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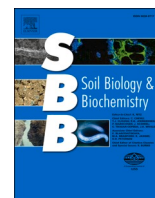
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Experimental evidence that soil fauna drives plant root exudation patterns

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ABSTRACT

Soil fauna is an essential component of soil. However, its influences on root exudates are unknown. Through both direct and indirect interactions, soil fauna can affect the dynamics of interactions between plants, microbes, and soil environments. In this study, we used an original approach called EcoRoots, which features rhizoboxes with sorption traps and allows exudates on roots to be sampled with minimum soil and plant disturbance. We designed an experiment to determine the effect of springtails (*Folsomia candida* W.), earthworms (*Lumbricus terrestris* L.), or both on plant root metabolites of *Poa annua* L., a gramineous species. After nine weeks of experiments, soil fauna increased the nutrients in soil, but *P. annua* growth was reduced mainly in the presence of earthworms. With regard to *P. annua* exudation, we found specific assemblages depending on the fauna identity, with the interaction between springtails and earthworms bringing different exudates than the sum of the two single treatments. Plants in presence of soil faunal groups produced a higher richness of exudate metabolite than without soil fauna. We also found that earthworms had the greatest influence on metabolite patterns. The changes in root exudation were associated with differences in abiotic factors and microorganism communities, suggesting that both direct and indirect effects might explain these patterns. Nevertheless, distinguishing the mechanisms involved in the process remains complex. To conclude, we strongly recommend studying living soil or more real ecosystems to advance our understanding of root exudation and, more globally, soil interactions and ecology, whether in natural or agricultural ecosystems.

1. Introduction

Biotic interactions (e.g., competition, predation, facilitation) affect fitness and structure communities, thereby driving key ecological and evolutionary processes at the ecosystem level (Wardle, 2006). Exploring such interactions is not an easy task, particularly for species that live in poorly accessible compartments such as the soil system, where the majority of species living on Earth are likely to be found (Anthony et al., 2023). Among soil organisms, soil fauna contributes to multiple ecological functions belowground (Brussard et al., 1998; Ross et al., 2022), such as plant growth promotion and regulation (Wardle, 1999; Briones, 2014), and interacts with plants closely either directly through root feeding, for example, or indirectly by improving soil fertility and structure or modifying rhizosphere microbial assemblages (Coulibaly et al., 2019). In parallel, plants deliver trophic resources at the basis of soil food webs through senescent tissues or root exudates. The

rhizosphere is a dynamic and complex zone with numerous interactions between plant roots, soil, and biota (Hiltner, 1904; del Carmen Orozco-Mosqueda et al., 2022). Outcomes of rhizospheric interactions are important as they can regulate plant growth and flowering period (Lu et al., 2018), and they also shape microbiota or surrounding soil fauna and eventually determine ecosystem trajectories.

Exudates or more broadly rhizodeposition may represent 5%–21% of total photosynthetically fixed carbon (Simon and Haichar, 2019), composing a high diversity of metabolites such as sugars, amino acids, organic acids, and enzymes (Narasimhan et al., 2003). These metabolites are highly important as chemical interfaces to communicate with the soil environment (Badri and Vivanco, 2009). Many studies investigate root–microbial interactions, particularly symbiotic interactions with mycorrhizae and nitrogen-fixing nodules, which have an important effect on plant nutrient absorption (Harley, 1997; Zheng et al., 2022). Relationships between pathogens or rhizophagous organisms and plant

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roots are also well studied. For example, pathogenic nematodes locate their food on the basis of root-induced chemical gradients and may alter the plant behavior to create feeding sites (Bonkowski et al., 2009). Plant root responses to faunal attack have also been reported and include different strategies such as misdirecting aggressors by secreting lure made of detached root border cells or secreting (E)- β -caryophyllene, a specific volatile that attracts entomopathogenic nematodes (reviewed by Bonkowski et al., 2009).

The relationship between non-pathogenic soil fauna and plant exudation is less studied than these examples of direct physical interactions, even though recent studies suggested that most soil detritivore invertebrates obtain their carbon from roots (i.e., senescent tissues or rhizodeposition) and not from aboveground litter (Pollierer et al., 2007). Dominant soil faunal groups such as earthworms (*Lumbricus terrestris* L.) and springtails (i.e., collembola) have been shown to promote plant growth and influence root architecture or biomass (Forey et al., 2015; Medina-Sauza et al., 2019; Chauvat and Forey, 2021), which could ultimately modify exudation patterns in terms of quality and quantity. This hypothesis is supported by recent studies that used transcriptomic analyses that highlighted the close relationship between springtails and plant physiology (Graf et al., 2019; Pu et al., 2022). Thus, earthworms and springtails could have the potential to influence plant metabolism and thereby exudation through different mechanisms (Fig. 1). They could directly influence root exudation by modifying soil

physicochemical conditions through bioturbation (e.g., by earthworms) or by altering root growth/architecture. They could also indirectly influence root exudation either by modifying soil abiotic properties such as N and P availability or by modifying rhizospheric microbial communities (Fig. 1). Indeed, both faunal groups are known to disseminate rhizosphere microorganisms (Bonkowski et al., 2009) or to regulate them (Fig. 1). For example, springtails were shown experimentally to regulate the composition and associated functions of soil microbial communities according to their functional groups (Coulibaly et al., 2019), while earthworms generally promote the growth of fast-growing bacteria (Medina-Sauza et al., 2019). With regard to fungi, earthworm activities enhanced root colonization by mycorrhizal fungi in more than 60% of the reviewed cases by Medina-Sauza et al. (2019). Springtails were also shown to feed preferentially on soil phytopathogenic fungi (Bourgeois et al., 2023). Although these mechanisms may be diverse, no experimental evidence to date shows that soil fauna communities can affect root exudation patterns. Investigating this potential influence appears crucial to advance our understanding of plant–soil interactions.

The notorious difficulty of collecting exudates in soil conditions might explain our weak knowledge of the role of soil organisms and especially fauna on exudation processes. Various methods are used to collect and identify root exudates (Oburger and Jones, 2018; Williams et al., 2021), with leaching and hybrid collection methods mainly being compatible with studies of root in soil. However, these two common methods are not satisfactory. The leaching method collects a whole soil chemical signal instead of only root exudates (Bohm et al., 2023). Hybrid methods, which involve growing plants in soil before digging them up, washing the roots, and transferring the plants to a hydroponic solution for exudate collection, introduce bias because root washing causes unavoidable stress and root damage (Oburger and Jones, 2018; Williams et al., 2021). A third but less well-known and developed methodology has been proposed to avoid these biases: the use of exudation traps (e.g., specialized resins, filter papers) directly localized at the root surface in the soil without involving digging (Read et al., 2003; Phillips et al., 2008; Neumann et al., 2014; Zickenrott et al., 2016). However, the efficiency of this methodology has been rarely tested. Recently, we developed a methodology called EcoRoots, which uses exudation traps coupled with inert rhizoboxes and allows the root exudates in soil to be sampled successfully and their quality to be analyzed with limited disturbance (Bohm et al., 2025).

