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

Maryam Chelkha [a,b], Rubén Blanco-Pérez [b], Ignacio Vicente-Díez [b], Francisco Ángel Bueno-Pallero [c], Souad Amghar [a], Abdellatif El Harti [a], Raquel Campos-Herrera [b,*]

[a] Research Team "Lombricidae, Improving Soil Productivity and Environment" (LAPSE), Ecole Normale Supérieure (E.N.S.), Centre « Eau, Ressources Naturelles, Environnement et Développement Durable (CERNE2D), Mohammed V University, Avenue Mohamed Bel Hassan El Ouazzani, BP : 5118, Takaddoum – Rabat, Morocco
[b] Instituto de Ciencias de la Vid y del Vino (CSIC-Universidad de La Rioja-Gobierno de La Rioja), Finca La Grajera, Ctra. de Burgos Km. 6, 26007 Logroño, Spain
[c] UDIT MED—Mediterranean Institute for Agriculture, Environment and Development, Pólo, Universidade do Algarve, Campus de Gambelas, Ed 8, 8005-139 Faro, Portugal

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ABSTRACT

Earthworms are ecological engineers that can contribute to the displacement of biological control agents such as the entomopathogenic nematodes (EPNs) and fungi (EPF). However, a previous study showed that the presence of cutaneous excreta (CEx) and feeding behavior of the earthworm species *Eisenia fetida* (Haplotaxida: Lumbricidae) compromise the biocontrol efficacy of certain EPN species by reducing, for example, their reproductive capability. Whether this phenomenon is a general pattern for the interaction of earthworms-entomopathogens is still unknown. We hypothesized that diverse earthworm species might differentially affect EPN and EPF infectivity and reproductive capability. Here we investigated the interaction of different earthworm species (*Eisenia fetida, Lumbricus terrestris*, and *Perionyx excavatus)* (Haplotaxida) and EPN species (*Steinernema feltiae, S. riojaense*, and *Heterorhabditis bacteriophora*) (Rhabditida) or EPF species (*Beauveria bassiana* and *Metarhizium anisopliae*) (Hypocreales), in two independent experiments. First, we evaluated the application of each entomopathogen combined with earthworms or their CEx in autoclaved soil. Hereafter, we studied the impact of the earthworms' CEx on entomopathogens applied at two different concentrations in autoclaved sand. Overall, we found that the effect of earthworms on entomopathogens was species-specific. For example, *E. fetida* reduced the virulence of S. *feltiae*, resulted in neutral effects for *S. riojaense*, and increased *H. bacteriophora* virulence. However, the earthworm *P. excavates* increased the virulence of *S. feltiae,* reduced the activity of *H. bacteriophora,* at least at specific timings, while *S. riojaense* remained unaffected. Finally, none of the EPN species were affected by the presence of *L. terrestris.* Also, the exposure to earthworm CEx resulted in a positive, negative or neutral effect on the virulence and reproduction capability depending on the earthworm-EPN species interaction. Concerning EPF, the impact of earthworms was also differential among species. Thus, *E. fetida* was detrimental to *M. anisopliae* and *B. bassiana* after eight days post-exposure, whereas *Lumbricus terrestris* resulted only detrimental to *B. bassiana*. In addition, most of the CEx treatments of both earthworm species decreased *B. bassiana* virulence and growth. However, the EPF *M. anisopliae* was unaffected when exposed to *L. terrestris* CEx, while the exposure to *E. fetida* CEx produced contrasting results. We conclude that earthworms and their CEx can 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. Additional studies are needed to verify these interactions under natural conditions.

1. Introduction

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 organisms ranging from microscopic size (bacteria, archaea, oomycetes,

* Corresponding author.
E-mail address: raquel.campos@icvv.es (R. Campos-Herrera).

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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 the maintenance of soil structure and the decomposition of organic matter, are supported and modulated by soil biota (Wall, 2012; Bardgett and van der Putten, 2014). Understanding and preserving soil biodiversity (organisms and their activities) is critical to prevent their loss and enhance their beneficial services.

Earthworms (Annelida: Oligochaeta), the most 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, earthworms' gut contains bacteria that can modify soil properties that would stimulate certain plant functions and change micro and mesofauna communities (Brown et al., 2000; Aira et al., 2016, 2015). Thus, earthworms can contribute to reduce the numbers 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, earthworms are categorized into three main groups: epigeic, anecic, and endogeic (Bouché, 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, feeding mostly 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 upper part of the ground (Bouché, 1977; Lavelle, 1988).

