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

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Abstract: Earthworms influence soil ecosystem functions such as decomposition and regulation of nutrient cycles by burrowing, feeding, or secreting their cutaneous excreta. Earthworms can also modulate soil biodiversity and their interactions. Recent research has revealed that earthworm feeding activity or excretions can alter the performance of entomopathogenic nematodes (EPNs) and entomopathogenic fungi (EPFs), known biological control agents. Still, the impact of earthworm cadavers on the growth and reproduction of other biological control agents such as nematophagous fungus (NFs) and EPFs has been poorly explored. We suggest that earthworm body tissue could be used as a food supply by certain fungal species. We hypothesize that fungi with a high adaptive capacity as pathobionts such as NF will use earthworm cadavers to a certain extent, while the growth and reproduction of fungi with limited or specific nutrition needs, such as EPFs, will be restricted. In this study, we evaluated two NF species: a nematode-trapping fungus *Arthrobotrys musiformis* Drechsler (Orbiliales: Orbiliaceae) and a nematode egg or cyst parasite *Purpureocillium lilacinum* (Thom) (Hypocreales: Ophiocordycipitaceae), and one EPF species: *Beauveria bassiana* (Balsamo). We exposed each fungus to two different *Aporrectodea molleri* extract media at six concentrations in agar medium (n = 3 per treatment) and liquid medium (n = 5): (i) earthworms devoid of intestinal contents (EDG) (C1...C6), and (ii) fresh earthworms (FE) (C1...C6). The use of FE and EDG was designed to discriminate the effect of earthworms' intestinal content on fungal growth. These treatments were also compared to alternative conventional media potato dextrose agar (PDA) and brain heart infusion (BHI) as the positive controls. We evaluated the vegetative growth capacity daily for 18 days, registering the reproduction at the end of the experiment. We observed that EPF *B. bassiana* did not grow in FE and EDG solid medium but showed germination in their liquid media. However, both NF species could grow and reproduce in both media but following a species-specific pattern. In all cases, FE and EDG reduced fungal growth compared to PDA and BHI. Our results suggest that the use of earthworm as a resource in natural conditions might depend on the expected specialization, with EPF *B. bassiana* with the lowest use, NF *A. musiformis* moderately, and *P. lilacinum* widely used. The study illustrates the complexity of the soil organisms' interactions and highlights the necessity to advance the understanding of the contribution of all the beneficial soil organisms in a comprehensive manner.

Key words: *Arthrobotrys musiformis, Beauveria bassiana, Purpureocilium lilacinus*, earthworms

1. Introduction

Earthworms are bioindicators of soil biodiversity and health (Paoletti, 1999; Fründ et al., 2011; Fusaro et al., 2018). They are involved in a variety of ecological functions and services through feeding, burrowing, and castings, such as decomposition (Lubbers et al., 2017; Frouz, 2018; Barthod et al., 2020) and nutrient cycling (Bohlen et al., 2004; Domínguez et al., 2004; Blouin et al., 2013). Burrows retain a large amount of oxygen and organic matter, and the castings are high in assimilable C and various nutrients (Lavelle et al., 2001; Curry and Schmidt, 2007).

Studies on the earthworm community claim that their presence ranges from 1 g to 150 g per m² (Buckerfield et al., 1997; Phillips et al., 2019), and are considered among the most abundant soil biomass (Owen and Galbraith, 1989; Briones and Schmidt, 2017; Li et al., 2020). In addition to organic matter, earthworms can feed on fungi (Bonkowski et al., 2000; Curry and Schmidt, 2007; Song et al., 2020), nematodes (Dash et al., 1980; Demetrio et al., 2019), and protozoa (Bonkowski and Schaefer, 1997; Monroy et al., 2008), so their activity can modulate the soil community. Specifically, entomopathogenic fungi (EPF)

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can be disseminated on the surface of the earthworm body (phoresy) or after surviving transit through the gut (Shapiro-Ilan and Brown, 2013). However, this interaction is not always positive, because recent studies pointed out that earthworm cutaneous excreta might alter their vitality, pathogenicity, and reproduction (Chelkha et al., 2021; Zhou et al., 2021). This impact is related to earthworm species, spore viability, and drilospheric impacts, which is the zone of earthworm influence, including midden litter and the soil volume descending along the burrow wall (Brown, 1995; Brown et al., 2000).

The endogenous earthworm species *Aporrectodea molleri* and their effects on microbial abundance have been less studied than other ecological categories of earthworms (Medina-Sauza et al., 2019; Yakkou et al., 2021a). Therefore, there is little evidence on the impact of other species, rather than *Eisenia fetida* or *Lumbricus terrestris*, species available in commercial stocks and accessible to research. However, the presence of other species, such as *Pontoscolex corethrurus,* increase bacterial abundance in soil, as shown by soil bacterial community analysis in metataxonomic approaches (16S rRNA gene) (Braga et al., 2016). In addition, there is evidence that earthworms can increase certain proteobacteria, such as acidobacteria, in their gut or cuticles (Gong et al., 2018). Therefore, due to bacteria in their guts and castings, earthworms might significantly affect the soil (Andriuzzi et al., 2016). Indeed, the gut provides a distinct microenvironment with controlled moisture conditions and various nutrient reservoirs that can function as a biological filter for ingested microbial communities, selecting, preferring, and eliminating groups of microorganisms (Horn et al., 2003; Drake, 2007). Therefore, understanding the effect of the gut on fungal proliferation will expand our knowledge regarding the effect of earthworms on soil ecosystem function (Horn et al., 2003; Drake, 2007).

