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Enhanced plant growth in the presence of earthworms correlates with changes in soil microbiota but not nutrient availability

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ABSTRACT

Earthworms enhance plant growth but the precise mechanism by which this occurs is not known. An understanding of the mechanism could potentially support changes in agricultural management reducing fertiliser usage and therefore costs and the carbon footprint of agriculture. We conducted a factorial experiment in which 5 strains of wheat were grown in the presence and absence of earthworms under regular watering and droughted conditions. The different wheat strains all responded in a similar fashion. Plant biomass was greater in the presence of earthworms and under regular watering. The presence of earthworms reduced the impact of drought on plant biomass and also slowed down the rate of drying of the droughted soils. Plant nutrient content (N, P, Si) showed no consistent pattern with treatments but plant total N, P and Si mirrored plant biomass and decreased in the order earthworm-present watered > earthworm-present droughted > earthworm-absent watered > earthworm-absent droughted. Nutrient availability in the soil, as assessed by chemical extractions showed no consistent pattern with treatments. Differential gene expression of plants was greater between watering treatments than between earthworm treatments. Genes that were differentially expressed between the earthworm treatments predominantly related to plant defences, abiotic stress and control of plant growth though a couple were linked to both nitrogen cycling and stress responses. The soil microbiome of the earthworm-present treatments was more associated with nutrient-rich environments, the promotion of plant growth and the suppression of plant pathogens whilst that of the earthworm-absent treatments included a variety of plant pathogens. Our data are consistent with enhanced plant growth being due to changes in the microbiome brought about by earthworm processing of the soil rather than changes in nutrient availability directly due to earthworm activity.

1. Introduction

Earthworms are ecosystem engineers that have a significant impact on a variety of soil processes and through this can influence plant growth (Blouin et al., 2013). It has been hypothesised that earthworms may promote plant growth by a variety of mechanisms including increased nutrient availability, increased abundance and activity of beneficial micro-organisms in the soil, reduced populations of pathogens, production of plant growth promoting hormones and, the modification of soil structure (Scheu, 2003; Brown et al., 2004). Evidence for the role of earthworms impacting plant productivity through soil nutrient availability is presented by van Groenigen et al. (2014). Through meta-analysis they concluded that the presence of earthworms increased above ground plant biomass by, on average, 23%, predominantly through the release of nitrogen from organic matter. Although van Groenigen et al. (2014) reported little evidence for a role of earthworms in mobilising phosphorus, recent studies have suggested otherwise (e.g. Ros et al., 2017; Vos et al 2019). Silicon (Si) accumulation by plants can

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improve growth under stress (Cooke et al., 2016; Debona et al., 2017; Singh et al., 2020; Thorne et al., 2020). However, the beneficial effects of Si depend critically on both soil conditions (Wade et al., 2022) and plant genotype (Thorne et al 2021; Thorne et al., 2022); some studies indicate that earthworms can increase plant available Si (Bityutskii et al., 2016; Hu et al., 2018). Thus changes in the availability of a number of key nutrients can help explain increased plant growth in the presence of earthworms.

Van Groenigen et al. (2014) found similar earthworm impacts on above ground biomass for a range of different crop types though within each crop type there was a significant range in the extent of those effects. One component of this variation may be due to the use of different cultivars or strains of a particular crop type in the different studies. For example, different strains are known to differ in their response to different levels of nitrogen fertilisation or availability (e.g. Manschadi and Soltani, 2021; Belete et al., 2018), their uptake of Si and the impact that this has on drought tolerance (e.g. Thorne et al., 2021) and their response to different levels of P (e.g. Lin et al., 2020).

Earthworms can impact the soil microbiome in a number of ways (Brown, 1995). Soil conditions can be changed via digestion of organic matter and the release of nutrients and via burrowing activities that can change soil aeration, oxygen levels and moisture contents; changes in soil conditions can in turn change which microbes can thrive in an environment. Earthworms are known to exhibit feeding preferences (Bonkowski et al., 2000, Goncharov et al., 2020) which can lead to both direct changes in abundance and increased abundance of otherwise competitively excluded taxa. The varying chemical environments experienced by microbiota during gut transit can also lead to changes in diversity (e.g. Brown, 1995; Furlong et al., 2002). Changes in microbial diversity due to consumption by earthworms have been reported to help control fungal pathogens either by direct consumption of the pathogens (e.g. Jorge-Escudero et al., 2021; Goncharov et al., 2020) or by altering the bacterial community which in turn impacts on the fungi (e.g Clapperton et al., 2001). Earthworm mucus has also been found to have a negative effect on fungi (e.g. Plavšin et al., 2017) and plant-feeding nematodes (Yu et al., 2019). Whilst there are various reports in the literature of changes in the bacterial population due to the activity of earthworms, there is significant variation amongst studies as to which bacterial taxa are enriched and which diminished by earthworms, relating to differences in other soil properties (Medina-Sauza et al., 2019). Changes in the microbiome can lead to increased mineralisation of C and N and increased solubility of P which could, in turn, result in increased plant growth (Medina-Sauza et al., 2019). Additionally, earthworm-stimulated plant growth may, in part, be due to the release of signal molecules that ultimately lead to the production of phytohormones involved in plant immune systems and growth regulation, either direct from the earthworm or from the earthworm-altered microbiome (e.g. Puga-Freitas et al., 2012a; Puga-Freitas and Blouin, 2015).

In previous studies earthworms have been determined to stimulate plant defences. For example, increases in jasmonic acid, a defence-related phytohormone, and phenolic compounds in tomato plants in the presence of earthworms have been linked to resistance to the western flower thrip (Xiao et al., 2017) and nematodes, but not to other plant herbivores that are chewers or phloem-feeders rather than cell feeders (Xiao et al., 2018). Blouin et al. (2005) found that increased tolerance of rice to nematodes was linked to the presence of earthworms inducing changes in expression of stress genes involved in the jasmonate signalling pathway in plants and suggested that this occurred due to physical damage caused by earthworms on plant roots. Similarly, Lohmann et al. (2009) found that the presence of earthworms increased defence compounds in plants, countering the effects of nematodes. Puga-Freitas et al. (2012a) observed upregulation of a range of genes associated with interactions between plants and other organisms, including genes involved in plant defences, in the presence versus the absence of earthworms. They noted that changes in the plant transcriptome were consistent with responses to the molecule flagellin and suggested that earthworms produce small molecules that can directly stimulate plant defences. We also note that many plant defences are triggered by changes in the microbiome including changes in the abundance of potential pathogens and non-pathogens (Nishad et al., 2020; Singh et al., 2016; Martinez-Hidalgo et al., 2015).

There is a growing trend towards the uptake of reduced tillage and regenerative agriculture (Schreefel et al., 2020; Giller et al., 2021). These practices should lead to increased earthworm numbers in arable settings (e.g. Eriksen-Hamel et al., 2009; Prendergast-Miller et al., 2021). The enhanced plant growth observed in the presence of earthworms is unlikely to remove the need for fertiliser applications to arable crops under such systems, but reduced fertiliser inputs may be possible which could help reduce the C footprint and financial cost of modern agriculture. Similarly as droughts become more frequent due to climate change (Milly et al 2002; Prudhomme et al., 2003) the presence of increased earthworm populations, and the increased plant growth that these usually engender, may mitigate the impacts of reduced plant growth during the actual droughts. Furthermore, depending on the mechanism by which earthworms impact on plant growth it is possible that despite earthworm activity decreasing during droughts (e.g. Bohlen et al., 1995; Plum and FIlser, 2005) a legacy effect might reduce the impact of drought on plant-growth although studies suggest the opposite may also be the case (Blouin et al., 2007). A full understanding of how earthworms boost plant growth is necessary to determine whether or not earthworm effects should be incorporated into crop growth models, to maximise the benefits that earthworms may deliver to modern agriculture and to quantify their economic benefit. This may then guide incentives-based policies for earthworm-friendly farming practices (e.g. Plaas et al., 2019). Furthermore, understanding how earthworms boost plant growth may allow farmers to modify management practices to increase this effect.