Entomopathogenic nematodes (EPNs) and fungi (EPF) 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 them are also available to implement in IPM programs and organic production (Campos-Herrera, 2015; Lacey et al., 2015). 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). The infective juvenile (IJ) is the stress-resistant stage of EPNs, responsible for the dispersion, penetration in the host, and release of the bacteria (Gaugler and Kaya, 1990). The EPN-bacteria complex copes with the host within 24–48 h by producing specific compounds (Boemare, 2002; Bode, 2009; Lu et al., 2017). Then, the EPNs complete 2–3 generations inside the insect cadaver, and when conditions are limiting (scarce food, excess of excretory products), the IJs emerge to start the search again (Gaugler and Kaya, 1990; Kaya and Gaugler, 1993; Campos-Herrera, 2015). Similarly, 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). EPF can 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 re-start the life cycle again.

Few studies have explored the interaction between earthworms and entomopathogen organisms in soils. The first studies of this kind evaluated the indirect impact of earthworm 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). Moreover, the cutaneous excreta (CEx) of earthworms is 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 and other compounds of antibiotic nature (Dales and Kalaç, 1992; Bilej et al., 1995, 1991; Kasschau et al., 2007; Fiołka et al., 2012) that 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 *Hererorhabditis 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). Finally, Chelkha et al. (2020) observed that the presence of the CEx produced by *E. fetida* reduced the virulence and reproductive capability of certain EPN species, especially steinernematids over 600 μm size. However, it is still unknown if these negative impacts might be expanded to other entomopathogens or earthworm species. The current study aimed to investigate how earthworms and their CEx could alter the virulence and reproductive capability of EPNs and EPF. We expect that different earthworm/CEx species will affect the EPN and EPF virulence and reproductiveness differently. Moreover, we suppose that the exposure to the individuals of 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,* medium ~ 800 μm, and large ~ 1200 μm size, respectively) 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.

2. Materials and methods

2.1. 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). 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 (22–24 °C) 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.

The EPN populations used for this study were native to vineyards from La Rioja, Spain (Blanco-Pérez et al., 2020): two steinernematids 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 14 °C 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., 1997). The conidia suspensions were prepared using PDA culture (3–4 week 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 40 °C with ventilation, and stored under laboratory conditions for over a week before being used (Chiriboga et al., 2017).

2.2. 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 as experimental units Petri dishes (9 cm diam.) filled with 45 g sterilized soil moistened to 24% (w/v) with distilled water (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 in 48 IJs per Petri dish (0.8 IJs/cm^2) (see Chelkha et al., 2020) and the EPF inoculum in 2–4 × 10^7 conidia per Petri dish (3.2–6.3 × 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 or EPF growth (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.

2.3. 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/cm^2) and 20 IJs per well (10 IJs/cm^2); and two EPF concentrations (determined after a preliminary test): 1–2 × 10^7 conidia/ml (1–2 × 10^6 conidia per well) and 1–2 × 10^8 conidia/ml (1–2 × 10^7 conidia per well). Plates were incubated at 24 °C in the dark for 24 h. Then, we added 1 g 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 48 h 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 48 h incubation at 24 °C in the dark, we made three serial dilutions from each Eppendorf tube to reach a final dilution of 1–2 × 10^4 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 1 l 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 1 l 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 22–24 °C for the growth of EPF colonies, we counted for the germination of conidia. The experiment was conducted twice with