Nematophagous fungi (NF) are predominantly recognized as natural inhabitants in the soil environment (Gray, 1987). They are carnivorous fungal species 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 eggs and cysts (Nordbring-Hertz, 2004). In particular, 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). In addition, *Purpureocillium lilacinum* (Hypocreales: Ophiocordycipitaceae) is an egg-parasitic fungus active against root-knot and cyst nematodes but also can be a pathogen of insects and cause some human mycoses (Luangsa-Ard et al., 2011; Toledo-Hernández et al., 2019).

On the other hand, other critical fungal inhabitants are the groups of the EPF in the order Hypocreales (Ascomycota), considered the most numerous natural enemies of arthropod pests in the agroecosystem (Barra-Bucarei et al., 2019). For example, *Beauveria bassiana* (Hypocreales: Clavicipitaceae) is often used as a biological control agent (Meyling and Eilenberg, 2007; Baki et al., 2021). It infects the host through the cuticle by conidia. Once the insect dies, EPF enters the saprophytic phase, begins active growth of hyphae and reproductive structures, and generates aerial mycelia for dispersal to begin the life cycle again (Feng et al., 1994; Ownley et al., 2008). The behaviour of EPFs and NF and their requirements for nutrient sources are distinct. In this ecological context, we hypothesized that decomposing earthworms could promote the growth of NF and EPFs by altering the availability of underground nutrients. However, their effectiveness will differ depending on the degree of the saprophytic capacity of the fungus.

In this study, we included two NF species and one EPF as an example to start elucidating these complex interactions in a proof-of-concept approach. All our experiments were performed in vitro to avoid possible interference with other soil components. We used different scenarios and compared the growth parameters of each fungal species with a conventional medium suitable to all these species. In order to elucidate the relevance of the gut contents and decomposition of the earthworm as a resource for beneficial soil fungi, we addressed the following objectives: (i) evaluate the mycelial growth of different fungal species in different concentrations of earthworm extract medium, (ii) compare fungal growth upon exposure to earthworm extract with the presence or absence of earthworm gut content, and (iii) evaluate the production and germination of conidia in the presence of earthworm extract at different concentrations.

2. Material and methods

2.1. 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 a.m., 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 (Houida et al., 2021). Individuals of *A. molleri* were kept in good condition in a plastic pot with soil, food, 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.

2.2. 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, and 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, 500 g, Oxoid) to solidify the media. 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. In addition, potato dextrose agar (PDA, C = 24 g/L) and heart-brain agar

(BHI Brain Heart Infusion Agar, dehydrated, Oxoid, C = 3.7 g/L) 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).

2.3 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 fungi 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 the Malassez counting chamber to $5–6 \times 10^6$. 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 ungerminated conidia were counted microscopically by Malassez chamber (n = 5), and we calculated the germination percentage. The experiment was performed twice.

2.4. 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 (with the following 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.

3. Results

3.1. 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* (supplementary data, Table S1). Specifically, the differences on the growth in each media were expanding the differences while advancing in time (Figure 1a). 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 1a). In addition, both worm extract-based media dilutions promoted higher fungal growth (Figure 1b). 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 1b).

Concerning sporulation, *A. musiformis* produced 4 to 10 times more conidia on PDA than on any other medium (Figure 1c). 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 1d). 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 approximately 70% germination (Figure 1e). 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 1e). When comparing the effect of earthworm extract media concentrations in detail, we observed an opposite trend in conidial production (Figure 1f). 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 1f), while in EDG media, the germination percentage decreased with the dilution of the medium (Figure 1f).

Finally, overall, mycelial density was low at the low concentrations (C3 and C4) and almost zero for C5 and C6 (data not shown) (Figure 2).

