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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

Table 1
Nucleotidic sequences and melting temperatures of the five primer pairs used in RT-PCR reactions.

Genes	Sequences of primers	Tm
HBT (AtHBT-f)	5′GATAGAAGGAAGAATGCTGC3′	52 °C
HBT (AtHBT-r)	5′TACTGCTTTTGAATGGAGAGAG3′	
ICK1 (AtICK1-f)	5′GGTTATTTATTTGACTCTCTCT3′	47.5 °C
ICK1 (AtICK1-r)	5′ATTCTTCTTTCTCCTCCTCT3′	
PLD alpha (AtPLDα-f)	5′CCAAAACAAGGAGGAGATG3′	52 °C
PLD alpha (AtPLDα-r)	5′CAGGGTTACGAGGACACAAAA 3′	
RUBISCO (AtpRUB-f)	5′GTTGAAGGAAGTGGAAGAGT 3′	50 °C
RUBISCO (AtpRUB-r)	5′TACACAAAAGCAAAGGGAAA 3′	
SOD (AtSOD-f)	5′TGTCTACTGGTCCACATTTCAAC3′	57 °C
SOD (AtSOD-r)	5′TTTCCGAGGTCATCAGGGTCT3′	
S19 (AtS19-f)	5′TCCAGGAAGCAGTTCGTTATTGAT3′	60 °C
S19 (AtS19-r)	5′CTGGTGATGCCAAGAAGAAGTGA3′	

transcriptase (Qiagen, France) and 10 μM of oligo-dT primers, according to the manufacturer's instructions. Transcript abundance of the *Arabidopsis* genes listed in Table 1 was analyzed by semi-quantitative RT-PCR using 1 μL of cDNA obtained from leaves and roots and the primers shown in Table 1. 20 μL PCR reactions were performed in a Master Cycler Gradient thermocycler (Eppendorf AG, Germany), using the Taq PCR Master mix (Promega, France). For each primer pair, the optimal number of cycles was determined during preliminary reactions. PCR reactions were as follows: 5 min at 94 °C followed by 30–40 cycles (30 s at 94 °C, 30 s at annealing temperature, 30 s at 72 °C) and 10 min at 72 °C. PCR products were analyzed after separation on ethidium bromide stained 1% agarose gels. Fluorescence images of PCR products were digitized and quantified, using the Gel-Doc Quantity One software (BioRad, France).

2.7. DNA cloning and sequencing

PCR products were cloned in the pGEM-Teasy vector plasmid system (Promega, France), following the manufacturer's instructions. Plasmidic DNA preparation was carried out using the Wizard Plus SV minipreps DNA purification kit (Promega, France). Sequencing was performed on both strands using the AbiPrism system (Genoscreen, France).

2.8. Macroscopic measurements

For each treatment, plant biomass analysis was carried out on three of the six replicates. Rosette diameter was measured upon forming of the floral bud. At the end of plant cycle (approximately two months after transfer of the seedlings to the microcosms), fresh weight and maximal length of floral stems and roots were determined. Roots were washed to remove soil particles. For each plant, the number of bolts and mature siliques, the mass of total seed production and the weight of 1000 seeds were determined. Clean vegetative organs were dried for two days at 70 °C and weighed. Carbon and nitrogen contents (C/N ratios) were determined using a CHN elemental analyzer (Thermo Finnigan Flash EA1112) in roots, leaves, bolts and seeds, separately.

Root biomass distribution between diameter classes was established according to the method of Blouin et al. (2007) on dried root systems. Briefly, shredded dry roots were sieved on a column of sieves with decreasing mesh sizes; biomass distribution according to root diameter was assessed by weighing the biomass recovered in each sieve (Blouin et al., 2007).

2.9. Soil analyses

Soil nitrate and ammonium contents were determined by KCL extraction and spectrocolorimetry at the INRA "Laboratoire d'Analyse des Sols" in Arras (France). For each treatment, approximatively 50 g of soil were taken from three separate microcosms and used for analysis.

2.10. Statistical analysis

Analyses were performed using the SAS software (SAS, 1989). Output variables (plant growth parameters and soil nitrogen contents) were analyzed using a two-way ANOVA testing for soil and earthworm effects and the interaction between these two factors. To determine the direction of significant effects and the combinations of treatment and soil responsible for these effects, multiple comparisons of Least Square Means (SAS, 1990) were made. LSMEANS differences are summed-up in the Figures, with letters indicating significant differences between treatments.

3. Results

ANOVA for all vegetative and reproductive parameters (except for the parameter 1000 seed weight) showed that over 80% of result variability was explained by the statistical model (soil type, earthworm presence, interactions between these factors). This suggested that the experimental conditions were efficiently controlled.

3.1. Earthworm vitality in the microcosms

The earthworms spend three months in the microcosms. At the end of the experiment, earthworms from the different microcosms were carefully collected and weighed together. In the rich soil, earthworm biomass had increased by 20% (n=6, SD=3.13) whereas it showed a 10% ($n = 6$, SD = 4.02) decrease in the poor soil. No death was recorded. Moreover, earthworm activity appeared to be superior in the poor soil than in the rich one: in the former, the surfaces of the microcosms were completely covered with casts, whereas fewer casts were observed on top of the rich soil.

3.2. Effects of earthworms and soil quality on plant vegetative growth

Earthworms and soil type had significant impact on all plant vegetative growth parameters and several significant soil × earthworms interactions were observed (Table 2). For example, earthworms induced a three-fold increase in rosette diameter in the poor soil whereas they had no significant effect on this parameter, in the rich soil (Fig. 1a; Table 2). As a result, rosette diameters were equivalent in the rich soil and in the poor soil with the earthworms. Furthermore, the leaves of the plants grown in the poor soil without earthworms were purple and scrawny, whereas in the presence of earthworms, they were bright green and well developed (Fig. 2), as were the leaves of the rich soil plants.

