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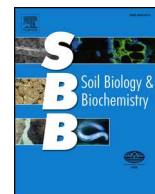
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## New approaches using mass spectrometry to investigate changes to cytokinin and abscisic acid (ABA) concentrations in soil



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### ABSTRACT

Phytohormones such as cytokinins, abscisic acid (ABA) and auxins play a vital role in plant development and regulatory processes. Their role within the plant is a focus for much research, with studies using recent advances in mass spectrometry performance allowing the quantification of low levels of phytohormones extracted from plant tissues. Despite these advances, external factors influencing the production of phytohormones are less well studied. Here, a new approach is presented for the extraction of a range of phytohormones from plant growth media (soil and hydroponic solution), their identification using high mass accuracy mass spectrometry and subsequent quantification using multiple reaction monitoring (MRM). The ability to detect phytohormones in matrices other than plant tissue presents the opportunity to study further the influence of factors such as below ground organisms and soil bacteria on phytohormone production.

This novel approach was therefore applied to the plant growth media from a series of experiments comparing plant growth in the presence and absence of earthworms. A small but significant increase in ABA concentration was observed in the presence of earthworms, increasing even further when plants were also present. This finding suggests that earthworms could stimulate plant ABA production. This experiment and its outcomes demonstrate the value of studying phytohormones outside plant tissue, and the potential value of further research in this area.

### 1. Introduction

Phytohormones are a wide range of compounds including cytokinins, abscisic acid (ABA), auxins, gibberellins and a number of other biologically active acids that play fundamental roles in plant development (reviewed by Shan et al., 2012 and Schaller et al., 2015). These compounds often mediate antagonistic or co-acting processes, which include regulatory processes such as cell division, cell enlargement and tissue expansion, associated with increased plant biomass (Hanano et al., 2006). Phytohormones such as cytokinins and ABA are ubiquitous throughout plant species and tissues and investigating their role remains an enormous research area (e.g. Werner et al., 2001; Hanano et al., 2006). However, despite extensive characterisation, phytohormone roles are still not fully understood. Beyond the *in planta* mode of phytohormone action, external factors influencing their production are currently an under-researched area. The ability of plant growth-promoting rhizo- and endophytic bacteria to elicit or mediate

production of these compounds is understood to be a major factor affecting plant growth (Brown, 1972; Santoyo et al., 2016), as are environmental stresses such as drought (Arkhipova et al., 2007), salinity (Cao et al., 2016) or toxic metal contamination (Barceló and Poschenrieder, 1990).

Recently, advances in sensitivity and selectivity of state-of-the-art mass spectrometric techniques have allowed the identification and quantification of a wide range of phytohormones including cytokinins, IAA and ABA in plant tissues when combined with separation by gas or liquid chromatography (Pan et al., 2010; Farrow and Emery, 2012). Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) is an MS technique which generates high mass accuracy (1–2 ppm) data from all ionisable compounds in a sample. This high mass accuracy means that the chemical composition of individual compounds can be identified based solely on their mass. Furthermore, the high resolution of the technique means that signals from compounds of similar mass do not overlap, and it is therefore possible to analyse a complex mixture

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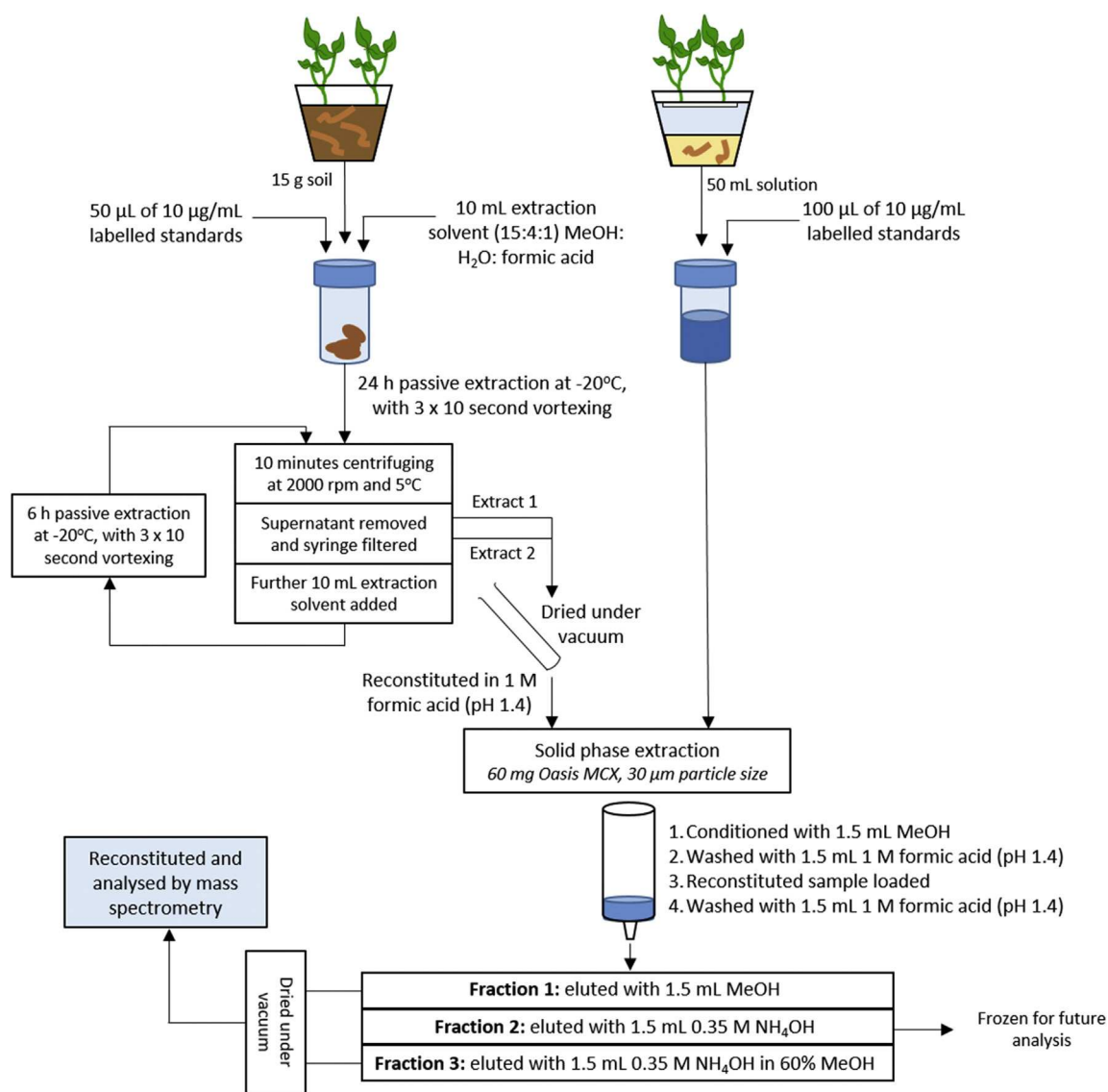


Fig. 1. Schematic showing the experimental set up, extraction, and purification methods for both soil and hydroponic solutions.

without prior separation. Used with electrospray ionisation (which ionises with minimal fragmentation of compounds), FT-ICR-MS is therefore a powerful technique for the rapid detection of a large range of unknown analytes. Multiple reaction monitoring (MRM) is an MS method typically carried out using a triple quadrupole instrument with prior separation by liquid chromatography. MRM is a tandem technique, where a specific ion is targeted in the first quadrupole, and then specific transitions (the fragmentation of selected precursor ions to specific product ions) are recorded in the second and third quadrupoles. This approach produces data with high specificity, low background, and very low limits of detection and quantification; it is thus ideally suited to quantification. Conducting internal standardisation using isotopically labelled standards at known concentrations allows high accuracy of quantification. The use of a combination of these mass spectrometric techniques offers substantial improvement in terms of sensitivity, accuracy and speed of analysis compared to traditional methods for analysing phytohormones, such as enzyme-, radioimmuno- or gas chromatography-MS-based assays (Ma et al., 2008; Farrow and Emery, 2012).