3.2. 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* (supplementary data, Table S2). Specifically, the earthworm medium produced such differences since day 6 (Figure 3a). In detail, from day 9 to day 12, EDG media supported the highest growth of the fungus (Figure 3a), 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 3b).
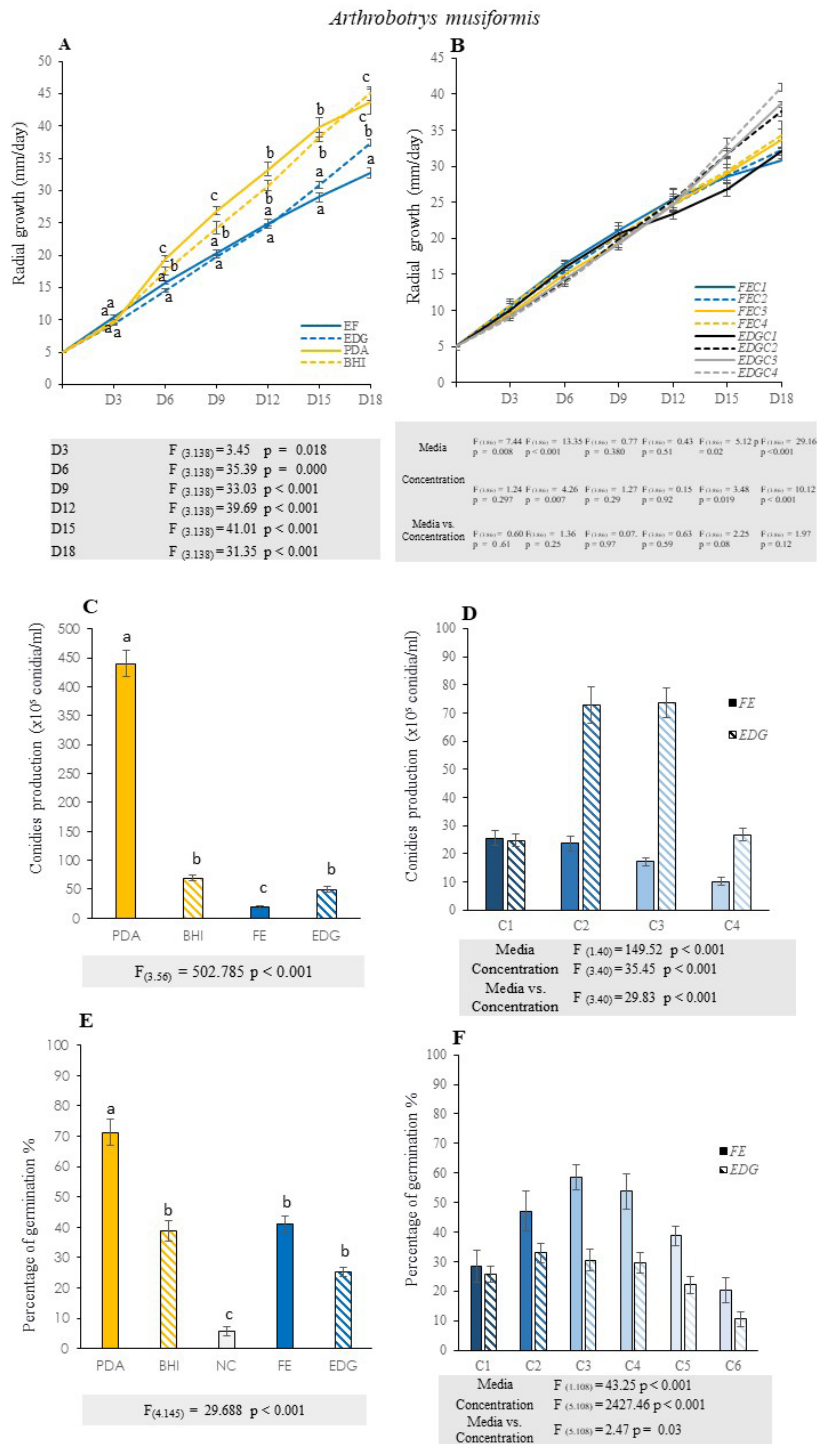
Conidial production in earthworm media (FE and EDG) was lower than that observed in conventional PDA and BHI media (Figure 3c). Conidial production was affected by both factors (concentration/medium) and interaction (Figure 3d), with an opposite pattern observed for *A. musiformis*. Second, the FE medium showed an optimum at C3, whereas conidia production in the EDG medium decreased when the medium was diluted (Figure 3d). Finally, the germination percentage was more than twice as high in the conventional PDA medium as in BHI or any other earthworm medium (Figure 3e). 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 3f).

Finally, overall, the mycelial morphology was different depending on the treatment and invisible for concentrations C5 and C6 (data not shown) (Figure 4).
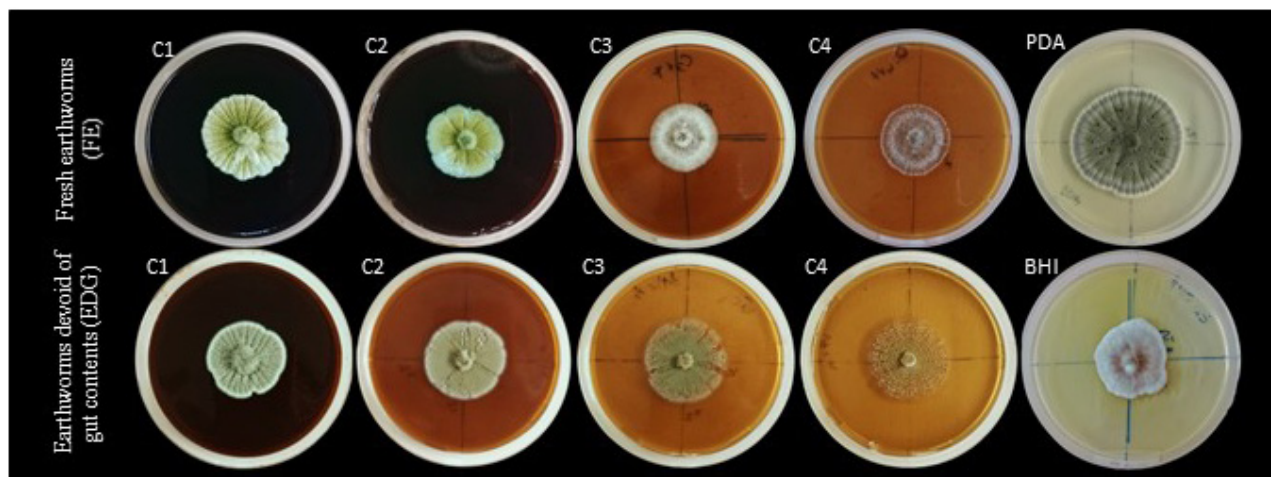
3.2. The effect of earthworm extract on the growth, production, and germination of *Beauveria bassiana* conidia