Significant soil × earthworm interaction was also observed for root dry biomass. This parameter was reduced in both soils in the presence of the earthworms. However, this was statistically significant in the poor soil only (Fig. 1b; Table 2). Generally, very low root biomasses were recorded in the rich soil (Fig. 1b). Regardless of soil type, earthworms induced a significant reduction (>50%) in the maximal length of root systems (Fig. 1c, Table 2).

3.3. Effect of earthworms and soil quality on plant reproductive parameters

In the poor soil, earthworms delayed the forming of floral buds since inflorescences emerged after 21 days of growth in their presence whereas plants growing without earthworms formed floral buds after 15 days. In the rich soil, plants growing with and

Table 2
Earthworms and soil effects on reproductive and vegetative parameters (*P*-values from a two-way ANOVA).

	df	Rosette diameter	Root system maximal length	Root dry biomass
R^2		0.97	0.98	0.97
Soil	1	<0.0001	0.0017	<0.0001
Earthworm	1	<0.0001	<0.0001	0.0014
Soil × Earthworm	1	<0.0001	0.7430	0.0030

	df	Maximum bolt length	Above ground biomass	Total seed production	Number of silliques	Weight of 1000 seeds	Number of bolts par plant
R^2		0.98	0.92	0.85	0.94	0.15	0.93
Soil	1	<0.0001	<0.0001	0.0075	0.0002	0.5268	0.0011
Earthworm	1	0.0053	0.0002	0.0021	0.0003	0.8661	0.0011
Soil × Earthworm	1	0.0006	0.0022	0.0070	0.0001	0.3581	0.0011

	df	Shoot/Root	Allocation to seeds
R^2		0.66	0.82
Soil	1	0.0109	0.0037
Earthworm	1	0.1631	0.0050
Soil × Earthworm	1	0.1797	0.0566

without earthworms formed their floral buds after 21 days of growth in the microcosms (Fig. 2).

As was the case for most vegetative growth parameters, significant effects of soil × earthworm interaction on reproductive parameters (number of siliques, total seed weight, number of bolts per plant, maximum bolt length, Fig. 3) were found, suggesting that earthworm impact was dependent on soil type (Table 2). In the rich soil, no significant impact was recorded (Table 2). In the poor soil, however, earthworms increased bolt length by 74% (Fig. 3a), aboveground biomass by 68% (Fig. 3b), total seed weight by 136% (Fig. 3c), the number of siliques per plant by 201% (Fig. 3d), and the number of bolts per plant by 100% (Fig. 3e, Table 2). The values found with earthworms in the poor soil were equivalent to those measured in the rich soil, with or without earthworms, except for the average bolt length that remained 33% lower than that of the rich soil plants (Fig. 3a). Neither soil type nor earthworm presence influenced the 1000 seed weight (Fig. 3f, Table 2). As described previously, earthworm-derived benefits on plant productivity were limited to the poor soil.

3.4. Changes in plant resource allocation induced by earthworms

A consequence of the presence of earthworms was a dramatic change in the distribution of biomass within the plants. In the poor soil, earthworms doubled global plant biomass allocation to seeds (Table 2). As a result, this parameter was equivalent in the poor soil with the earthworms and in the rich soil with or without earthworms (Fig. 4a; Table 2).

There was no general effect of earthworms or earthworm × soil interaction on shoot/root ratio (Table 2, Fig. 4b). However, when earthworm effects were tested separately in the two soils, it appeared that they significantly modified plant morphology in the poor soil, as shown by the significant increase in shoot/root ratios (ANOVA, $P < 0.05$). In this soil, earthworms also influenced the

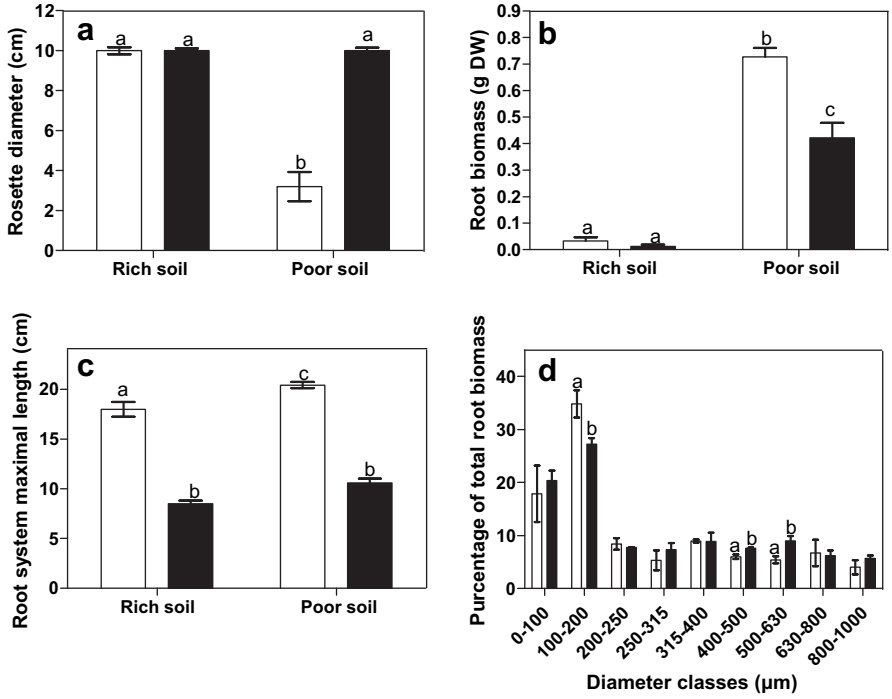

Fig. 1. Changes in (a) rosette diameter, (b) root system biomass, (c) root system maximal length and (d) root biomass distribution between diameter classes in *Arabidopsis thaliana* plants grown in rich or poor soil with (black bars) or without (control; white bars) earthworms *Aporrectodea caliginosa*. Vertical bars indicate ± s.e.m. ($n = 3$). Significant differences between earthworms vs control as evaluated by LSMEANS ($P < 0.05$) are indicated by different letters.