This study aims to test if the presence of soil fauna might influence root exudation patterns. This elementary question is essential to advance our understanding of plant functioning and plant–soil interactions. Recently, it had been demonstrated that adding collembola species to plants may induce strong changes in soil chemical signals (Bohm et al., 2023), but these soil leachates were not exclusively produced by root exudates. To achieve this aim, we design an experiment using the EcoRoots method to properly sample root exudates with soil fauna (Bohm et al., 2025). We investigate how the presence of two dominant soil faunal groups, i.e., earthworms and springtails, can affect the exudation quality of *Poa annua*, an annual grass known to respond to the presence of fauna (Forey et al., 2015; Chauvat and Forey, 2021). Earthworms and springtails are the most documented taxa in terms of their effect on plant physiology (Scheu, 2003; Puga-Freitas and Blouin, 2015). They differ in size and body mass (macro- and mesofauna, respectively) and role in soil ecology (engineer species and microbial grazers, respectively). We applied four treatments of soil fauna presence (combination of earthworms and/or springtails versus no fauna) and measured plant growth, rhizospheric properties (soil physicochemistry and microbial communities), and root exudate composition using the metabolomic approach. We hypothesized that adding soil fauna to microcosms will affect the composition of exuded root metabolites, with a greater number of metabolites exuded in the presence of soil faunal groups due to more complex interactions. We also hypothesized that earthworms, springtails, and their interactions will induce different specific exudation patterns, with earthworms having the biggest effect according to the

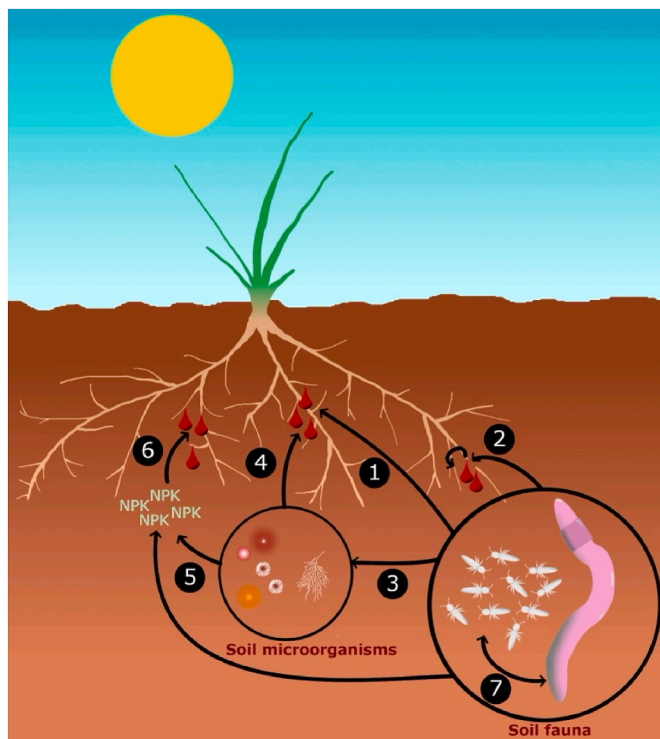


Fig. 1. Hypothetic effects of two main groups of soil fauna, Earthworms and Springtails on root exudates. (1) Direct effect through hormone-like effect modifying plant physiology (Puga-Freitas and Blouin, 2015). (2) Direct effect by mechanical interactions (e.g. bioturbation) that alter plant rhizodeposition (Williams et al., 2021). (3) Indirect effect, regulating soil microorganisms which modify both (4) direct exudation by releasing compounds that stimulate root exudation (Phillips et al., 2008) and (5) indirectly, along with soil fauna, by modifying soil abiotic properties as N and P availability (Filsler et al., 2016; Rengel and Marschner, 2005), humidity (Staszal et al., 2022) or pH (Dakora and Phillips, 2002). Indeed, (6) plants adapt their exudation to nutrient availability (Rengel and Marschner, 2005). Moreover, (7) soil fauna species may interact with each other's modifying their abundance (McLean and Parkinson, 2000; Salem et al., 2022) which could differently modify exudation. Effects of exudates on soil ecosystem are not represented here.

mass ratio hypothesis (Grime, 1998). This hypothesis states that ecosystem properties are driven by the characteristics of the mass-dominant species.

2. Materials and Methods

2.1. Experimental setup

The experiment was conducted with rhizoboxes made of glass or plastic hemispheric cylinders (46 mm radius and 200 mm height) angled at $45^\circ \pm 4'$ to make the roots grow close to a removable glass window, thus making them accessible without soil perturbation (Supplementary Fig. 1). Glass material is preferred to plastic because the latter may cause phthalate contamination but we had not a sufficient number of glass rhizoboxes and consequently used plastic ones. Each rhizobox was sealed with a thin layer of water between the window and the hemispheric cylinder. The base of each rhizobox was composed of either rigid glass concrete filter for half of the rhizoboxes or of plastic grid for the other half. Both glass and plastic filters had a mesh size smaller than 1 mm to facilitate leaching and prevent soil animals from escaping. We included these two types of filters in the following analyses as a random factor. These rhizoboxes were covered with an opaque film to avoid light exposure and subsequently algae development. Eight treatments were designed: plant without soil fauna (P), plant with earthworm (PE), plant with springtails (PS), plant with springtails and earthworm (PSE), and the same treatments but without plant, namely, Ctrl, E, S, and SE. Each treatment was replicated six times, for a total of 48 rhizoboxes.