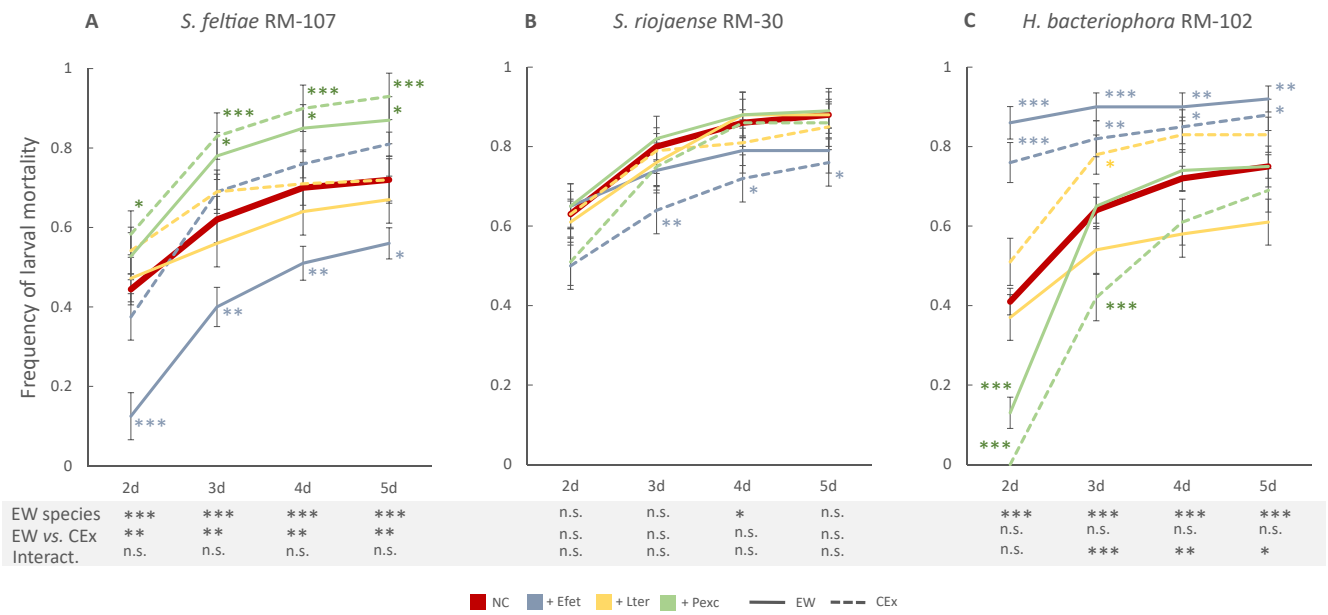

Fig. 1. Cumulative *Galleria mellonella* larval mortality (2–5 days) after exposure to 48 infective juveniles (IJs) of the entomopathogenic nematode species (A) *Steinernema feltiae*, (B) *S. riojaense*, and (C) *Heterorhabditis bacteriophora*, IJs only applied or combined with two earthworms (EW) or 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.

freshly produced CEx and new culture/suspension for the two EPF species.

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.

3. Results

3.1. 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* (Fig. 1), but not on EPN reproductive capability (Fig. 2). 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* (Figs. 1 and 2). 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 (Fig. 1A). We observed opposite results for *H. bacteriophora*: its virulence was significantly higher for *E. fetida* treatments and lower when combined with *P. excavatus* CEx (Fig. 1C). Lastly, *S. riojaense* virulence only decreased with *E. fetida* CEx (Fig. 1B). 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 (Fig. 1C). Indeed, earthworms did not affect EPN reproductive rates except by increasing it for *S. feltiae* when combined with *E. fetida* CEx (Fig. 2A).

The two earthworm species and their CEx differently affected EPF virulence and growth (Figs. 3 and 4). First, individuals of *E. fetida* only reduced *B. bassiana* virulence after eight days of exposure (Fig. 3A), while *M. anisoliae* showed an overall negative impact from day 8 until 14 (Fig. 3B), which translated in a reduction of the reproductive potential (Fig. 4B). Exposure to *L. terrestris* reduced *B. bassiana* virulence (Fig. 3A), while *M. anisopliae* was unaffected (Fig. 3B). Concerning CEx exposure, *E. fetida* CEx reduced *B. bassiana* virulence (Fig. 3A) and reproductive potential (Fig. 4A), whereas *M. anisopliae* exhibited an increase in its virulence (Fig. 3B). On the other hand, *L. terrestris* treatments only affected *B. bassiana* by decreasing its virulence (Fig. 3A).

3.2. 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 reproductive rates (Figs. 5 and 6). 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 (Fig. 5E and F), and the opposite for the frequency of larvae producing *S. feltiae* offspring (Fig. 6A 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* (Fig. 5A and B) and *S. riojaense* (Fig. 6C and D), respectively. In contrast, the infectivity of *S. riojaense* (Fig. 5C and D) and reproductive rates of *H. bacteriophora* (Fig. 6E 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 (Fig. 5A and 6A). Besides, for the high *S. feltiae* inoculum,

