez, María del Mar González-Trujillo, Souad Amghar, Abdellatif El Harti, Raquel Campos-Herrera. Virtual Conference of the Society of Nematologists, December 15th-16th 2020.
- Poster: **The earthworm mucus and their feeding activity can decrease the biological control action by entomopathogenic nematodes and entomopathogenic fungi**. **Maryam Chelkha**, Rubén Blanco-Pérez, Francisco ángel Bueno-Pallero, Abdellatif Abdellatif El Harti, Souad Amghar, Raquel Campos-Herrera. International Congress on Invertebrate Pathology and Microbial Control & 52nd Annual Meeting of the Society for Invertebrate Pathology & 17th Meeting of the IOBC-WPRS Working Group "Microbial and Nematode Control of Invertebrate Pests" 28th July - 1st August 2019. Valencia, from 28th July to 1st August of 2019.
- Poster: **virulence of entomopathogenic nematodes against two aerial pests: frankliniella occidentalis (thysanoptera: thripidae) and tuta absoluta (lepidoptera: gelechiidae): intra- and interspecific variability**. Ignacio Vicente-Díez, María del Mar González-Trujillo, Magda Galeano, **Maryam Chelkha**, José Eduardo Belda, Javier Calvo, Raquel Campos-Herrera. Virtual Conference of the Society of Nematologists, December 15th-16th 2020.
- Poster: **Enhancing organic viticulture: insecticidal effect of entomopathogenic nematodes and the cell-free supernatant from *Xenorhabdus* and *Photorhabdus* bacteria against *Philaenus spumarius* (Hemiptera: Aphrophoridae), vector of Xylella Fastidiosa (Proteobacteria: Xanthomonadaceae).** Ignacio Vicente-Díez, Rubén Blanco-Pérez, **Maryam Chelkha**, María del Mar González-Trujillo, Alicia Pou, and Raquel Campos-Herrera. Virtual Conference of the Society of Nematologists, December 15th-16th 2020.
- Poster: **The presence of earthworm mucus secretion could altered entomopathogenic nematodes activity as biological control agents. Maryam Chelkha**, Rubén Blanco-Pérez, Francisco ángel Bueno-Pallero, Abdellatif El Harti, Souad Amghar, Raquel Campos-Herrera. 33 rd European Society of Nematologist meeting (ESN), Ghent, Belgium, September 9 th -13 th 2018.

- Poster: **Earthworm feeding activity and mucus secretion can decrease entomopathogenic nematodes activity as biological control agents. Maryam Chelkha**, Rubén Blanco-Pérez, Francisco ángel Bueno-Pallero, Abdellatif El Harti, Souad Amghar, Raquel Campos-Herrera. The Fourth International American Moroccan Agricultural Sciences Conference (AMAS) Conference IV, May 9th to 11th, 2018, Qualipôle-Agropolis, Meknes, Morocco.
- Poster: **Identification of the Coelomic Microbial Community of *Lumbricus terrestris* (Annelida: Lumbricidae).** Lamia Yakkou, **Maryam Chelkha**, Mohammed Raouane, Souad Amghar, and Abdellatif El Harti. The Fourth International American Moroccan Agricultural Sciences Conference (AMAS), May 9th to 11th , 2018, Qualipôle-Agropolis, Meknes, Morocco.
- Poster: **Isolation and Identification of Aerobic Bacterial Community in Earthworm (Lumbricus terrestris) chloragogen tissue**. Lamia Yakkou, **Maryam Chelkha**, Mohammed Raouane, Souad Amghar, and Abdellatif El Harti. The Fourth International American Moroccan Agricultural Sciences Conference (AMAS), May 9th to 11th, 2018, Qualipôle-Agropolis, Meknes, Morocco.
- Poster: **Earthworms' cocoons harbour beneficial bacteria for soil fertility**. Sofia houida, Lamia yakkou, **Maryam chelkha**, Mohammed raouane, Abdellatifel harti and souad amghar. The first edition of the doctoral days organized by the Water, Natural Resources, Environment and Sustainable Development Research Center of the Mohammed V University of Rabat under the theme: Water, Energy & Environment Nexus from June 12th to 14th, 2019 at the Ecole Normal Superior of Technical Education - Rabat, Morocco.
- Poster: **Dissemination of solubilizing phosphorus bacteria by the earthworm *lumbricus terrestris* through the coelomic fluid**. Lamia yakkou, Sofia houida, **Maryam chelkha**, Mohammed raouane, Abdellatifel harti and souad amghar. The first edition of the doctoral days organized by the Water, Natural Resources, Environment and Sustainable Development Research Center of the Mohammed V University of Rabat under the theme: Water, Energy & Environment Nexus from June 12th to 14th, 2019 at the Ecole Normale Superior of Technical Education - Rabat, Morocco.