Each rhizobox was filled with 510 ± 10 g of fresh soil. The soil used for this experiment was Rendzic Leptosols (according to the soil classification of IUSS Working Group, 2007; soil organic carbon = 6.17%; pH $H_2O = 7.79$; humidity of fresh soil = 40%) collected in September 2017 from a chalk grassland (1.703000E, 49.2202200 N) in Normandy, France. Prior to the rhizoboxes being filled, the soil was defaunated as in Chauvat and Ponge (2002) by repeated cycles of freezing and thawing. Prior to the inoculation of soil fauna, microorganisms (fungi and bacteria) were extracted from the remaining chalk grassland soil and re-inoculated in this defaunated soil as in Forey et al. (2015). Two weeks later, within half of the 48 rhizoboxes, a seedling of *Poa annua* L. (two-week-old seedling having two leaves) was transplanted in all treatments that included a plant. The day after, soil fauna was introduced with the following treatments: S, E, SE, or Ctrl. For the earthworms, we selected *Lumbricus terrestris* L., an anecic species commonly found in the chalk grassland in Normandy (Decaëns et al., 2008). These earthworms were purchased in a store. Prior to their introduction in rhizoboxes, the earthworms were placed on moistened filter paper for 48 h in Petri dishes stored in a dark room with a temperature of $15^\circ C$ to void their guts and to prevent the introduction of too much external bacteria in the earthworm treatments (Eisenhauer et al., 2009). Then, they were acclimated for three days in the same soil used in the rhizoboxes. A single juvenile *L. terrestris* (weight standardized from 1.75 g to 2.20 g) was inoculated in each E and SE rhizobox. This is equivalent to 330 earthworms per m^2 , which is slightly more than the mean density observed in the chalk grasslands in Normandy (Decaëns et al., 2008), but this experiment was maintained for only nine weeks. For the springtails, we needed a few hundred animals for the experiment. Thus, we selected *Folsomia candida* W., which is not common in the field, but it is a cosmopolitan species that can reach high abundances in different soils. Individuals were obtained from our laboratory populations. Ten of them were introduced four times (2, 15, 23, and 34 days after seedling transplantation) to prevent mortality caused by the introduction method (i.e., using a pooter). The rhizoboxes were incubated in a phytotron for nine weeks (climate chamber temperature: $18.5^\circ C/64.4^\circ F$; daily light/dark cycles of 18 h/6 h). They were watered every two days to keep them approximately at 40% humidity, with the same soil moisture between treatments. At the end of the experiment, the springtails were extracted from each rhizobox by using the Berlese-Tullgren device

(Tullgren, 1918), allowing us to confirm that populations were maintained throughout the whole experiment and that no contamination occurred in the treatment without springtails.

2.2. Exudation collection and analysis

Metabolites from root exudates were sampled at the end of the experiment, i.e., after growing in rhizoboxes for nine weeks. We set a relatively short experimentation period to avoid initiation of flowering, which could profoundly modify the root metabolism. Springtails are known to highly affect the flowering phenology of *Poa annua* after 10 weeks of growth (Forey et al., 2015). We used the methodology developed by Neumann et al. (2014) and recently improved by Bohm et al. (2025), which consists of placing 20 nitrocellulose traps with a 5 mm diameter on the roots of *Poa annua* in each rhizobox in a dark room. The roots were accessible once the glass window was removed. Traps were placed on the root tips and root hairs, which are two parts of the roots that have a high rate of exudation (Holz et al., 2018; Oburger and Jones, 2018). We tried to position the traps in a way that limits contact with the surrounding soil of roots, but we cannot prevent some soil from being sampled. Faced with this challenge, we also positioned traps on bare soil to analyze their metabolite content and separate them from the root exudates (see methodology below). For the rhizoboxes without plant, the traps were randomly placed directly on the soil after the glass window was removed. After 60 min, the 20 traps of a single rhizobox were all removed with tweezers, gathered in an amber vial, and kept at $-20^\circ C$ before processing.

For exudate analysis, traps were derivatized using the silylating agent BSTFA (N, O-bis(trimethylsilyl)trifluoroacetamide) with a protocol equivalent to Neumann et al. (2014). The derivation results in more volatile and less polar metabolites, thereby allowing them to be analyzed by gas chromatography coupled with high resolution mass spectrometry (GC-HRMS).

Traps were ultrasonicated for 30 min in 2 mL of methanol. 1 mL supernatant was then collected to be dried at $20^\circ C$ (room temperature) under constant nitrogen flow. Dry samples were then resolubilized in a 500 μL hexane/acetone mixture (1:1) and derivatized adding 100 μL pyridine and 100 μL BSTFA under nitrogen flow to be magnetically stirred for 2 h. This solution is then 0.2 μm PTFE filtered and kept at $5^\circ C$ before analysis. Extracted-metabolites were analyzed with GC-Q-Orbitrap (Trace 1310 GC and Q-Exactive, Thermo Scientific, Bremen, Germany) system. This system was completed with an autosampler (MPS from Gerstel Mülheim, Germany) and a programmed temperature vaporizer injector with a cool injection system. The injection volume was of 1 μL and was vaporized with the following injector temperature program in splitless mode: $30^\circ C$ for 0.2 min then $10^\circ C/s$ up to $300^\circ C$ for 18 min. Metabolites were separated with a BP5MS-UI analytical column (30 m \times 0.25 mm and 0.25 μm of film thickness from SGE Analytical Science (Milton Keynes, UK)) under a constant $1.7 mL min^{-1}$ helium flow at $40^\circ C$ for 3 min. The oven temperature program was as follows: 3 min at $40^\circ C$ followed by temperature increase at $15^\circ C/min$ up to $325^\circ C$ hold for 3 min. Electronic ionization was performed at 70 eV with a temperature of source kept at $250^\circ C$. Mass spectroscopy (MS) acquisition was then performed in full scan mode with a mass to charge (m/z) 50 to 550 range (Bohm et al., 2025). Each analysis was performed in triplicates. 20 freshly created and unused nitrocellulose traps were equally processed as blanks. For technical reason, only half of the traps, 3 set of traps corresponding to three randomly chosen rhizoboxes per treatment were analyzed.

Xcalibur 4.0 was used for processing purpose and metabolites were deconvoluted and assigned with Compound Discoverer 3.3 software. The NIST 1.4 Mass Spectral Library & Search Software (NIST, 2014/EPA/NIH) version 2.2 build version June 10, 2014, was used to compare the Orbitrap spectra with the library spectra.

2.3. Plant growth and soil biochemical properties

On the same day that the exudates were sampled, several plant traits were measured on the six replicates. The leaf number was counted. Three root traits (total root length, mean root diameter, and number of root tips) were assessed using WinRHIZO™ image analysis software (V. reg. 2009c, Regent Instruments, Canada). To estimate plant allocation, both root and shoot biomasses were measured after being dried at 65 °C for 48 h. Each shoot and root sample was powdered to measure nutrient content. The nitrogen content of roots and shoot was measured by chromatography with a CHN pyrolysis micro-analyzer (Flash, 2000 Series, CHNS/O Analyzers, Thermo Fisher Scientific, France). The mineral elements of the shoots (potassium [K], phosphorus [P], calcium [Ca], and magnesium [Mg]) were analyzed by atomic absorption spectrophotometry (AAS, ICE 3000 SERIES, Thermo Scientific, China).

Soil microbial community was determined by means of phospholipid fatty acid (PLFA) analysis within each rhizobox. PLFA extraction and analysis was performed using the protocol described in Coulibaly et al. (2019). The PLFA concentrations were expressed as nmol PLFA g⁻¹ dry soil. We used different PLFAs as indicators of the microbial community structure following Willers et al. (2015). Five PLFAs were used as indicators of Gram-positive bacteria (i15:0, a15:0, i16:0, a17:0 and i18:0); two PLFAs were Gram-negative indicators (16:1ω11 and 16:1ω9); one was a fungal indicator (18:2ω9); and one was an Actinobacteria indicator (10Me16:0).