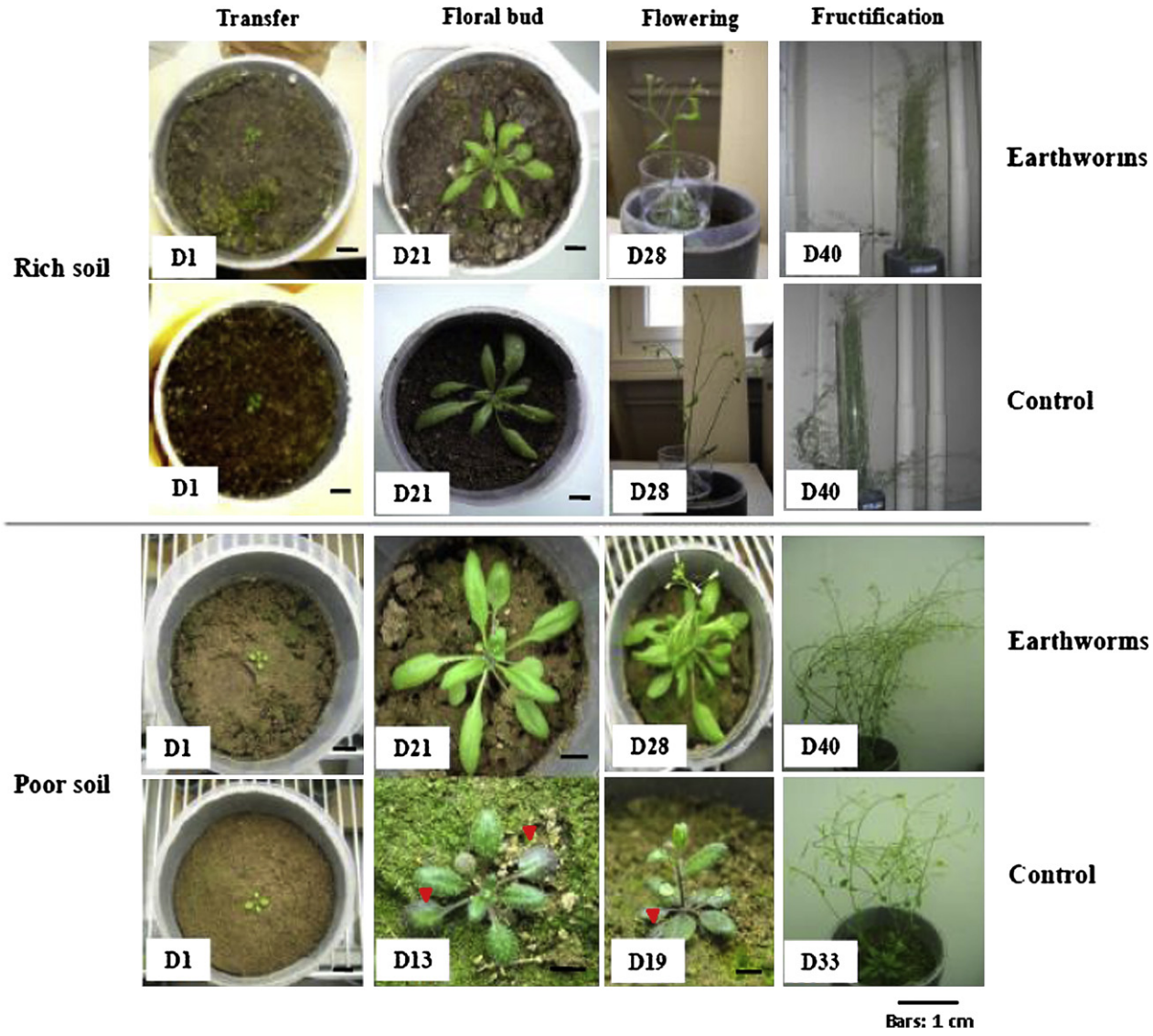

Fig. 2. Phenological plate of *Arabidopsis thaliana* cultivated with or without earthworms in the rich or poor soil. Transfer: day 1 (D1) after transfer of seedlings to the microcosms; Floral bud: development of floral buds, Flowering: flower development, Fructification: silique development. Red arrows indicate stress-related anthocyanin build up in control plants grown on poor soil (D13, D19).

architecture of the root systems. The biomass corresponding to fine roots (100–200 μm in diameter) was significantly reduced in their presence whereas the biomass allocated to larger roots (400–500 μm and 500–630 μm in diameter) was increased (Fig. 1d).

3.5. Effect of earthworms and soil quality on plant C/N ratios

Plant C/N ratios were significantly affected by the earthworms (Table 3). Overall, they were lower in aerial organs but the difference was significant only in the bolt stems as shown by significant organ × treatment interaction (Fig. 5, Table 3).

3.6. Effects of plants and earthworms on soil nitrogen status

Our experimental design did not allow analysis of a full-model testing simultaneously for the effects of earthworms, the presence of a plant and the soil type, on soil nitrogen status (ammonium and nitrogen contents). As a result, soil-specific models testing for the effects of earthworms and plants (but not for their interaction) were analyzed separately (Table 4).

In the rich soil, neither the earthworms nor the plants significantly impacted ammonium or nitrate contents (Table 4). In the poor soil on the other hand, a 4.5-fold increase in nitrate content and a 0.8-fold decrease in ammonium content were observed with the earthworms (Fig. 6, Table 4). Compared with earthworms, plants had opposite impact on nitrogen contents: ammonium concentrations increased (+100%) and nitrate contents were almost depleted (−90%) (Fig. 6).