In this study we carried out plant growth experiments in which different strains of wheat were grown in the presence and absence of the endogeic earthworm *Allolobophora chlorotica* and under regular or drought watering regimes. Plant growth, nutrient content and gene expression, soil nutrient availability and the soil microbiome were measured. This allowed us to test the following hypotheses. Earthworm activity:

1. results in increased nutrient availability which in turn leads to increased plant growth,
2. increases the relative abundance of microbiota beneficial to plant growth,
3. reduces the impact of drought on plant growth, and
4. affects different strains of wheat differently.

2. Methods

2.1. Experimental design

A factorial experiment was designed with wheat strain, earthworm presence and, water availability as the experimental conditions manipulated. Five diverse Northern European wheat strains were selected for this study; 3 landraces from the AE Watkins Collection (with Accession identifiers 1190336-1, 1190451-1, 1190779-1), Shamrock from the Gediflux Northern European Wheat collection (identifier 40037), and commercially available Skyfall (RAGT) identified as strains 1 through 5 respectively (n = 5; see Table S3 for complete list of accession identifiers). Two wheat plants of each genotype were grown in soil either in the presence or absence of the green morph of *Allolobophora chlorotica* earthworms (E for earthworm-present, N for earthworm-absent; n = 2) subjected to "normal" or "drought" conditions (W for "normal" conditions, D for "drought" conditions; n = 2) after the plants were established. Each treatment comprised 4 replicates giving 80 individual plant pots in total which are referred to as XYZA where X identifies the wheat strain, Y the presence / absence of earthworms, Z the watering condition

and A the replicate number e.g. 2ED3 indicates the 1190336–1 strain (strain 2), in the presence of earthworms under drought conditions and is the third replicate of the treatment. The experiment was carried out in a glasshouse with set temperature points of 20 °C daytime and 16 °C night time with a typical range of ± 5 °C. High-pressure sodium lamps (Philips 400 W), programmed to maintain a 16-hour photoperiod coinciding with the daytime temperature setpoint were used to supplement ambient sunlight conditions when these fell below 150 W m^{-2}. Pots were arranged randomly in four 4 × 5 pot blocks with each block comprising one pot of each treatment.

2.2. Soil

Soil was collected in the early autumn (Oct 2019) from BSSE field, Leeds experimental farm. This field had been arable for at least 16 years, predominantly under winter wheat but with potatoes, vining peas, oilseed rape and beets as rotation breaks. The soil is a silt loam, has a pH of 7.69 ± 0.01 and an organic matter content as determined by loss on ignition at 350 °C of 3.20 ± 0.06% (±std. dev, n = 3) (Hallam et al., 2020).

Soil was hand sorted to remove earthworms, large stones and roots. 853.8 ± 0.9 g (mean, ± stdev, n = 80) field moist soil was added to each of 80 one litre pots. 5 mm diameter drainage holes were present at the base of the pots so prior to adding the soil the pots were lined with fine nylon mesh to ensure that earthworms could not escape through the holes. Similarly, the tops of the pots were lined with hook and loop fastener to reduce the likelihood of earthworms escaping over the pot edges (Lubbers and van Groenigen, 2013).

2.3. Wheat

The wheat seeds were germinated on wet tissue paper at room temperature. After 6 days, germination efficiency was 94, 84, 82, 75 and 100% for strains 1 – 5 respectively. Two seedlings of a specific wheat strain were added to each pot.

2.4. Earthworms

Clittelate *Allolobophora chlorotica* were collected from Warren paddock, a pasture field at Leeds experimental farm close to BSSE field. This species was the most common species found in the BSSE field from which the soil was collected (Prendergast-Miller et al., 2021). Earthworms were rinsed, weighed (0.20 ± 0.05 g, mean ± stdev, n = 120) and, two days after transferring the seedlings to the pots, three earthworms were added to each earthworm treatment pot to give an earthworm biomass of 0.59 ± 0.07 g per pot (n = 40), equivalent to c. 300 earthworms m^{-2} and a biomass of c. 60 g m^{-2} which was similar to that found in the BSSE field (Prendergast-Miller et al., 2021).

2.5. Watering regime and in-experiment measurements

Plants were watered with deionised water on an *ad hoc* basis for 38 days. After this time the drought pots were no longer watered whilst watering of the normal pots continued until Day 53 when all the plants were harvested.

On Days 38 and 53 the length of the two longest leaves of each plant were measured with a ruler and the chlorophyll content of the same leaves was measured with an atLEAF CHL BLUE chlorophyll meter. Chlorophyll content is reported as the raw C values obtained from this instrument. They can be converted into SPAD values using the relationship SPAD = (0.93 × C value) = 7.6, $R^2 = 0.78$ as determined by Zhu et al. (2012). The moisture content of the soil was measured on Days 38, 45 and 53 using a ML3 Theta probe connected to a HH2 meter (both Delta T devices Ltd); moisture content was recorded in mV and also as volumetric water content using the default mineral soil settings and is reported here as %vol.

2.6. Plant processing and measurement

On Day 53 above ground biomass was harvested by cutting the plant stems 0.5 cm above the soil surface. One leaf each from both plants in a plant pot was placed in the same Eppendorf. The leaves were flash frozen in liquid nitrogen and stored at −80 °C. Subsequently the material was ground whilst frozen using a Retsch MM200 ball mill and extracted for RNA using Omega Bio-Tek EZNA plant RNA kits. After extraction, RNA concentrations were quantified and equal amounts of RNA were pooled from plants of the same accession and treatment to control for the effect of environment on the transcriptome, with different accessions providing biological replicates for each treatment. The 20 resulting RNA samples were sequenced using the Illumina Novaseq platform, reads mapped to the IWGSC V1.1 reference (https://wheat-urgi.versailles.inra.fr/Seq-Repository/Assemblies), and transcript read counts generated by Novogene Co Ltd.

The remaining above ground biomass was weighed, placed into preweighed paper bags, dried to constant weight at 60 °C and weighed again. The oven-dried plant material was ground in a Retsch MM200 ball mill and subsampled for analysis of C and N using an Elementar Vario macro elemental analyser and for Si and P by X-ray fluorescence. For the CN analyser, samples of c. 50 mg were analysed. Elemental Analysis Birch leaf standard OAS, catalogue number B2166, certificate number 136,621 was run as a certified reference material and gave recoveries of 99.8 ± 0.8 and 101.0 ± 6.4% (n = 4, ± stdev) for the reported C and N concentrations of 48.09 and 2.12 wt% respectively. For the Si and P analysis, samples of c. 0.15 g were analysed as pressed pellets (produced using a Specac Atlas manual 15 ton hydraulic press) using a Niton XL3t900 GOLDD Analyser (Themo Scientific UK) portable X-ray fluorescence instrument (Reidinger et al., 2012). Both sides of the pellet were analysed and reported values are an average of these values. For 22 of the samples (1ED1, 1ND1, 1ND2, 1ND4, 2ED1, 2NW1, 2NW3, 2ND2,3NW3, 3ND3, 4ED1, 4NW1, 4NW3, 4NW4, 4ND1, 4ND2, 5ED4, 5NW1, 5ND1, 5ND2, 5ND3, 5ND4) there was insufficient sample to produce a pressed pellet that held together and in these cases a pellet was made with a KBr base and the plant side of the pellet analysed twice. Material for sample 2ND1 was lost. Two in house pressed pellets of plant samples with Si concentrations in the range 0.53 – 0.67 wt% were used for quality control; both sides of each pellet were analysed every 10 – 15 samples and mean measured concentration was 0.67 ± 0.03% (±stdev, n = 158).

2.7. Soil processing and measurement

Following harvesting of the above ground biomass, soil was emptied from the pots and homogenised. Earthworms were retrieved from the pots, counted and weighed. A 50 mL centrifuge tube was filled with soil and this material was freeze dried and stored in a sealed container with desiccant (silica gel) for c. 8 months. DNA was subsequently extracted from 0.242 ± 0.008 g (mean ± std dev, n = 80) of this material using a Qiagen DNeasy PowerSoil Kit following the manufacturer's instructions, eluted in SIGMA water (W4502-1L) and stored at −20 °C prior to PCR amplification.