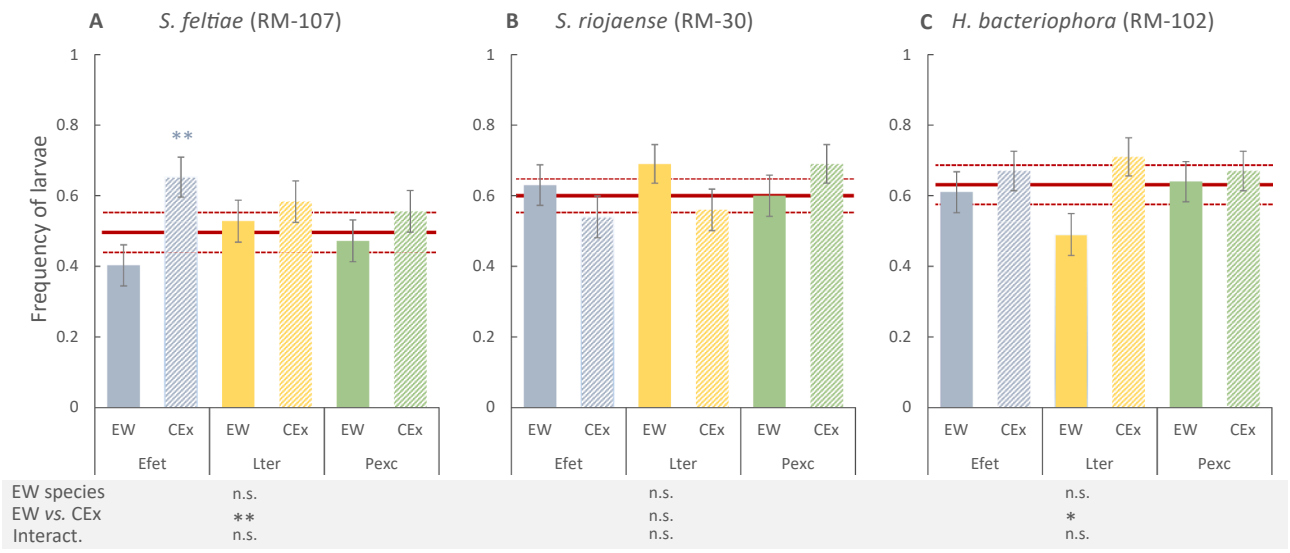

Fig. 2. Frequency of *Galleria mellonella* larvae producing nematode offspring after exposure to 48 infective juveniles (IJs) of the entomopathogenic nematode species (A) *Steinernema feltiae*, (B) *S. riojaense*, and (C) *Heterorhabditis bacteriophora*, IJs only applied or combined with two earthworms (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.

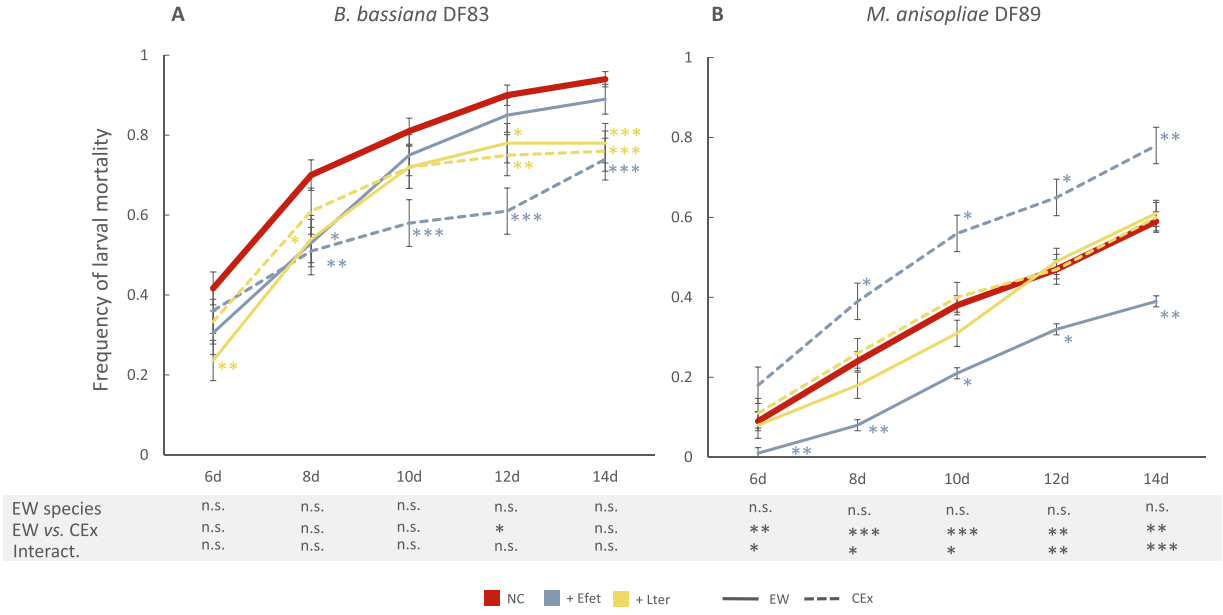

Fig. 3. Cumulative *Galleria mellonella* larval mortality (6–14 days) after exposure to 10^7 conidia per Petri dish of the entomopathogenic fungi species (A) *Beauveria bassiana* and (B) *Metarhizium anisopliae*, conidia only applied or combined with two earthworms (EW) or their cutaneous excreta (CEx) 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.