Finally, the major soil nutrients in each rhizobox were investigated. Concentrations of soil mineral N (NO₃⁻ and NH₄⁺) were determined colorimetrically (Sequential Analyzer Gallery, Thermo Fisher Scientific). Briefly, soil samples (10 g fresh weight) were shaken with 50 ml KCl (1 M) on an orbital shaker for 1 h. The suspension was filtered first through a Whatman 42 filter and then through a 0.45-NYL filter before being analyzed. P, K, Ca, Mg, and Mn were measured by inductively coupled plasma analysis of acid digestion of soil with the iCAP 7000 series (Thermo Fisher Scientific).

2.4. Data analyses

Metabolites were annotated from the chromatograms on the basis of two parameters: *m/z* ratio and retention time (*tr*). Their areas were given by the areas under the chromatographic peaks. To remove metabolites coming directly from the chemical composition of the disc traps, we retrieved 10 times the values of the blanks (unused traps) from each rhizobox chromatogram. The same threshold was used to remove the soil chemical information (with or without fauna) from the plant treatments (with or without fauna), calculating the mean of each treatment without plant (three replicates) to remove their signal from the rhizoboxes with plant for data treatments (Supplementary Fig. 2). The obtained deconvoluted chromatograms were associated with new treatments called P', PE', PS', and PSE'. This approach was expected to facilitate the analysis specifically of the effect of fauna rather than confounding springtail or earthworm signals that may have been trapped, in case the animals wandered close to the traps during exudate collection. Multivariate analysis of parameters was performed using MetaboAnalystR package version 3.3.0 (Pang et al., 2022) computed on RStudio for Windows, R version 4.2.2 (R Core Team, 2023). The results were visualized using 3D sPLS-DA, heat maps, and dendrograms. Treatment differences using the metabolite data were tested with PERMANOVA. Canberra distance from the *dist* function in the *stats* package was used to create a distance matrix that was tested with *adonis2* from the *vegan* package (Oksanen et al., 2024) using the Bray–Curtis method. For clarity, heat maps were produced on the basis of the first 10% of the most discriminant metabolites (called hereafter variable importance in projection [VIP]) according to ANOVA tests with treatments as the factor and each metabolite as the dependent variable. Metabolites and treatments were clustered using the Ward clustering algorithm and Euclidean distance. To determine the exudation similarities between

treatments according to their metabolites, a dendrogram was created using Euclidean distance with the *dist* function and *hclust* as the clustering algorithm, both from the *stats* package. The three replicates per treatment were averaged prior to the similarity calculation.

To examine the mechanisms behind exudation differences across treatments, we investigated the effect of fauna on plant growth, microbial communities, and soil nutrients in treatments with plant (P, PS, PSE, PE). Plant variables (biomasses, root and leaf traits, and plant chemistry), soil microorganisms (based on PLFA), and soil nutrients were tested between treatments using GLMs (R Core Team, 2022). PLFA and soil nutrients were also analyzed with Principal component analysis (PCA) using the *factmineR* package of R (Le et al., 2008) to depict the general trend between these two soil components. As previously described, while performing the GLMs, the rhizoboxes' bottom material (glass or plastic) were included as random effect. Tukey's HSD post hoc tests were used to identify differences between treatments, if necessary.

3. Results

3.1. Effect of soil fauna on plant variables and soil nutrient properties

At the end of the experiment, the presence of different soil fauna assemblages was found to significantly influence *Poa annua* growth for 4 of the 13 measured variables (Supplementary Table 1). More precisely, in the presence of both springtails and earthworms (PSE treatment), root biomass was reduced by 75% (Fig. 2b), root length was reduced by 66% (Fig. 2c), leaf number was reduced by 60% (Fig. 2a), and leaf Mg content was reduced by 25% (Fig. 2d). Although we observed the same tendency in the PE and PS treatments, this effect was weaker and not significantly different from controls (P), with the exception of the PE treatment having a smaller root length (Supplementary Table 1 and Fig. 2).

Regarding soil chemistry, pH, NH₄⁺, Ca, Mn, and P were not significantly different between the four treatments (Supplementary Table 2). Conversely, NO₃⁻, K, and Mg showed significant variation between treatments, with the smallest concentrations always found in treatments without soil fauna and the highest ones always including PSE (Supplementary Table 2). More precisely, NO₃⁻ was significantly higher in both PSE and PE (+35%) than in the P treatment, with PS in the intermediate position (+13%). K was significantly higher in faunal treatments (PE, PS, PSE, +44%) than in the P treatment (Supplementary Table 2). Lastly, Mg was higher in PSE (+8%), with PS and PE in the intermediate position (+6%).

3.2. Effect of soil fauna on soil microbial properties

The PCA performed on PLFA showed that microbial community composition differed between P on one side of the first axis with 72.6% of the information and PE, PS, PSE on the other side of this first axis (Fig. 3a). According to the first axis (72.6% of inertia), the variance of the P treatment is much higher than that of the treatment with fauna. The difference between the treatments was confirmed by the clustering analysis, with the P treatment rooting clearly outside the cluster of the three other treatments (Fig. 3b).

Except Gram-positive bacteria, all PLFA markers were significantly affected by the different treatments (Table 1). Total PLFAs, Gram-negative bacteria, and Actinobacteria showed the same pattern, being all significantly lower in both PSE and PE than in the P treatment, with PS in the intermediate position. Compared with P, total PLFAs, Gram-negative bacteria, and Actinobacteria showed a decrease from around 1/3 with PE (-33%, -36%, and -33%, respectively) and a decrease from around 3/8 with PSE treatment (-37%, -39%, and -37%, respectively). Fungi also had a significantly lower biomass in all faunal treatments (PE, PS, PSE) ranging from -59% in PE to -45% in PS compared with P treatment. Conversely, bacteria over fungi ratio and Gram-positive over Gram-negative ratio were both significantly higher in faunal treatments and particularly in PE treatment (+70% and 19%,