3.7. Earthworms affect transcript accumulation for target genes in Arabidopsis leaf development

Regardless of soil type, earthworms triggered an over-accumulation of *HBT* (NM_104135) transcripts in the leaves (Fig. 7a and e). Cu/Zn *SOD* transcripts were more abundant in the leaves of poor soil plants than in those growing in the rich soil (Fig. 7a and c). Leaf Cu/Zn *SOD* transcript accumulation decreased slightly in response to earthworms (Fig. 7a and c). Contrasting earthworm effects were observed on leaf *PLDα* steady-state transcript levels: a strong increase and a reduction in the poor and rich soil, respectively

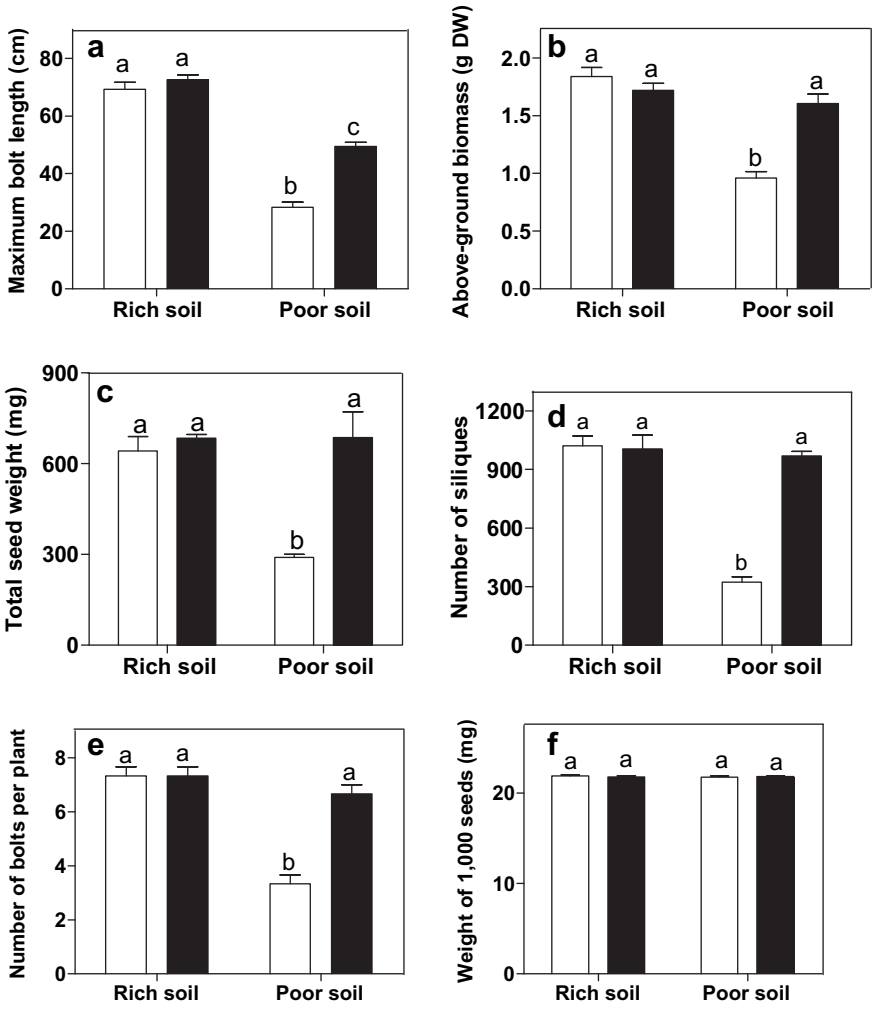

Fig. 3. Changes in (a) maximum bolt length, (b) aboveground biomass, (c) number of siliques per plant, (d) total seed production, (e) number of bolts per plants and (f) 1000 seed weight in *Arabidopsis thaliana* plants grown in rich or poor soil with (black bars) or without (control; white bars) earthworms *Aporrectodea caliginosa*. Vertical bars indicate ± s.e.m. ($n = 3$). Significant differences between earthworms vs control as evaluated by LSMEANS ($P < 0.05$) are indicated by different letters.

(Fig. 7a and b). The reverse situation was observed for *ICK1* leaf transcripts: they were much more abundant in the plants growing in the poor soil and the earthworm treatment reduced their abundance whereas earthworms had a slight boosting effect on *ICK1* transcript accumulation in the rich soil (Fig. 7a and d). Generally, transcript accumulation for the small nuclear-encoded sub-unit of Rubisco (*RbcS*) remained unaffected by the various treatments (Fig. 7a).

3.8. Earthworms affect transcript accumulation for target gene in Arabidopsis root tissues

As was observed in the leaves, transcriptional influence of earthworms was gene specific in root tissues. They induced an over-accumulation of *PLDα* transcripts (Figs. 7a and 5b). This was more pronounced in the poor soil. Earthworms induced a strong decrease in the accumulation of *ICK1* transcripts in the rich soil

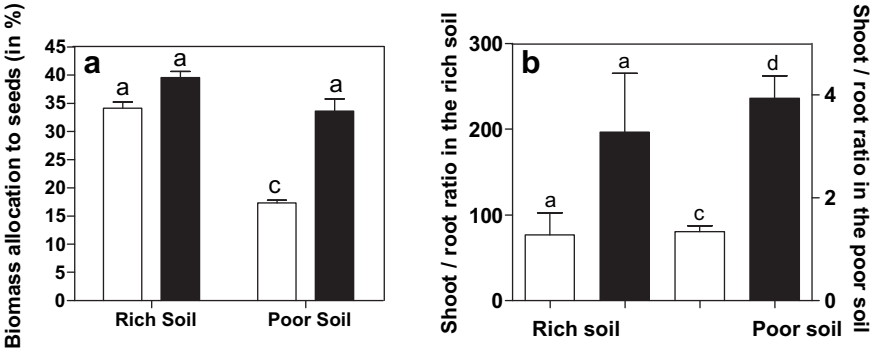

Fig. 4. Changes in (a) allocation to seeds and (b) shoot : root ratio in *Arabidopsis thaliana* plants grown in rich or poor soil with (black bars) or without (control; white bars) earthworms *Aporrectodea caliginosa*. Vertical bars indicate ± s.e.m. ($n = 3$). Significant differences between earthworms vs control as evaluated by LSMEANS ($P < 0.05$) are indicated by different letters.

Table 3
ANOVA for C/N values. Interactions between soil, earthworms and organ.