Bacterial DNA was amplified using primers 515F-Y-ill (TCGTCGGCAGCGTCAGATGTGTATAAGAGACA-GANNNHNNNWNNNHGTGYCAGCMGCCGCGGTAA) (Parada et al., 2016) and 806rmod-ill (GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGG ACT ACN VGG GTW TCT AAT) (Apprill et al., 2015). The primers include Illumina sequencing tags and the forward primer includes a random dodecamer to facilitate cluster analysis. The 50 μL amplification reaction comprised 5 μL of DNA extract, 1X Green GoTaq buffer, 0.2 mM dNTPmixture, 0.2 μM of each primer, and 0.025 U μL^{-1} of GoTaq G2 DNA Polymerase made up to a 50 μL volume with 31.75 μL of molecular grade H_2O. PCR reaction conditions were initial denaturation at 95 °C for 2 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 45 s and extension at 72C° for 1 min 30 s followed

by a final extension at 72 °C for 10 min.

Fungal DNA was amplified by semi-nested PCR. The first round of amplification used a 1:10 dilution of the DNA extract, the primers were ITS1f (CTTGGTCATTTAGAGGAAGTAA) (Gardes and Bruns, 1993) and ITS4 (TCCTCCGCTTATTGATATGC) (White et al., 1990); for the second amplification the primers were gITS7-ill (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGANNNHNNNWNNNHGTGARTCATCGARTCTTTG) (Ihrmark et al., 2012) and ITS4-ill (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TCCTCCGCTTATTGATATGC) with Illumina sequence tags as described above. The first 20 μL amplification reaction comprised 5 μL of x10 diluted DNA extract, 1X buffer, 0.2 mM dNTPmixture, 0.2 μM of each primer, and 0.0625 U μL^{-1} of GoTaq G2 DNA Polymerase made up to 20 μL with 9.55 μL of molecular grade H_2O. The second amplification reaction comprised 1 μL of the primary PCR product, 1X Green GoTaq buffer, 0.2 mM dNTPmixture, 0.2 μM of each primer, and 0.025 U μL^{-1} of GoTaq G2 Polymerase made up to 50 μL with 35.75 μL of molecular grade H_2O. PCR reaction conditions were initial denaturation at 95 °C for 2 min, 25 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 45 s and extension at 72C° for 1 min 30 s followed by a final extension at 72 °C for 10 min.

The size of the final PCR products (c. 300 bp) was confirmed by agarose gel electrophoresis. The amplified DNA was purified using Beckman Coulter Agencourt AMPure XP beads with a 1:0.8 sample:bead mix and two washes in 70% ethanol then eluted in 20 μL of molecular grade H_2O. Purified PCR products were quantified using a Quant-iT™ dsDNA Assay fluorescence kit (Invitrogen, Life Technologies, Carlsbad, CA, USA). PCR products were diluted to 10 ng μL^{-1} where possible, though for some, concentrations were as low as 2.94 ng μL^{-1} for the bacterial DNA and 2.41 ng μL^{-1} for the fungal DNA. c. 20 μL of PCR product were provided for Illumina Miseq sequencing (Illumina Inc., San Diego, CA, USA) at the University of York, UK. Amplicon libraries by sample and gene target were generated using cutadapt (Martin, 2011) to select for primer sequences. The resulting sample sets for bacteria and fungi were analysed separately using DADA2, with default parameters (Callahan et al., 2016).

After subsampling for DNA extraction the remaining soil was weighed, oven dried at 105 °C and reweighed to determine moisture content. pH was measured. Available nitrate was determined using a 1 M KCl extraction with 8 g soil shaken in 40 mL of solution for 1 h (Rowell, 1994). Olsen P was determined using a 0.5 M $NaHCO_3$ extraction adjusted to pH 8.5 with 5 g soil shaken in 100 mL of solution for 30 min (Olsen et al., 1954). Nitrate and Olsen P solutions were analysed using a Seal AA3 autoanalayser. For nitrate analyses instrumental accuracy was determined as 103% through analysis of an in-house 0.5 mg L^{-1} reference material, precision determined by repeat analysis of 10% of the samples (Gill et al., 1997) was 4.4% and the detection limit, determined by ten repeat analyses of the calibration blank (Walsh, 1997) was 0.03 mg L^{-1}, equivalent to c. 0.145 mg kg^{-1}. Method blank concentrations were just above detection (0.04 ± 0.01 mg L^{-1}, n = 4, stdev) and so results were blank corrected. For phosphate analysis instrumental accuracy was determined as 106% through analysis of an in-house 1.0 mg L^{-1} reference material, precision determined as above was 1.0% and the detection limit, determined as above, was 0.004 mg L^{-1}, equivalent to c. 0.077 mg kg^{-1}. Method blank concentrations were below detection. Readily available Si was determined using a 0.01 M $CaCl_2$ extraction (Georgiadis et al., 2013) with 6 g soil shaken in 30 mL of solution for 24 h. Si was measured using a Thermo iCAP 7000 inductively coupled plasma-optical emission spectrometer. Instrumental accuracy was determined as 107% through analysis of an in house 0.5 mg L^{-1} reference material, precision determined as above was 3.9% and the detection limit, determined as above was 0.18 mg L^{-1}, equivalent to c. 0.91 mg kg^{-1}. Method blank concentrations were below detection.

2.8. Statistics

All data other than the plant RNA and soil DNA data, and non-normally distributed data were analysed in SigmaPlot for Windows 14.5 by 3-way Analysis of Variance (ANOVA) followed by Holm-Sidak post hoc tests to compare between treatments with wheat strain, presence or absence of earthworms and regular watering or drought as factors. Normality and equal variance were assessed using the Shapiro-Wilks and Brown-Forsythe tests respectively. Of the data sets investigated several were not normally distributed (above ground dry biomass, plant %N, plant %C, plant total P, soil nitrate, fungal alpha diversity at genus level, pre- and post-drought leaf length, pre-drought leaf chlorophyll content and soil moisture, and final earthworm mass) and four (above ground dry biomass, total plant P, soil nitrate, and final earthworm mass) did not have equal variance. Transformations converted some of the data sets to a normal distribution (square root for above ground dry biomass, inverse for plant %N, log10 for soil nitrate and square for pre-drought moisture levels) and given the robustness of ANOVA to heterogeneous variance and our aim to look at interactions between our factors, 3-way ANOVA was still used in our analysis (Underwood, 1996) for these data. However, we were unable to transform the remaining data to obtain normal distributions and therefore, for those data sets, the non-parametric Scheirer Ray Hare test (Holmes et al., 2016) was used for comparison between our factors. Data were ranked in Excel prior to carrying out 3-way ANOVA on the ranked data in SigmaPlot. The sum of squares and degrees of freedom output from the ANOVA was then used to calculate the Scheirer Ray Hare H factor, significance was determined used the chi squared distribution function with the corresponding degrees of freedom. For the soil DNA data, the phyloseq (v. 1.36.0, McMurdie and Holmes, 2013) and vegan (v. 2.5.7, Oksanen et al., 2022) packages were used in RStudio for Windows (version 2021.09.0) running R (v. 4.1.1) to calculate Bray-Curtis distance matrices for the genus level data and perform ecological analysis. PERMANOVA analysis was carried out to determine significant differences between total communities found between treatments. Differences between treatments were also visualized through non-metric dimensional scaling (NMDS) ordinations based on the Bray-Curtis distances between samples. Significant differences in the relative abundance of taxa were assessed in RStudio using ANOVA with a Benjamani-Hochberg correction for multiple comparisons in the tidyverse (version 1.3.1, Wickham et al., 2019) package. For the plant RNA data, differential expression analysis to compare earthworm-present vs earthworm-absent, and drought vs watered treatments as well as their interactions was performed using the DESeq2 package (Love et al., 2014) incorporating the ashr shrinkage function from Stephens (2016). For all statistical analysis significant differences were deemed to be present when $p \leq 0.05$. For the plant RNA analysis a cut off value of ± 2 for the log fold change was also applied. In the Results sections only significant differences are reported. To compare the function of differentially expressed genes, gene ontology (GO) terms were analysed using AgriGO V2.0 (Tian et al., 2017) singular enrichment analysis (SEA) and cross comparison of SEA tools.