P. excavatus CEx negatively affected EPN virulence (Fig. 5B) while *L. terrestris* CEx positively affected EPN reproductive capability (Fig. 6B). Regarding *S. riojaense*, *E. fetida* and *L. terrestris* CEx reduced mortality rates for low and high IJ inoculum, respectively (Fig. 5C and D). Moreover, *S. riojaense* reproductive rates decreased when exposed to *E. fetida* CEx at low IJ inoculum (Fig. 6C) but increased in the presence of the *L. terrestris* and *P. excavatus* CEx at high IJ inoculum (Fig. 6D). Finally, CEx from earthworms only affected *H. bacteriophora* by reducing its virulence when exposed to *L. terrestris* CEx at high IJ inoculum (Fig. 5F).

As observed for EPNs, higher conidial inoculum resulted in higher larval mortality and fungal growth rates (Figs. 7 and 8). Overall, the CEx from earthworms reduced the EPF virulence and growth, although not significantly for the combination of *L. terrestris* and *M. anisopliae* (Figs. 7

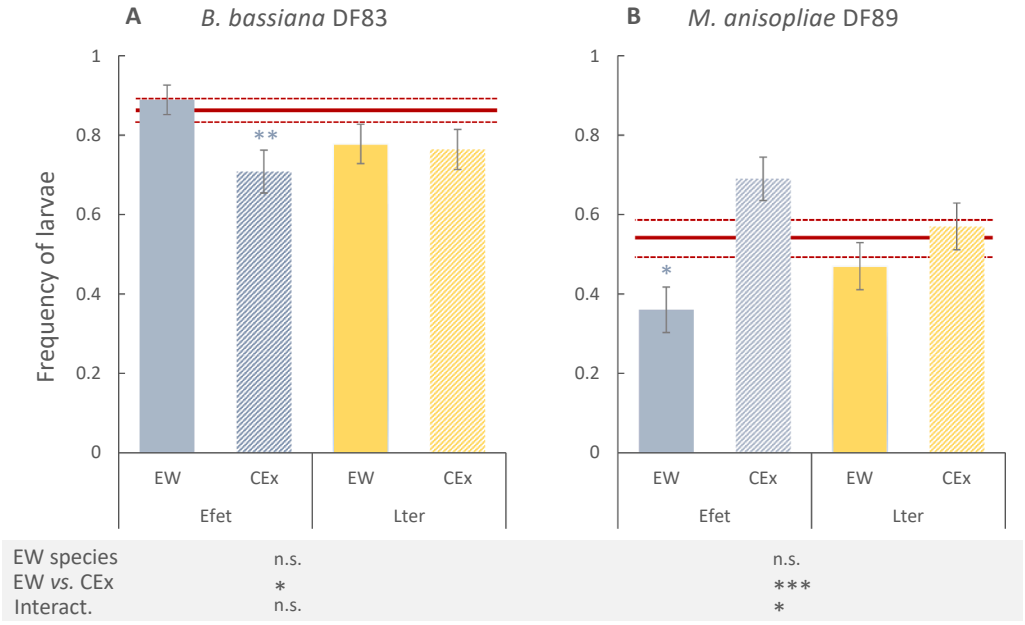

Fig. 4. Frequency of *Galleria mellonella* larvae producing mycelia after exposure to 10^7 conidia per Petri dish of the entomopathogenic fungi species (A) *Beauberia bassiana* and (B) *Metarhizium anisopliae*, conidia only applied or combined with two earthworms (EW) or their cutaneous excreta (CEx) 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.