The *B. bassiana* fungus did not grow in either earthworm medium. However, *B. bassiana* conidia germinated

Figure 1. 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.** Cumulative growth from 3 to 18 days according to conventional and earthworm-based media. **B.** Cumulative growth as a function of concentration and earthworm-based medium. **C.** Conidia production (×10⁵ conidia/mL) according to conventional and earthworm-based media. **D.** Conidia production (×10⁵ 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 = 10 g/L, and C4 = 5 g/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 2. Growth of *Arthrobotrys musiformis* after 20 days postexposer 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 = 40 g/L, C2 = 20 g/L, C3 = 10 g/L, C4 = 5 g/L , and two rich media: potato dextrose agar (PDA), and brain heart infusion (BHI).

on both earthworm-based media at a rate similar to that of the conventional BHI medium (approximately 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 5b), with maximum germination for C4 = 45.2 ± 3.55 for EDG medium (supplementary data, Table S3).

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
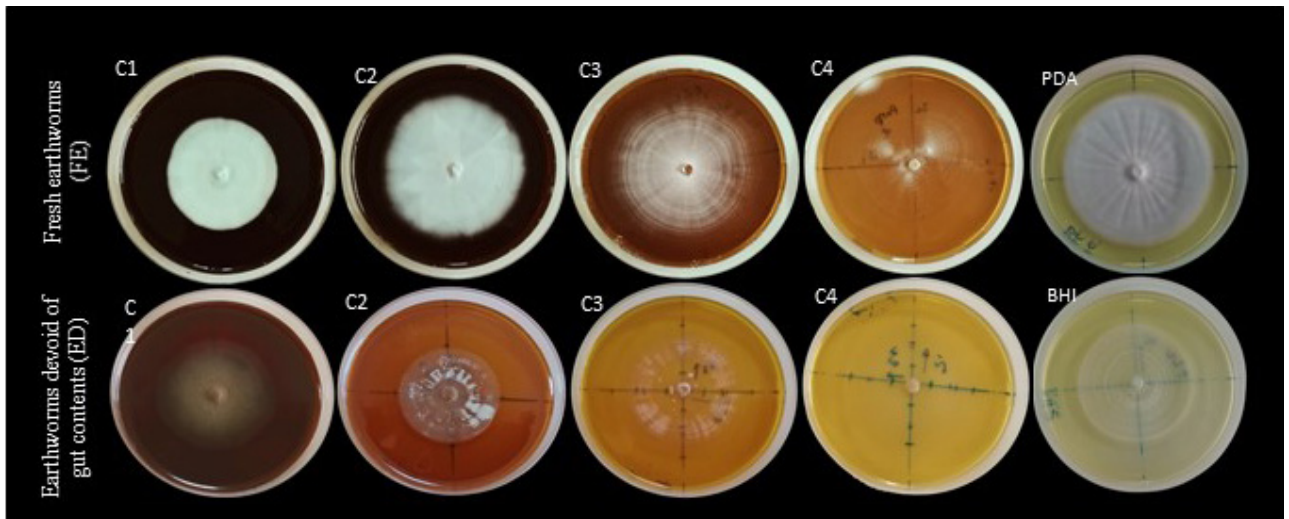
and coelomic fluid or a combination of all these factors may alter their activity (Zhou et al., 2021; Chelkha et al., 2021). Indeed, inhibitory growth activity was observed when the phytopathogenic fungi *Berkeleyomyces basicola*, *Fusarium culmorum*, *Fusarium oxysporum*, *Globisporangium irregulare, 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