3. Results

3.1. Changes in soil moisture content

Moisture levels in all treatments are reported in Table S1. Prior to the droughting there were small differences in the moisture content of the earthworm-present vs earthworm-absent treatments (Fig. 1a, Table S2a). After 6 days of droughting, moisture content was reduced in the droughted treatments; earthworm-present treatments contained more moisture than the earthworm-absent treatments and there were no interactions (Fig. 1b, Table S2b). At the end of the drought period the soil moisture contents of four drought samples (1ED4 22.8 %vol, 2ED2 27.8 %vol, 4ND2 25.5 %vol and 4ND4 33.2 %vol) suggested that these

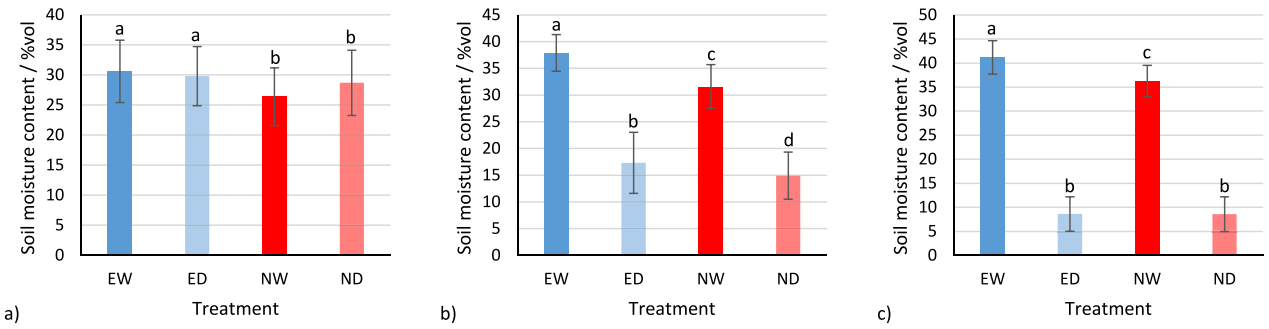

Fig. 1. Average soil moisture contents of the earthworm and watering treatments a) immediately before the start of droughting (Day 38), b) mid-way through the drought (Day 45) and c) at the end of the experiment (Day 53). EW = earthworm-present, watered; ED = earthworm-present, droughted; NW = earthworm-absent, watered; ND = earthworm-absent, droughted. Values are averages ± standard deviations. n = 20 for the pre-drought conditions and for the EW and DW post drought treatments, n = 18 for the ED and ND post drought treatments. For each graph different letters above the bars indicate a significant difference at $p \leq 0.05$.

had been watered by accident; average moisture content of the other drought treatments was 8.6 ± 3.5 %vol (n = 36, stdev) and the watered treatments had an average moisture content of 38.7 ± 4.2 %vol (n = 40, stdev). These samples were therefore removed from further analysis. The moisture contents of the droughted earthworm-present and earthworm-absent treatments were not different but in the watered treatments the earthworm-present treatment contained more moisture than the earthworm-absent treatment (Fig. 1c, Table S2c).

3.2. Earthworm mass and survival

Initial mass of individual earthworms was 0.197 ± 0.046 g (n = 120, stdev). There were differences in survival between the watering treatments. At the end of the experiment average earthworm numbers were 2.4 ± 0.8 (n = 20, stdev) in the watered treatments and lower (2 way ANOVA, $p \leq 0.01$) at 1.0 ± 1.1 (n = 18, stdev) in the droughted treatments; the majority of surviving earthworms in the drought treatments were aestivating. There was no difference in survival between wheat strains.

At the end of the experiment there were differences in earthworm mass between the watered and droughted treatments and an interaction between watering treatment and the initial and final masses of the earthworms. Over the duration of the experiment earthworm mass increased to 0.315 ± 0.080 g (n = 48, stdev) in the watered treatments and decreased to 0.112 ± 0.029 g (n = 18, stdev) in the droughted treatments relative to the initial mass but showed no difference between wheat strains.

3.3. Plant physical and chemical parameters

Full details of plant leaf length, chlorophyll content and biomass on a by treatment basis are given in Table S3. Up to the point that the drought conditions were imposed on the plants, leaf length showed no difference between the watered and droughted treatments or the earthworm treatments (Fig. 2a) but there were differences between wheat strains (Tables S3 and S4a). Similarly, there was no difference in the

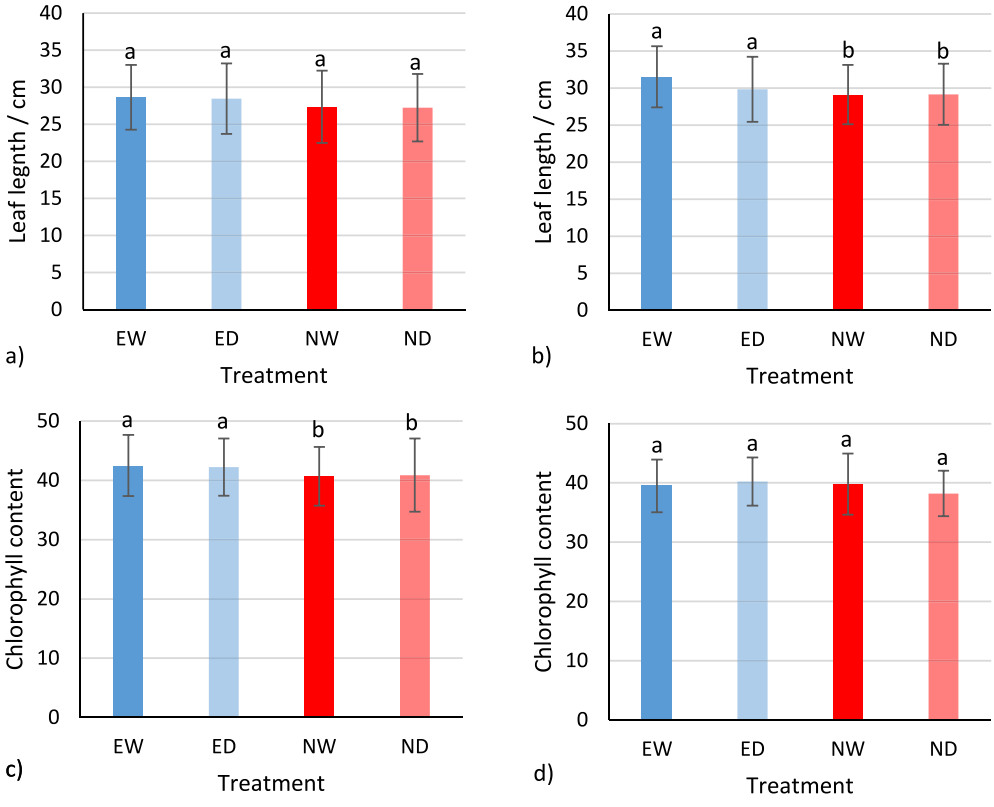

Fig. 2. Average leaf lengths (cm) a) before the start of droughting and b) at the end of the experiment and chlorophyll contents (as raw C values from the atLEAF CHL BLUE meter) c) before the start of droughting and d) at the end of the experiment. EW = earthworm-present, watered; ED = earthworm-present, droughted; NW = earthworm-absent, watered; ND = earthworm-absent, droughted. Values are averages ± standard deviations. n = 40 for a) and c). For b) and c) n = 40 for EW and NW and 36 for ED and ND. For each graph different letters above the bars indicate a significant difference at $p \leq 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

chlorophyll content between the watered and droughted treatments but there were differences between strains (Tables S3 and S4c), between earthworm treatments (Fig. 2c) and an interaction term between all three factors. At the end of the drought period none of the plants showed any visible signs of stress. Leaf length still showed no difference between the watered and droughted treatments and was still greater in the presence of earthworms than in their absence (Fig. 2b, Table S4b). There were differences in the lengths of leaves between most of the wheat strains but no interactions between earthworm presence, watering regime and wheat strain. The only difference in the chlorophyll content between treatments at the end of the experiment was between wheat strains (Fig. 2d, Table S4d).

