

# Earthworms suppress thrips attack on tomato plants by concomitantly modulating soil properties and plant chemistry

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## ABSTRACT

How plants cope with herbivore attack is partially modulated by the biotic and abiotic environment where the plant lives. For instance, theory predicts that soil fertility should drive patterns of plant resource allocation and defensive strategies. Earthworms, by their burrowing and casting activities, modify soil physicochemical properties and soil fertility. Therefore, earthworm-mediated changes in soil properties could alter plant physiology, plant nutritional quality, and ultimately, plant resistance against insect herbivores. We tested this hypothesis by measuring the combinatorial effects of two earthworm species, an epi-endogeic earthworm (*Amyntas corticis*) and an endo-aneic earthworm (*Metaphire guillelmi*), on soil properties, tomato plants' physiological traits and plant resistance against the western flower thrips (*Frankliniella occidentalis*). We found that *A. corticis* alone increased plant resistance more than *M. guillelmi* alone or the combination of two species. The increased plant resistance was associated with a significant increase in the defence-related phytohormone jasmonic acid and the production of phenolic compounds. Furthermore, we observed a strong link between earthworm-mediated changes in soil properties and plant eco-physiological traits. Our results thus build toward a better predictive model of how earthworms can simultaneously influence soil parameters, plant productivity and resistance against herbivore pests.

## 1. Introduction

In response to herbivory, plants have evolved a wide variety of physical and chemical defence strategies (Schoonhoven et al., 2005), which can be constitutively expressed, or can only be induced after herbivory (Karban and Baldwin, 2007). Plant defences are not only highly species-specific, but also strongly depend on the biotic and abiotic environment. Among the classically-postulated factors that drive plant defence trait variation is soil resource availability (Coley et al., 1985; Puga-Freitas and Blouin, 2015). In other words, how much nutrient a soil can provide directly influences plant physiology, and indirectly affects plant defences against higher trophic levels thorough eco-physiological trait trade-offs and resource allocation changes (Fine et al., 2004; Endara and Coley, 2011). Soil biota influences soil productivity (Barrios, 2007), in turn potentially altering plant chemical defence traits (Lillo et al., 2008; Smoleń and Sady, 2009; Puga-Freitas

and Blouin, 2015), and eventually influencing aboveground trophic interactions (Johnson et al., 2011; Xiao et al., 2018). Therefore, a better understanding of how soil biota modulates soil properties, the production of plant secondary metabolites and phytohormone biosynthesis, could foster natural crop protection and biological control strategies for sustainable agriculture (Lyon et al., 2014).

It is now widely recognized that the activity of soil invertebrates can modify soil physical, chemical and biological properties, and in turn influence plant growth and physiology (Puga-Freitas and Blouin, 2015; Kergunteuil et al., 2016). As the major soil ecosystem engineers, earthworms facilitate soil physical structure development (e.g. porosity formation and macro-aggregation process) and related soil ecosystem functions (Angst et al., 2017). Earthworms, particularly, have been shown to alter plant available nutrients through their feeding and casting activities (Blouin et al., 2013; Andriuzzi et al., 2016). Thereby, earthworms could alter plant performance, resource allocation, and

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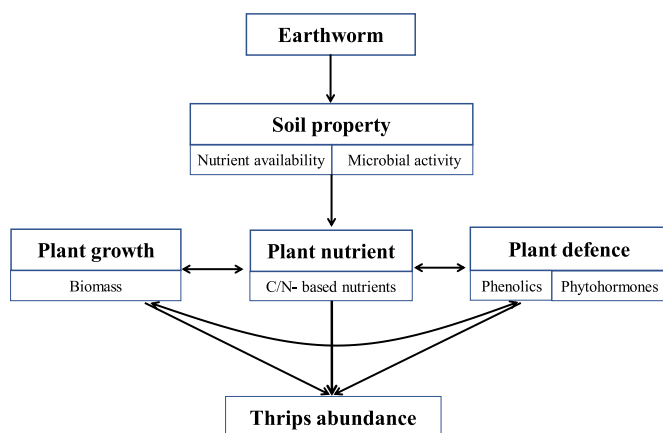


Fig. 1. Conceptual model describing the links and indirect effects between earthworm activity, soil properties, plant resource allocation to growth and defences, and ultimately, resistance against insect herbivores.

ultimately, plant resistance to herbivores (Fig. 1) (Lohmann et al., 2009; Wurst, 2013; Xiao et al., 2018). For instance, the earthworm *Aporrectodea caliginosa* has been shown to influence aboveground plant chemical defences by modifying stress-related gene expression (Jana et al., 2010; Puga-Freitas et al., 2012, 2016), and subsequent production of phytohormones (Puga-Freitas and Blouin, 2015), and plant secondary metabolites (Newington et al., 2004; Wurst et al., 2006; Trouve et al., 2014).

Nonetheless, the effect of earthworms on plant-herbivore interactions is highly variable, ranging from negative to neutral to positive, by means of a meta-analysis, we have recently shown that earthworm presence overall decreases plant resistance by 15% (Xiao et al., 2018). However, the magnitude of earthworm effects on plant resistance is strongly influenced by herbivore feeding guild, as well as by the species or ecological types of earthworms (Xiao et al., 2018). Indeed, earthworms can be classified into three ecological types with distinct burrowing patterns and food preferences. Epigeic earthworms live in the litter layer above the mineral soil and often feed intensively on surface litter. Endogeic earthworms, predominantly geophagous, form non-permanent horizontal burrows and feed on organic matter in the soil and the associated microbial biomass. Finally, anecic earthworms build permanent vertical burrows and feed on large organic matter debris, which they drag from the soil surface into their burrows (Bouché, 1977). Thus, the effects of different earthworm species on soil structure, organic matter decomposition or nutrient mobilization, and activity of microbial communities, as well as on affecting plant nutrient uptake and aboveground biomass accumulation will vary both qualitatively and quantitatively (Bossuyt et al., 2006; Butenschoen et al., 2009; Felten and Emmerling, 2009; Andriuzzi et al., 2016). However, when different earthworm ecological types co-exist in soils, they could exploit niche complementarity for increasing soil aggregation (Bossuyt et al., 2006; Felten and Emmerling, 2009), and nutrient mineralization processes (Postma-Blauw et al., 2006). While various earthworm species naturally co-occur in nature, the combined effect of different earthworm species on soil properties, and how this cascades up to influence plant resistance against herbivores, has so far been neglected. Thereby, we here hypothesized that earthworm-driven modifications of soil properties, plant physiological properties and ultimately, plant resistance, would be species-specific and combination-specific.

Specifically, we had the following expectations: 1) Earthworms increase plant physiological performance via modulating soil biochemical properties; 2) The effect of earthworms on plant resistance against herbivores is mainly mediated by changes in the chemical defence traits including phenolics and phytohormones; 3) The effects of earthworms on soil properties, plant physiological traits, and plant resistance are species specific, but stronger when different earthworm ecological

types co-exist in the soil.