These methodological advances have opened up new routes to understanding the roles that phytohormones play within plant tissues and how these respond to factors such as stress (e.g. Cao et al., 2016).

However, the direct identification and quantification of phytohormones in matrices other than plant tissue, such as soils and other plant-growth media, has thus far been neglected, partly due to the difficulties in isolating these compounds from a complex matrix (Puga-Freitas et al., 2012). Another factor known to influence plant growth is the presence and behaviour of below-ground organisms; in particular, Scheu (2003) summarised that earthworms increase plant biomass in the majority (70–80%) of reported studies relating to this phenomenon. The possible mechanisms by which they do this include modifications to rhizobacterial populations, alteration of soil structure, changing nutrient availability (particularly nitrogen), or by ingestion-induced ‘priming’ of seed germination (Blouin et al., 2013; Xiao et al., 2017). However, there is also some evidence that humic substances extracted from earthworm faeces exhibit auxin or cytokinin-like activity (for example influencing signalling pathways and plant metabolism), indicating a link between the presence of earthworms and the synthesis of these compounds (Pizzeghello et al., 2013). In particular, the auxin indole-3-acetic acid (IAA) has been isolated from earthworm compost (Muscolo et al., 1998). Controlled studies further suggest that earthworms influence plant growth via modification or production of phytohormones either directly, or by stimulating the bacterial populations that produce them (Puga-Freitas et al., 2012).

Building upon existing extraction and analytical methods commonly used to quantify phytohormones in plant materials (Pan et al., 2010; Farrow and Emery, 2012), new methodologies have been developed by which a range of compounds can be extracted, identified and quantified directly from plant growth media. To demonstrate the application of these methodologies, a series of laboratory-based experiments was carried out aimed at testing the hypothesis that the presence of earthworms alters the concentrations of phytohormones present in the immediate growth environment, building on evidence from studies such as those by Muscolo et al. (1998) and Puga-Freitas et al. (2012). Experiments compared the effects of the presence of plants, earthworms, and a combination of the two on the concentrations of a range of phytohormones, first in hydroponic solutions to control for the presence of existing phytohormones, and to provide a relatively simple matrix for extraction and analysis. The studies were then repeated in soil to provide a more realistic system and to test whether extraction is possible from more complicated matrices. Finally, to further investigate the mechanisms by which earthworm-driven modification of the phytohormone concentrations may occur, small scale molecular biological studies were carried out to investigate whether changes in the expression of genes known to be involved in phytohormone biosynthesis could be detected in the plants from the hydroponic experiments.

## 2. Materials and methods

### 2.1. Sample preparation and purification

The analytical procedure for both soil and hydroponic experiments is summarised in Fig. 1. The complexity of the soil matrix demanded appropriate extraction conditions to first be determined. Extraction from hydroponic solution was anticipated to be relatively straightforward. All reagents were analytical grade (Sigma) unless otherwise stated.

#### 2.1.1. Preparation of soil samples

Phytohormone extraction was based on reported methods for the extraction of compounds from plant tissue (e.g. Farrow and Emery, 2012; Novák et al., 2008). However, as extraction of phytohormones from soil had not been previously reported, appropriate extraction conditions were first tested. Two 5–6 g samples of soil were spiked with 100 µL of a mixture of authentic standards containing 10 µg/mL each of: zeatin (Z), isopentenyladenosine (iP), indole-3-acetic acid (IAA), abscisic acid (ABA) and adenosine (Ade). Two further unspiked samples were extracted in parallel, so that the natural, or background, phytohormone content could be accounted for. Samples were first extracted using a 15: 4: 1 (v: v: v) mixture of methanol: water: formic acid (according to Farrow and Emery, 2012) for 4 h at –20 °C, after which the solvent was removed. The pellet was then re-extracted with another addition of extraction solution and the process repeated for a further 18 h; a further 24 h; and a further 72 h, providing a total of four time points over a 5-day extraction period. For each timepoint the solvent was dried and the five spiked compounds quantified by MRM (Section 2.2.2). Analysis of each sample indicated that quantifiable levels of each of the five compounds were removed after 24 h, even from the unspiked samples. As prolonged extraction could cause the degradation of some compounds (Pan et al., 2010), a 24 h extraction time was therefore adopted for all experimental samples as summarised in Fig. 1: samples were first spiked with 50 µL of a solution of a mixture containing 10 µg/mL each of the isotopically labelled standards <sup>2</sup>H<sub>6</sub> N<sub>6</sub>-isopentenyladenine, 6-<sup>15</sup>N trans-zeatin, <sup>13</sup>C<sub>6</sub> indole-3-acetic acid, <sup>2</sup>H<sub>6</sub> trans-abscisic acid (all OlChem Lm, Czech Republic) and <sup>2</sup>H<sub>6</sub> adenosine (QMX Laboratories Ltd, UK) to allow internal standardisation. Passive extraction was then carried out for 24 h at –20 °C using 10 mL of a 15: 4: 1 (v: v: v) mixture of methanol: water: formic acid pre-cooled to –20 °C, vortexing for 10 s three times over this period (at approximately 4, 18 and 22 h). After 24 h, the samples were centrifuged at 5 °C

for 10 min at 2000 rpm, the supernatant removed, and syringe filtered using 0.2 µm PVDF filters (17 mm diameter, Phenomenex). A further 10 mL of the pre-cooled extraction solution was then added to the pellet, vortexed and extracted for a further 6 h before repeating the centrifuge and filtering steps, and pooling the supernatants. The resultant solution was dried under vacuum.

#### 2.1.2. Preparation of hydroponic solutions

Recovery of authentic standards from the hydroponic solutions proved straightforward, and therefore no extraction solvent was required. To allow internal calibration, 50 mL aliquots of the hydroponic solutions were spiked with 100 µL of the 10 µg/mL solution containing isotopically labelled phytohormones (Section 2.1.1; Fig. 1).

#### 2.1.3. Solid phase extraction

Due to the complexity of the soil matrix, sample clean-up was required before analysis of the extracted phytohormones by mass spectrometry. Several studies demonstrate the benefits of using mixed mode cation exchange (MCX) cartridges for the analysis of phytohormones (Novák et al., 2008; Dobrev et al., 2005). The use of MCX cartridges also allows separation of basic and acidic compounds so that different MS ionisation modes can be used, which is beneficial if large numbers of compounds are to be analysed. Therefore, the MCX methods described by Dobrev et al. (2005) were adopted as summarised in Fig. 1 for both the soil extracts and the hydroponic samples: 60 mg, 3 cc Oasis MCX SPE cartridges with a 30 µm particle size (Waters) were conditioned with 1.5 mL methanol followed by 1.5 mL of 1 M formic acid (adjusted to pH 1.4 using HCl). For hydroponic solutions, the entire 50 mL sample was loaded onto the cartridge, which was then washed with 1.5 mL 1 M formic acid (pH 1.4). For soil samples, dry samples (produced as in Section 2.1.1) were reconstituted in 1.5 mL 1 M formic acid (pH 1.4), loaded onto the column and washed with a further 1.5 mL formic acid. Three separate fractions were eluted using: 1.5 mL methanol (Fraction 1, acidic compounds); 1.5 mL 0.35 M NH<sub>4</sub>OH (Fraction 2, cytokinin ribosides) and 0.35 M NH<sub>4</sub>OH in 60% methanol (Fraction 3, cytokinin free bases). All three fractions were dried under vacuum, then Fraction 1 reconstituted in 200 µL 1:1 (v: v) methanol: water with 0.1% formic acid and Fraction 3 reconstituted in 200 µL water with 0.1% formic acid. (Fraction 2 was archived at –20 °C for future analysis and not analysed as part of this study).

## 2.2. Mass spectrometric methods

### 2.2.1. Compound identification using FT-ICR-MS

For several samples from both soil and hydroponic experiments, both Fractions 1 and 3 (See Fig. 1) were directly analysed by FT-ICR-MS; from these data, target analytes were then identified for quantification by MRM.