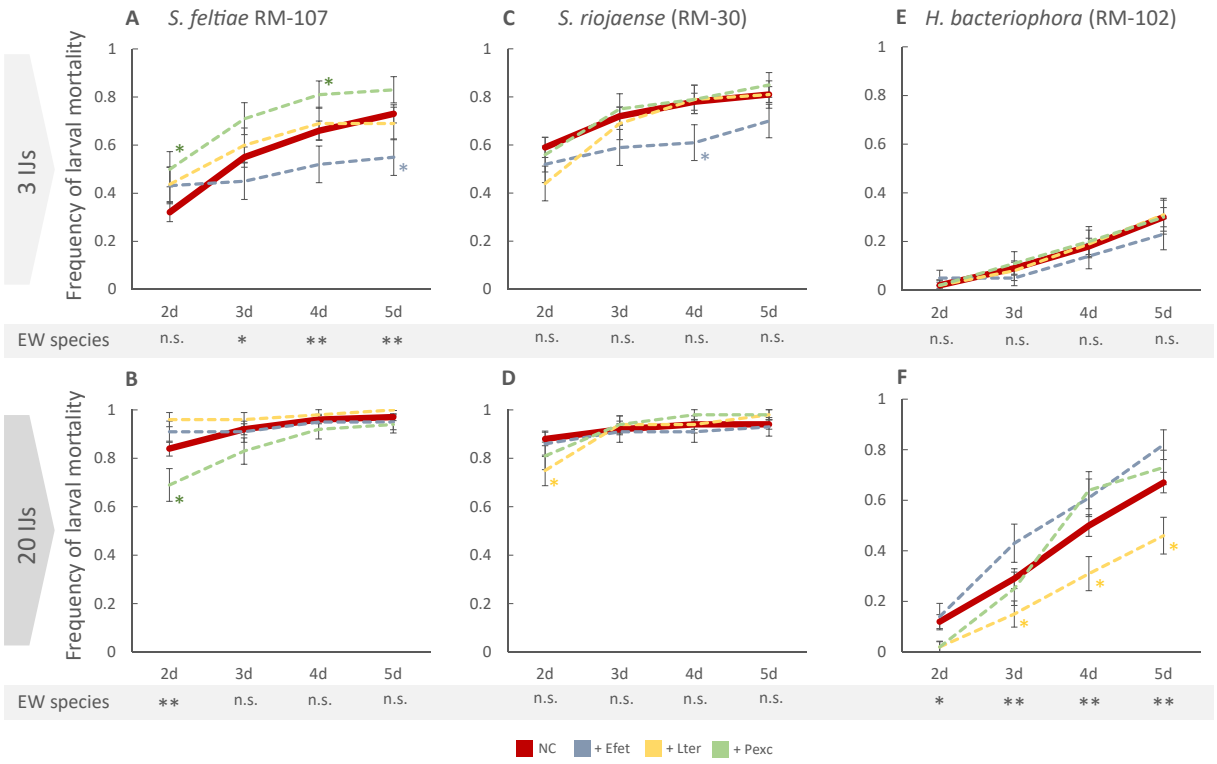

Fig. 5. Cumulative *Galleria mellonella* larval mortality (2–5 days) after exposure to 3 or 20 infective juveniles (IJs) of the entomopathogenic nematode species: (A-B) *Steinernema feltiae*, (C-D) *S. riojaense*, and (E-F) *Heterorhabditis bacteriophora*, IJs only applied or combined with the cutaneous 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.

and 8). However, except for the low conidial inoculum of *M. anisopliae* (Fig. 8C), *E. fetida* CEx decreased the EPF virulence and growth significantly more than *L. terrestris* CEx (Figs. 7 and 8). Finally, the EPF conidial viability was significantly higher for *B. bassiana* combined with *L. terrestris* CEx (Fig. 9A) and lower for *M. anisopliae* combined with *E. fetida* CEx (Fig. 9B).