Source	DF	F	P > F
Earthworm	1	9.15	0.0045
Organ	3	40.02	<0.0001
Soil	1	0.40	0.5333
Soil × Earthworm	1	0.02	0.8859
Organ × Earthworm	3	4.88	0.0058
Organ × Soil	3	3.23	0.0330
Organ × Earthworm × Soil	3	0.16	0.9226

(Fig. 7a and d). Cu/Zn *SOD* transcript levels were not influenced by their presence (Fig. 7a and c). However, *SOD* transcript accumulation was higher in the roots of poor soil plants than in those growing in the rich soil, as was observed in the leaves.

4. Discussion

4.1. In the poor soil without earthworms, Arabidopsis plants exhibit mineral deficiency characteristics

Compared to those growing in the rich soil, the plants cultivated in the poor soil without earthworms showed phenotypic and developmental responses typical of mineral -particularly nitrogen (N)-deficiency: early reproductive switch, smaller shoot to root ratio, as a result of a higher allocation of assimilates to the roots and severely reduced seed yield (Eaton, 1935; Scheible et al., 1997; Hirai et al., 2004; Hermans et al., 2006; Mantelin et al., 2006; Remans et al., 2006). The almost complete depletion of the initial NO_3^- content in the poor soil by the end of the experiment support the hypothesis of N-starvation. In comparison, the growth conditions in the rich soil could be considered as near optimum. This new experimental system, using both a rich and a poor soil, led to the development of contrasted plant phenotypes that should facilitate the identification of earthworm effects in relation with soil quality.

4.2. Earthworms can reverse most effects of poor soil quality on Arabidopsis growth and development

All mineral/nitrogen deficiency symptoms observed in the plants growing in the poor soil without earthworm were absent in their counterparts cultivated in the presence of earthworms. As a result, vegetative biomass and seed production with the earthworms were equivalent in the poor and rich soil plants. Rosette diameter was significantly increased and comparable to that of the rich soil plants. *ICK1* and *PLDα* gene expression analyses suggested that this was mediated through enhanced cell proliferation in the leaf tissues.

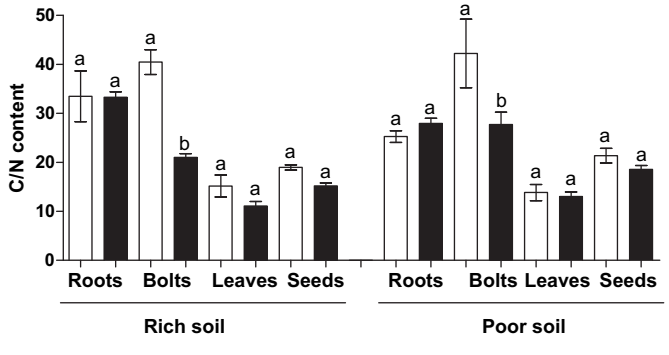

Fig. 5. Changes in C/N ratios in the roots, bolt stems, leaves and seeds of in *Arabidopsis thaliana* plants grown in rich or poor soil with (black bars) or without (control; white bars) earthworms *Aporrectodea caliginosa*. Vertical bars indicate ± s.e.m. ($n = 3$). Significant differences due to earthworm effects on each organ and each soil as evaluated by LSMEANS ($P < 0.05$) are indicated by different letters.

Table 4
ANOVA for nitrate and ammonium contents in the rich and the poor soil (RS and PS respectively). The total degrees of freedom is 11 for the rich soil and 8 for the poor soil.

	df	Nitrate		Ammonium	
Soil		RS	PS	RS	PS
R^2		0.28	0.82	0.08	0.95
Earthworm	1	0.0335	<0.001	0.7812	<0.001
Plant	1	0.1040	0.0473	0.4872	0.0294

The dramatic increase in the nitrate content of the poor soil with earthworms was most likely a determining factor in the comparatively dramatic increase in *Arabidopsis* biomass production. In addition, earthworm-induced high nitrate concentrations were probably responsible for the significant reduction in the number of fine roots observed in the poor soil plants, as was previously reported (Zhang and Forde, 2000; Remans et al., 2006). Since decaying earthworms were not a possible source of additional N (worms survival rates were 100%), the majority of the extra N was probably formed through mineralization of soil organic matter by gut-associated and cast-associated micro-organisms, in accordance with Brown's hypothesis that earthworms mostly increase plant growth through N mineralization (Brown et al., 1999). However, recent evidence have started an ongoing and fascinating debate of whether enhanced mineralization alone provides a full explanation for the growth stimulation (Brown et al., 2004; Scheu, 2003; Blouin et al., 2006).

4.3. Some effects of earthworms on Arabidopsis are independent of soil quality

An effect of earthworms similarly observed in both soils was the decrease in C/N ratios of the aboveground tissues (particularly in bolt stems). Elevated nitrate contents have a negative effect on the

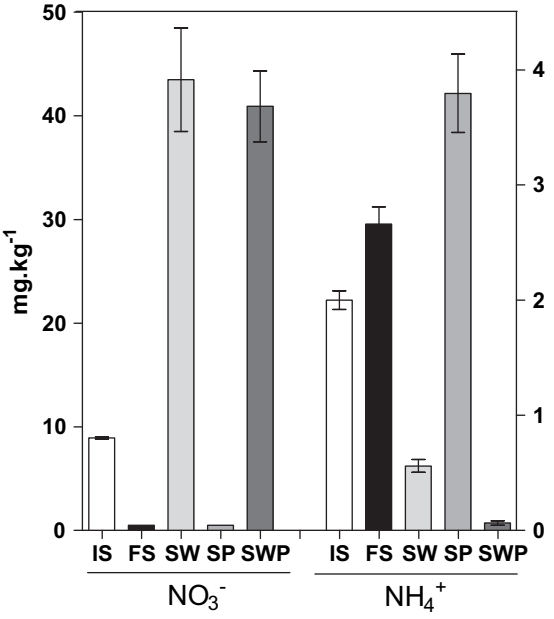

Fig. 6. Changes in (a) nitrate (mg kg^{-1}) and (b) ammonium (mg kg^{-1}) contents in the poor soil (IS) at the end of the experiment (FS) induced by the presence of earthworms (*Aporrectodea caliginosa*) (SW), the presence of *Arabidopsis thaliana* plants (SP), and the combination of both (SPW). Vertical bars indicate ± s.e.m. ($n = 3$).