Analysis was carried out using a solariX XR FT-ICR-MS with electrospray ionisation (Bruker) using direct infusion at a flow rate of 120 µL/h. Instrument calibration was carried out using 10% sodium formate solution. Fraction 1 (acidic compounds) was analysed in negative ion mode over the *m/z* range 100–2000 with eight scans averaged and an ion accumulation time of 0.2 s. The ESI source had a nebulizer gas pressure of 1 bar and a capillary voltage of 4500 V. Fraction 3 (cytokinin free bases) was analysed in positive ion mode over the *m/z* range 100–3000, averaging eight scans and using an ion accumulation time of 0.2 s. The ESI gas pressure was again 1 bar, and ionisation achieved by applying a capillary voltage of 4500 V.

Mass spectra were acquired and processed using Compass Data Analysis (version 4.4, Bruker), with a mass accuracy < 1 ppm allowing the identification of elemental formulae of analytes based on accurate mass.

### 2.2.2. Multiple reaction monitoring (MRM)

Following identification by FT-ICR-MS, quantification of IAA and

**Table 1**

Summary of experimental conditions used for each of the five compounds analysed and their isotopically labelled standards during quantification by MRM.

Compound	Transition ( <i>m/z</i> to <i>m/z</i> )	'Collision energy' setting (V)	Internal standard	Transition ( <i>m/z</i> to <i>m/z</i> )	'Collision energy' setting (V)
IAA	174.1 -> 130.1	14.15	<sup>13</sup> C <sub>6</sub> IAA	180.21 -> 165.1	19.45
ABA	263.2 -> 153.1	10.25	<sup>2</sup> H <sub>6</sub> ABA	269.2 -> 225.1	14.5
iP	204.2 -> 136.1	16.37	<sup>2</sup> H <sub>6</sub> iP	210.2 -> 137.1	16.55
Ade	268.2 -> 136.1	17.94	<sup>2</sup> H <sub>1</sub> Ade	269.2 -> 137.1	18.09
Z	220.2 -> 136.1	18.09	N <sub>15</sub> Z	221.2 -> 137.1	18.14

ABA (Fraction 1) and Z, Ade and iP (Fraction 3) was carried out using internal standardisation and MRM using a TSQ Endura triple quadrupole mass spectrometer (Thermo Scientific) with LC separation, following methods modified from Farrow and Emery (2012).

Commercially purchased standards of IAA, ABA, Ade, iP and Z and the equivalent isotopically labelled standards (Section 2.1.1) were first used to determine appropriate precursor-to-product transitions for quantification by MRM (Table 1). Collision induced dissociation conditions were optimised to maximise the signal produced. The transitions used for each compound are summarised in Table 1. Fraction 1 was analysed in negative ion mode with a 1 s cycle time, 1.5 mTorr collision gas pressure, ion transfer tube temperature of 350 °C and a spray voltage of 2700 V. Fraction 3 was analysed in positive ion mode with a 1 s cycle time, 1.5 mTorr collision gas pressure, ion transfer tube temperature of 350 °C and a spray voltage of 3500 V.

Compounds were separated by HPLC using a Kinetex C18 core-shell column (2.6 µm particle size, 100 × 3 mm; Phenomenex) and an Ultimate 3000 HPLC system (Thermo Fisher), allowing a flow rate of 450 µL min<sup>-1</sup> and an injection volume of 20 µL. Both SPE fractions were eluted using 95: 5 (v: v) water: methanol with 0.1% formic acid with an increasing gradient of methanol. Fraction 1 was separated in a total of 7.5 min as follows: an increase from 0% to 70% methanol by 0.8 min; increase to 100% by 4.5 min; 1-min wash at 100% methanol; 2-min wash with 100% aqueous phase. Separation of Fraction 3 was achieved by: an increase from 0% to 80% methanol by 5 min; increase to 100% by 5.1 min; 1.4-min wash at 100% methanol; 1-min wash with 100% aqueous phase (total time of 7.5 min). For both fractions, the column was kept at room temperature (25 °C).

### 2.3. Plant growth experiments in the presence and absence of earthworms

#### 2.3.1. Hydroponic solutions

To control for the presence of existing phytohormones in soil and to provide a relatively simple matrix for extraction and analysis, plant growth experiments were first conducted in hydroponic solution for a period of 42 days. Four replicates of four experiments (earthworms plus plants; earthworms only; plants only; neither earthworms nor plants) were conducted in 580 mL glass Weck jars containing 250 g of acid washed silica sand and 250 mL of a hydroponic solution based on groundwater (100 mg/L NaHCO<sub>3</sub>, 20 mg/L KHCO<sub>3</sub>, 200 mg/L CaCl<sub>2</sub>, 180 mg/L MgSO<sub>4</sub> in deionised water; Arnold et al., 2007). For each plant-present experiment, 15 *Sinapis alba* (formerly *Brassica alba*; white mustard) seeds were first propagated in a Petri dish in 4.5 mm diameter agar plugs in floating discs (8 cm diameter, 8 mm thick, made of PVC foam; Kumari et al., 2008). After 5 days, 10 plants or non-germinated seeds were removed, to give a total of 5 plants in each experiment, and then the discs added to the solutions. No prior treatment of the seeds was carried out. Empty discs were also added to the plant-absent controls to ensure consistency. To allow the development of significant root systems, plants were then grown for a further 4 weeks.

Although earthworms have been shown to survive in hydroponic solution, stress or death may occur without constant aeration and replacement of the solution (Arnold et al., 2007). Thus, earthworms were only added for a duration of 24 h at the end of the plant growth period, with aeration carried out using a syringe every 8 h. *Eisenia fetida* were used due to their known ability to survive submersion in solution

(Arnold et al., 2007). Mature specimens were purchased from Blade Biological, Cowden, Edenbridge, UK. Two specimens per earthworm-present experiment were first dehydrated on moist filter paper for 48 h, changing the filter paper after 24 h, and then added to the jars (Arnold and Hodson, 2007). Earthworms were weighed both before and after the experiment, and a similar biomass of earthworms added to each experiment as far as possible.

#### 2.3.2. Soil

Soil experiments were carried out after the hydroponic experiments, introducing the potential effects of soil microfauna and creating a more realistic environment for both plants and earthworms. In contrast to the hydroponic experiments *Lumbricus terrestris* were used because they are naturally occurring, common UK earthworms that are frequently reported in UK earthworms field surveys (Carpenter et al., 2012). Additionally, when we have investigated the impacts of earthworm species on other plant-soil-earthworm interactions (e.g. Sizmur and Hodson, 2008; Sizmur et al., 2011) *L. terrestris* have had a more readily detected impact than smaller species such as *Eisenia veneta* (a species similar to *E. fetida*) and *Allolobophora chlorotica*. Finally, the earthworm density we used in the experiment (see below) was far higher than that typically observed in pasture or arable fields. Thus, we hoped to amplify potential plant responses to the presence of the earthworms.

Plant growth experiments were carried out over a period of 42 days in a controlled temperature room (18 °C) with a 12 h photo period. Soil (a sandy loam Eutric Cambisol (WBR, 2006) with a pH of 6.5 (measured following the method described in BS7755-3.2, 1995) and an organic matter content of 4.6 ± 0.2% (n = 3, mean ± standard deviation, determined by loss on ignition at 400 °C, Nelson and Sommers, 1996) was obtained from a local area of grassland, air dried, and sieved to 2 mm. 300 g of soil was added to each of 20 glass jars (five replicates of each of the four experimental conditions), and 160 mL of deionised water added (to achieve 80% maximum water-holding capacity). Fifteen *S. alba* seeds (undergoing no prior treatment) were planted directly into the jars for each of the plant-present experiments and left for 17 days to allow the plants to become established before addition of the earthworms, thus reducing the effect of earthworm burrowing on plant growth. Ten plants or non-germinated seeds were removed to give a total of 5 plants per experiment. Three mature *L. terrestris* per earthworm-present experiment (purchased from Blade Biological and first dehydrated as described for the hydroponic experiments) were added to each earthworm-present jar, and the experiments left for a further 25 days. Experiments were watered every 2–3 days for the duration of the experiment, and earthworms weighed before and after the experiments.