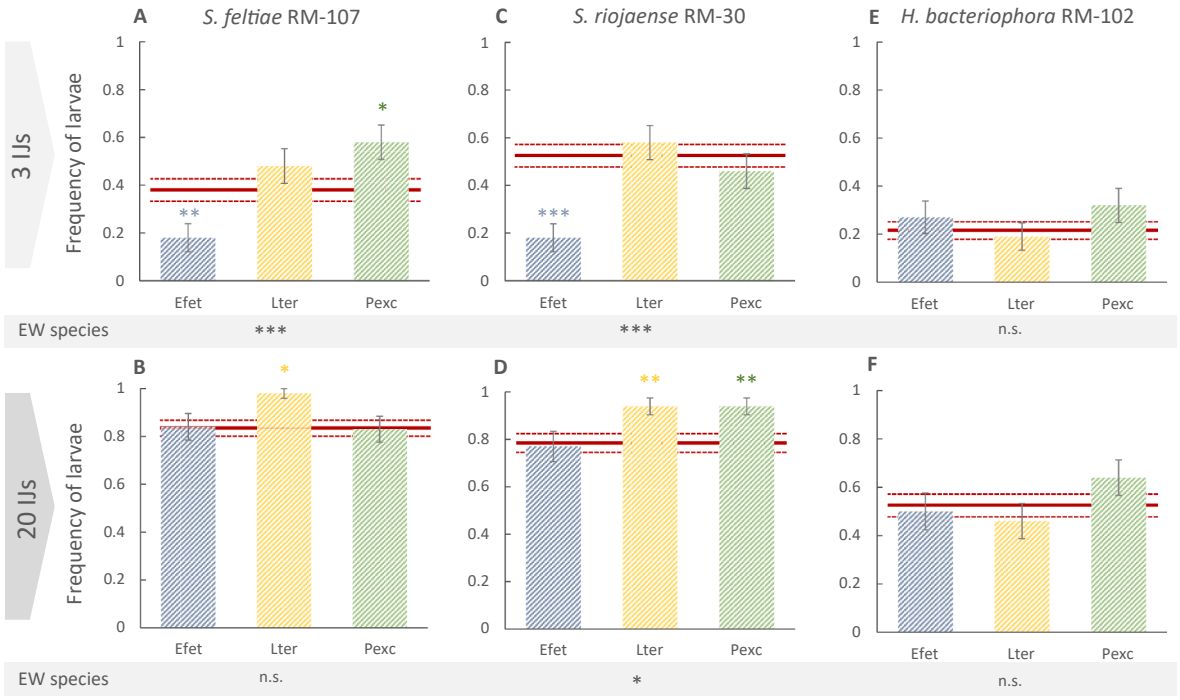

Fig. 6. Frequency of *Galleria mellonella* larvae producing nematode offspring after exposure to 3 or 20 infective juveniles (IJs) of the entomopathogenic nematode species (A-B) *Steinernema feltiae*, (C-D) *S. riojaense*, and (E-F) *Heterorhabditis bacteriophora*, IJs only applied or combined with the 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.

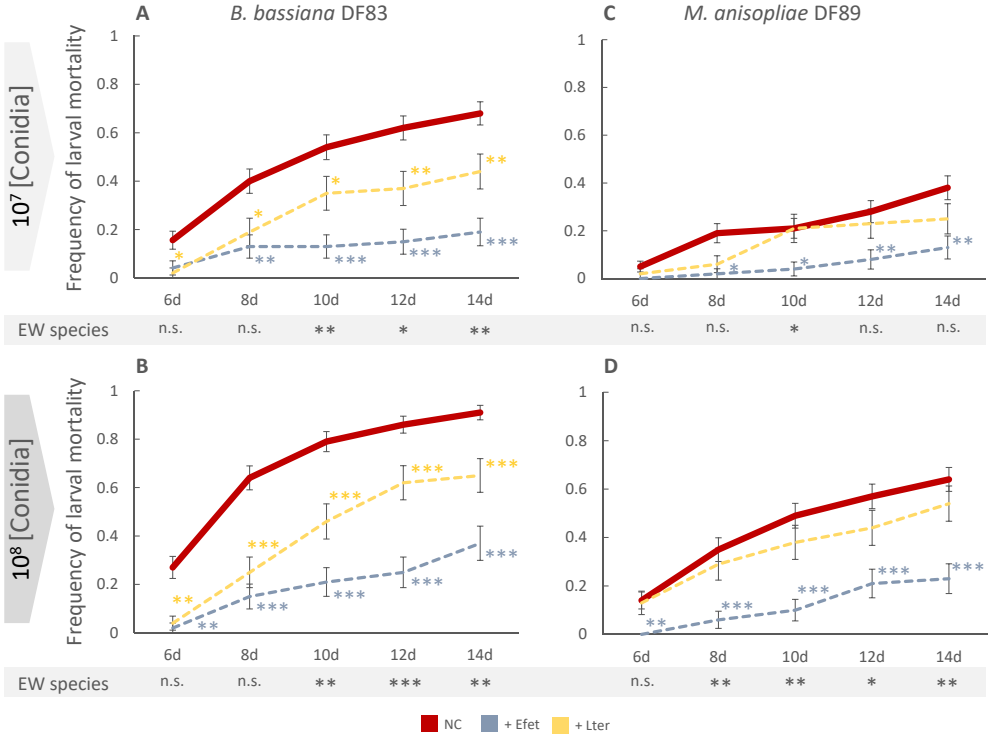

Fig. 7. Cumulative *Galleria mellonella* larval mortality (6–14 days) after exposure to 10^7 or 10^8 conidia per well of the entomopathogenic fungi species (A-B) *Beauberia bassiana* and (C-D) *Metarhizium anisopliae*, conidia only applied or combined with the cutaneous excreta (CEx) of the earthworm 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 (NC) (pictured on graphs). Values are least-square means ± SE.