Purpureocillium lilacinum
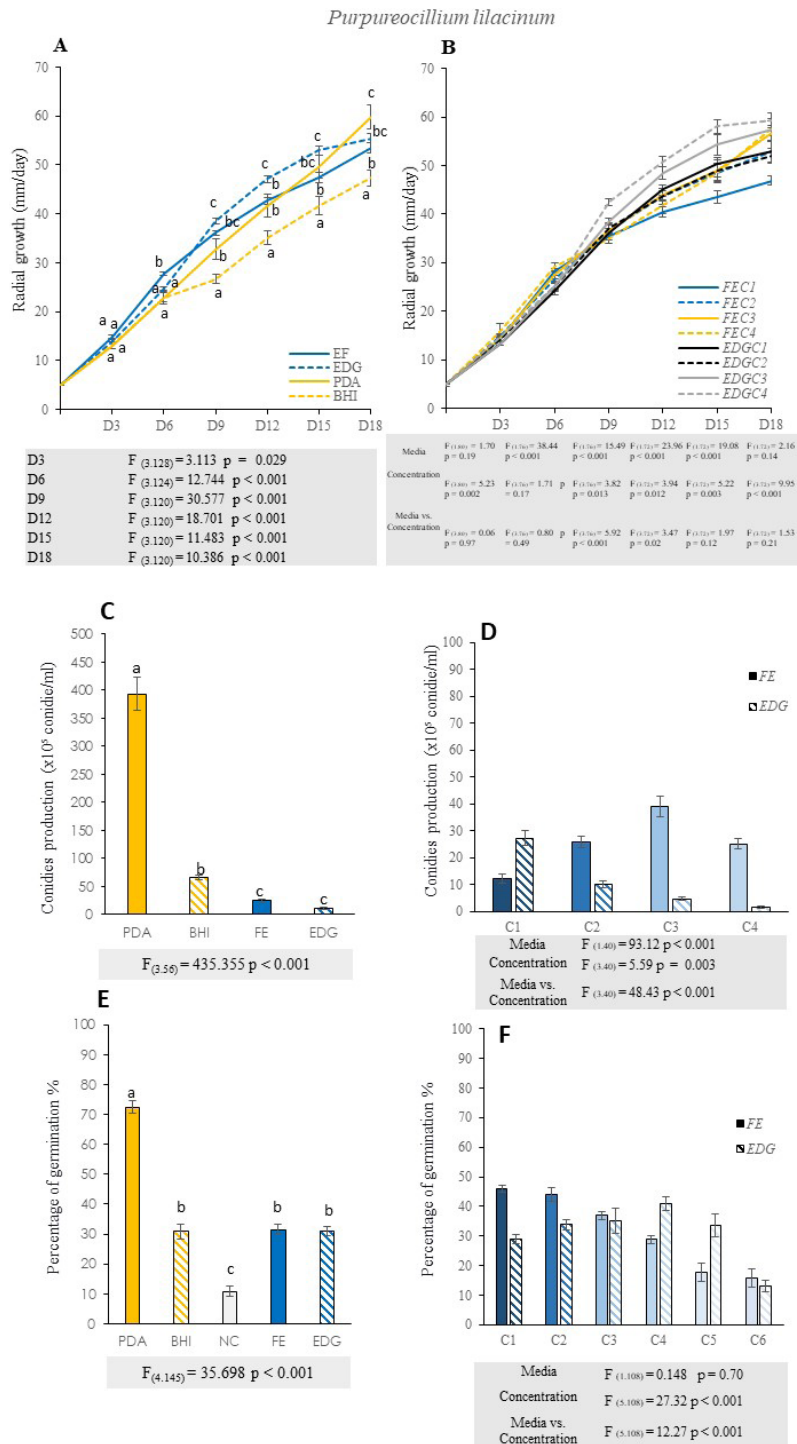
Figure 3. Evaluation of vegetative growth, conidial production and germination in the fungus *Purpureocillium lilacinum* exposed to two earthworm extracts: fresh earthworm (FE), earthworms devoid of intestinal contents (EDG) and two conventional media: potato dextrose agar (PDA), and brain heart infusion agar (BHI). **A.** Cumulative growth from 3 to 18 days according to conventional and earthworm-based media. **B.** Cumulative growth as a function of concentration and earthworm-based medium. **C.** Conidia production (×10⁵ conidia/mL) according to conventional and earthworm-based media. **D.** Conidia production (×10⁵ 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 = 10 g/L, and C4 = 5 g/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 4. Growth of *Purpureocillium lilacinum* after 20 days postexposer 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 = 40 g/L, C2 = 20 g/L, C3 = 10 g/L, C4 = 5 g/L , and two rich media: potato dextrose agar (PDA), and brain heart infusion (BHI).

Beauveria bassiana
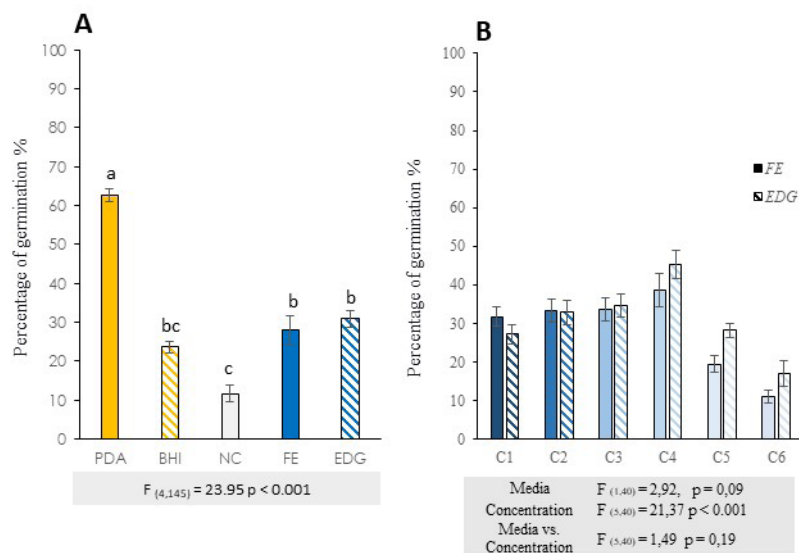
Figure 5. Evaluation of conidial germination of the fungus *Beauveria bassiana* exposed to two different earthworm extracts: fresh earthworms (FE) and earthworms without gut contents, EDG, and two conventional media: potato dextrose agar (PDA), and brain heart infusion agar (BHI). **A.** Percentage germination 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 = 10 g/L, and C4 = 5 g/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".

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.