## 2. Materials and methods

### 2.1. Study system

We tested our hypothesis using the tomato-thrips system. Tomato plants are among the most widely cultivated crops worldwide (FAO, 2014), and particularly under the condition of protected cultivation are generally attacked by thrips (Insecta: Thysanoptera) (Puche et al., 1995). Western flower thrips (*Frankliniella occidentalis*, WFT) are highly polyphagous species, directly feeds on epidermal and subepidermal cell contents of both meristematic and mature leaf and flower tissues, and indirectly damage plants by transmitting tospoviruses such as tomato spotted wilt virus (Reitz, 2009). Consequently, WFT has become one of the most serious pests of cultivated plants and causes significant economic losses in greenhouses and open-field plant production worldwide (Morse and Hoddle, 2006; Reitz, 2009). Interestingly, previous studies have shown that WFT feeding on tomato plants significantly increased the expression of marker genes involved in jasmonic acid (JA) biosynthesis (*VSP2*, *LOX2*, *AOS* and *AOC2*) (Abe et al., 2009) and JA-induced defence genes (*PIN2* and *LAP1*) (Kawazu et al., 2012). Thus, JA is not only generally induced upon chewing herbivore attack (Howe and Jander, 2008), but also involves in mediating plant resistance against cell-content feeder such as thrips.

The tomato (*Solanum lycopersicum*) cultivar Jinpeng-1 (Xi'an Jinpeng Seedlings Co., LTD) was chosen as being commonly used in fields and under greenhouse conditions in China. Specifically, this cultivar is highly resistant against viral (e.g. tomato mosaic virus) and fungal diseases (e.g. leaf mold, Fusarium wilt) (Li, 2004), but is susceptible to cell-content feeders such as root-knot nematode *Meloidogyne incognita* (Xiao et al., 2016).

The experimental WFT population was obtained from a stock culture kindly provided by the Laboratory of Insect Informatic Ecology (College of Plant Protection, Nanjing Agricultural University, China). From the initial stock culture, 40 female and 40 male adult WFT were inoculated on young fresh green bean pods (*Phaseolus vulgaris*) in 1500 ml volume gauze-covered plastic jars in an incubator at constant conditions ( $27 \pm 1$  °C, 16 h/8 h (day/night) photoperiod and 70% relative humidity) (Berndt et al., 2004). To obtain uniformly aged thrips, after 5 days, all the egg-infested bean pods were transferred to four jars containing fresh green bean pods as described above. Ten days later, the newly hatched adults served as the experimental WFT population for the bioassays (see below).

To measure the effect of earthworms on tomato plant resistance against WFT, we chose two species of earthworms having different ecological strategies: the epi-endogeic *Amyntas corticis* (Kinberg, 1867) and the endo-anecic *Metaphire guillelmi* (Michaelsen, 1895). Generally, epi-endogeic earthworms mainly build shallow and tortuous burrows inside the topsoil and often feed intensively on surface litter, whereas endo-anecic earthworms build numerous vertically deep and horizontally orientated burrows within the top and subsoil, and particularly feed and decompose organic matter (Felten and Emmerling, 2009). *A. corticis* and *M. guillelmi* are widely distributed in China and often they co-occur (Sun, 2013; Jiang, 2016). *A. corticis* has been shown to significantly impact soil carbon cycle and microbial activity (Snyder et al., 2009), while *M. guillelmi* has been shown to facilitate soil nitrogen (N) mineralization (Tao et al., 2009; Wu et al., 2015). Both species of earthworms were collected from a vegetable production soil in Suzhou City, Jiangsu Province, China (N 31°24', E 120°26') and maintained on the experimental soil (see below) in a climate chamber at 10 °C until the onset of experiment. Finally, before the bioassay, earthworms were placed in a plastic container with wet filter paper for 48 h to remove soil from their guts, standardize their water content (Dalby et al., 1996; Eisenhauer et al., 2009), and assess their general health according to Fründ et al. (2010).

## 2.2. Experimental design for the bioassay

To measure the effect of earthworms on tomato-thrips interaction, we set up a two-factor completely randomized design. Factors were 1) “earthworm” with four levels: control without earthworm (C), with the epi-endogeic earthworm *A. corticis* (Ac), with the endo-aneic earthworm *M. guillelmi* (Mg), with both earthworms *A. corticis* and *M. guillelmi* simultaneously (Ac + Mg), and 2) “western flower thrips” with two levels: without thrips (-Thrips) and with thrips (+Thrips). Five replicates were set up per treatment for a total of 40 containers.

The experimental soil (silty clay loam) was collected from the plough layer (0–20 cm) of an abandoned cropland that showed limited to no trace of earthworm activity in Suzhou City, Jiangsu province, China (N 31°26', E 120°28'). We here aimed at reducing a previous potential effect of earthworms on the experimental soil. The composite fresh soil collected from the field was sieved through a 5 mm diameter mesh to exclude coarse fragments and homogenized. Macrofauna was manually removed during the sieving process. The initial experimental soil characteristics were:  $\text{pH}_{\text{water}} = 5.86$ , electrical conductivity =  $0.08 \text{ dS m}^{-1}$ , mineral N (nitrate and ammonium) =  $42.8 \text{ mg kg}^{-1}$ , available phosphorus =  $68.9 \text{ mg kg}^{-1}$ , available potassium =  $187.6 \text{ mg kg}^{-1}$ , total organic carbon =  $2.8 \text{ g kg}^{-1}$ , and total N =  $0.15 \text{ g kg}^{-1}$ .

Tomato seedling plants were prepared in advance by surface-sterilizing the seeds in 10%  $\text{H}_2\text{O}_2$  (v/v) for 30 min, washing and then soaking them in sterile distilled water for 12 h before being germinated on damp gauze at 25 °C for one week. Equally-sized tomato seedlings were individually transplanted into plastic containers (upper diameter 22 cm, lower diameter 14 cm, 19 cm height), filled with 4.0 kg experimental soil. After one week, the four-level earthworm treatments were applied as follows: 1) four similar size *A. corticis* (total average fresh weight:  $9.62 \pm 0.39 \text{ g}$ ), 2) four similar size *M. guillelmi* (total average fresh weight:  $9.78 \pm 0.31 \text{ g}$ ), 3) the combination of two *A. corticis* and two *M. guillelmi* (total average fresh weight:  $9.59 \pm 0.27 \text{ g}$ ), and 4) control plants without earthworms. The amount of earthworm placed in each container corresponds to an average earthworm density ( $105 \text{ individuals m}^{-2}$ ) found in local vegetable fields. To prevent earthworms from escaping, the inside bottoms of the containers were lined with two layers of fleece fabric, while the upper rims were covered by a nylon mesh surface pierced in the centre for the trunk of the tomato plants to pass. Each plant was watered with 50 ml distilled water every second day, and the containers were redistributed randomly within a custom-made climate chamber ( $24 \pm 1^\circ\text{C}$ ,  $70 \pm 5\% \text{ RH}$ , 16h/8h (day/night), 15,000 LX of active radiation) every two weeks.

Four weeks after the beginning of the earthworm treatment, one randomly-chosen branch per plant bearing 5–7 fully expanded leaves was covered with a nylon mesh bag ( $61 \mu\text{m}$  pore size). Prior to thrips inoculation, the thrips rearing jars were placed in a refrigerator at 6 °C for 20 min to reduce their movement. Twenty, one-week old adult WFT (♀: ♂ = 1: 1) were randomly selected from the four rearing jars and transferred into a clean Eppendorf tube (2 ml) using a fine hairbrush. The Eppendorf tubes were then fixed on the petiole of the tomato branch, and then opened for allowing WFT adults to crawl/fly out of the tube and infest the plant. Control (non-infested) plants received the same bag but no thrips were added.