Above-ground dry biomass varied with presence of earthworms, watering regime and wheat strain and there was an interaction between the presence of earthworms and watering regime (Tables S3 and S4e). In the watered and droughted treatments dry biomass was greater in the presence than absence of earthworms (Fig. 3a). However, although dry biomass was greater in the watered than droughted treatments in the presence of the earthworms, in the absence of earthworms there was no difference in the biomass between the watered and droughted treatments. Wheat strain 3 produced more biomass than strain 5 but otherwise there were no differences in the biomass produced by the different strains.

The nitrogen concentration in the biomass varied with watering regime with an interaction between watering regime and earthworm presence; there was no variation between wheat strains (Tables S3 and S4f). In the presence of earthworms there was no difference between the nitrogen concentration of the biomass in the watered and droughted treatments, but in the absence of earthworms the nitrogen concentration of the biomass was lower in the droughted compared to the watered treatments (Fig. 3b). Accounting for biomass (Fig. 3c, Table S4g), the mass of total above ground N (i.e. biomass × concentration) was greater in the earthworm-present than earthworm-absent treatments and in the watered compared to the droughted treatments.

The carbon concentration in the biomass varied with wheat strain (Strain 1 had a slightly lower %C content than Strain 3) and there were interactions between wheat strain and the other two factors but there were no significant differences between earthworm or watering treatments (Fig. 3d, Table S3 and S4h).

The C:N ratio of the biomass varied with wheat strain and watering regime and there was an interaction between the presence of earthworms and the watering regime (Tables S3 and S4i). The droughted wheat had a higher C:N ratio than the watered wheat, though this varied with earthworm treatment (Fig. 3e). In the presence of earthworms there was no difference in the C:N ratio of the watered and droughted wheat but in the absence of earthworms the droughted wheat had a higher C:N ratio than in their presence.

The P concentration in the biomass varied between wheat strains and there were interactions between the earthworm presence and watering treatments and between all 3 factors (Tables S3 and S4j). The P concentration in the biomass was greater in the absence than the presence of earthworms in the watered treatments but there was no difference between earthworm treatments in the droughted treatments (Fig. 3f). Taking into account biomass, mass of total above ground P was greater in the presence than absence of earthworms and in the watered compared to the droughted treatments; there were no significant interactions between the earthworm and watering treatments (Fig. 3g, Table S4k).

The Si concentration in the biomass varied with the presence and absence of earthworms, watering or droughting and between wheat strains; there were interactions between the presence of earthworms and watering (Table S3 and S4l). In the watered treatments, Si concentration was lower in the presence of earthworms than in their absence (Fig. 3h). Taking into account biomass, mass of total above ground Si was greater in the presence than absence of earthworms and in the watered compared to the droughted treatments with no interactions (Fig. 3i, Table S4m).

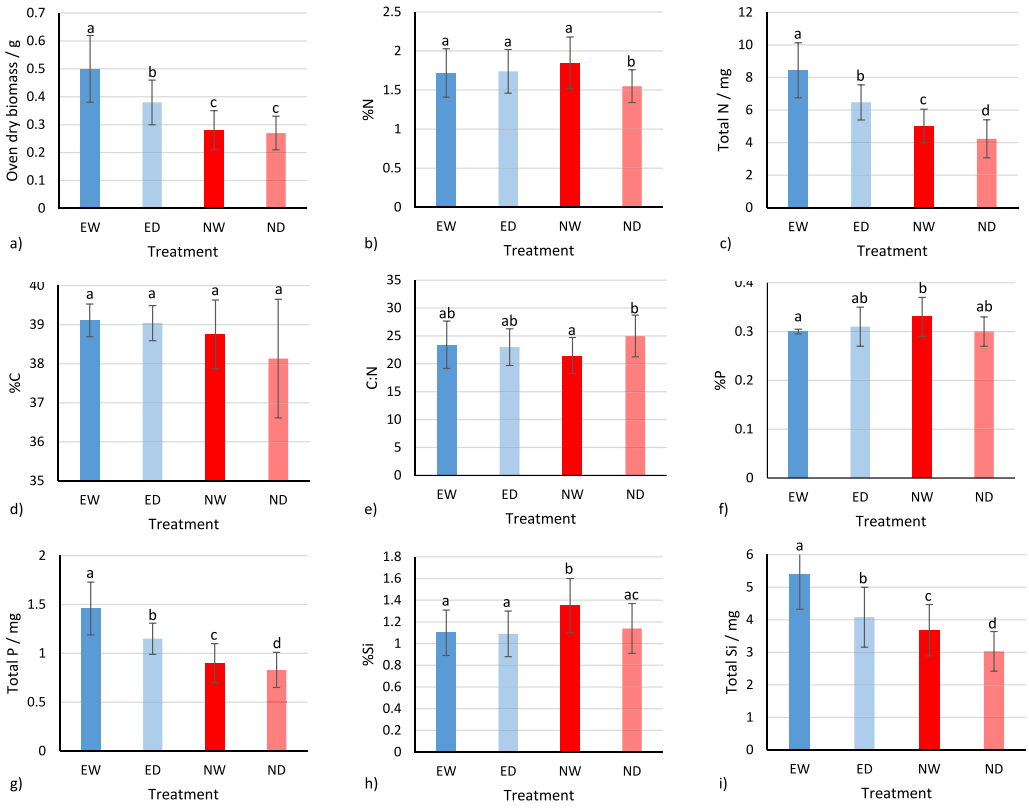

Fig. 3. Average a) above-ground dry biomass, b) plant %N, c) plant total N (mg), d) plant %C, e) plant C:N, f) plant %P, g) plant total P (mg), h) plant %Si and i) plant total Si (mg) at the end of the experiment. Total mass values calculated from concentration × oven dry biomass. EW = earthworm-present, watered; ED = earthworm-present, droughted; NW = earthworm-absent, watered; ND = earthworm-absent, droughted. Values are averages ± standard deviations. n = 20 for EW and NW and 18 for ED and ND for dry biomass and chemical composition expect for P and Si where n = 17 for ND. For each graph different letters above the bars indicate a significant difference at $p \leq 0.05$.

3.4. Plant RNA response

Differences were present in the RNA expression between both the earthworm and the watering treatments; there were also interactions in RNA expression between the earthworm and watering treatments (Table S5). The number of differentially expressed genes (DEGs) between the earthworm treatments compared to between the watering treatments was relatively small. There were two DEGs between the earthworm-present and earthworm-absent treatments compared to 20,731 between the watered and droughted treatments. This trend was also present when a two factor model with interaction was used (Table 1), suggesting that overall, the watering treatments had a greater impact on plant gene expression than the presence or absence of earthworms with plants focussing resources on responses to abiotic stress. However, as this study is concerned with the potential impacts of earthworms on plant growth, here we focus on the differences in gene expression in the presence and absence of earthworms. There are a greater number of differences in gene expression when comparing earthworm-present and earthworm-absent treatments within the droughted treatments compared to within the watered treatments. Information regarding the function of earthworm treatment DEGs is summarised in Table S5, and results of the gene ontology analysis are presented in (Table S6); there were too few differentially expressed genes to perform this analysis on the watered earthworm-present vs earthworm-absent treatments. In general, for differences between the earthworm treatments, the genes appear to be related to plant defence, which may indicate responses to changes in the microbiome or directly to the presence of earthworms, abiotic stress or the control of plant growth.