### 2.4. Plant and growth matrix analysis

#### 2.4.1. Plant biomass and soil characterisation

At the completion of the experiments, the above-ground plant parts were harvested and weighed. Those from soil samples were dried for 24 h and reweighed to obtain both wet and dry masses, whilst those from hydroponic solutions were quickly frozen at -80 °C for molecular analysis.

For the soil experiments, soil moisture content was calculated by weighing a sub-sample of soil at the end of the experiment, drying at 105 °C for 24 h and reweighing. This was used to correct phytohormone

concentrations to the mass of oven dry soil.

Sub-samples of the soil were frozen at  $-20^{\circ}\text{C}$  before chemical extraction and analysis. 50 mL aliquots of the hydroponic solution were removed and frozen prior to sample preparation and analysis as described in Section 2.1.

#### 2.4.2. Fluorescein diacetate hydrolysis (FDH) assay

Fluorescein diacetate hydrolysis (FDH) assays were carried out immediately following completion of the soil-based experiments, on fresh soil sieved to 2.8 mm using a protocol described by Shaw and Burns (2005). The FDH assay colourimetrically measures the hydrolytic capacity of soil as a proxy for biological activity. Briefly, approximately 1 g soil was equilibrated at room temperature with sterile potassium phosphate buffer (60 mM; pH 7.6) and reacted with 0.1 mL of a 1000  $\mu\text{g}/\text{mL}$  solution of fluorescein diacetate in acetone for 30 min. To terminate the reaction, the solution was vortexed with 15 mL of 2:1 chloroform:methanol (v:v), before centrifugal separation of the layers. The top layer containing the residual fluorescein was removed, centrifuged at 16,500 g for 5 min before analysis using a PerkinElmer Lambda 25 UV-vis spectrophotometer at 490 nm. Biological activity was expressed as  $\mu\text{g}$  fluorescein/g dry soil/30 min, calculated using external calibration with solutions containing known concentrations of fluorescein.

#### 2.4.3. pH analysis of hydroponic solution

Arnold et al. (2007) observed a decrease in pH in hydroponic solutions over time in their study, potentially due to earthworm secretions, which may be exacerbated by stress. As a broad indicator of whether the earthworms had a significant impact on the chemistry of the solution, the pH of the hydroponic solutions at the completion of the experiment were therefore recorded using a calibrated glass pH probe (Denver instruments).

#### 2.5. Statistical analysis

Earthworm-plant experimental data were analysed using SigmaPlot for Windows (version 14) and Excel 2016. Outliers in the data were identified as being those values which were more than 1.5 times the interquartile range above the third quartile or below the first quartile and were removed prior to further statistical analysis. Plant biomass in the presence and absence of earthworms in both the hydroponic and soil-based experiments, and pH in the hydroponic experiments were normally distributed (Shapiro-Wilk test) and were compared using a *t*-test. The impacts of plants and earthworms on FDH activity in the soils and phytohormone concentration in the hydroponic experiments were determined using two-way analysis of variance (ANOVA) with the presence or absence of earthworms and plants as the two factors and a *p* value of  $\leq 0.05$  taken to indicate significance differences. Phytohormone concentration data from the hydroponic experiments were not normally distributed and could not be normalised using standard transformations. However, because we wished to determine interaction effects between earthworms and plants on FDH activity and the concentration of phytohormones, and because ANOVA is robust to violations of assumptions of normality and equal variance, we analysed our data using two-way ANOVA followed by Holm Sidak post hoc testing, rather than one-way ANOVA on ranks considering first the impact of earthworms and then the impact of plants.

#### 2.6. Molecular biology

Although the genome of *S. alba* is not well studied, comparative genome mapping has shown that much of its genome is conserved in the closely related *Brassica* species *B. napus*, with many regions of conserved gene order in the well-studied *Arabidopsis thaliana* (Nelson et al., 2011). In both *A. thaliana* and *B. napus*, a number of studies have identified genes upregulated in response to stress (Shamloo-

Dashtpagerdi et al., 2015; Wei et al., 2016). Therefore, using the *B. napus* genome as a guide, DNA primer sequences were designed, and tested to see if these primers could amplify *S. alba* orthologues in both the plant shoots and roots from the hydroponic experiments.

Gene expression studies were conducted using quantitative PCR (qPCR) to quantify levels of expression from genes in the ABA synthesis pathway (9-cis-epoxycarotenoid dioxygenase; NCED3, NCED5), and genes upregulated in response to abiotic stresses including NaCl, drought or cold (CIPK6, NHX1, RD29A). Full details of the genes, and primer sequences used, can be found in the Supplementary information. Prior to qPCR, primer sequences were blasted against *L. terrestris* and *E. fetida* genomes to ensure sequences were specific to *S. alba*.

To assess whether earthworms are capable of directly synthesising phytohormones, a search for diagnostic genes associated with biosynthesis of phytohormones was conducted. The *E. fetida* genome was downloaded from <http://ryanlab.whitney.ufl.edu/genomes/Efet/> and the *L. terrestris* genome was obtained from <http://ngenomes.org/index.html>. The protein sequences for the *A. thaliana* genes ABA1, NCED2, NCED3, NCED5, NCED6, NCED9 and AAO3 (which encode enzymes of the ABA synthesis pathway) were then used to search both genomes using the NCBI standalone BLAST software after indexing.

### 3. Results

#### 3.1. Assessment of analytical methods

##### 3.1.1. Untargeted analysis by FT-ICR-MS

FT-ICR-MS analysis was used to survey the range of phytohormones in the hydroponic and soil samples. Typical spectra of Fractions 1 and 3 from soil extracts are shown in Fig. 2A and B; analysis of hydroponic solutions resulted in less complex spectra in which phytohormone signals could be easily identified. For Fraction 1, ABA was detected in most (but not all) of the hydroponic and soil samples analysed (Fig. 2A for soil). IAA was detected in only very few samples, despite it being widely reported in similar analysis of plant tissues. IAA is reported to be unstable at certain light levels, pHs and temperatures (Nissen and Sutter, 1990; Farrow and Emery, 2012); therefore, to determine whether IAA survived our extraction procedure, a small amount of an authentic IAA standard was spiked onto soil and analysed after extraction. IAA was detected at only very low intensity, suggesting that it cannot be extracted and detected effectively using this procedure, despite the low extraction temperatures.

Analysis of Fraction 3 yielded a complex spectrum, attributed to the variety of compounds present within soil. However, the high mass accuracy analysis allowed the identification of several compounds related to plant growth including Ade, iP and Z as well as benzyladenine (BAP) and other adenosine-related products (Fig. 2B). Although iP was often present at very low intensity, it was detected in all samples analysed using FT-ICR-MS.

As a result of the data from the untargeted FT-ICR-MS analyses, it was decided to proceed to quantification by MRM of Z, iP, Ade and ABA, using labelled standards of each as an internal standard. Analysis of IAA was also attempted, but in most cases this compound was not detected.

##### 3.1.2. Quantification using MRM

Addition of known amounts of isotopically labelled internal standards prior to extraction allowed the quantification of levels of the four target compounds (ABA, Ade, Z and iP). The amount present in each sample (in  $\mu\text{g}$ ) was determined by comparison of the peak areas of the diagnostic product ion, with those of the relevant internal standards. This method accounts for losses during extraction and chromatography, as losses of the labelled standard are assumed to be equivalent to loss of the target compounds. Concentrations of the target compounds were then corrected to give values per gram of dry soil or per mL of hydroponic solution.