## 2.3. Sampling

Three weeks after the inoculation of the WFT, destructive sampling of each treatment was carried out. Thrips were collected in custom-made traps lined with sticky yellow cards on the bottom (Fig. S1). Specifically, by vigorously shaking the experimental nylon mesh bags in the trap container, thrips were forced to fall at the bottom and being tapped on the sticky card, and later were counted under a dissecting microscope. Immediately after thrips removal, branches were weighted fresh, and flash-frozen in liquid nitrogen. A 500 mg frozen leaf sample

per plant was next stored at  $-80^\circ\text{C}$  until phytohormone and secondary metabolite analyses (see below). The remaining tissues of each branch were freeze-dried for 4 days under vacuum ( $-50^\circ\text{C}$  collector temperature; Christ ALPHA 1–4 LD plus, Germany) and ground to powder using a ball mill (MM200, Retsch, Germany) for primary and nutritional metabolite analyses (see below). Then all shoots were cut at ground level and fresh biomass was recorded. Subsamples of fresh materials were dried at 65 °C for 3 days and then weighed to calculate shoot water content (SWC) and shoot dry biomass (SDB). Finally, 400 g fresh soil samples were collected near roots and stored at 4 °C for soil property analyses (see below). Roots were gently separated from the soil, and earthworms were collected and weighed.

## 2.4. Soil property measurements

Soil pH was determined with a 1:2.5 mass/volume (g/ml) soil-water suspension. For mineral N ( $\text{NH}_4^+\text{-N}$  and  $\text{NO}_3^-\text{-N}$ ) content measurement, 10 g fresh soil was extracted with 50 ml of 2 M KCl and then filtered through ashless filter paper. The concentrations of  $\text{NH}_4^+\text{-N}$ ,  $\text{NO}_3^-\text{-N}$  were determined by a continuous flow analyzer (Skalar Analytical, Breda, the Netherlands). Available phosphorus (AP) content was determined colorimetrically using molybdate after extracting samples with 0.50 M  $\text{NaHCO}_3$  (Olson, 1954). Available potassium (AK) was extracted with ammonium acetate solution, and then determined by a flame photometry (Lu, 1999). Soil basal respiration (SR) was determined using gas chromatography (Alef and Nannipieri, 1995). Briefly, fresh soil (equivalent to 5.0 g dry mass) was weighed into jars and the basal respiration was represented by the  $\text{CO}_2\text{-C}$  evolution for 12 h at 25 °C. Soil microbial biomass carbon (MBC) was determined by the fumigation-extraction method (Vance et al., 1987). Briefly, three replicates of each fresh sample (equivalent to 12.5 g dry mass) were extracted with 50 ml 0.5 M  $\text{K}_2\text{SO}_4$  immediately after non-fumigated and fumigation with  $\text{CHCl}_3$  for 24 h. The extraction efficiency coefficients used to convert the difference in extracted organic carbon between the fumigated and the non-fumigated soil to MBC was 0.38. Soil total carbon (STC) and nitrogen (STN) were determined on dry soil powder using an automated elemental analyzer (Vario MAX CNS, Elementar, Germany).

## 2.5. Plant primary and nutritional metabolite measurements

Leaf total N (LTN) were determined on dry tissue powder using an automated elemental analyzer (Vario MAX CNS, Elementar, Germany). Leaf total phosphorus (LTP) was determined colorimetrically after  $\text{HNO}_3\text{-HClO}_4$  digestion using vanadate-molybdate yellow colour method (Chapman and Pratt, 1962). Leaf free amino acids (LAA) were extracted according to the method described by Rohsius et al. (2005) with slight modification. For each sample, 50 mg of freeze-dried leaf powder were weighed and extracted in 5 ml 10% (v/v) acetic acid with 5.6% (w/v) polyvinylpyrrolidone (PVPP) to remove phenolic compounds, and constantly shaken for 90 min at room temperature (20 °C). The extracts were centrifuged at 10000 g for 10 min at 4 °C. The supernatant was used for total free amino acid determination using Cd-nihydrin method as described in Fisher et al. (2001). Calibration curves were prepared using L-glutamine as standard for amino acid content analysis. Absorbance at 570 nm was measured using a multifunctional Microplate Reader (TECAN Infinite M200, Switzerland).

## 2.6. Plant phytohormone and secondary metabolite analyses

The accumulation of phytohormones jasmonic acid (JA) and salicylic acid (SA) in plant leaves was measured using a modified HPLC-MS/MS method (Pan et al., 2010; Balmer et al., 2013). Briefly, phytohormones from 150 mg fresh weight tissue were extracted using 1 ml EtOAc/formic acid (99.5/0.5 v/v) in 2 ml Eppendorf tubes. All tubes were shaken at 4 °C for 15 min, and then extracted ultrasonically with

ice-bath supersonic device for 15 min. After centrifugation at 13000g for 5 min at 4 °C, the supernatant was transferred to a new tube and evaporated to dryness on a nitrogen evaporator. After dryness, the solid residue was resuspended in 100 µl aqueous methanol (70%). A 10 µl aliquot of this solution was then directly injected into an HPLC-MS/MS system (Agilent 1290/6460 LC-MS system, USA) equipped with a 250 mm × 4.6 mm × 5 µm C<sub>18</sub> Column (Agilent XDB-C<sub>18</sub>, USA). The 1290/6460 LC/MS system was operated at a flow rate of 0.5 ml min<sup>-1</sup>. A mobile phase composed of solvent A (50% UP water) and solvent B (50% methanol) was used in gradient mode for separation. The compounds were detected in the ESI negative mode. Molecular ions (M-H) with m/z 209 (JA) and 137 (SA) were fragmented and relevant daughter ions 59 (JA) and 93 (SA) were recorded for quantification. The collision energy was 15 eV for JA and 10 eV for SA. The concentration was quantified using a calibration equation obtained by linear regression from five calibration points for each analysis. In addition, leaf constitutive defence compounds total phenolics were extracted and measured by the Folin–Ciocalteu colorimetric method as described previously (Ainsworth and Gillespie, 2007; Xiao et al., 2016).

## 2.7. Statistical analyses

All statistical analyses were performed with the R software v3.3.2 (R Development Core Team, 2016). Principal component analyses (PCA) of both soil and plant trait was performed to check for collinearity among traits and remove redundancies (Fig. S2). In total we retained 9 soil variables (pH, NH<sub>4</sub><sup>+</sup>-N, NO<sub>3</sub><sup>-</sup>-N, AP, AK, SR, MBC, STC, STN) and 8 plant traits (SWC, SDB, LAA, LTN, LTP, JA, SA and phenolics). Differences in soil properties and plant traits among treatments (earthworm and thrips treatments) and their interactions were tested using a permutational multivariate ANOVA (PERMANOVA) using the *adonis* function in the *vegan* package in R (Oksanen et al., 2013), and PERMANOVAs were run on the Bray-Curtis distance with 999 permutations per analysis. Results were visualized by non-metric multidimensional scaling (NMDS) using the *vegan* package in R (Oksanen et al., 2013) for all the traits taken together. The effect of earthworms on thrips abundance was assessed with one-way ANOVA. The effects of earthworm and thrips on each variable of soil properties, plant growth traits, plant chemical traits (i.e. nutrient and defence compounds) were assessed with a two-way MANOVAs. Differences within earthworm and thrips treatments were determined by Tukey's post-hoc tests ( $p < 0.05$ ).