References

Amador JA, Görres JH, Savin MC (2006). Effects of *Lumbricus terrestris* L. on nitrogen dynamics beyond the burrow. Applied Soil Ecology 33: 61-66. doi: 10.1016/j.apsoil.2005.09.008

Andriuzzi WS, Schmidt O, Brussaard L, Faber JH, Bolger T (2016). Earthworm functional traits and interspecific interactions affect plant nitrogen acquisition and primary production. Applied Soil Ecology 104: 148-156. doi: 10.1016/j.apsoil.2015.09.006

Barra-Bucarei L, France Iglesias A, Pino Torres C (2019). Entomopathogenic Fungi. In: Souza B., Vázquez L., Marucci R. (eds) Natural Enemies of Insect Pests in Neotropical Agroecosystems. Springer, Cham. 123-136. doi: 10.1007/978-3-030-24733-1_11

Barthod J, Dignac MF, le Mer G, Bottinelli N, Watteau F et al. (2020). How do earthworms affect organic matter decomposition in the presence of clay-sized minerals?. Soil Biology and Biochemistry 143: 107730. doi: 10.1016/j.soilbio.2020.107730

Baki D, Tosun HŞ, Erler F (2021). Indigenous entomopathogenic fungi as potential biological control agents of rose sawfly, *Arge rosae* L. (Hymenoptera: Argidae). Turkish Journal of Zoology 45: 517-525. doi: 10.3906/ZOO-2105-15

Bhorgin AJ, Uma K (2014). Antimicrobial activity of earthworm powder (*Lampito mauritii*). International Journal of Current Microbiology and Applied Sciences 3: 437-443.

Bityutskii NP, Solov'eva AN, Lukina EI, Oleinik AS, Zavgorodnyaya YA et al. (2007). Stimulating effect of earthworm excreta on the mineralization of nitrogen compounds in soil. Eurasian Soil Science 40: 426-431. doi: 10.1134/S1064229307040096

Blouin M, Hodson ME, Delgado EA, Baker G, Brussaard L et al. (2013). A review of earthworm impact on soil function and ecosystem services. European Journal of Soil Science 64: 161-182. doi: 10.1111/ejss.12025

Bohlen PJ, Parmelee RW, Blair JM (2004). Integrating the effects of earthworms on nutrient cycling across spatial and temporal scales, In: Earthworm Ecology, Second Edition, 161-180. doi: 10.1201/9781420039719

Bonkowski M, Griffiths BS, Ritz K (2000). Food preferences of earthworms for soil fungi. Pedobiologia, 44: 666-676. doi: 10.1078/S0031-4056(04)70080-3

Bonkowski M, Schaefer M (1997). Interactions between earthworms and soil protozoa: A trophic component in the soil food web. Soil Biology and Biochemistry 29: 499-502. doi: 10.1016/ S0038-0717(96)00107-1

Braga LP, Yoshiura CA, Borges CD, Horn MA, Brown GG et al (2016). Disentangling the influence of earthworms in sugarcane rhizosphere. Scientific Reports, 6: 1-13. doi: 10.1038/srep38923

Briones MJI, Schmidt O (2017). Conventional tillage decreases the abundance and biomass of earthworms and alters their community structure in a global meta-analysis. Global Change Biology, 23: 4396-4419. doi: 10.1111/gcb.13744

Brown GG, Edwards CA, Brussaard L (2004). How earthworms affect plant growth: Burrowing into the mechanisms. In: Earthworm Ecology, Second Edition 2: 13-49 doi: 10.1201/9781420039719

Brown GG (1995). How do earthworms affect microflora and faunal community diversity? Plant and soil 170: 209-231. doi: 10.1007/BF02183068

Brown GG, Barois I, Lavelle P (2000). Regulation of soil organic matter dynamics and microbial activity in the drilosphere and the role of interactions with other edaphic functional domains. European Journal of Soil Biology 36: 177-198. doi: 10.1016/ S1164-5563(00)01062-1

Buckerfield JC, Lee KE, Davoren CW, Hannay JN (1997). Earthworms as indicators of sustainable production in dryland cropping in Southern Australia. Soil Biology and Biochemistry 29: 547-554. doi: 10.1016/S0038-0717(96)00033-8

Chauhan PS, Tomar J, Prasad GBKS, Agrawal OP (2014). Evaluation of antimicrobial activity of earthworm tissue extract *Eudrilus eugeniae*. Journal of Chemical and Pharmaceutical Research 6: 28-38.

Chelkha M, Blanco-Pérez R, Vicente-Díez I, Bueno-Pallero FÁ, Amghar S et al. (2021). Earthworms and their cutaneous excreta can modify the virulence and reproductive capability of entomopathogenic nematodes and fungi. Journal of Invertebrate Pathology 184: 107620. doi: 10.1016/j. jip.2021.107620

Curry JP, Schmidt O (2007). The feeding ecology of earthworms - a review. Pedobiologia 50: 463-477. doi: 10.1016/j. pedobi.2006.09.001

Dash MC, Senapati BK, Mishra CC (1980). Nematode Feeding by Tropical Earthworms. Oikos 322-325. doi: 10.2307/3544291

Demetrio WC, Dionísio JA, Maceda A (2019). Negative effects of earthworms on soil nematodes are dependent on earthworm density, ecological category and experimental conditions. Pedobiologia 76: 150568. doi: 10.1016/j.pedobi.2019.150568

Domínguez J, Bohlen PJ, Parmelee RW (2004). Earthworms increase nitrogen leaching to greater soil depths in row crop agroecosystems. Ecosystems 7: 672-685. doi: 10.1007/s10021-004-0150-7

