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Earthworms influence the production of above- and belowground biomass and the expression of genes involved in cell proliferation and stress responses in *Arabidopsis thaliana*

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ABSTRACT

To better understand the complex mechanisms of action of earthworms on plants, we set up an experimental system using the model plant *Arabidopsis thaliana* (L.) Heynh, *Aporrectodea caliginosa* a common temperate earthworm and two types of soil with contrasted contents in organic matter and nutrients. Changes in plant biomass, biomass allocation to roots, leaves and stems and C/N ratios were related to variations in the expression of several plant genes involved in cellular division and stress responses and with earthworm-induced alterations in soil mineral status.

In the poorest soil, i.e. with low contents in mineral nutrient and organic matter, earthworms increased soil nitrate content very significantly and boosted plant aboveground biomass production. This correlated with changes in leaf transcript accumulation suggesting enhanced cell division and lesser incidence of reactive oxygen species. In the richer soil, earthworms had no significant effect on the production of aerial biomass. However, several plant responses were observed regardless of soil quality: enhanced accumulation of an auxin-responsive transcript in the leaves, a strong decrease in root length and biomass and a reduction in C/N values, particularly in the bolt stems. Although these results pointed out earthworm-induced enhancement of mineralization as a determining factor in the formidable plant growth responses, the release in the drilosphere of phytohormone-like compounds by earthworm-activated bacteria was most likely implicated as well in this process and resulted in "forced" nitrogen uptake by the plants. The herein demonstrated sensitivity of the model plant *A. thaliana* to earthworms shows that such new experimental set up could become a central key to the development of multidisciplinary investigations on plant–soil interactions.

1. Introduction

Earthworms are generally regarded as beneficial to plant growth (Brown et al., 1999; Scheu, 2003). Their mechanisms of action include changes in soil structure that affect root growth and water balance (Blanchart et al., 1999). Earthworms allow plants to better resist parasitic nematode attacks, either by decreasing nematode population density (Yeates, 1981; Senapati, 1992), by enhancing the capacity of plants to tolerate these parasites (Blouin et al., 2005; Lafont et al., 2007) or by stimulating microbes that are antagonistic to root pathogens (Clapperton et al., 2001). Mostly, earthworms are known to induce changes in nutrient spatiotemporal availability (Barois et al., 1999) through fragmentation and burying of soil litter (Brown et al., 2000) and microbe-based mineralization of soil organic matter (Postma-Blaauw et al., 2006). According to some authors, the latter leads to the release of mineral nitrogen essentially and represents the major mechanism of action of earthworms responsible for increases in plant biomass production (Brown et al., 1999). It could explain how greater benefits on productivity have mostly been observed in poor soils (Brown et al., 2004). However, in an experimental system combining rice plants and the earthworm *Millsonia anomala*, increasing the availability of mineral nutrients did not suppressed the positive effect of the earthworm on plant growth (Blouin et al., 2006). This meant that other mechanisms than mineralization were involved. The stimulation by earthworms of bacteria producing phytohormone-like compounds (Krishnamoorthy and Vajranabhaiah, 1986) has been suggested. Auxin-like

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compounds have indeed been identified in earthworm casts (Muscolo et al., 1998, 1999). Furthermore, these molecules appeared to be potent mediators of plant nitrogen metabolism since they systemically stimulated nitrate transport into plants and its assimilation by plant cells (Muscolo et al., 1999; Canellas et al., 2002; Quaggiotti et al., 2004).

What emerges from this rapid overview of the literature is that plant–earthworms relations are extremely complex, due to the number of mechanisms involved, and the fact that soil characteristics, plant physiology and earthworm behaviour are likely to influence these mechanisms. As a result, efficient contributions to their understanding should address the physiological and molecular processes underlying the macroscopic changes in plant growth and morphology observed in the presence of earthworms. In this context, we designed an experimental set up combining the peregrine endogeic earthworm *Aporrectodea caliginosa* (Lee, 1985; Scheu, 2003) and the plant *Arabidopsis thaliana* (L.) Heynh. This plant species was chosen for its value as a model organism extensively studied at both physiological and genetic levels. Its responsiveness to earthworms was tested here for the first time through analysis of variations in C/N ratios and in root, leaf and seed biomass production. At the same time, the possible effects of earthworms on various plant cell processes was examined at the molecular physiology level by studying the steady-state levels of *ICK1*, *PLDα*, Cu/Zn *SOD*, *HBT* and *RubcS* gene transcripts. When overexpressed in *Arabidopsis* plants, *ICK1*, which encodes a potent inhibitor of cell cycle cyclin-dependent protein kinases (CDKs) (Wang et al., 1998; Francis, 2007) induced a significant reduction in leaf size and rosette diameter (Bemis and Torii, 2007). A high *ICK1* transcript level was therefore considered an indicator of poor cell division. HBT protein functions have been related to IAA-regulated cell division and differentiation (Blilou et al., 2002). *PLDα* and Cu/Zn *SOD* transcripts both encode proteins that are transcriptionally responsive to stresses, such as wounding (Wang, 2002) and excess of reactive oxygen species (Sakamoto et al., 1995; Kaminaka et al., 1999), respectively. They were used here as cell stress indicators. It is noteworthy that high levels of *PLD* gene expression have been observed in dividing and growing plant cells suggesting that it may play an essential role in cell proliferation (Xu et al., 1997). The *RubcS* transcripts that encode the small sub-unit of the ribulose 1,5-diphosphate carboxylase were studied here to assess the possible transcriptional impact of earthworms on the carbon fixing enzyme (Nielsen et al., 1998).