To assess how earthworms could simultaneously affect soil and plant chemical traits, we tested for a shared structure between the matrices of soil and plant chemical traits by performing a coinertia analysis (the *coa* function in the *ade4* package in R (Dray et al., 2003; Dray and Dufour, 2007)). The significance of the shared variance was assessed using a Monte Carlo test implemented in the *ade4* package. In other words, we here tested whether the matrices of soil properties and plant chemical traits vary across earthworm treatments. If these were the case, it would lead us to conclude that earthworm treatments impose a similar co-variation between soil and plant chemical traits. Because the coinertia analysis resulted to be significant (see Results), we next fitted the soil property matrix on the plant chemical trait NMDS ordination (function *envfit* in the package *vegan* in R) to explore which soil factors were influenced by earthworm treatments, and shaped changes in plant phenotypes. Afterwards, we performed a linear regression to investigate whether the main axis of the coinertia analysis, representing the shared co-variation between soil properties and plant chemical traits was significantly associated with thrips abundance. Additionally, to assess the relative contribution of the bottom up force of earthworms on patterns of plant resource allocation in response to thrips attack, we performed structural equation modeling (SEM) of earthworm biomass, soil properties, plant growth, plant nutrient and plant chemical defence variables. SEM is a comprehensive statistical method used in testing hypotheses about causal relationships among observed and unobserved (latent) variables and has been proved to be

useful in investigating complex networks of relationships and formulating theoretical constructions (Grace, 2006). Prior to the SEM procedure, we reduced the number of variables for soil properties (9 variables: pH, NH<sub>4</sub><sup>+</sup>-N, NO<sub>3</sub><sup>-</sup>-N, AP, AK, SR, MBC, STC and STN), plant nutrients (3 variables: LAA, LTN and LTP), and plant defences (3 variables: JA, SA and phenolics) through principal component analyses (PCA) on each group of variables separately (Veen et al., 2010). To test how earthworms modulate soil and plant physiological traits in response to thrips attack, we performed the SEM based on the conceptual model of hypothetical relationships as shown in Fig. 1, and based on theoretical expectations (Wurst, 2010). SEM analyses were performed with the *lavaan* package in R (Rosseel, 2012) using maximum likelihood estimation procedures. Model fit was estimated by Pearson's chi-square goodness of fit test ( $\chi^2$ ) ( $p > 0.05$  indicate statistically significant model fit), comparative Fit Index (CFI) ( $> 0.90$  indicates good model fit), and goodness of fit index (GFI) ( $> 0.90$  indicates good model fit) (Grace, 2006). The root mean square error of approximation (RMSEA), which is adjusted for degrees of freedom, and is considered an index of the parsimonious and good-fitting model, was also estimated (Browne and Cudeck, 1992). Standardized path coefficients were presented only when the significance level was less than 0.05. R<sup>2</sup> values were obtained for each dependent matrix, showing the amount of the variance explained by the model (Grace, 2006).

Finally, we tested for the relative contribution of different plant chemical traits on thrips abundance. First, we computed a capscale analysis implemented in the *vegan* package in the R environment to analyse the relationships between different plant chemical traits and thrips abundance. This analysis computes the Bray-Curtis distance between individual plants based on their chemical traits, and tests whether there is a correlation with thrips numbers. Second, to corroborate the capscale analysis, we performed a random forest analysis with all plant chemical traits as predictive variables and thrips number as response variable using the *randomForest* package (Liaw and Wiener, 2002). Random Forest (RF) model analyses are becoming widely used methods for extrapolating the relative importance of predictor variables via multi-tree sampling and self-adjustment (Breiman, 2001; Cutler et al., 2007).