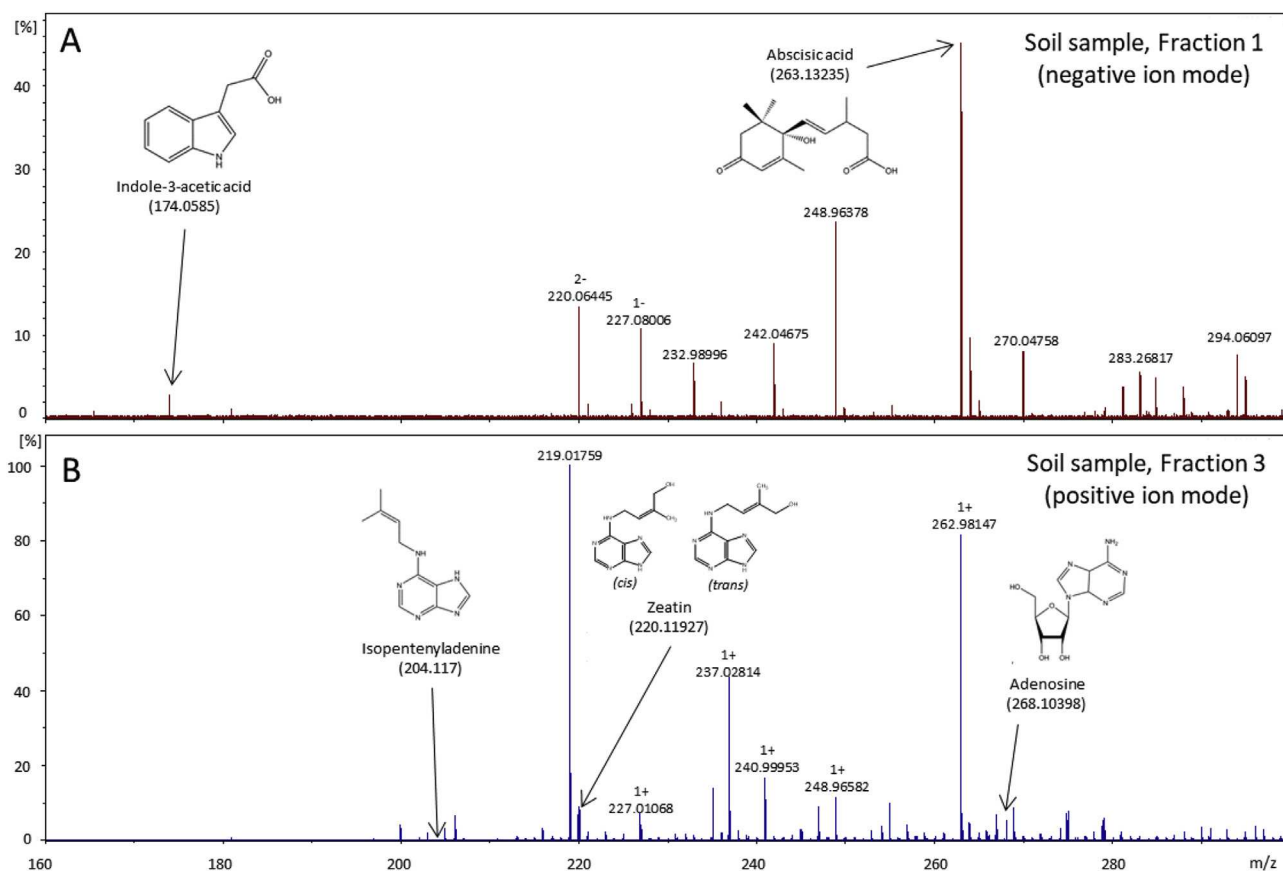


Fig. 2. FT-ICR-MS spectra of Fraction 1 (A) and Fraction 3 (B) extracts of soil, indicating the peaks corresponding to the plant-related compounds then quantified using MRM. Compounds were identified in the spectrum based on their accurate masses.

Table 2

Analytical parameters for the four compounds analysed and their isotopically labelled standards. For all  $R^2$  values, p-values are  $< 0.05$ . The repeatability is derived from multiple injections of a standard, and the LoD and LoQ values derived from the slope and standard deviation of the linear calibration curves.

Phytohormone				Isotopically labelled standard					
Analyte	Linearity ( $R^2$ )	Repeatability (% RSD)	LoD ng/g soil	LoQ ng/g soil	LoD ng/mL HP	LoQ ng/mL HP	Analyte	Linearity ( $R^2$ )	Repeatability (% RSD)
ABA	0.9899	8%	$8.0 \times 10^{-3}$	$2.4 \times 10^{-5}$	$4.8 \times 10^{-4}$	$1.4 \times 10^{-3}$	$^2\text{H}_6$ ABA	0.9899	7%
iP	0.9941	17%	$1.5 \times 10^{-5}$	$4.5 \times 10^{-5}$	$8.9 \times 10^{-7}$	$3.0 \times 10^{-3}$	$^2\text{H}_6$ iP	0.9956	8%
Ade	0.9966	7%	$1.8 \times 10^{-5}$	$5.5 \times 10^{-5}$	$1.1 \times 10^{-6}$	$3.3 \times 10^{-6}$	$^2\text{H}_1$ Ade	0.9997	11%
Z	0.9986	7%	$2.0 \times 10^{-6}$	$6.5 \times 10^{-6}$	$1.3 \times 10^{-7}$	$3.9 \times 10^{-7}$	$\text{N}_{15}$ Z	0.9970	5%

Linearity of response for all compounds was demonstrated by injecting solutions containing both isotopically labelled and authentic standards at concentrations ranging from 0.1 to 100 ng/mL. Linearity was above 0.98 for all compounds (Table 2) across the concentration range except for IAA; this has been attributed to the potential decay of IAA during the extraction process. Results for IAA have thus been reported only as either detected or undetected (SI Table 1). Having demonstrated linearity, relative response factors (RRF) were calculated for each target compound from the peak area ratio of labelled standard: target compound in a solution containing a known quantity of each. This provides a correction based on the response expected from the target compound compared to the internal standard.

Repeatability was determined by three replicate injections of solutions prepared at 0.01 ng/mL and expressed as the % relative standard deviation (% RSD; also termed coefficient of variation; Table 2). The repeatability varied between compounds, with that for iP being comparatively weak but with all other compounds being under 10%.

Limits of detection (LoD) are defined as the lowest concentration of a compound that can be reliably detected, and limit of quantification

(LoQ) the lowest concentration at which the compound can be reliably quantified. Both parameters were calculated based on the standard deviation and slope of the calibration curves, according to the following equations:

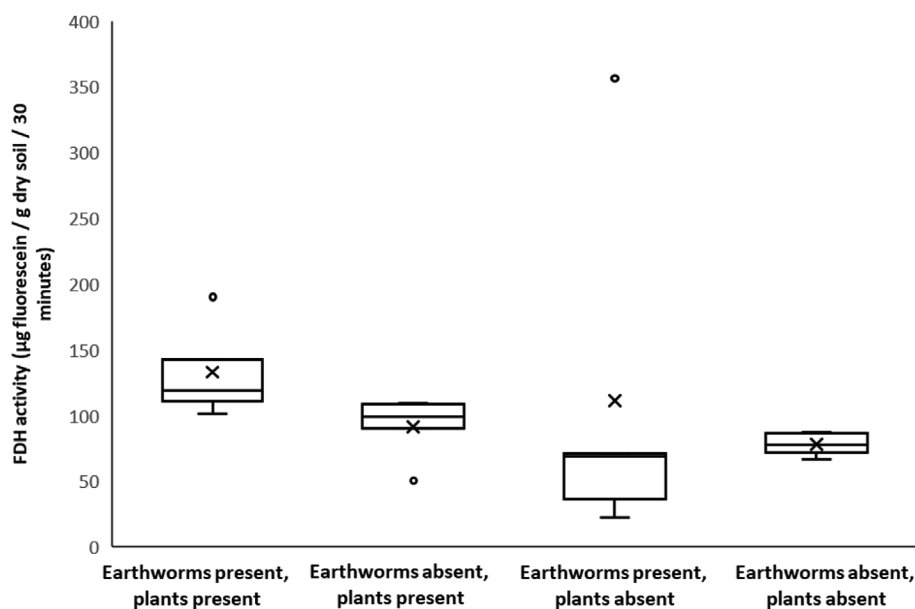
$$\text{LoD} = 3.3\sigma/s; \text{LoQ} = 10\sigma/s$$

Where  $\sigma$  is the standard deviation of the y-intercept and  $s$  is the slope of the calibration curve (Shrivastava and Gupta, 2011). These values were then adjusted to provide approximate values for the LoD per gram of dry soil or per mL of hydroponic solution based on the extraction methods outlined above (Table 2).