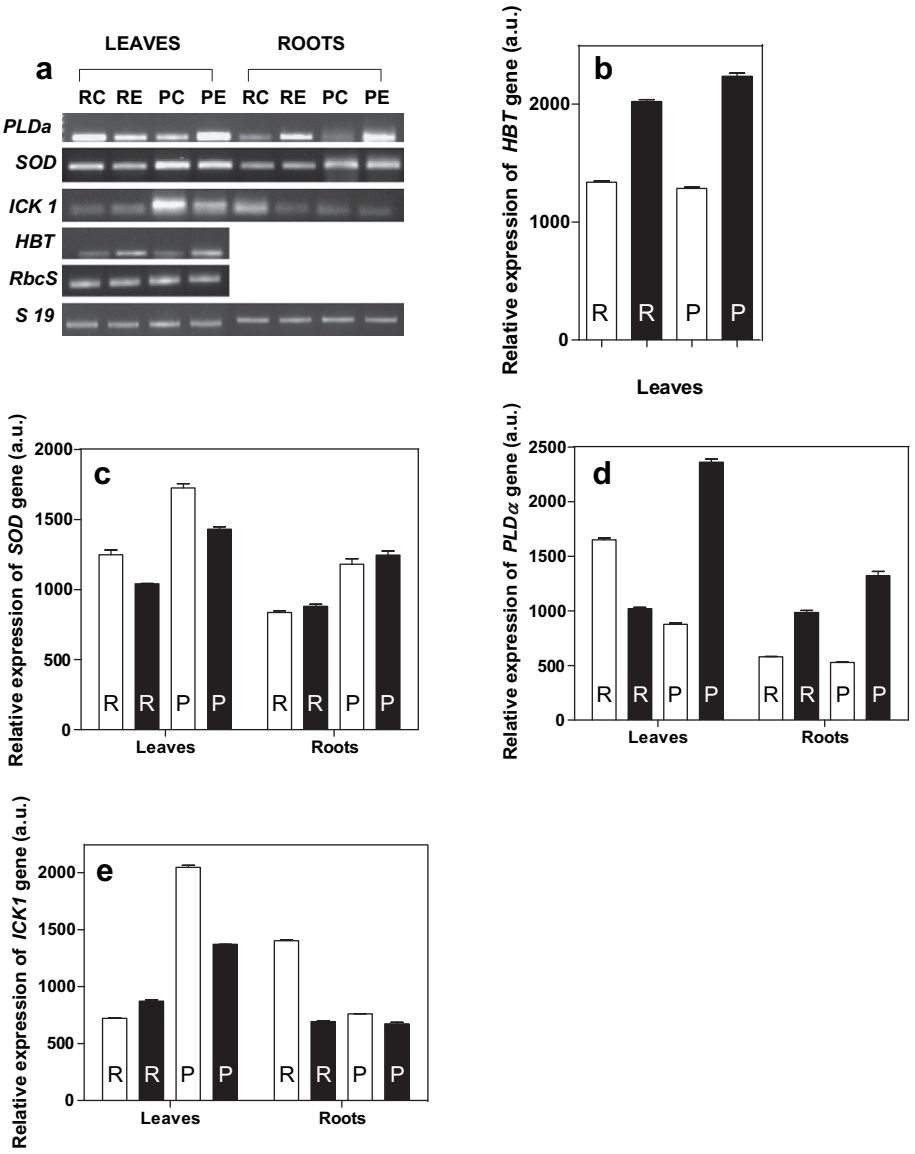

Fig. 7. RT-PCR analysis of (a and b) *HBT*, (a and c) Cu/Zn *SOD*, (a and d) *PLDα* and (a and e) *ICK1* gene expression in the leaves and roots of *Arabidopsis thaliana* plants grown in rich (R) or poor (S) soil with (black bars) or without (control; white bars) earthworms *Aporrectodea caliginosa*. The S19 gene was used as an amplification control. Relative gene expression (arbitrary units, a.u.) was determined using the Quantity One programme (Bio-Rad).

transport of shoot-derived auxin to roots and, as a consequence, alter auxin metabolism in shoot tissues (Caba et al., 2000; Walch-Liu et al., 2006). In the present experiment, leaf auxin metabolism seemed affected, as shown by the over-accumulation of *HBT* transcripts in the leaves of *Arabidopsis* plants exposed to earthworms. At the same time, the change in plant nitrogen status alleviated the oxidative stress in the leaves, as indicated by the general reduction in leaf transcript accumulation for a *Cu/Zn SOD*.

In the poor soil, the decrease in C/N ratio could easily be ascribed to the stimulation by earthworms of organic matter mineralization – as mentioned before – and enhanced nitrification processes (Rizhiya et al., 2007). In the rich soil, on the other hand, the nitrate content was not significantly affected. To understand how apparently N-replete plants, such as the rich soil plants, absorbed additional N in the presence of earthworms, one should refer to the work by Quaggiotti et al. (2004). These authors observed a decrease in leaf C/N resulting from enhanced nitrate influx in N-fed maize plantlets exposed to purified extracts of earthworm casts. They concluded that this phenomenon was triggered by the auxin-like compounds (with auxin-like activity) found in the earthworm casts (Tomati et al., 1988). These results and ours show that, in the presence of earthworms, control over mineral/nitrogen nutrition is not necessarily dependant on the plant mineral status, as is the case with inducible uptake systems that respond to mineral deficiency (Chrispeels et al., 1999).

Other general effects of earthworms were a reduction in root maximum length and in global root biomass, leading to higher shoot/root ratios. In the confined space of the microcosms, repeated root abrasion by earthworms leading to wounding stress could contribute to the limiting of root development. The high levels of *PLDα* transcripts as compared to no-worm controls suggested that such stress occurred since this gene is responsive to wounding in *Arabidopsis* (Wang, 2002). However, the lack of root expansion could be a result of the earthworm-induced enhanced auxin supply at the root level, since it is acknowledged that root tissues are sink organs for auxin and rapidly stop elongating when exposed to increasing concentrations of the hormone (Chadwick and Burg, 1966).