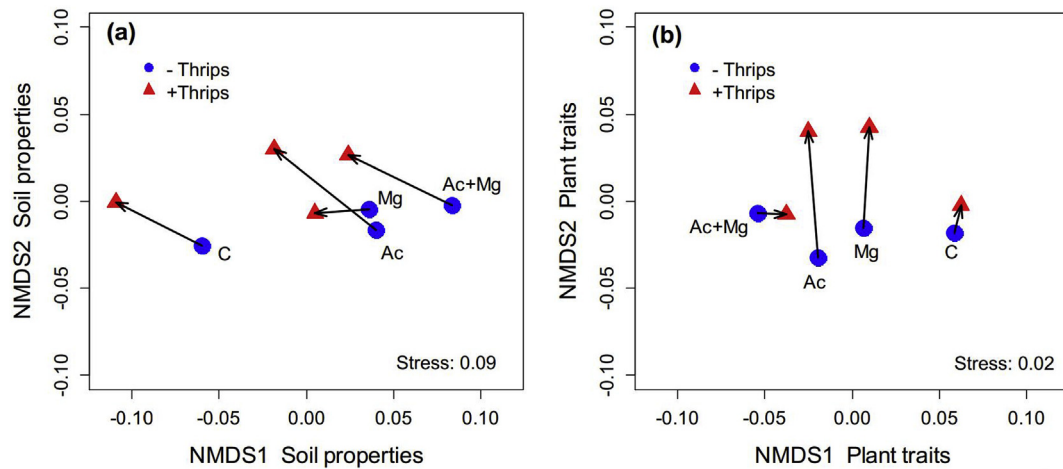
## 3. Results

### 3.1. Earthworm effects on soil properties

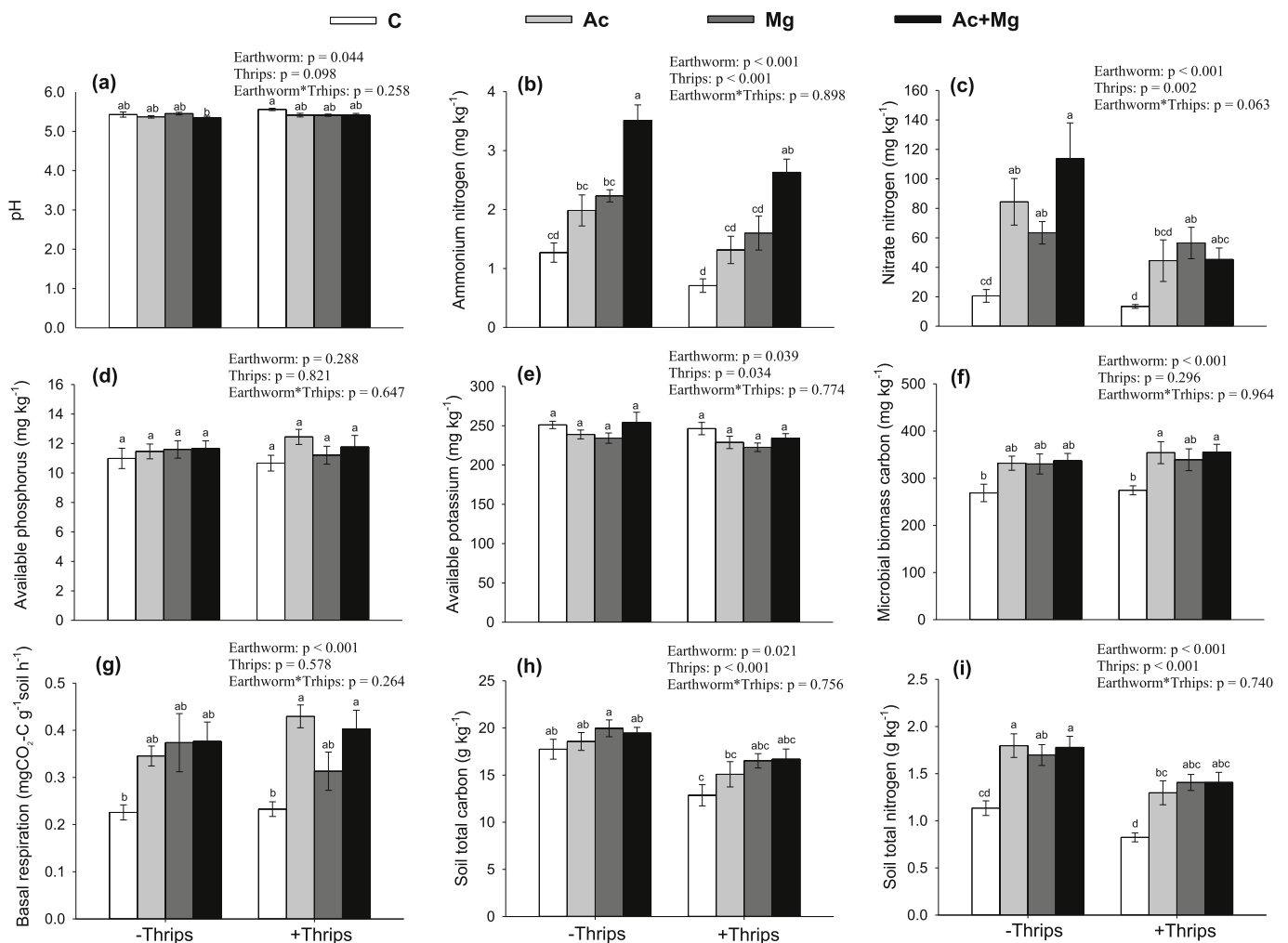
All earthworms survived at the end of the experiment, but the earthworm biomass slightly decreased overall (*A. corticis*: by 7%, *M. guillelmi*: by 15%, the combination of *A. corticis* and *M. guillelmi*: by 10%). Soil properties were significantly influenced by earthworms and thrips, but not by their interactions (Fig. 2a, Fig. S3a; PERMANOVA, earthworm effect:  $F_{3,32} = 8.12$ ,  $p = 0.001$ ,  $R^2 = 0.38$ ; thrips effect:  $F_{1,32} = 5.39$ ,  $p = 0.007$ ,  $R^2 = 0.08$ ; earthworm × thrips effect:  $F_{3,32} = 0.54$ ,  $p = 0.822$ ,  $R^2 = 0.02$ ). More specifically, earthworm *A. corticis* significantly increased soil MBC (Fig. 3f), SR (Fig. 3g) and STN (Fig. 3i) in the presence of thrips; earthworm *M. guillelmi* significantly increased soil NN (Fig. 3c) and STN (Fig. 3i) in the presence of thrips; the combination of earthworm *A. corticis* and *M. guillelmi* impacted soil properties more, and particularly significantly increased soil AN (Fig. 3b), NN (Fig. 3c), MBC (Fig. 3f), SR (Fig. 3g) and STN (Fig. 3i) in the presence of thrips.

### 3.2. Earthworm effects on plant traits

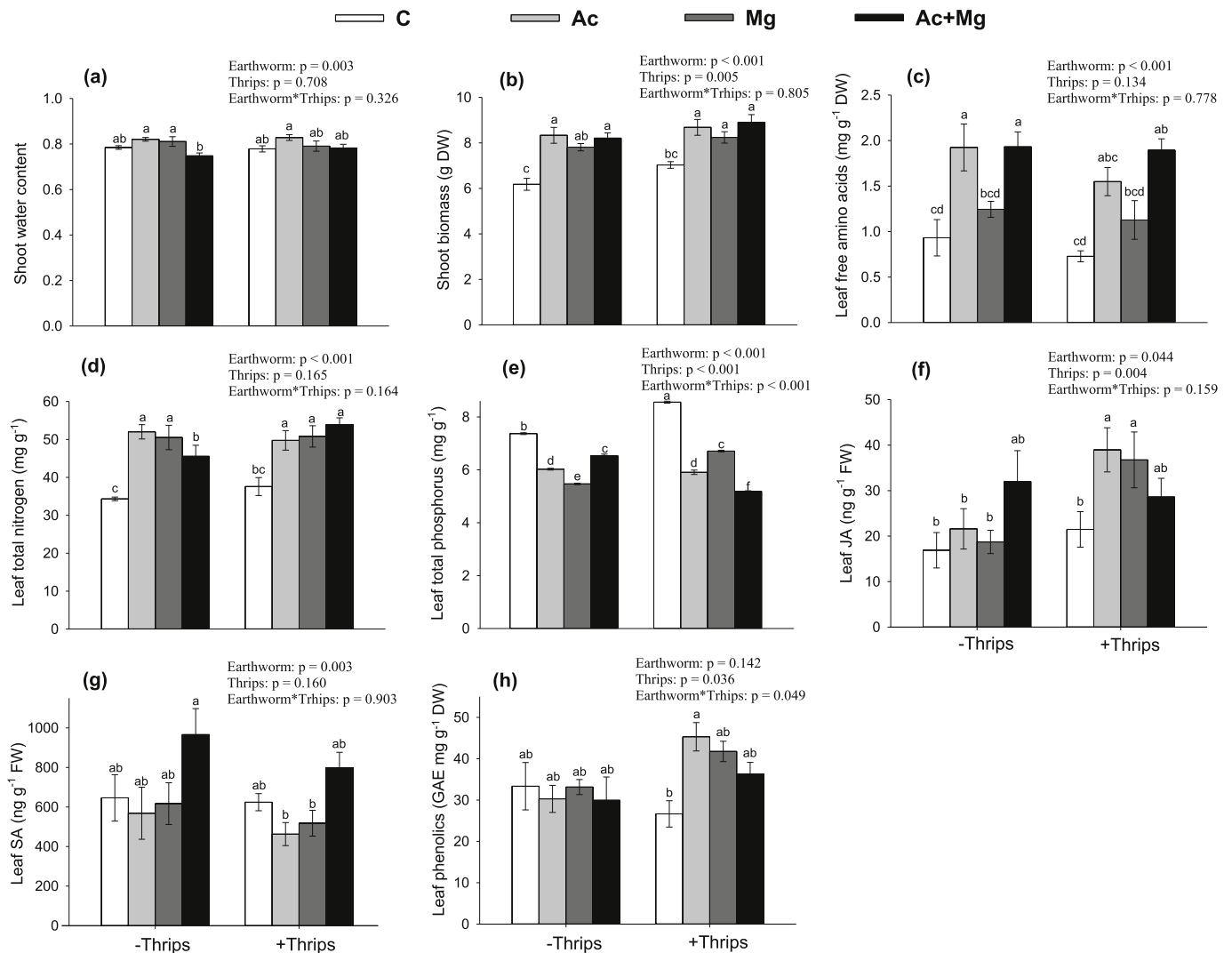
Plant traits was significantly influenced by earthworm treatments, but not by thrips or their interactions (Fig. 2b, Fig. S3b; PERMANOVA, earthworm effect:  $F_{3,32} = 6.14$ ,  $p = 0.001$ ,  $R^2 = 0.35$ ; Thrips:  $F_{1,32} = 1.62$ ,  $p = 0.179$ ,  $R^2 = 0.03$ ; earthworm × thrips effect:  $F_{3,32} = 0.36$ ,  $p = 0.839$ ,  $R^2 = 0.02$ ). For plant growth under thrips attack, earthworm *A. corticis*, *M. guillelmi* and their combination