Nine DEGs were found to respond differently to watering treatment depending on earthworm presence or absence (the interaction term). After inspection of normalised read counts, only one of these genes was found to have clear differences in expression. Normalised expression of TraesCS4A02G099000 was highest under watered conditions in the absence of earthworms but showed a 7-fold decrease in expression under drought conditions (Fig. 4). Conversely, when earthworms were present, normalised expression was slightly higher (1.6X) under drought conditions. Gene TraesCS4A02G099000 is a cysteine-rich receptor-kinase-like protein, which has been found to respond to both nitrogen stress and soil-borne fungal infections (Sultana et al., 2020, Guo et al., 2020).

3.5. Soil chemistry

Soil chemistry data are summarised in Fig. 5; individual treatment data are given in Table S7. There was a small decrease in pH in the presence of earthworms compared to in their absence (Fig. 5a). Similarly, the watered treatments had a slightly lower pH compared to the droughted treatments. There was no difference between the wheat strains and no interactions (Table S8a).

Available nitrate showed a high level of within-treatment variation (Fig. 5b, Tables S7 and S8b). Samples 2EW2 (10.70 mg kg^{-1}), 3EW1 (18.12 mg kg^{-1}), 4EW2 (13.18 mg kg^{-1}) and 5NW2 (18.43 mg kg^{-1}) were excluded from further analysis as they were greater than 3 standard deviations from the mean of the remaining samples (1.99 ± 1.95 mg kg^{-1}, n = 72). Other parameters measured on these samples fell within the range of values obtained from other samples from the same treatment, so it appears that these individual nitrate extracts were contaminated. The watered treatments had more available nitrate than the drought treatments (Fig. 5b). Significant differences were also present between wheat strains (Table S8b), possibly reflecting differing uptake of nitrate by the different strains.

Table 1
Numbers of differently expressed genes between treatments filtered at a log2-foldchange level of ± 2.

Contrast	Number of differentially expressed genes
Water vs drought (earthworm-present)	2058
Water vs drought (earthworm-absent)	1828
Earthworm-present vs earthworm-absent (watered)	5
Earthworm-present vs earthworm-absent (droughted)	32
Interaction	9

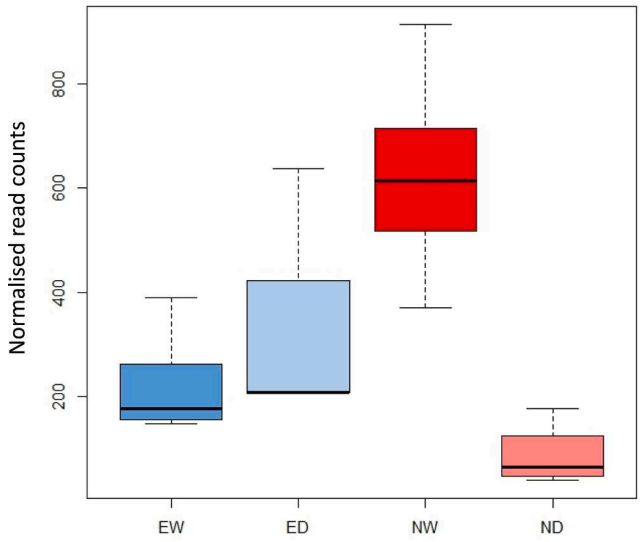

Fig. 4. Normalised read counts generated in DESeq2 compared across earthworm and watering treatments for gene TraesCS4A02G0099000. Plots show minimum, median, maximum, 25th and 75th percentile values. EW = earthworm-present, watered; ED = earthworm-present, droughted; NW = earthworm-absent, watered; ND = earthworm-absent, droughted.

There were no differences in Olsen P between treatments (Fig. 5c, Table S8c).

Available Si varied with wheat strain and watering treatment and there was an interaction between earthworm and watering treatments (Tables S7 and S8d). Within the watered treatments there was no difference between earthworm-present and earthworm-absent treatments but in the droughted treatments the earthworm-present treatments contained more available Si than the earthworm-absent treatments (Fig. 5d). In the earthworm-present treatments the watered soils contained less available Si than the droughted soils; there was no difference between the available Si in the earthworm-absent watered and droughted soils.

3.6. Soil microbiome

The relative abundance of different taxa varied between both the earthworm and the watering treatments with a small number of interactions between them, a full list of relative abundances and statistical significance is given in the (Tables S9 and S10). Tables S11 and S12 provide information on the function of selected taxa.

Within the bacteria, the dominant phyla, in order of decreasing abundance were Chloroflexi (26.5%), Actinobacteriota (20.2%), Acidobacteriota (12.3%), Proteobacteria (9.8%), Planctomycetota (9.8%), Verrucomicrobiota (6.0%), Cyanobacteria (4.1%), Firmicutes (2.7%), Bacteroidota (2.3%), and Gemmatimonadota (1.4%); these represent the typical dominant bacteria in soils (Fierer et al., 2007; Wei et al., 2018). The earthworm-present treatment contained more Actinobacteriota (22.8 vs 17.6%) and fewer Acidobacteriota (10.8 vs 13.9%) and Gemmatimonadota (1.2 vs 1.7%) than the earthworm-absent treatment at phylum level.

At the bacterial genus level, the dominant genera, in order of decreasing abundance were *Gaiella* (2.5%), *RB41* (2.4%), *Bacillus*

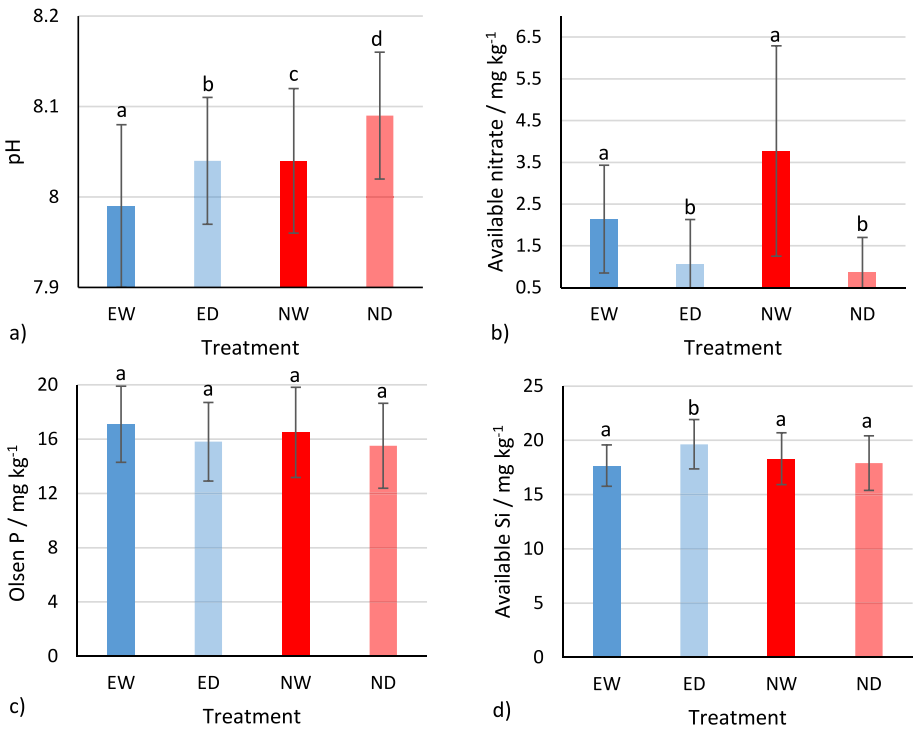

Fig. 5. Average a) pH, b) available nitrate (mg kg^{-1}), c) Olsen P (mg kg^{-1}) and d) available Si (mg kg^{-1}) in the soil at the end of the experiment. EW = earthworm-present, watered; ED = earthworm-present, droughted; NW = earthworm-absent, watered; ND = earthworm-absent, droughted. Values are averages ± standard deviations. For pH, Olsen P and available Si n = 20 for EW and NW and 18 for ED and ND. For available nitrate n = 17 for EW, 18 for ED and ND, 19 for NW. For each graph different letters above the bars indicate a significant difference at $p \leq 0.05$.