### 3.2. Growth experiments

#### 3.2.1. Plant biomass

The presence of earthworms typically leads to an increase in plant biomass (Scheu, 2003). However, in both our hydroponic and soil-based experiments no significant difference in biomass was observed between *S. alba* grown with earthworms present and those without (SI



**Fig. 3.** Box plots showing the mean (crosses) and median, the interquartile range and the minimum and maximum data points for biological activity measured using the FDH assay for each of the four soil treatments. Circles represent outliers, which are accounted for in the displayed means, but removed from the data set before statistical analysis. Although there was no significant difference between experiments with/without earthworms, there was a significant increase when plants were present.

**Table 2;** t-tests,  $p = 0.095$  for the soil data,  $p = 0.630$  for the hydroponic data).

### 3.2.2. Biological activity using FDH assay

Fluorescein diacetate can pass into cells where it is hydrolysed enzymatically, leading to a fluorescent colour (Bandick and Dick, 1999). The assay provides a broad approximation of the biological activity present in the soil, but is not diagnostic of the nature of that activity. However, the analysis aimed to provide an assessment of the extent to which earthworms might modify microbial activity, or indicate relationships between microbial activity and plant growth.

There is a significant increase in biological activity in the presence of plants but not in the presence of earthworms (SI Table 3; Fig. 3) (2-way ANOVA,  $p \leq 0.001$  and  $p = 0.478$  respectively). FDH levels are significantly greater in the presence of earthworms and plants than in their absence ( $p = 0.03$ , *t*-test) but although FDH levels are greater in the presence of earthworms and plants than in the presence of just plants (118 vs 101) this difference is not significant ( $p = 0.16$ , *t*-test).

### 3.2.3. pH analysis of hydroponic solution

No significant difference was found between pH in plant present/absent ( $p = 0.226$ ) or earthworm present/absent ( $p = 0.818$ ) experiments (SI Table 4).

## 3.3. Changes in phytohormone concentrations between experimental samples

### 3.3.1. Experiments in hydroponic solution

In all hydroponic experiments, ABA, iP, Z and Ade were detected, albeit at very low concentrations (SI Table 1; Fig. 4). IAA was not detected, although this may be due to its instability leading to breakdown during extraction (Nissen and Sutter, 1990; Farrow and Emery, 2012). For Ade (*t*-test,  $p = 0.002$ ), significantly more phytohormone was detected in the presence compared to the absence of plants (mean concentration =  $0.025 \mu\text{g/mL}$ , standard deviation =  $0.012$  with plants, compared to  $0.005 \mu\text{g/mL}$ , standard deviation =  $0.006$  without; SI Table 1). However, the same is not seen for Z or iP (*t*-tests,  $p = 0.142$  and  $0.463$  respectively). The presence or absence of earthworms had no significant effect on the concentrations of these cytokinins (*t*-tests, Ade,  $p = 0.281$ ; Z,  $p = 0.318$ ; iP,  $p = 281$ ). However, in earthworm present experiments a significantly higher concentration of ABA was observed when plants were also present (two-way ANOVA,  $p < 0.001$ ), and in

plant present experiments a higher concentration was observed when earthworms were also present (two-way ANOVA,  $p = 0.006$ ).

### 3.3.2. Experiments in soil

The experiments in soil aimed to be comparable with the experiments in hydroponic solution but be carried out in a more realistic system. All five compounds were identified in soil samples at least twice, with levels ranging from  $0.2 \text{ ng/g}$  dry soil for zeatin to over  $20 \mu\text{g/g}$  dry soil for iP (SI Table 1). However, the instances of observation were greatly reduced in comparison to those in the hydroponic solutions, particularly for ABA. Although all compounds were detected, no significant statistical relationship between phytohormone concentrations and the presence/absence of earthworms ( $p > 0.600$ ) or plants ( $p > 0.0985$ ) were detected.

## 3.4. Molecular biological analysis

Although the presence of earthworms significantly increased levels of ABA in the hydroponic solution in the presence of plants, there was no upregulation of NCED3, NCED5, CIPK6 or NHX1. A significant decrease in the expression of RD29A was seen in roots in the presence of earthworms (Fig. SI 1).

A search of the genomes of both *E. fetida* and *L. terrestris* revealed no sequences known to be associated with ABA synthesis.

## 4. Discussion

### 4.1. Analytical methods for quantifying phytohormones in soil

Whilst several studies have reported the extraction and detection of a range of phytohormones from plants (Novák et al., 2008; Pan et al., 2010; Farrow and Emery, 2012), similar analysis in other matrices, such as soil and other growth media, has been neglected. However, the ability to examine the range and concentrations of phytohormones present in soil could potentially open up routes to understanding the influences of below-ground organisms (for example earthworms) and rhizobacteria on plant growth.

In this study, it has been demonstrated that a range of phytohormones can be extracted from both soil and hydroponic growth solution and detected using untargeted FT-ICR mass spectrometry. Furthermore, three commonly occurring cytokinins (Z, iP, Ade) as well as ABA were then quantified by MRM. Limits of detection ranged



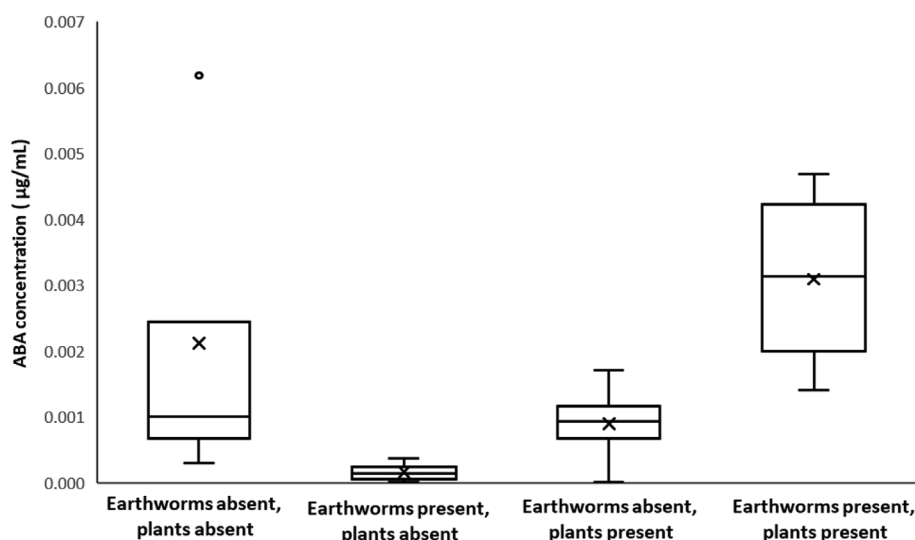


Fig. 4. Box plots showing the mean (crosses) and median, the interquartile range and the minimum and maximum data points for ABA concentration for each of the four hydroponic treatments. Circles represent outliers, which were removed from the data set prior to data analysis. Significant increases were seen in earthworm present experiments when plants were also present, and in plant present experiments when earthworms were also present.

between 0.08 ng/g fresh soil for Ade and 2 ng/g fresh soil for iP, with much lower concentrations detectable in the hydroponic solution, as a much larger volume of matrix could be processed for analysis. The LoD for soil could potentially be improved by using larger masses of soil, although this was experimentally limited by the need to rapidly remove, without heating, the larger volumes of solvent that would be used. In addition, larger volumes of soil would have increased animal usage in the experiments.