**Fig. 2.** Nonmetric Multidimensional Scaling (NMDS) ordination plot of the first two axes of soil properties (a), and plant traits (b). Earthworm treatments: C, without earthworm; Ac, with epi-endogeic earthworm *Amyntas corticis*; Mg, with endo-aneic earthworm *Metaphire guillelmi*; Ac + Mg, with earthworm *A. corticis* and *M. guillelmi* simultaneously. Blue dots are plants without thrips (-Thrips), while red triangles represent plants with thrips (+Thrips). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)



**Fig. 3.** Effects of earthworms on soil properties in the absence (-Thrips) and presence (+Thrips) of western follower thrips. (a) Soil pH, (b) ammonium nitrogen (AN), (c) nitrate nitrogen (NN), (d) available phosphorus (AP), (e) available potassium (AK), (f) microbial biomass carbon (MBC), (g) basal respiration (SR), (h) soil total carbon (STC), and (i) soil total nitrogen (STN). Earthworm treatment labels as Fig. 2. Error bars represent means ± SE (n = 5). Two-way MANOVA results were shown in figures and different letters indicate statistically significant differences between thrips and earthworm treatments (Tukey's HSD test, p < 0.05).



**Fig. 4.** Effects of earthworms on plant traits in the absence (-Thrips) and presence (+Thrips) of western flower thrips. (a) Shoot water content (SWC), (b) shoot dry biomass (SDB), (c) leaf free amino acids (LAA), (d) leaf total nitrogen (LTN), (e) leaf total phosphorus (LTP), (f) leaf jasmonic acid (JA), (g) leaf salicylic acid (SA), (h) leaf total phenolics (phenolics). Earthworm treatment labels as Fig. 2. Error bars represent means  $\pm$  SE (n = 5). Two-way MANOVA results were shown in figures and different letters indicate statistically significant differences between thrips and earthworm treatments (Tukey's HSD test,  $p < 0.05$ ).

significantly increased shoot biomass by 23%, 17% and 27%, respectively (Fig. 4b).

For plant chemical nutrients, earthworm presence significantly affected plant nutritional metabolites (e.g. amino acids, total N and total phosphorus) in leaves (Two-way MANOVAs,  $p < 0.01$ , Fig. 4c–e). Particularly, in the presence of thrips, the two combined earthworm species significantly increased soluble amino acids, total N concentration and decreased total phosphorus concentration of leaves (Fig. 4c–e).

Concerning plant chemical defence traits in response to thrips attack, particularly *A. corticis* increased JA concentration by 76% in leaves (Fig. 4f), and decreased SA concentration by 26% in leaves (Fig. 4g). Earthworm presence affected the concentration of leaf phenolics, particularly, earthworm *A. corticis* notably increased leaf total phenolics by 70%, while the earthworm *M. guillelmi* and the combination of these two species tended to increase leaf phenolics by 57% and 36%, respectively (Fig. 4h).

### 3.3. Earthworm effects on thrips abundance

At the end of experiment, the earthworm *A. corticis* significantly decreased thrips abundance by 67% compared to the treatment without earthworms ( $p < 0.05$ ), earthworm *M. guillelmi* tended to decrease

thrips abundance, and the combination of *A. corticis* and *M. guillelmi* did not influence thrips abundance (Fig. 5).

### 3.4. Earthworm-mediated the relationship between soil properties and plant chemical traits

The coinertia analysis showed a significant correlation between soil property matrix and plant chemical trait matrix across earthworm treatments (Monte Carlo test on 999 replicates,  $r = 0.65$ ,  $p = 0.001$ ). Specifically, plant-soil chemical variation aligned along a major axis beginning with high soil pH and AK, and high LTP in leaves, and ending with high SR, NN, STC and STN for soil properties and high LTN, phenolics and JA in leaves (Fig. S4). Taken together, these soil changes resulted in plant chemical traits within the same treatments to significantly cluster in three groups (a cluster with both the single inoculations “Ac” and “Mg”, mainly characterized by high NN and SR, and a cluster obtained from the double inoculation “Ac + Mg”, mainly characterized by large amounts of AN, and a control “C” cluster, characterized by general low amounts of nutrients, MBC and SR) (Fig. 6a). The main axis of the coinertia analysis between soil properties and plant chemical traits was significantly and negatively associated with thrips abundance ( $t = -2.72$ ,  $r = -0.54$ ,  $p = 0.014$ , Fig. 6b).

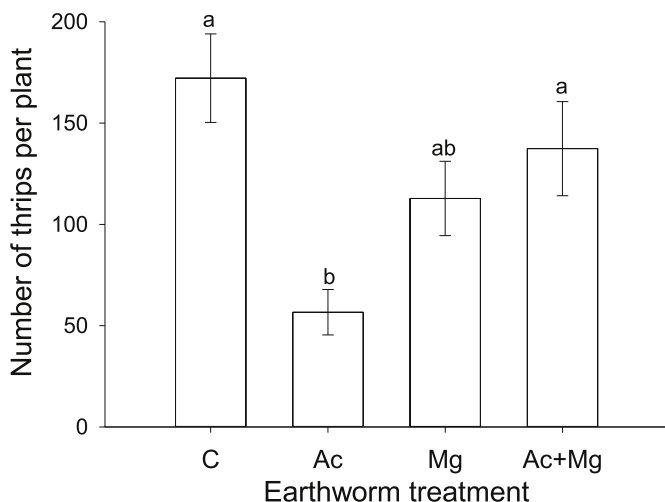


Fig. 5. Effects of earthworms on the abundance of western flower thrips *Frankliniella occidentalis*. Earthworm treatment labels as Fig. 2. Error bars represent means  $\pm$  SE (n = 5) and different letters indicate statistically significant differences among earthworm treatments (Tukey's HSD test,  $p < 0.05$ ).

Furthermore, the SEM analysis showed that the earthworm effects on soil properties ( $p < 0.001$ ) positively influenced plant growth ( $p < 0.001$ ), plant nutrients ( $p < 0.001$ ) and plant chemical defences ( $p < 0.05$ ) (Fig. 7). Thrips abundance was negatively related to plant growth ( $p < 0.001$ ) and plant chemical defences ( $p < 0.001$ ) but was positively related to plant nutrients ( $p < 0.05$ , Fig. 7).