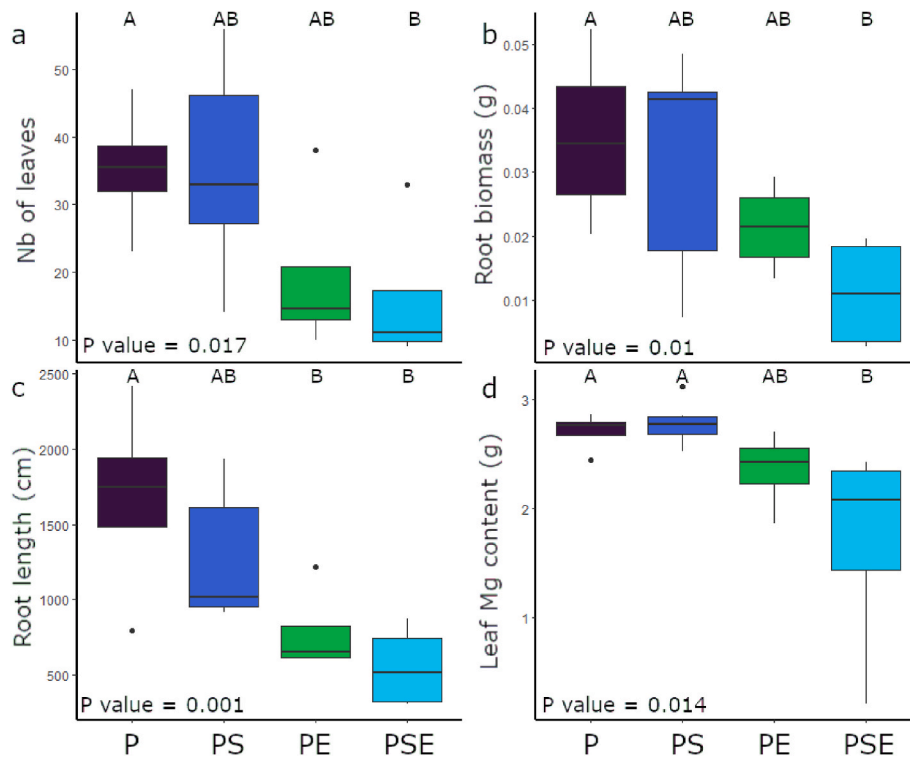


Fig. 2. Effect of Soil fauna on *Poa annua* on four plant variables: **a/leaf number**, **b/root biomass**, **c/root length**, and **d/leaf Magnesium content**, after 9 weeks of growth in rhizoboxes (n = 6)

Data are means ± SD. Within a single variable, different letters denote significantly different values according to Tukey HSD Posthoc tests. Name of treatments: P= Plant, PS = Plant and Springtails, PE= Plant and Earthworms and PSE = Plant, Springtails and Earthworms.

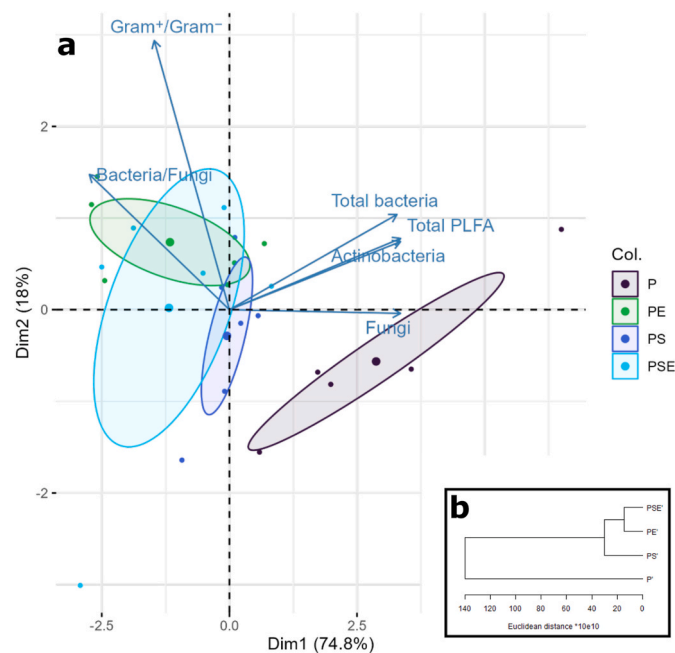


Fig. 3. **a/PCA** representing the influence of plant and soil fauna on soil microbial communities described by PLFAs (Phospholipids Fatty Acids) across four treatments Plant (P), Plant and Earthworm (PE), Plant and Springtails (PS) and Plant, Springtails and Earthworm (PSE). Ellipse represents a 95% confidence interval. Actinobact: actinobacteria, Bact/Fungi: bacteria over fungi ratio, Gram+/Gram-: bacterial Gram + over Gram-ratio, and Tot PLFA: the sum of all PLFAs. **b/Euclidean distance** of soil PLFAs amount between 4 treatments.

respectively) with PS and PSE in intermediate values compared with P treatment.

3.3. Effect of soil fauna on exudated metabolites

In total, 1412 metabolites were retrieved from the trap analyses, of which 919 were kept after the average blank trap signal was removed. Withdrawing the signal of the treatment without plants (Ctrl, S, E, and SE) to the respective treatments with plants (P, PS, PE, and PSE; see Materials and Methods) obtained a total of 737 metabolites in the different treatments (P', PS', PE', PSE'), which were used further in the following analyses.

The total metabolite richness differed between treatment with P' having the lowest number of metabolites (160 metabolites for the three replicates), PS' and PE' had an intermediate metabolite richness (respectively 345 and 368) and PSE' the highest value with 452 metabolites, i.e. three times the richness of P'. The average number of metabolites per rhizoboxes followed the same patterns with 110 ± 8 metabolites for P', 163 ± 126 for PS', 140 ± 147 for PE' and 254 ± 162 for PSE' but there was not significant variation between traitement ($F = 0.724$, $p = 0.565$). Regarding their metabolite profiles, all the 4 treatments (P', PS', PE', PSE') were significantly different (PERMANOVA, $F = 1.62$; $p = 0.0042$).

According to their metabolites, all four treatments (P', PS', PE', PSE') were significantly different (PERMANOVA, $F = 1.62$; $p = 0.0042$). The first three axes of the sPLS-DA represented 61.9% of the total inertia and allowed differences between treatments to be visualized (Fig. 4). The three replicates of P' had higher values on both axes 2 and 3 compared with the replicates of all other treatments, meaning that 26.4% of the data differentiated them. Moreover, PS' and PSE' treatments were distinguishable from each other, the PSE' treatment being differentiated mostly by the first axis and the PS' treatment being differentiated by the third one. Furthermore, on the basis of Euclidean distance, two clusters

Table 1

Means and standard deviations of soil PLFAs across four treatments: Plant (P), Plant and Earthworm (PE), Plant and Springtails (PS) and Plant, Springtails and Earthworm (PSE). F and p-value are given. Statistical significance was set at $p < 0.05$. Within a single variable, different letters denote significantly different values according to Tukey HSD Posthoc tests. Actinobact: actinobacteria, Bact/Fungi: bacteria over fungi ratio, Gram+/Gram-: bacterial Gram + over Gram-ratio, and Tot PLFA: total PLFAs. All soil PLFA indicators are in nmol.g^{-1} . For clarity, variables significantly different between treatments are highlighted in bold.