The repeatability of analyses varied, with the data for iP being prone to high levels of error but those for ABA and zeatin showing particularly good repeatability and linearity (Table 2). There is scope for further refining these methods; previous studies report the detection of phytohormones in plant tissue at the picomole per gram fresh weight level (Farrow and Emery, 2012). The fast analysis time (7.5 min per fraction) reported here offers the ability to expand the method to examine additional compounds that may be present in soil, such as gibberellins and other auxins. Although these compounds were not detected by FT-ICR-MS in our experiments, and therefore not targeted for quantification, this may not be the case for others using our approaches.

Applications for the ability to detect phytohormones in plant growth media are far reaching, including developing understanding of interactions between soil microbes and plants, and understanding the potential uses of plant growth-promoting bacteria, for example in agriculture. Despite the obvious potential value in understanding these relationships in terms of advancing land management practices to maximise crop growth (e.g. Sturz et al., 2000; Bhattacharyya and Jha, 2012), little research has been previously conducted in this area.

#### 4.2. Comparison of earthworm present/absent experiments

Applying the developed analytical methods, experiments were carried out aimed at investigating the impact of the presence of earthworms with and without plants on the concentrations of phytohormones within the growth media.

Despite no measurable differences in plant biomass in the presence of earthworms, a significant increase in the presence of ABA was detected when earthworms were present in hydroponic solution together with plants. This suggests that there could be interactions between the earthworms and plants that cause ABA to be produced. The possibility that the presence of earthworms alters the regulation pathways of certain phytohormone-related genes was tested for by molecular biology methods. A search of the earthworm genome for genes related to ABA production revealed no matches, indicating that earthworms are unlikely to be able to directly produce ABA. We hypothesise instead that the increase in ABA we observed in our earthworm-present

experiments in hydroponic solution was caused by indirect influence. Further research would need to be carried out in order to fully assess the mechanisms by which earthworms may be involved in ABA (and possibly other phytohormone) regulation in plants.

An increase in ABA production in the presence of earthworms could be attributed to a range of indirect factors including increased competition for nutrients, or the chemical modification of the solution by earthworms. As ABA is frequently associated with abiotic or biotic stress, this seems the most obvious explanation for its increased presence (Baron et al., 2012; Cao et al., 2016). Analysis of the pH of the solutions did not reveal significant differences, although this is only a very broad measure of the degree to which the earthworms may have altered the environment. It is also possible that the presence of earthworms induced changes in the expression of genes known to be involved in plant stress responses. For example, in addition to affecting plant roots through burrowing, and physiological activities such as excretions (Brown et al., 2004), there is some evidence that earthworms also feed on living plant root material (e.g. Baylis et al., 1986; Gunn and Cherrett, 1993).

A small-scale study was therefore conducted to see if genes known to be involved in stress responses, or in the biosynthesis of ABA, were upregulated in either the plant roots or plant shoots grown in hydroponic solution in the presence of earthworms. However, only a few genes were tested and in only one case (RD29A) was a significant difference (decrease) seen between the presence/absence of earthworms. Whilst this may be related to the observed differences in ABA concentrations, the metabolic pathway of ABA production is complex and as such this requires further investigation (Milborrow, 2001; Baron et al., 2012). In particular, transcriptomic-based studies could be employed to assess the effect of earthworms on global plant metabolic pathways.

Substantial evidence exists that earthworms benefit crop yields (reviewed by Scheu, 2003 and Van Groenigen et al., 2014). However, the observations from our hydroponic experiment suggest that under some circumstances, for example in an already stressed system, earthworms may in fact cause greater levels of stress to plants, resulting in higher levels of ABA in the soil. To our knowledge, no study has effectively dismantled the effects of earthworms in systems with limited nutrient availability.

Whilst the increase in ABA was not replicated in soil-based experiments, we did observe an increase in biological activity (FDH assay) when both earthworms and plants were present. This could indicate a potential synergistic relationship between plants, soil microbes and earthworms, which could be further investigated using the developed methods. Differences between the results of the hydroponic and soil

experiments may in part be due to the use of different earthworm species. *E. fetida* are litter feeders and *L. terrestris* are an anecic (burrow dwelling) species, and consequently they will interact with the soil differently. Differences between the two species in terms of e.g. sensitivity to toxicants (Asensio et al., 2007; Dittbrenner et al., 2011) and biochemistry (e.g. Rejnek et al., 1986; Zimmerman et al., 2017) in addition to behavioural differences are well established in the literature.

The additional complexity of a soil matrix compared to hydroponic solutions will inevitably increase associated difficulties in the extraction. It is also possible that increased biological activity in soils compared to hydroponic experiments leads to degradation or conversion of phytohormones during extraction. There is therefore scope to improve the extraction method to achieve better recovery, allowing the observation of more subtle changes in phytohormone concentrations within soils.

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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.2019.04.017>.