Drake HL, Horn MA (2007). As the worm turns: the earthworm gut as a transient habitat for soil microbial biomes. Annual Review of Microbiology 61: 169–189. doi: 10.1146/annurev.micro.61.080706.093139

Ečimović S, Vrandečić K, Kujavec M, Žulj M, Ćosić J et al. (2021). Antifungal activity of earthworm coelomic fluid obtained from *Eisenia andrei, Dendrobaena veneta* and *Allolobophora chlorotica* on six species of phytopathogenic fungi. Environments 8: 102. doi: 10.3390/environments8100102

Feng MG, Khachatourians GG, Poprawski TJ (1994). Production, formulation and application of the entomopathogenic fungus *Beauveria bassiana* for insect control: current status. Biocontrol Science and Technology 4: 3-34. doi: 10.1080/09583159409355309

Frouz J (2018). Effects of soil macro- and mesofauna on litter decomposition and soil organic matter stabilization. Geoderma 332: 161-172. doi: 10.1016/j.geoderma.2017.08.039

Fründ HC, Graefe U, Tischer S (2011). Earthworms as bioindicators of soil quality. In: Biology of earthworms. Springer, Berlin, Heidelberg, 261-278. doi: 10.1007/978-3-642-14636-7_16

Fusaro S, Gavinelli F, Lazzarini F, Paoletti MG (2018). Soil biological quality index based on earthworms (QBS-e). A new way to use earthworms as bioindicators in agroecosystems. Ecological Indicators 93: 1276-1292. doi: 10.1016/j.ecolind.2018.06.007

Gong X, Jiang Y, Zheng Y, Chen X, Li H et al. (2018). Earthworms differentially modify the microbiome of arable soils varying in residue management. Soil Biology and Biochemistry 121: 120-129. doi: 10.1016/j.soilbio.2018.03.011

Gray NF (1987). Nematophagous fungi with particular reference to their ecology. Biological Reviews, 62: 245-304. doi: 10.1111/j.1469-185X.1987.tb00665.x

Guhra T, Stolze K, Schweizer S, Totsche KU (2020). Earthworm mucus contributes to the formation of organo-mineral associations in soil. Soil Biology and Biochemistry 145: 107785. doi: 10.1016/j.soilbio.2020.107785

Herrera-Estrella A, Casas-Flores S, Kubicek CP (2016). Nematophagous Fungi. In: Environmental and microbial relationships. Springer, Cham, 247-267. doi: 10.1007/978-3-319-29532-9_13

Horn MA, Schramm A, Drake HL (2003). The earthworm gut: an ideal habitat for ingested N2O-producing microorganisms. Applied and Environmental Microbiology 69: 1662-1669. doi: 10.1128/AEM.69.3.1662-1669.2003

Houida S, Yakkou L, Bilen S, Raouane M, El Harti A et al. (2021). Taxonomic and functional characteristics of aerobic bacteria isolated from the chloragogenous tissue of the earthworm *Aporrectodea molleri*. Archives of Microbiology 203: 4805-4812. doi: 10.1007/s00203-021-02396-2

Khomyakov NV, Kharin SA, Nechitailo TY, Golyshin PN, Kurakov Av et al. (2007). Reaction of microorganisms to the digestive fluid of earthworms. Microbiology, 76: 45-54. doi: 10.1134/S0026261707010079

Lavelle P, Barros E, Blanchart E, Brown G, Desjardins T et al. (2001). SOM management in the tropics: Why feeding the soil macrofauna? In: Managing Organic Matter in Tropical Soils: Scope and Limitations. Springer, Dordrecht, 53-61. doi: 10.1007/978-94-017-2172-1_6

Li J, Zhang Z, Wang H, Wang S, Chen Q (2020). Urban land-use impacts on composition and spatiotemporal variations in abundance and biomass of earthworm community. Journal of Forestry Research 31: 325-331. doi: 10.1007/s11676-018-0807-2

Liu X, Xiang M, Che Y (2009). The living strategy of nematophagous fungi. Mycoscience 50: 20-25. doi: 10.1007/s10267-008-0451-3

Luangsa-Ard J, Houbraken J, van Doorn T, Hong SB, Borman AM et al. (2011). *Purpureocillium*, a new genus for the medically important *Paecilomyces lilacinus*. FEMS Microbiology Letters 321: 141-149. doi: 10.1111/j.1574-6968.2011.02322.x

Lubbers IM, Pulleman MM, van Groenigen JW (2017). Can earthworms simultaneously enhance decomposition and stabilization of plant residue carbon? Soil Biology and Biochemistry 105: 12–24. doi: 10.1016/j.soilbio.2016.11.008

Lussenhop J (1992). Mechanisms of microarthropod-microbial interactions in soil. Advances in Ecological Research 23: 1-33. doi: 10.1016/S0065-2504(08)60145-2

Medina-Sauza RM, Álvarez-Jiménez M, Delhal A, Reverchon F, Blouin M et al. (2019). Earthworms building up soil microbiota, a review. Frontiers in Environmental Science 7: 81. doi: 10.3389/fenvs.2019.00081

Meyling NV, Eilenberg J (2007). Ecology of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* in temperate agroecosystems: Potential for conservation biological control. Biological Control 43: 145-155. doi: 10.1016/j.biocontrol.2007.07.007

Monroy F, Aira M, Domínguez J (2008). Changes in density of nematodes, protozoa and total coliforms after transit through the gut of four epigeic earthworms (Oligochaeta). Applied Soil Ecology 39: 127-132. doi: 10.1016/j.apsoil.2007.11.011