	P	PS	PE	PSE	Stat. Test:	
					F	P value
Tot PLFA	351.15 ± 95.96 A	248.69 ± 30.80 AB	234.45 ± 54.02 B	221.43 ± 62.67 B	4.61	0.014*
Fungi	6.83 ± 2.59 A	3.77 ± 0.47 B	2.79 ± 1.07 B	2.83 ± 1.28 B	8.67	0.001***
Gram+	57.70 ± 16.16	44.29 ± 7.4	43.16 ± 10.07	40.31 ± 14.20	2.11	0.133
Gram-	46.7 ± 12.97 A	32.43 ± 4.26 AB	30 ± 8.34 B	28.46 ± 8.87 B	4.63	0.014*
Actinobact	22.85 ± 6.31 A	16.51 ± 1.99 AB	15.24 ± 3.38 B	14.24 ± 3.77 B	4.89	0.011*
Bact/Fungi	16.16 ± 3.62 C	20.47 ± 2.99 BC	27.43 ± 4.47 A	25.60 ± 4.50 AB	9.08	0.001***
Gram+/Gram-	1.23 ± 0.05 B	1.36 ± 0.06 AB	1.46 ± 0.09 A	1.39 ± 0.15 AB	4.70	0.013*

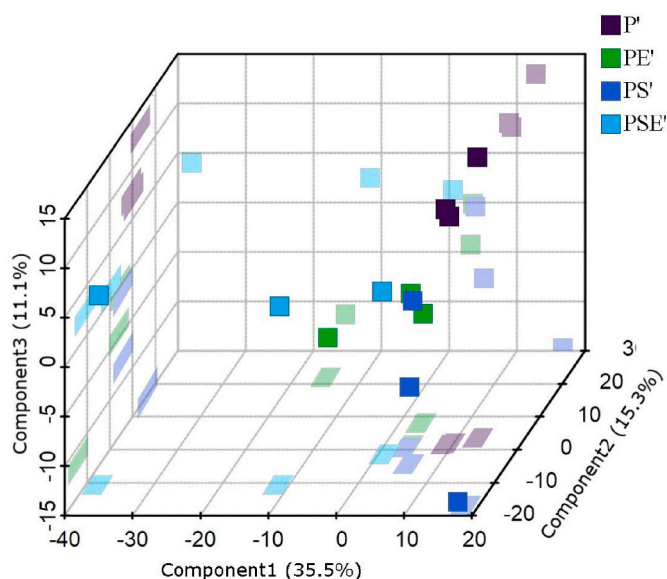


Fig. 4. 3D mapping of the 737 metabolites produced in the different combination of soil fauna treatments using a 3D sPLS-DA. The four treatments are Plant (P'), Plant and Earthworm (PE'), Plant and Springtails (PS') and Plant, Springtails and Earthworm (PSE'). Inertia of each of the 3 axes is given in % into brackets.

were revealed—one cluster with PE' and PSE' with the highest similarity and a second cluster with PS' and P' (Fig. 5).

Further clustering analysis based on the 10% VIP metabolites (i.e., 73

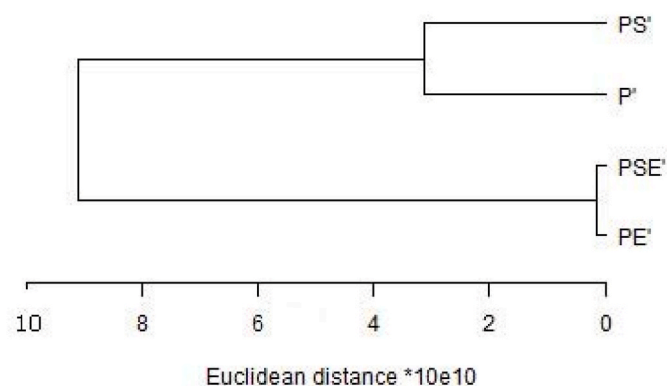


Fig. 5. Euclidean distance of the 737 mean metabolites' abundance exuded by a plant (*Poa annua*) between 4 treatments, i. e Plant (P'), Plant and Earthworm (PE'), Plant and Springtails (PS') and Plant, Springtails and Earthworm (PSE').

metabolites) clearly grouped together the three replicates of P' and one replicate of PS' (Fig. 6). The three replicates of the PSE' were well clustered. The associated heat map clearly shows a group of metabolites that were abundant in one or another of the four treatments. P' and PSE' were characterized by an abundance of 26 and 33 specific metabolites, respectively, equivalent to 35% and 45% of the total amount of VIP metabolites. Conversely, PE' and PS' produced less specific and abundant metabolites when considering these 10% VIP metabolites.

4. Discussion

To investigate the effect of soil fauna on root exudates, we applied an original methodology called EcoRoots, which uses rhizoboxes equipped with exudation traps. We collected metabolites successfully and demonstrated that soil fauna may alter *Poa annua* exudation with a specific exudation pattern depending on soil fauna assemblages. Before discussing changes in plant exudation patterns induced by different soil fauna assemblages, we first evaluate the impact of soil fauna on plant growth, soil properties, and microbial assemblages.

4.1. Effects of soil fauna on plant growth, soil abiotic variables, and microbiome

Adding soil fauna (i.e., earthworms or/and springtails) increased the soil concentration of several nutrients (NO_3^- , K, and Mg). Many studies reported that soil fauna, especially earthworms and springtails, increased the rates of soil nutrient mineralization either directly by releasing enriched excreta or mostly through their interactions with the soil microbial communities (Crowther et al., 2012; Forey et al., 2015). The interplay between microorganisms and microbivores is known as the microbial loop (Clarholm, 1985), which determines the rate of nutrient release within the rhizosphere. Briefly, the release of root exudates increases the bacterial growth, with nutrients such as N being locked into microbial biomass during this intense growth phase. This process continued until microbial consumption at higher trophic levels. Nevertheless, this soil enrichment did not stimulate either shoot or root plant biomass, contrary to previous studies (Cole et al., 2004; Forey et al., 2015; Chauvat and Forey, 2021). Indeed, in the presence of soil fauna, plants had a smaller number of leaves and shoot and root biomasses; this effect was particularly visible in treatments with earthworms (PSE and secondly PE). Such unexpected results could be explained by the short duration of our experiment, which did not allow the plants to benefit from the increased nutrient content in the faunal treatments. A short incubation period was designed to avoid flowering initiation, which would have completely changed the exudation pattern according to the phenological stage. Another possibility is that earthworms, by limiting plant growth in our case, also limited soil nutrient uptake by plants, resulting in higher concentration of several soil nutrients.