## References

- Arkhipova, T.N., Prinsen, E., Veselov, S.U., Martinenko, E.V., Melentiev, A.I., Kudoyarova, G.R., 2007. Cytokinin producing bacteria enhance plant growth in drying soil. *Plant and Soil* 292, 305–315.
- Arnold, R.E., Hodson, M.E., Comber, S., 2007. Effect of organic complexation on the toxicity of Cu to the earthworm *Eisenia fetida*. *Applied Geochemistry* 22, 2379–2405.
- Arnold, R.E., Hodson, M.E., 2007. Effect of time and mode of depuration on tissue copper concentrations of the earthworms *Eisenia Andrei*, *Lumbricus rubellus* and *Lumbricus terrestris*. *Environmental Pollution* 148, 21–30.
- Asensio, V., Kille, P., Morgan, A.J., Soto, M., Marigomez, I., 2007. Metallothionein expression and Neutral Red uptake as biomarkers of metal exposure and effect in *Eisenia fetida* and *Lumbricus terrestris* exposed to Cd. *European Journal of Soil Biology* 43, S233–S238.
- Bandick, A.K., Dick, R.P., 1999. Field management effects on soil enzyme activities. *Soil Biology and Biochemistry* 31, 1471–1797.
- Barceló, J.U.A.N., Poschenrieder, C., 1990. Plant water relations as affected by heavy metal stress: a review. *Journal of Plant Nutrition* 13, 1–37.
- Baron, K.N., Schroeder, D.F., Stasolla, C., 2012. Transcriptional response of abscisic acid (ABA) metabolism and transport to cold and heat stress applied at the reproductive stage of development in *Arabidopsis thaliana*. *Plant Science* 188–189, 48–59.
- Baylis, J.P., Cherrett, J.M., Ford, J.B., 1986. A survey of the invertebrates feeding on living clover roots (*Trifolium repens* L.) using <sup>32</sup>P as a radiotracer. *Pedobiologia* 29, 201–208.
- Bhattacharyya, P.N., Jha, D.K., 2012. Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. *World Journal of Microbiology and Biotechnology* 28, 1327–1350.
- Blouin, M., Hodson, M.E., Delgado, E.A., Baker, G., Brussaard, L., Butt, K.R., Dai, J., Dendooven, L., Peres, G., Tondoh, J.E., Cluzeau, D., Brun, J.J., 2013. A review of earthworm impact on soil function and ecosystem services. *European Journal of Soil Science* 64, 161–182.
- BS7755 – 3.2, 1995. Soil Quality – Chemical Methods – Determination of pH.
- Brown, M.E., 1972. Plant growth substances produced by micro-organisms of soil and rhizosphere. *Journal of Applied Bacteriology* 35, 443–451.
- Brown, G.G., Edwards, C.A., Brussaard, L., 2004. How earthworms affect plant growth: burrowing into the mechanisms. In: In: Edwards, C.A. (Ed.), *Earthworm Ecology*, vol. 2. pp. 13–49.
- Cao, D., Lutz, A., Hill, C.B., Callahan, D.L., Roessner, U., 2016. A quantitative profiling method of phytohormones and other metabolites applied to barley roots subjected to salinity stress. *Frontiers of Plant Science* 7 article 2070.
- Carpenter, D., Sherlock, E., Jones, D.T., Chiminoideis, J., Writer, T., Neilson, R., Boag, B., Keith, A.M., Eggleton, P., 2012. Mapping of earthworm distribution for the British isles and Eire highlights the under-recording of an ecologically important group. *Biodiversity & Conservation* 21, 475–485.
- Dittbrenner, N., Schmitt, H., Capowiez, Y., Triebkorn, R., 2011. Sensitivity of *Eisenia fetida* comparison to *Aporrectodea caliginosa* and *Lumbricus terrestris* after imidacloprid exposure. Body mass change and histopathology. *Journal of Soils and Sediments* 11, 1000.
- Dobrev, P.I., Havlíček, L., Vágner, M., Malbeck, J., Kamínek, M., 2005. Purification and determination of plant hormones auxin and abscisic acid using solid phase extraction and two-dimensional high performance liquid chromatography. *Journal of Chromatography A* 1075, 159–166.
- Farrow, S.C., Emery, R.J.N., 2012. Concurrent profiling of indole-3-acetic acid, abscisic acid, and cytokinins and structurally related purines by high-performance-liquid-chromatography tandem electrospray mass spectrometry. *Plant Methods* 42.
- Gunn, A., Cherrett, J.M., 1993. The exploitation of food resources by soil meso- and macro-invertebrates. *Pedobiologia* 37, 303–320.
- Hanano, S., Domagalska, M.A., Nagym, F., Dabis, S.J., 2006. Multiple phytohormones influence distinct parameters of the plant circadian clock. *Genes to Cells* 11, 1381–1392.
- Kumari, M., Taylor, G.J., Deyholos, M.K., 2008. Transcriptomic responses to aluminum stress in roots of *Arabidopsis thaliana*. *Molecular Genetics and Genomics* 279, 339–357.
- Ma, Z., Ge, L., Lee, A.S.Y., Hong Yong, J.W., Ngai Tan, S., Shi Ong, E., 2008. Simultaneous analysis of different classes of phytohormones in coconut (*Cocos nucifera* L.) water using high-performance liquid chromatography and liquid chromatography-tandem mass spectrometry after solid-phase extraction. *Analytica Chimica Acta* 610, 274–281.
- Milborrow, B.V., 2001. The pathway of biosynthesis of abscisic acid in vascular plants: a review of the present state of knowledge of ABA biosynthesis. *Journal of Experimental Botany* 52, 1145–1164.
- Muscolo, A., Cutrupi, S., Nardi, S., 1998. IAA detection in humic substances. *Soil Biology and Biochemistry* 30, 1199–1201.
- Nelson, D.W., Sommers, L.E., 1996. Total carbon, organic carbon and organic matter. In: *Methods of Soil Analysis, Part 3. Chemical Methods*. Soil Science Society of America and American Society of Agronomy, Madison, USA.
- Nelson, M.N., Parkin, I.A., Lydiate, D.J., 2011. The mosaic of ancestral karyotype blocks in the *Sinapis alba* L. genome. *Genome* 54, 33–41.
- Nissen, S.J., Sutter, E.G., 1990. Stability of IAA and IBA in nutrient medium to several tissue culture procedures. *HortScience* 25, 800–802.
- Novák, O., Hauserová, E., Amakorová, P., Doležal, K., Strnad, M., 2008. Cytokinin profiling in plant tissues using ultra-performance liquid chromatography – electrospray tandem mass spectrometry. *Phytochemistry* 69, 2214–2224.
- Pan, X., Welti, R., Wang, X., 2010. Quantitative analysis of major plant hormones in crude plant extracts by high-performance liquid chromatography-mass spectrometry. *Nature Protocols* 5, 986–992.
- Pizzeghello, D., Francioso, O., Ertani, A., Muscolo, A., Nardi, S., 2013. Isopentenyladenosine and cytokinin-like activity of different humic substances. *Journal of Geochemical Exploration* 129, 70–75.
- Puga-Freitas, R., Barot, S., Tacconat, L., Renou, J.-P., Blouin, M., 2012. Signal molecule mediates the impact of the earthworm *Aporrectodea caliginosa* on growth, development and defence of the plant *Arabidopsis thaliana*. *PLoS One* 7, e49504.
- Rejnek, J., Tucková, L., Sima, P., Kostka, J., 1986. The proteins in *Lumbricus terrestris* and *Eisenia foetida* coelomic fluids and on coelomocytes reacting with sheep and goat IgG molecules. *Developmental & Comparative Immunology* 10, 467–475.
- Santoyo, G., Moreno-Hagselsieb, G., Orozco-Mosqueda, Mdel, C., Glick, B.R., 2016. Plant growth promoting bacterial endophytes. *Microbiological Research* 183, 92–99.
- Schaller, G.E., Bishopp, A., Keiber, J.J., 2015. The yin-yang of hormones: cytokinins and auxin interactions in plant development. *The Plant Cell Online* 27, 44–63.
- Scheu, S., 2003. Effects of earthworms on plant growth: patterns and perspectives. *Pedobiologia* 47, 846–856.
- Shan, X., Yan, J., Xie, D., 2012. Comparison of phytohormone signalling mechanisms. *Current Opinion in Plant Biology* 15, 84–91.
- Shamloo-Dashtgarderi, R., Razi, H., Ebrahimie, E., 2015. Mining expressed sequence tags of rapeseed (*Brassica napus* L.) to predict the drought responsive regulatory network. *Physiology and Molecular Biology of Plants: an International Journal of Functional Plant Biology* 21, 329–340.
- Shaw, L.J., Burns, R.G., 2005. Enzyme activity profiles and soil quality. In: Bloem, J., Hopkins, D., Benedetti, A. (Eds.), *Microbiological Methods for Assessing Soil Quality*. CAB, Wallingford, pp. 158–172.
- Shrivastava, A., Gupta, V.B., 2011. Methods for the determination of limit of detection and limit of quantitation of the analytical methods. *Chronicles of Young Scientists* 2, 21–25.
- Sizmur, T., Hodson, M.E., 2008. The impact of *Eisenia veneta* on As, Cu, Pb and Zn uptake by ryegrass (*Lolium perenne* L.). *Mineralogical Magazine* 72, 495–499.
- Sizmur, T., Tilston, E.L., Charnock, J., Palumbo-Roe, B., Watts, M.J., Hodson, M.E., 2011. Impacts of epigeic, anecic and endogeic earthworms on metal and metalloid mobility and availability. *Journal of Environmental Monitoring* 13, 266–273.
- Sturz, A.V., Christie, B.R., Nowak, J., 2000. Bacterial Endophytes: potential role in developing sustainable systems of crop production. *Critical Reviews in Plant Sciences* 19 (1), 1–30.
- Van Groenigen, J.W., Lubber, I.M., Vos, H.M.J., Brown, G.G., De Deyn, G.B., van Groenigen, K.J., 2014. Earthworms increase plant production: a meta-analysis. *Scientific Reports* 4, 6365.
- WBR, 2006. World Reference Base for Soil Resources, 2 ed. ISRIC-FAO, Rome.
- Wei, Y., Shi, H., Xia, Z., Tie, W., Ding, Z., Yan, Y., Wang, W., Hu, W., Li, K., 2016. Genome-wide identification and expression analysis of the WRKY gene family in cassava. *Frontiers of Plant Science* 7, 25.
- Werner, T., Motyka, V., Strnad, M., Schülling, T., 2001. Regulation of plant growth cytokinin. *Proceedings of the National Academy of Sciences* 98, 10487–10492.
- Xiao, S., Wang, X., Koricheva, J., Kergunteuil, A., Le Bayon, R.-C., Liu, M., Hu, F., Rasmann, S., 2017. Earthworms affect plant growth and resistance against herbivores: a meta-analysis. *Functional Ecology* 32, 150–160.
- Zimmerman, D., Dilusto, M., Dienes, J., Abdulmalik, O., Elmer, J.J., 2017. Direct comparison of oligochaete erythrocrurins as potential blood substitutes. *Bioengineering and Translational Medicine* 2, 212–221.