Needham AE (1957). Components of nitrogenous excreta in the earthworms *Lumbricus terrestris*, L. and *Eisenia foetida* (Savigny). Journal of Experimental Biology 34: 425-446. doi: 10.1242/jeb.34.4.425

Nordbring-Hertz B (2004). Morphogenesis in the nematode-trapping fungus *Arthrobotrys oligospora* - an extensive plasticity of infection structures. Mycologist 18: 125-133. doi: 10.1017/S0269915X04003052

Nordbring-Hertz B, Friman E, Veenhuis M (1989). Hyphal fusion during initial stages of trap formation in *Arthrobotrys oligospora*. Antonie van Leeuwenhoek 55: 237-244. doi: 10.1007/BF00393852

Nordbring-Hertz B, Jansson HB, Tunlid A (2001). Nematophagous Fungi, In: eLS. doi: 10.1038/npg.els.0004293

Owen RB, Galbraith WJ (1989). Earthworm biomass in relation to forest types, soil and land use: implications for woodcock management. Wildlife Society Bulletin (1973-2006) 17: 130-136. doi: 10.2307/3782638

Ownley BH, Griffin MR, Klingeman WE, Gwinn KD, Moulton JK et al. (2008). *Beauveria bassiana*: Endophytic colonization and plant disease control. Journal of Invertebrate Pathology 98: 267–270. doi: 10.1016/j.jip.2008.01.010

Paoletti MG (1999). The role of earthworms for assessment of sustainability and as bioindicators. Agriculture, Ecosystems and Environment 74: 137-155. doi: 10.1016/S0167-8809(99)00034-1

Phillips HR, Guerra CA, Bartz ML, Briones MJ, Brown G et al. (2019). Global distribution of earthworm diversity. Science 366: 480-485. doi: 10.1126/science.aax4851

Plavšin I, Velki M, Ečimović S, Vrandečić K, Ćosić J (2017). Inhibitory effect of earthworm coelomic fluid on growth of the plant parasitic fungus *Fusarium oxysporum*. European Journal of Soil Biology 78: 1-6. doi: 10.1016/j.ejsobi.2016.11.004

Punu G, Ansari A, Jaikishun S, Seecharran D (2016). Effect of earthworm (*Perionyx excavatus*) powder on selected bacteria and fungi. Journal of Advances in Biology & Biotechnology 5: 1-5. doi: 10.9734/jabb/2016/22572

Scheu S (1987). Microbial activity and nutrient dynamics in earthworm casts (Lumbricidae). Biology and Fertility of Soils 5: 230-234. doi: 10.1007/BF00256906

Scheu S (1991). Mucus excretion and carbon turnover of endogeic earthworms. Biology and Fertility of Soils 12: 217-220. doi: 10.1007/BF00337206

Sethulakshmi K, Ranilakshmi K, Thomas A (2018). Antibacterial and antifungal potentialities of earthworm *Eudrilus eugeniae* paste and coelomic fluid. Asian Journal of Biology 5: 2456-7124. doi: 10.9734/ajob/2018/39786

Shapiro-Ilan DI, Brown I (2013). Earthworms as phoretic hosts for *Steinernema carpocapsae* and *Beauveria bassiana*: Implications for enhanced biological control. Biological Control 66: 41-48. doi: 10.1016/j.biocontrol.2013.03.005

Song K, Sun Y, Qin Q, Sun L, Zheng X et al. (2020). The effects of earthworms on fungal diversity and community structure in farmland soil with returned straw. Frontiers in Microbiology 3129. doi: 10.3389/fmicb.2020.594265

Toledo-Hernández RA, Toledo J, Valle-Mora J, Holguín-Meléndez F, Liedo P et al. (2019). Pathogenicity and virulence of *Purpureocillium lilacinum* (Hypocreales: Ophiocordycipitaceae) on Mexican fruit fly adults. The Florida Entomologist 102: 309-314.

Whalen JK, Parmelee RW (2000). Earthworm secondary production and N flux in agroecosystems: A comparison of two approaches. Oecologia,124: 561-573. doi: 10.1007/s004420000413

Yakkou L, Houida S, Dominguez J, Raouane M, Amghar S, El Harti A (2021a). Identification and characterization of microbial community in the coelomic fluid of earthworm (*Aporrectodea molleri*). Microbiology and Biotechnology Letters 49: 391-402. doi: 10.48022/mbl.2104.04013

Yakkou L, Houida S, Raouane M, Amghar S, El Harti A (2021b). Study of the effect of the earthworm (*Aporrectodea molleri*) cutaneous excreta on bacterial growth. International Journal of Ecology 2021: 5518568. doi: 10.1155/2021/5518568

Zhang W, Cao J, Zhang S, Wang C (2016). Effect of earthworms and arbuscular mycorrhizal fungi on the microbial community and maize growth under salt stress. Applied Soil Ecology 107: 214-223. doi: 10.1016/j.apsoil.2016.06.005

Zhou X, Liang W, Zhang Y, Ren Z, Xie Y (2021). Effect of earthworm *Eisenia fetida* epidermal mucus on the vitality and pathogenicity of *Beauveria bassiana*. Scientific Reports 11: 1-11. doi: 10.1038/s41598-021-92694-y