Soil fauna affected the soil microbiome community, potentially reducing or boosting plant pathogens and/or plant growth-promoting

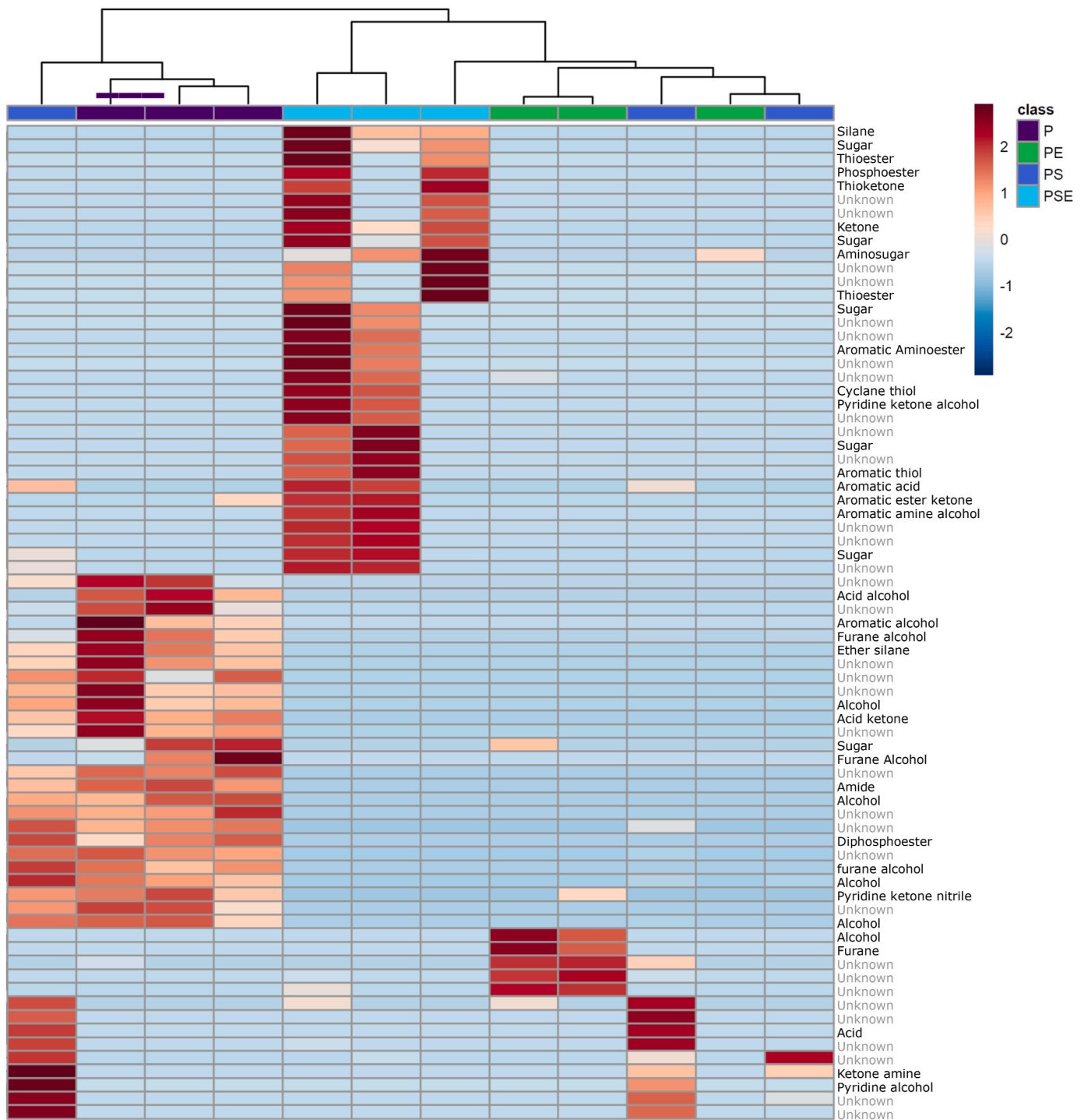


Fig. 6. Cluster heatmap analysis combined with hierarchical clustering analysis of VIP trapped-metabolites exudated by *Poa annua* without (P) and with soil fauna (PE = Plant and Earthworm, PS = Plant and Springtails, PSE = Plant, Springtails and Earthworm). Metabolites from the soil were removed from this analysis. Clustering for metabolites and ecoroots was performed using ward algorithm and Euclidean distance. The groups on the right were given according to the metabolite found when confidence number was greater than 5. Color represented the metabolites abundance scaled so the abundance sum of a single metabolite across treatments was null.

taxa (Innocenti and Sabatini, 2018; Goncharov et al., 2020). Our PCA results clearly showed that soil fauna addition led to a specific microbial assemblage compared with treatment without fauna and that these assemblages were specific to each treatment. We also clearly showed a decrease in the total microbial biomass following earthworm introduction either alone or in combination with springtails. In the literature, earthworms are known to strongly interact with the microbial assemblages, but there are inconsistencies about whether or not they promote

the microbial biomass in controlled experiments. The results seem to depend on several factors such as the presence or absence of plant, the species of earthworms, or the soil characteristics (Enami et al., 2001; Chang et al., 2016; Hedéneec et al., 2020; Zhong et al., 2024). Total microbial biomass is a coarse variable that may poorly reflect the outcomes of biotic interactions. However, the presence of earthworms clearly affects the microbial composition by promoting bacteria biomass to fungi's cost. Growing evidence suggests that earthworms rely on the

bacterial energy channel (Crotty et al., 2011; Larsen et al., 2016; Zhong et al., 2024) to acquire their carbon. Bacteria are assumed to represent the fast energy channel in soil, being responsible for most of the processing of labile carbon compounds such as root exudates (Wolters, 2000; Moore et al., 2003). Thus, bacteria likely serve as an important link for earthworms to acquire root-derived resources (Briones et al., 2019). The single presence of springtails only affected the fungal biomass by drastically reducing it by almost two times compared with the control without fauna, supporting the results of previous studies showing top-down regulation (Ek et al., 1994; Mikola and Setälä, 1998; Coulibaly et al., 2019). However, other studies demonstrated that springtails may trigger compensatory growth of the fungi on which they graze (Hanlon, 1981; Hedlund et al., 1991). Overall, the outcome of springtail–fungi interaction is largely dependent on the species composition and population density of fungivores (Thimm and Larink, 1995).

4.2 Presence of soil fauna on exudation patterns.

In this experiment, we showed for the first time that the metabolite signature of plant exudates (here *P. annua*) was influenced by the presence of soil fauna. For decades, interactions between roots and soil fauna were considered through the prism of root herbivory in an agricultural context. However, recent studies have highlighted that the majority of soil invertebrates mainly rely on the carbon inputs from roots (Pollierer et al., 2007). Indeed, active interactions and feedback may occur between plant roots and soil fauna.

With regard to the 737 metabolites trapped in this experiment, the spls-da demonstrated that all four treatments had significant different metabolite signatures. The PS', PE', and PSE' treatments differ from P' treatment by 26.1% of the inertia. The treatments with soil fauna had two to three times more total number of metabolites compared to control without fauna. As hypothesized, the highest exudated metabolite richness was found in treatment with the highest number of faunal groups (PSE'). On the 10% VIP metabolites, we also found that each treatment had a specific exudation pattern. In the presence or absence of soil fauna, metabolites produced by plants showed significant and different abundances with 33% of the metabolites specific to only the P treatment.