5. Conclusion

This new experimental set up confirms that organic matter mineralization and release of phytohormone-like compounds are complementary mechanisms stimulated by earthworms. The fact that plants are able to integrate both processes at the molecular level points at the enormous potential of earthworms in adjusting plant phenotypes in response to environmental stresses. In addition, this original model plant system opens fascinating new possibilities for follow-up investigation in the area of plant/earthworm interaction. Its small size constitutes a great asset for the quick and easy screening of candidate microbes with plant growth enhancing capabilities. The unique diversity in *Arabidopsis* mutants (in nitrate uptake and hormonal signaling, for example) offers key-tools for the identification of active ingredient(s) responsible for molecular and phenotypic adjustments. To conclude, this experimental set up could be viewed as an essential model system to investigate plant/macrofauna and plant/microfauna interactions in particular and soil ecology in general.

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References

Barois, I., Lavelle, P., Brossard, M., Tondoh, J., Martinez, M.A., Rossi, J.P., Senapati, B.K., Angeles, A., Fragoso, C., Jimenez, J.J., Decaëns, T., Lattaud, C., Kanyonyo, J., Blanchart, E., Chapuis, L., Brown, G., Moreno, A., 1999. Ecology of earthworm species with large environmental tolerance and/or extended distributions. In: Lavelle, P., Brussaard, L., Hendrix, P. (Eds.), Earthworm Management in Tropical Agroecosystems. CABI Publishing, Wallingford, UK, pp. 57–86.

Bemis, S.M., Torii, K.U., 2007. Autonomy of cell proliferation and developmental programs during *Arabidopsis* aboveground morphogenesis. Developmental Biology 304, 367–381.

Blanchart, E., Albrecht, A., Alegre, J., Duboisset, A., Pashanasi, B., Lavelle, P., Brussaard, L., 1999. Effects of earthworms on soil structure and physical properties. In: Lavelle, P., Brussaard, L., Hendrix, P. (Eds.), Earthworm Management in Tropical. CAB International, Wallingford, UK, pp. 139–162.

Blilou, I., Frugier, F., Folmer, S., Serralbo, O., Willemsen, V., Wolkenfelt, H., Eloy, N.B., Ferreira, P.C., Weisbeek, P., Scheres, B., 2002. The *Arabidopsis* HOBBIT gene encodes a CDC27 homolog that links the plant cell cycle to progression of cell differentiation. Genes Development 16, 2566–2575.

Blouin, M., Barot, S., Roumet, C., 2007. A quick method to determine root biomass distribution in diameter classes. Plant and Soil 290, 371–381.

Blouin, M., Barot, S., Lavelle, P., 2006. Earthworms (*Millsonia anomala*, Megascolecidae) do not increase rice growthy through enhanced nitrogen mineralization. Soil Biology and Biochemistry 38, 2063–2068.

Blouin, M., Zuily-Fodil, Y., Pham-Thi, A.T., Laffray, D., Reversat, G., Pando, A., Tondoh, J., Lavelle, P., 2005. Belowground organism activities affect plant aboveground phenotype, inducing plant tolerance to parasites. Ecology Letters 8, 202–208.

Brown, G., Edwards, C.A., Brussaard, L., 2004. How earthworms affect plant growth: burrowing into the mechanisms. In: Edwards, C.A. (Ed.), Earthworm Ecology. CRC Press, Boca Raton, USA, pp. 13–49.

Brown, G., 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 26, 177–198.

Brown, G., Pashanasi, B., Villenave, C., Patron, J.C., Senapati, B.K., Giri, S., Barois, I., Lavelle, P., Blanchart, E., Blakemore, R.J., Spain, A.V., Boyer, J., 1999. Effects of earthworms on plant production in the tropics. In: Lavelle, P., Brussaard, L., Hendrix, P. (Eds.), Earthworm Management in Tropical Agroecosystems, Wallingford, pp. 87–137.

Caba, J.M., Centeno, M.L., Fernandez, B., Gresshoff, P.M., Ligero, F., 2000. Inoculation and nitrate alter phytohormone levels in soybean roots: differences between a supernodulating mutant and the wild type. Planta 211, 98–104.

Canellas, L.P., Olivares, F.L., Okorokova-Facanha, A.L., Facanha, A.R., 2002. Humic acids isolated from earthworm compost enhance root elongation, lateral root emergence, and plasma membrane H^+-ATPase activity in maize roots. Plant Physiology 130, 1951–1957.

Chadwick, A.V., Burg, S.P., 1966. An explanation of the inhibition of root growth caused by Indole-3-Acetic Acid. Plant Physiology 42, 415–420.

Chrispeels, M., Crawford, N., Schroeder, J., 1999. Proteins for transport of water and mineral nutrients across the membranes of plant cells. The Plant Cell 11, 661–675.

Clapperton, M.J., Lee, N.O., Binet, F., Conner, R.L., 2001. Earthworms indirectly reduce the effects of take-all (*Gaeumannomyces graminis* var. tritici) on soft white spring wheat (*Triticum aestivum* cv Fielder). Soil Biology and Biochemistry 33, 1531–1538.

Eaton, S.V., 1935. The nature and properties of soils. Botanical Gazette 97, 68–100.

Francis, D., 2007. The plant cell cycle -15 years on. New Phytologist 174, 261–278.

Hermans, C., Hammond, J.P., White, P.J., Verbruggen, N., 2006. How do plants respond to nutrient shortage by biomass allocation? Trends in Plant Science 11, 610–617.

Hirai, M.Y., Yano, M., Goodenowe, D.B., Kanaya, S., Kimura, T., Awazuhara, M., Arita, M., Fujiwara, T., Saito, K., 2004. Integration of transcriptomics and metabolomics for understanding of global responses to nutritional stresses in *Arabidopsis thaliana*. Proceedings of the National Academy of Science of the United States of America 101, 10205–10210.