Another original feature of our experimental system, in addition to the molecular analyses, consisted in the use of two soils with contrasting properties: a sandy cambisol and a clayey leptosol, the cambisol being much poorer in mineral nutrients and organic matter than the leptosol. The objective was to differentiate between two types of plant responses to earthworms: those mediated through nutrient release and those related to other mechanisms of action. It was assumed that the uncoupling between these response mechanisms would lead to the identification of general earthworm effects independent of soil quality.

2. Materials and methods

2.1. Soil characteristics and microcosms preparation

Soils were collected from the top layer (0–20 cm), at the Museum National d'Histoire Naturelle in Brunoy (Essonne, France) and at the Centre de Recherche en Ecologie Expérimentale et Prédictive - CEREEP (Saint-Pierre-Lès-Nemours, France). One is a calcareous leptosol supporting a deciduous forest (total organic carbon content, 56.7 g kg^{-1}; total nitrogen content, 4.65 g kg^{-1}; pH, 7.45; CEC, 23.4 cmol kg^{-1}) with a loamy texture (34.4% clay, 39.2% silt, 27.4% sand). The second soil, much poorer than the other one, is a cambisol supporting a natural meadow (total organic carbon content, 14.7 g kg^{-1}; total nitrogen content, 1.19 g kg^{-1}; pH, 5.22; CEC, 4.08 cmol kg^{-1}) with a sandy texture (6.9% clay, 19.0% silt, 74.1% sand). The leptosol and cambisol collected will hereafter be referred to as "rich"(R) and "poor" (P) soils, respectively. Both soil samples were dried at 25 °C for a week, passed through a 2 mm mesh sieve and used to prepare microcosms. These growth units consisted in 10 cm diameter, 16 cm-high pots filled with 0.9 kg or 1.3 kg of the rich or poor soil, respectively, to occupy similar volumes in the pots. Soils were maintained at 80% of the field capacity with deionised H_2O.

2.2. Earthworms

A. caliginosa earthworms were collected at the IRD site in Bondy (Seine Saint Denis, France). Individuals of similar size and with a well developed clitellum were chosen. In all earthworm treatments, approximately 1.7 g of worms (around four animals), which correspond to a biomass of 200 g m^{-2} as was observed in some pastures (Zou and Gonzalez, 1996), were added to microcosms four weeks prior to the introduction of the plants (D0) in order to maximize earthworm effects. Control microcosms (without earthworms) also were prepared and incubated for four weeks before D0.

2.3. Plant growth

A. thaliana (L.) Heynh. ecotype Columbia seeds were germinated in the dark on wet Whatman paper. When cotyledons were fully open (six days after germination), plantlets were transferred to microcosms on the basis of one plant per microcosm. Plant growth was carried out under controlled conditions (Conviron growth chamber, Canada): 20 ± 1 °C and 18 ± 1 °C day and night temperatures, 70% ± 5% relative humidity, 400 μmol m^{-2} s^{-1} PPFD for 10 h per day.

2.4. Plant treatments

Arabidopsis plantlets were transferred to different types of microcosms containing the rich soil (with or without earthworms) or the poor soil (with or without earthworms). Six replicates were set up for each treatment combination. For both soils, additional "no-plant" control microcosms were set up (with or without earthworms). Three replicates were set up for each control. The distribution of the microcosms in the growth chamber was randomized and changed after each biweekly watering.

2.5. Plant sampling and total RNA extraction

To sample plant tissue at a similar developmental stage, all plant samples were collected upon formation of the floral buds. Total leaf and root materials were collected from three of the six replicates, snap-frozen in liquid nitrogen and stored at −80 °C. Leaf ribs were systematically removed from the leaf samples. Total RNA extraction was carried out using RNeasy Plant Minikit (Qiagen, France) on 100 mg and 50 mg of fresh leaf and root materials, respectively, following the manufacturer's instructions. DNAse I (Promega, France) treatment was applied to all RNA extracts. RNA quantification was done at 260 nm, using a Nanodrop® ND-1000 UV–Vis spectrophotometer (NanoDrop Technologies, Wilmington, USA).

2.6. RT-PCR analysis

First strand cDNA synthesis was performed in 20 μL reactions on 150 ng of total RNA using four units of Omniscript reverse