### 3.5. Relative importance of earthworm-mediated plant chemical traits to thrips abundance

The capscale analysis showed that the distance between individual plants based on their chemical traits was significantly correlated to the number of thrips ( $p = 0.001$ ), particularly, there was a direct negative correlation between the number of thrips and JA/phenolics content in plants (phenolics and thrips:  $r = -0.67$ ,  $p = 0.001$ ; JA and thrips:  $r = -0.64$ ,  $p = 0.002$ , Fig. 8a). This observation was corroborated with the random forest analysis, which identified phenolics and JA as the two most important predictors of plant resistance against thrips (Fig. 8b).

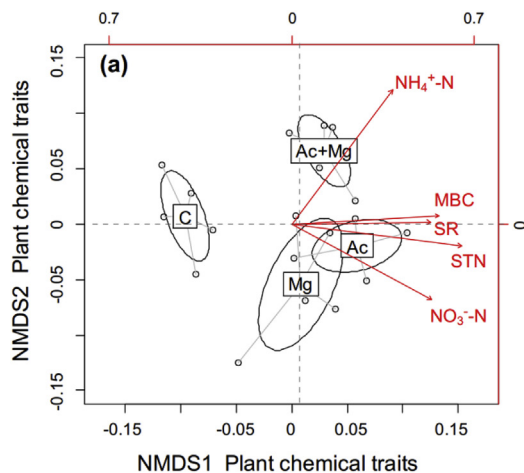


Fig. 6. Effects of earthworms on co-variation between soil properties and plant chemical traits in response to thrips. Earthworm treatment and soil parameters labels as Figs. 2 and 3.

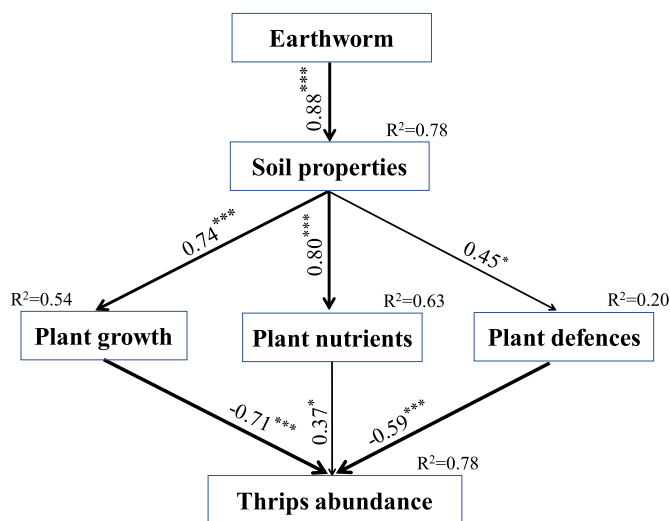
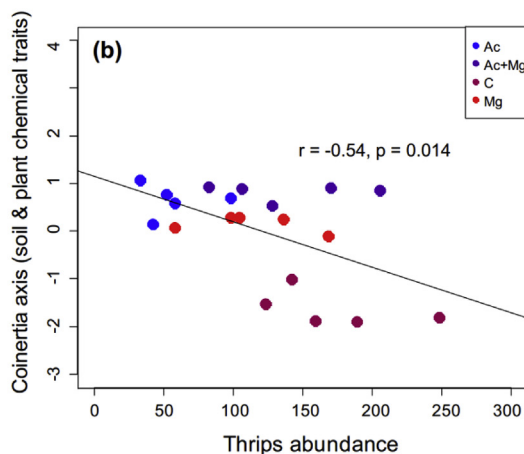


Fig. 7. Structural equation modeling (SEM) demonstrating the effects of earthworms on plant growth, plant nutrients and plant defences on thrips abundance. Model fit summary:  $\chi^2 = 7.21$ ,  $df = 5$ ,  $p = 0.21$ ; CFI = 0.98; GFI = 0.97; RMSEA = 0.14. Numbers above solid arrows are standardized path coefficients (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ) and width of an arrow indicates the strength of the relationships. Standardized path coefficients were presented only when the significance level was less than 0.05, and  $R^2$  values represent the proportion of variance explained for each variable by the model. Soil properties include pH, ammonium nitrogen ( $NH_4^+-N$ ), nitrate nitrogen ( $NO_3^-N$ ), available phosphorus (AP), available potassium (AK), soil basal respiration (SR), microbial biomass carbon (MBC), soil total carbon (STC) and soil total nitrogen (STN). Plant growth includes shoot dry biomass (SDB); Plant nutrients include leaf amino acids (LAA), leaf total nitrogen (LTN), leaf total phosphorus (LTP). Plant chemical defences include jasmonic acid (JA), salicylic acid (SA) and leaf phenolics (phenolics).

## 4. Discussion

This study confirmed that earthworms, independently of species identity, improved soil properties, especially by increasing soil nitrogen availability and microbial activity. However, their effect on above-ground plant-herbivore interactions depended on their identity. Single-species earthworm particularly *A. corticis* increased defence compounds (e.g. jasmonic acid, phenolics), while the combination of both earthworm species favoured accumulation of primary nutrient (e.g. amino acids) more than secondary defence compounds in leaves under thrips attack. This resulted in the earthworm *A. corticis* or *M. guillelmi* as single



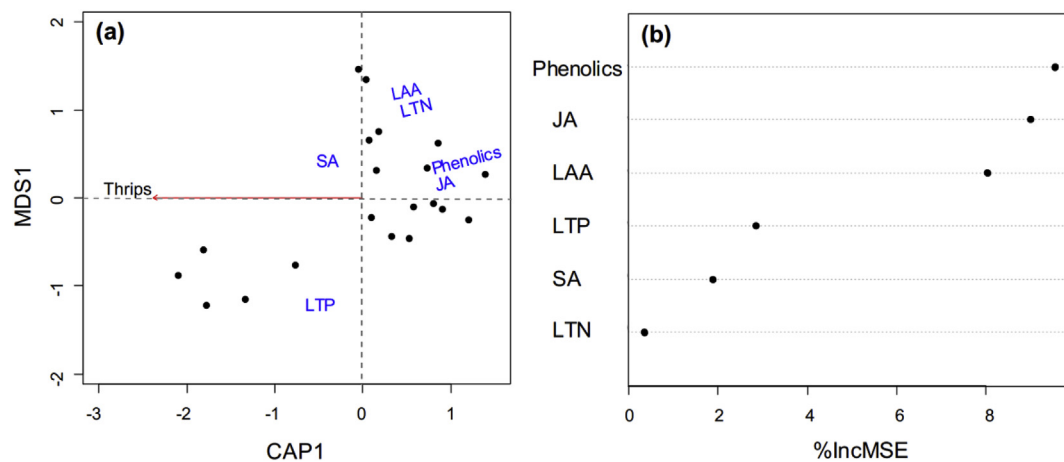


Fig. 8. Relative importance of earthworm-mediated plant chemical traits to thrips abundance. (a) Capscale analysis (CAP) shows that different plant chemical traits relate to thrips abundance. (b) Variable importance plots from random forest prediction model shows the predictor importance (% increase in MSE) of multiple plant chemical traits with respect to thrips abundance across all treatments. Leaf free amino acids (LAA), leaf total nitrogen (LTN), leaf total phosphorus (LTP), leaf jasmonic acid (JA), leaf salicylic acid (SA) and leaf total phenolics (phenolics).

species inoculations to decrease thrips abundance more than the dual-species soil treatment. In sum, variation in earthworm species composition affected soil N and microbial activity differently, and modified patterns of plant resource allocation to growth and chemical defences, in turn ultimately affecting plant resistance against aboveground herbivores.