NATIONAL MEETINGS

- Talk: **The presence of earthworms as cadaver could alter the biological control by nematophagous fungi (*Arthrobotrys musiformis, purpureocillium lilacinum*) and entomopathogenic fungi (*Beauveria bassiana*)**. **Maryam Chelkha**, Lamia yakkou, Sofia Houida, Mohamed Raouane, Souad Amghar, Raquel Campos-Herrera, Abdellatif El Harti. 7th Edition of the International School of Research "Biodiversity, Biotechnology and Sustainable Development" Training Center of the Faculty of Medicine and Pharmacy of Agadir 25-27April 2019.
- Talk: **Earthworms feeding activity and mucus secretion alter biological control activity. Maryam chelkha**, Rubén blanco-pérez, Francisco ángel Bueno-Pallero, Abdellatif El harti, Souad Amghar and Raquel Campos-Herrera. The first edition of the doctoral days organized by the Water, Natural Resources, Environment and Sustainable Development Research Center of the Mohammed V University of Rabat under the theme: Water, Energy & Environment Nexus from June 12th to 14th, 2019 at the Ecole Normale Superior of Technical Education - Rabat, Morocco.

- Seminar: **Biological interactions between earthworms and rhizosphere components: characterizing multi-trophic to improve biocontrol of insect pests**. Maryam CHELKHA. Instituto de las Ciencias de la Vid y el Vino-CSIC, Logroño (Spain) on July 6, 2021

INTERNSHIP

- **Research internship:** 06/07/2019–31/12/2019
    - IN-vid: Agroecological innovation of the vineyard. The Institute of Vine and Wine Sciences (ICVV), Logroño, Spain.
    - Objectives: as part of the CSIC ICOOP+ project, we unravel the impact of earthworms and their mucus on the biocontrol potential of entomopathogenic nematodes. Advisor: R. Campos–Herrera.
- **Research internship:** 09/03/2020–12/12/2020
    - IN-vid: Agroecological innovation of the vineyard. The Institute of Vine and Wine Sciences (ICVV), Logroño, Spain.
    - Objectives: as part of the CSIC ICOOP+ project, we evaluate the impact of earthworms, their mucus, entomopathogenic nematodes, or the two-by-two

combination on the bacterial soil community associated with tomato plants. Plant traits, biocontrol activity, and bacterial richness and diversity by Next Generation Sequencing approach. Advisor : R. Campos–Herrera.

- **Research internship:** 22/02/2021–21/08/2021
    - IN-vid: Agroecological innovation of the vineyard. The Institute of Vine and Wine Sciences (ICVV), Logroño, Spain.
    - Objectives: as part of the CSIC ICOOP+ project, we investigate whether earthworms or their mucus can generate Induced Systemic Resistance (ISR) to protect the plants against biotic threats. Advisor : R. Campos–Herrera.
- **Laboratory Assistant** : 01/09/2018-30/07/2019
    - Ecole Normale Supérieure (E.N.S.), Centre « Eau, Ressources Naturelles, Environnement et Développement Durable (CERNE2D), Mohammed V University.
    - Responsibilities: Training undergraduate students, preparing and assisting with practical courses on Cellular biology, plant physiology, animal physiology, and microbiology.
- **Research internship** :05/11/2017–03/05/2018
    - MeditBio, Centre for Mediterranean Bioresources and Food, Faculty of Science and Technology, University of Algarve, Campus de Gambelas, Faro, 8005-139, Portugal.
    - Objectives: Biological interactions between earthworms and rhizosphere components: characterizing multi-trophic interactions to improve biocontrol of insect pests" Advisor: R. Campos–Herrera.

AWARDS AND GRANTS

- Ph.D. student Bursary Award 2018; European society of nematologist (ESN).
- Ph.D. students award 2019; Society of invertebrate pathology, nematode division.
- The international mobility program "Mare-Nostrum" as a visiting student at the Universidade do Algarve (Portugal),2017/2018.
- The travel assistance grant associated with CSICI-COOP+2018 (COOPA20231), 2019/2021