Kaminaka, H., Morita, S., Tokumoto, M., Yokoyama, H., Masumura, T., Tanaka, K., 1999. Molecular cloning and characterization of a cDNA for an iron-superoxide dismutase in rice (*Oryza sativa* L.). Biosciences Biotechnology Biochemistry 63, 302–308.

Krishnamoorthy, R.V., Vajranabhaiah, S.N., 1986. Biological activity of earthworm casts: an assessment of plant growth promotor levels in casts. Proceedings of the Indian Academy of Sciences (Animal Science) 95, 341–351.

Lafont, A., Risede, J.M., Loranyer-Merciris, G., Clermont-Dauphin, C., Dorel, M., Rhino, B., Lavelle, P., 2007. Effects of the earthworm *Pontoscolex corethrurus* on banana plants infected or not with the plant-parasitic nematode *Radopholus similis*. Pedobiologia 51, 311–318.

Lee, K.E., 1985. Earthworms, Their Ecology and Relationships with Soils and Land Use. Academic Press, Sydney, Australia.

Mantelin, S., Desbrosses, G., Larcher, M., Tranbarger, T.J., Cleyet-Marel, J.C., Touraine, B., 2006. Nitrate-dependent control of root architecture and N nutrition are altered by a plant growth promoting *Phyllobacterium sp*. Planta 223, 591–603.

Muscolo, A., Cutrupi, S., Nardi, S., 1998. IAA detection in humic substances. Soil Biology and Biochemistry 30, 1199–1201.

Muscolo, A., Bovalo, F., Nardi, S., 1999. Earthworm humic matter produces auxin-like effects on *Daucus carota* cell growth and nitrate metabolism. Soil Biology and Biochemistry 31, 1303–1311.

Nielsen, T.H., Krapp, A., Röper-Schwarz, U., Stitt, M., 1998. The sugar mediated regulation of genes encoding the small subunit of Rubisco and the regulatory subunit of ADP glucose pyrophosphorylase is modified by phosphate and nitrogen. Plant, Cell and Environment 21, 443–454.

Postma-Blaauw, M.B., Bloem, J., Faber, J.H., van Groenigen, J.W., de Goede, R.G.M., Brussaard, L., 2006. Earthworm species composition affects the soil bacterial community and net nitrogen mineralization. Pedobiologia 50, 243–256.

Quaggiotti, S., Ruperti, B., Pizzeghello, D., Francioso, O., Tugnoli, V., Nardi, S., 2004. Effect of low molecular size humic substances on nitrate uptake and expression of genes involved in nitrate transport in maize (*Zea mays* L). Journal of Experimental Botany 55, 1–11.

Remans, T., Nacry, P., Pervent, M., Girin, T., Tillard, P., Lepetit, M., Gojon, A., 2006. A central role for the nitrate transporter NRT2.1 in the integrated morphological and physiological responses of the root system to nitrogen limitation in *Arabidopsis*. Plant Physiology 140, 909–921.

Rizhiya, E., Bertora, C., van Vliet, P.C.J., Kuikman, P.J., Faber, J.H., van Groeningen, J.W., 2007. Earthworm activity as a determinant for NO_2 emission from crop residue. Soil Biology and Biochemistry 39, 2058–2069.

Sakamoto, A., Okumura, T., Kaminaka, H., Sumi, K., Tanaka, K., 1995. Structure and differential response to abscisic acid of two promoters for the cytosolic copper/zinc- superoxide dismutase genes, SodCcl and SodCc2, in rice protoplasts. FEBS Letters 358, 62–66.

SAS, 1989. SAS/STAT User's Guide, Version 6, fourth ed. SAS Institute, Cary.

SAS, 1990. GLM procedure. In: SAS/GRAPH Software, Version 6, vol. 2. SAS Institute Inc., Cary, USA.

Scheible, W.R., Gonzales-Fontes, A., Lauerer, M., Müller-Röber, B., Caboche, M., Stitt, M., 1997. Nitrate acts as a signal to induce organic acid metabolism and repress starch metabolism in tobacco. Plant Cell 9, 783–798.

Scheu, S., 2003. Effects of earthworms on plant growth: patterns and perspectives: the 7th international symposium on earthworm ecology Cardiff Wales 2002. Pedobiologia 47, 846–856.

Senapati, B.K., 1992. Biotic interactions between soil nematodes and earthworms. Soil Biology and Biochemistry 24, 1441–1444.

Tomati, U., Grappelli, A., Galli, E., 1988. The hormone-like effect of earthworm casts on plant growth. Biology and Fertility of Soils 5, 288–294.

Walch-Liu, P., Ivanov, I.I., Filleur, S., Gan, Y., Remans, T., Forde, B.G., 2006. Nitrogen regulation of root branching. Annals of Botany 97, 875–881.

Wang, H., Qi, Q., Schorr, P., Cutler, A.J., Crosby, W.L., Fowke, L.C., 1998. ICK1, a cyclin-dependent protein kinase inhibitor from *Arabidopsis thaliana* interacts with both Cdc2a and CycD3, and its expression is induced by abscisic acid. Plant Journal 15, 501–510.

Wang, X., 2002. Phospholipase D in hormonal and stress signaling. Current Opinion in Plant Biology 5, 408–414.

Xu, L., Zheng, S., Zheng, L., Wang, X., 1997. Promoter analysis and expression of a phospholipase D gene from castor bean. Plant Physiology 115, 387–395.

Yeates, G.W., 1981. Soil nematode populations depressed in the presence of earthworms. Pedobiologia 22, 191–195.

Zhang, H., Forde, B.G., 2000. Regulation of *Arabidopsis* root development by nitrate availability. Journal of Experimental Botany 51, 51–59.

Zou, X., Gonzalez, G., 1996. Changes in earthworm density and community structure during secondary succession in abandoned tropical pastures. Soil Biology and Biochemistry 29, 627–629.