#### 4.1. Earthworm-mediated effects on soil properties and plant biomass production

In our study, earthworm presence didn't significantly influence soil available phosphorus and potassium contents, as was previously observed in soils inoculated with three earthworm ecological types: the epigeic *Lumbricus rubellus*, the endogeic *Aporrectodea caliginosa* and the anecic *Lumbricus terrestris* (Vos et al., 2014). However, earthworm presence overall led to a strong increase of soil mineral N availability, although the increase was not always significant in the presence of thrips. The increase in soil mineral N has been commonly found in previous experiments (Noguera et al., 2010; Blouin et al., 2013). Several possible mechanisms for explaining earthworm-stimulated N availability have been put forward, including direct and indirect effects. For instance, earthworm epidermal mucus contains  $\text{NO}_3^-$ -N and  $\text{NH}_4^+$ -N (Zhang et al., 2009), which could directly increase soil mineral N content. In addition, through casting and burrowing activities, earthworm could enhance microbial activity and accelerate nutrient mineralization (Sheehan et al., 2008; Ernst et al., 2009).

Moreover, the effects of different earthworm species on soil properties have been generally shown to be species-specific and combination-specific. For instance, the number of bacteria in manure was higher in the presence of epigeic earthworms (*Eisenia fetida*, *Eisenia andrei*, *Eudrilus eugeniae*) than in the presence of the anecic earthworm *Octodrilus complanatus* (Aira et al., 2009). Sheehan et al. (2006) observed synergistic effects on soil nitrate when co-inoculating both endogeic and anecic earthworm species. In the present study, the co-existence of *A. corticis* and *M. guillelmi* increased soil mineral N content more than those in the presence of *A. corticis* or *M. guillelmi* individually. As hypothesized, this result would confirm complementarity in feeding and burrowing activities between the two ecologically distinct earthworm species. Specifically, *A. corticis* has been shown to promote soil carbon cycle and microbial activity (Snyder et al., 2009), while *M. guillelmi* facilitated soil N mineralization (Tao et al., 2009; Wu et al., 2015). Such niche partitioning increases resource utilization, ultimately favouring increased soil structure formation and nutrient mineralization (Postma-Blaauw et al., 2006; Felten and Emmerling,

2009). Therefore, effect of multiple earthworm species on soil properties cannot be easily summarized from the individual species effects, and the synergistic, complementarity or antagonistic effects between the different earthworm species on soil properties should be generally investigated further.

The present study also showed that earthworm presence favoured plant aboveground biomass allocation in response to herbivory (Fig. 3b). This is in line with earlier studies showing that earthworms increased soil nutrient availability, and thereby contributed to the accumulation of plant biomass and nutrients aboveground (Poveda et al., 2005; Zhang et al., 2010; Li et al., 2013; van Groenigen et al., 2014). Interestingly, this effect was stronger when multiple species were inoculated together. As mentioned above, multiple earthworm species facilitate soil N mineralization more than when in isolation. For instance, Newington et al. (2004) found that nitrate concentrations in soils were higher with four earthworm species than that in the presence of single earthworm species, but the nitrate concentrations in throughflow water were lower with four earthworm species than that in the presence of single earthworm species. Thus, a more diverse earthworm community composition favours a generalized increase in soil available N and decreased N leaching under pot condition, which in turn facilitates plant biomass production (Postma-Blaauw et al., 2006; Zhang et al., 2010). The mechanisms that explain such effects might rely on a more efficient soil niche partitioning of the two earthworm species (Sheehan et al., 2006), but this needs to be further tested with further studies.

#### 4.2. Earthworm-mediated effects on plant defences and resistance to thrips

At the end of experiment, fewer thrips remained on tomato leaves when plants grew in the presence of earthworms. However, the inhibitory effects of earthworms on thrips abundance were species- or combination-specific. Particularly, the single earthworm inoculation (*A. corticis*) decreased thrips abundance better than *M. guillelmi* alone, or when in combination (*A. corticis* and *M. guillelmi*), this is contrary to our expectations. Previous studies showed that earthworms could increase plant resistance to pest by up-regulating the expression of defence-responsive genes, and subsequently promoting the production of toxic secondary metabolites (Blouin et al., 2005; Lohmann et al., 2009; Jana et al., 2010). Here, we found that the epi-endogeic *A. corticis* favoured more plant chemical defences (JA and phenolic compounds) accumulation in tomato leaves more than those tomato plants grew in the presence of both earthworm species. The variable effects of different earthworm inoculations on plant chemistry were correlated to changes



in soil properties. Based on the coinertia analysis results, the single earthworm species-mediated changes in plant chemical traits were positive correlated with an increase of soil nitrate, basal respiration, microbial biomass carbon and total N (Fig. 6a), ultimately resulting in a significant increase in the content of foliar defence compounds (e.g. phenolics and JA) (Fig. 4f, h). Conversely, when the two-earthworm species co-occurred, tomato leaves produced free amino acids and salicylic acid, which correlated with changes in soil ammonium. Taken together, these results indicate that the dual-species inoculations mediate an increase in plant nutrient content by promoting soil nitrogen availability. Such enhanced plant nutritional quality might have offset plant chemical defences, and in turn make plants more palatable to thrips. Accordingly, Pobożniak and Koschier (2013) found that higher leaf N and sucrose contents promote thrips population growth in pea (*Pisum sativum*) plants. Nonetheless, other studies showed that the presence of earthworms, such as *Octolasion tyraeum*, decreased the concentrations of chemical defence compounds (glucosinolates glucoiberin and glucoraphanin) in shoots of *Brassica oleracea* (Wurst et al., 2006). Therefore, earthworms can modify plant resource allocation to growth or defence, but such effects are highly context and species-dependent, which should be ultimately studied across a wide range of species and conditions.

Our results also indicated that soil microbial activity, as represented by basal respiration, in the presence of *A. corticis* was higher than that in the presence of earthworm *M. guillelmi* or *A. corticis* or *M. guillelmi* simultaneously. Therefore, the positive effect of *A. corticis* earthworms on plant chemical defence compounds could also be potentially linked to their positive effects on the increase in soil microbial activity. Indeed, a growing body of literature is showing that soil microorganisms can prime plants to respond more strongly and faster to aboveground herbivore attack (Badri et al., 2013; Pineda et al., 2013). Unfortunately, at the current stage this is only speculative, and future studies relating earthworm-mediated microbial community composition changes with plant defence expression during herbivore attack are needed to effectively address this hypothesis.

## 5. Conclusion and perspective

We found that single earthworm species soil inoculations indirectly suppressed thrips abundance more than soil inoculations composed of two earthworm species, mainly through facilitating plants to produce more defensive metabolites and less nutritional metabolites. Therefore, our results suggest that the earthworm-mediated effects on allocation patterns of plant resistance are strongly species- or combination-specific. However, why earthworms of different ecological types alter resource allocation patterns and plant resistance differentially is yet unknown. Studying the effect of earthworms on soil properties that are best linked to plant eco-physiological traits should provide insights in this direction. Such information could be used to improve environmental-friendly integrated pest management (Altieri, 1999).

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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.2018.11.023>.

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