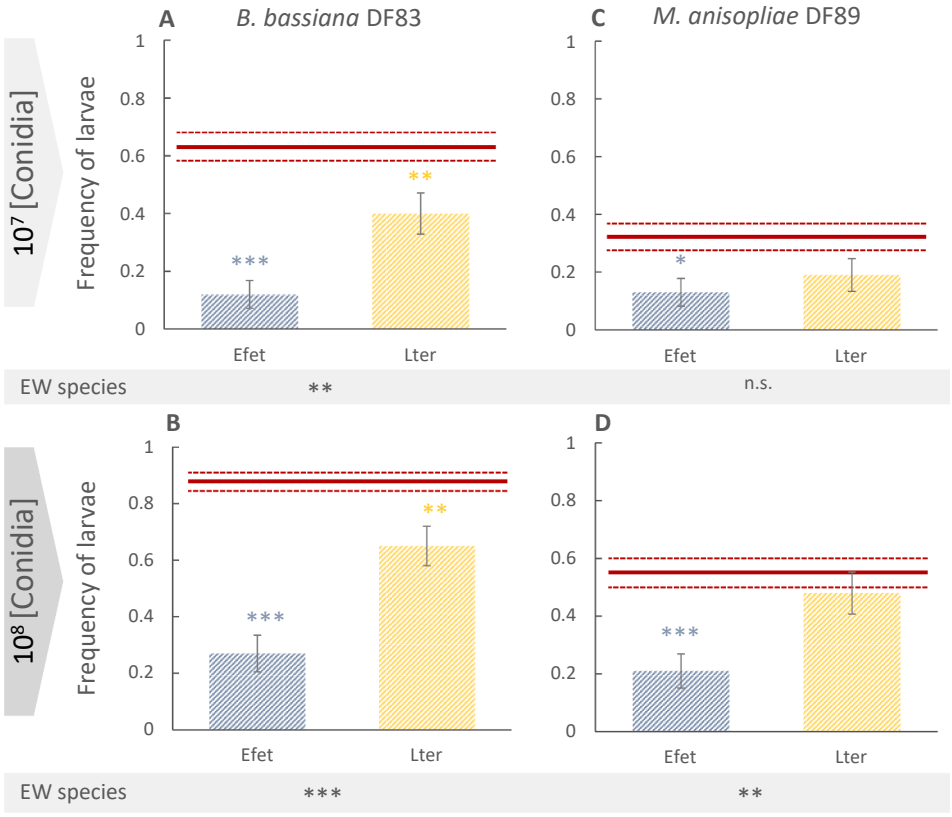

Fig. 8. Frequency of *Galleria mellonella* larvae producing mycelia after exposure to 10^7 or 10^8 conidia per well of the entomopathogenic fungi species (A-B) *Beauberia bassiana* and (C-D) *Metarhizium anisopliae*, conidia only applied or combined with the cutaneous excreta (CEx) of the earthworm species *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.

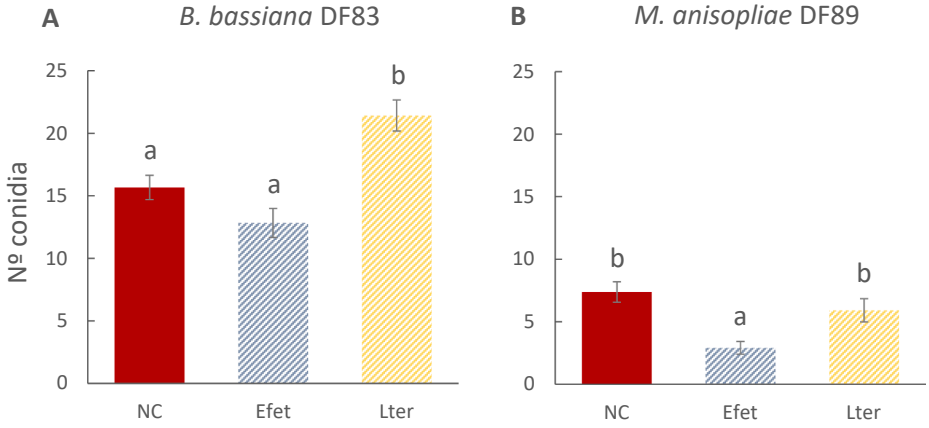

Fig. 9. Number of conidia germinated in selective media after exposure to 10^4 conidia per well of the entomopathogenic fungi species (A) *Beauberia bassiana* and (B) *Metarhizium anisopliae*, single applied (NC) or combined with the cutaneous excreta (CEx) of the earthworm species *Eisenia fetida* (Efet) and *Lumbricus terrestris* (Lter). Letter indicates different Tukey's test (HSD) subsets ($P < 0.05$). Values are least-square means ± SE.

4. 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,* characterize for a larger IJ size (Půža et al., 2020), was not affected 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 the feeding and movement activity of earthworms 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 1.17×10^7 conidia/cm^2 concentration employed by Shapiro-Ilan and Brown (2013), two orders of magnitude higher than tested in our study ($3.1–6.3 \times 10^5$ 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 provide effective crop management (for both implementation and conservation programs) based on beneficial soil organisms as entomopathogens.

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