These changes in the root exudation pattern of *P. annua* matching differences in PLFAs and nutrient availability suggest that plants adjust exudation to adapt to resources and biochemical conditions according to their need (Zhao et al., 2021). The exudation of metabolites may be directly induced by soil fauna through physical contact or hormone-like production, as found previously when insect larvae feed on roots (Van Dam and Bouwmeester, 2016). Differences in exudation could also come from indirect interactions between plants and fauna with soil microbiota or nutrient availability as mediators. As discussed earlier, soil fauna drives microbial assemblage with top-down control (Coulibaly et al., 2019), as supported by our results. These could be mediators, as both have been found to modify root exudation quality (Phillips et al., 2004; Rengel and Marschner, 2005) even if the exudation patterns that shape the rhizosphere are still unknown. Root exudates are known to also drive microbes that have distinct substrate preferences, and these preferences are dynamic depending on substrate availability and the presence of other microbes (Zhalnina et al., 2018). Thus, we can hypothesize that differences in microbiome between treatments could be the result of exudates that govern microbe–microbe interactions such as competition or cross-feeding aside from direct top-down control by soil fauna.

4.2. Soil fauna dependence of exudation patterns

In this study, we demonstrated that the faunal effect on rhizospheric metabolites was also dependent on the faunal combination. Earthworms had greater signature effects on rhizospheric metabolites than springtails did. The PS' treatment was more closely related to the P' treatment than to the PE' and PSE' treatments, which were very close to each other. Furthermore, the presence of earthworms alone or in combination with springtails affected soil microbial communities more strongly, with

a greater negative impact on fungi than that of springtails alone.

The effect of earthworms and springtails on soil abiotic properties was comparable except for nitrate content, which increased by around 22% more with earthworms than with springtails. All these effects on abiotic and microbial variables can be ascribed to earthworms, as we hypothesized a specific stronger signature that was different from that of springtails. Earthworm individual biomass is 1000 times higher than that of springtails. Additionally, they are recognized to be 'ecosystem engineers' because of their strong influence on soil properties and functional processes (Jones et al., 1994). Through bioturbation and feeding activities, they alter soil structure and microsites with implications for nutrient cycling, water infiltration, and overall soil health and plant growth (Lavelle and Spain, 2024). Springtails also affected root exudates but to a lesser extent than earthworms did, as shown on the heat map. Interestingly, we found that faunal combination (i.e., presence of both springtails and earthworms) generated a new exudation signature, without new metabolites but with differences in their concentrations producing an original signal that was not predictable from the single faunal treatments. Complexifying the system by enhancing biotic interactions (i.e., adding a soil faunal group) modified the exudate signature more than summing the exudates of two simpler systems did, suggesting a synergic effect or emerging properties, as observed with the overyielding concept that is long known in plant interactions (Huang and Xu, 1999). Similarly, increasing soil fauna biodiversity was also shown experimentally to result in overyielding (Hedde et al., 2010; Winck et al., 2020). Thus, we strongly encourage the use of diverse combinations of soil faunal assemblage to better understand the importance of these emerging properties on plants and soil functions.

4.3. Perspectives and conclusion

Understanding plant–soil interactions is a major challenge that needs to be addressed using holistic approaches, allowing ecological processes (i.e., soil functions and diversity) to be included. Using a soil-based method (i.e., EcoRoots) that does not cause disturbance rather than a hydroponic or semi-hydroponic method allowed us to demonstrate the importance of soil fauna on root exudation patterns and show that exudate sampling in soil is possible. Such methodology opens the field of possibilities in plant biology and ecology with potential manipulation of fauna, soil properties, or species interactions (e.g., plant–plant or plant–soil fauna). We also found that clarifying the mechanisms behind these exudation patterns is difficult because of multiple direct and indirect interactions between all trophic levels. A promising approach is to use isotopic labeling to trace the flow and fate of carbon and nutrients along trophic levels. Continuing to enhance this metabolomic approach would bring important information. This study was an exudation quality comparative method and not an exudation quantity method because for each metabolite, the obtained amount depends on the polarity of the metabolite, which defines its adsorption and desorption on the traps. Moreover, determining the metabolites is tedious, and the amount of identified metabolites is usually at most only a tenth of the metabolites that were found (Van Dam and Bouwmeester, 2016). Thus, identifying metabolites is an important step to advance our understanding of the involved mechanisms. In GC/MS, several mass spectral libraries of standard compounds have been established, but most existing metabolomics databases do not contain a satisfying proportion of plant metabolites (Salem et al., 2022). These identified metabolites are closer to the phenotype, thereby potentially directly reflecting the biochemical pathways (Salem et al., 2022). Coupling these metabolic pathways with other omics data, such as RNA-Seq-based transcriptome analysis, is a promising way to reveal potential functional pathways (Huang et al., 2021; Wang et al., 2022). Moreover, distinguishing plant-specific metabolites from those produced by associated microbes is a great challenge in root exudate analysis (Salem et al., 2022). We are aware that soil fauna might have induced different microorganisms' patterns in our soil and that some microorganisms' metabolites could have been

sampled in traps. Soil animal excreta, which vary from different treatments, might also be found in the traps. However, we tried to minimize this effect as much as possible by trapping metabolites only on the roots and not on the whole soil solution (Döll et al., 2024), and we removed microorganisms and soil fauna's metabolites from the treatments without plants. Reducing the size of traps (i.e., 1–2 mm instead of 5 mm) to better fit the root diameter would be a good approach to develop for future exudate sampling. Additionally, time sampling in EcoRoots was reduced to 60 min, which is shorter than the duration of most exudate collections, which typically range from 2 h to several days (Döll et al., 2024). Thus, we are confident that most of the trapped metabolites come from *P. annua* exudates. However, we cannot totally exclude the presence of some microorganisms' metabolites in our data. To confirm these results, plant metabolites can be labeled by, for example, ^{13}C plant labeling.

To conclude, our study is the first to provide evidence that soil fauna might drive exudation patterns. Clarifying the exudate-mediated ecology of belowground interactions is challenging, but it is possible. A next step would be to understand the involved mechanisms and determine which among plants, microorganisms, or/and soil fauna benefits from the changes in exudation. As discussed earlier, exudates are taken primarily by microbes and unlocked afterwards by soil animals/protists to be captured again by plants. One question is whether the release rate (consumption of microbes) could determine as feedback the amount and quality of metabolites released by the roots. Many issues remain unresolved regarding the interplay of numerous actors (fauna, protists, microbes, viruses, roots) in the rhizosphere.

CRediT authorship contribution statement

Antoine Levasseur: Writing – review & editing, Writing – original draft, Visualization, Formal analysis, Data curation. **Matthieu Chauvat:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Victoria Bohm:** Writing – review & editing, Validation, Resources, Data curation. **Pascal Cardinael:** Writing – review & editing, Validation, Methodology. **Guillaume Le Mer:** Validation. **Valérie Peulon-Agasse:** Writing – review & editing, Validation, Methodology. **Estelle Forey:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.soilbio.2025.109771>.

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