(1.6%), *Candidatus Udaeobacter* (1.4%), *Candidatus Xiphinematobacter* (1.3%), *Chthoniobacter* (1.2%), *Pirellula* (1.1%), *Pir4 lineage* (1.1%), *Intrasporangium* (1.0%), and *MND1* (0.62%). Of these *Gaiella* (2.8 vs 2.2%), *RB41* (2.1 vs 2.8%), *Intrasporangium* (1.1 vs 0.8%), and *Candidatus Udaeobacter* (1.3 vs 1.5%) were different between the earthworm-present and earthworm-absent treatments. Table S11a describes the function of the more abundant genera that differ significantly between treatments.

Within the fungi there were no differences in the relative abundance of the different phyla present between treatments. The most abundant phyla were the Ascomycota (67.1%), Basidiomycota (14.3%), and the Mortierellomycota (12.6%) which is a common observation in soils (Egidi et al., 2019; Grządziel and Gałązka, 2019). Similarly at Class and Order level the only difference between treatments was the higher abundance of Class Pezizomycetes (9.1 vs 7.6%) and Order Pezizales (9.1 vs 7.6%) in the earthworm-absent compared to the earthworm-present treatments (Table S12a); this order includes ectomycorrhizal fungi (Healy et al., 2013) and has been reported to favour habitats with available organic matter (Lin et al., 2019). At the fungal genus level, the dominant genera, in order of decreasing abundance were *Mortierella* (12.5%), *Tetracladium* (9.1%), *Ophiosphaerella* (7.5%), *Candida* (3.9%), *Schizothecium* (3.7%), *Thanatephorus* (3.0%), *Gibellulopsis* (2.9%), *Leucosporidium* (2.7%), and *Scutellinia* (2.6%) with only *Scutellinia* (Table S12a) differing in abundance between the earthworm-present (2.2%) and earthworm-absent (3.1%) treatments.

Bacterial α diversity (Simpson index) assessed at the genus level was lower in the earthworm-present than earthworm-absent treatments, consistent with e.g. Liu et al. (2019) and lower in the watered than droughted treatments and there were no interactions (Fig. 6a, Table S13). Fungal α diversity was also lower in the presence of earthworms (Fig. 6b, Table S14).

β diversity at the genus level was analysed by PERMANOVA analysis using the calculated Bray-Curtis matrices. Bacterial communities were different between the earthworm treatments, and between the watering treatments but not between wheat strains; there was no interaction between treatments (Table S15). The watering treatment explained 14.5%

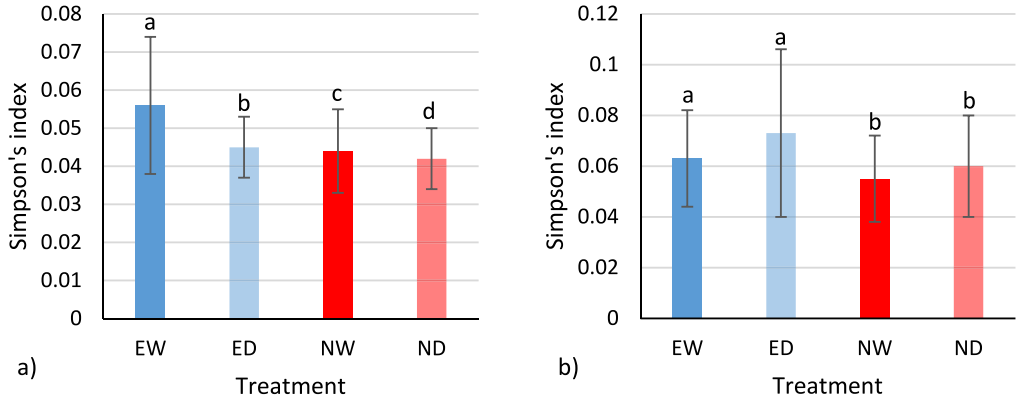

Fig. 6. Mean Simpson index ([Σn(n − 1)]/N(N − 1)] where n = number of individuals of a single genus and N = number of individuals in the total population) for a) bacterial and b) fungal diversity for the earthworm and watering treatments. Lower values indicate a greater diversity. n = 19 for EW (bacteria), n = 20 for EW (fungi) and NW, n = 18 for ED, ND. For each graph different letters above the bars indicate a significant difference at $p \leq 0.05$.

of the variation seen between samples and the earthworm treatment explained 7.51% of the variation. A similar pattern of β diversity was found when analysing fungal communities, wherein both earthworm and drought treatments were found to affect composition, with no interaction (Table S16). In this case a smaller proportion of variation was explained by watering (c. 3%) and earthworm (c. 2%) treatments than was seen for bacteria. Bacterial and fungal communities exhibited different patterns of β diversity across the five wheat strains. Wheat strain did not affect bacterial communities but was responsible for most of the explainable variation of fungal communities. Wheat strain explained 9.03% of between-sample variation for fungal communities. Strain 5 conditioned soils to contain different communities than those found when Strains 2, 3 and 4 were grown.

Bacterial and fungal community differences between treatments were visualised through NMDS based on the Bray-Curtis matrices. NMDS was also used to determine the centroids for all samples from the earthworm-present and earthworm-absent treatments (Figs. 7 and 8).

The ten closest taxa to the centroids of the earthworm-present and earthworm-absent treatments were then determined (Table S17 and S18). Of the ten bacterial taxa plotting closest to the centroid for the earthworm-present treatment, four belonged to the Actinobacteriota phylum, three to Chloroflexi, and one each to the Myxococcota, Bacteroidota and Firmicutes. For the earthworm-absent treatment there was a wider range of phyla found within this top ten list. This included four taxa from the Proteobacteria phylum, and one each from Chloroflexi, Verrucomicrobiota, Myxococcota, Bacteroidota, Armatimonadota, and the Actinobacteriota. Table S11b summarises the function of the bacterial taxa plotting closest to the centroids to the lowest level to which individual taxa were identified.

For the fungal taxa at phylum level the phyla that plot closest to the earthworm-present and earthworm-absent treatment centroids are dominated by the Ascomycota which are typically amongst the dominant fungal phyla in soils (Egidi et al., 2019; Grządziel and Gałązka, 2019). At the Class level, the earthworm-present treatment had 3 taxa that were members of the Tremellomycetes, 1 Orbiliomycetes, 2 Eurotiomycetes, 3 Dothideomycetes, and 1 Glomeromycetes whereas the

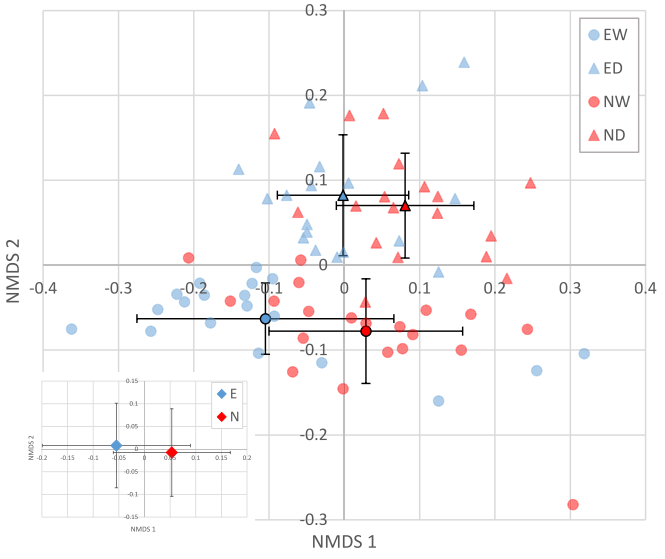

Fig. 7. NMDS plot based on the Bray-Curtis matrix for relative abundance of bacteria taxa for the earthworm-present and earthworm-absent, watered and droughted treatments. Centroids have black outlines and are plotted with standard deviations; individual samples (blue = earthworm-present samples, red = earthworm-absent, circles = watered, triangles = droughted) are also plotted. Inset shows centroids with standard deviations for the earthworm-present (E) and earthworm-absent (N) treatments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

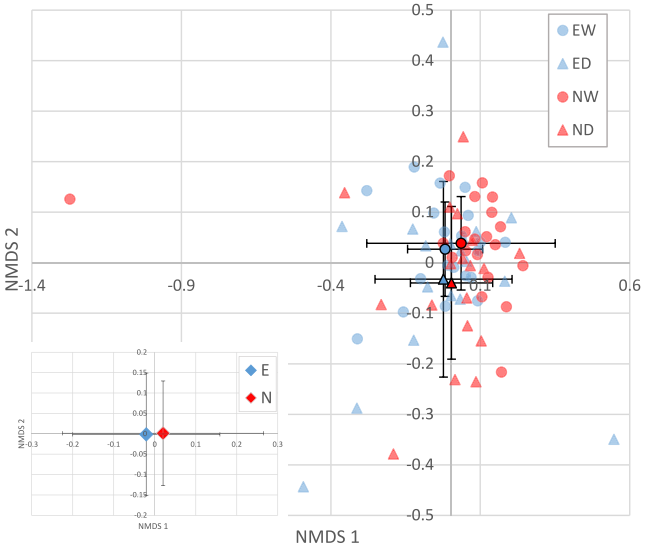

Fig. 8. NMDS plot based on the Bray-Curtis matrix for relative abundance of fungal taxa for the earthworm-present and earthworm-absent, watered and droughted treatments. Centroids have black outlines and are plotted with standard deviations; individual samples (blue = earthworm-present samples, red = earthworm-absent, circles = watered, triangles = droughted) are also plotted. Whilst the far left NW data point plots away from the main cluster of data, there was no justifiable reason for removing the sample from the analysis and its inclusion did not change the results of the PERMANOVA analysis. Inset shows centroids with standard deviations for the earthworm-present (E) and earthworm-absent (N) treatments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

earthworm-absent treatment had 5 Sordariomycetes, 2 Dothideomycetes, 2 Tremellomycetes, and 1 Glomeromycetes. Table S12b summarises the function of the fungal taxa plotting closest to the centroids to the lowest level to which individual taxa were identified.

4. Discussion

The presence of earthworms led to increased soil moisture retention and plant growth in both the watered and droughted treatments. The concentration of N, P and Si in the plant biomass showed no consistent trends but plant total N, P and Si, taking into account both biomass and tissue concentrations decreased in the order earthworm-present watered > earthworm-present droughted > earthworm-absent watered > earthworm-absent droughted. Available nitrate, Si and Olsen P in the soil showed no consistent trends. Differential gene expression was far greater between watering treatments than earthworm treatments. Between earthworm treatments, differential gene expression related to genes associated with plant defences (which may indicate responses to signal molecules produced by the earthworms themselves or changes in the microbiome), abiotic stress or control of plant growth. The soil microbiome varied between treatments with earthworm-present treatments being more associated with a microbiome that favoured nutrient rich environments and is typically associated with the promotion of plant growth and the suppression of plant pathogens. Generally, the 5 wheat strains responded to the earthworm and watering treatments in the same way (see SI tables and lack of interaction between strain and earthworm or watering treatment) and therefore the discussion focuses on the variation between earthworm and watering treatments.

4.1. Water availability

As is well established in the literature and shown by the reduced

biomass in our drought treatments (Fig. 3a), plant growth is reduced when water becomes limiting. This is both because of the necessity of water for plant growth *per se* and its role in supporting the transport of compounds through the soil and in the plant (e.g. He and Dijkstra, 2014). The increased water retention of the soil in the presence of the earthworms observed in this study most likely relates to earthworm processing of the soil, leading to increased aggregation and porosity (e.g. Blouin et al., 2013; Hallam et al., 2020), though in some studies earthworm-processing of soil can reduce water retention capacity (e.g. Blouin et al., 2007). However, the difference in moisture content is very small and is unlikely to explain the increased above ground biomass. Under drought, lower plant biomass when earthworms were absent may have been due to the faster drying of the earthworm-absent soil (Fig. 1) though in a similar pot experiment earthworm presence had no effect on evaporation rate of water from soil (Blouin et al., 2007). A larger effect of earthworm-presence was observed in the watered rather than the droughted treatments, which most likely reflects the water stressed conditions of the droughted treatments, as supported by relative differences in gene upregulation between watered and droughted treatments (Table 1). The biomass difference between the earthworm-present and earthworm–absent droughted-treatments might simply reflect the legacy of the impact of the earthworms during the watering phase. However, it is also possible that the reduced earthworm effect was due to the reduced earthworm numbers in the droughted treatments.

4.2. Chlorophyll content

Blouin et al. (2005) attributed increased growth in the presence of earthworms in part to higher chlorophyll concentrations due to increased nutrient availability resulting in more photosynthesis. In our experiment, chlorophyll concentrations were slightly greater in the earthworm-present treatments prior to the onset of drought (Fig. 2c) but, unlike our biomass data (Fig. 3a), showed no difference by the end of the experiment across all treatments (Fig. 2d). Therefore in our experiments the increased biomass in the presence of earthworms does not appear to be linked to chlorophyll content.

4.3. Nutrients

In broad terms the soil extractions provide little evidence for enhanced nutrient availability in the presence of earthworms (Fig. 5). A lack of increased extractability of nutrients (and thus apparent availability) could be due to increased nutrient plant uptake in the presence of the earthworms, for example by the stimulation of nutrient transporters (e.g. Quaggiotti et al., 2004). If this were the case an inverse correlation between plant and soil nutrient content might be expected, however, this is not supported by our data; there are no correlations, positive or inverse, between our measures of plant and soil nutrient content. Similarly, differential gene expression between earthworm treatments did not include genes uniquely associated with the uptake and movement of specific nutrients (Table S5 and S6). However, the microbiome of the earthworm-present treatments suggests a more nutrient rich environment than that of the earthworm-absent treatments (Table S11, 12). The phylum Actinobacteriota, previously found at enriched levels in earthworm-worked soil and earthworm guts and casts (e.g. Furlong et al., 2002; Schlatter et al., 2019; Medina-Sauza et al., 2019), is enriched in our earthworm-present treatments and is widely regarded as being copiotrophic, associated with environments enriched in soil nutrients and easily degradable organic matter (Trivedi et al., 2017; Barka et al., 2016; Fierer et al., 2007, 2012; Leff et al., 2015). Similarly the Acidobacteriota are enriched in our earthworm-absent treatments. Abundance of this phyla has previously been found to decrease in the presence of earthworms (e.g. Schlatter et al., 2019; Medina-Sauza et al., 2019). The Acidobacteriota are typically oligotrophic and show a decrease in abundance with increasing nutrient availability (Chiba et al., 2021; Ramirez et al 2012). However Gemmatimonadota, which are also more abundant in our earthworm-absent treatments are commonly found in nutrient rich soil (Li et al., 2022).

Van Groenigen et al. (2014) concluded that the presence of earthworms had no effect on the N concentration in above ground biomass; our results are consistent with this (Fig. 3b). However, in contrast to our soil extraction results, van Groenigen et al. (2014) concluded that earthworms increase N availability by enhanced breakdown of crop residues and organic matter and that, in N-limited systems, this causes the increase in plant growth typically observed in the presence of earthworms. Our available nitrate results are highly variable. However, available nitrate was not different between the earthworm-present and -absent treatments whilst biomass and total plant N were greatest in the earthworm-present watered treatment. Quaggiotti et al. (2004) characterised humic substances isolated from earthworm casts and showed that after exposing seedlings for 48 h to these substances and then a nitrate solution for 5 h, nitrate uptake and accumulation was enhanced. We did not observe enhanced concentrations of plant N in our earthworm-present treatments, the increased total plant N in this treatment appears to simply reflect the increased biomass. Furthermore, whereas Quaggiotti et al. (2004) observed the induction of some nitrate transporter genes following exposure to the humic substances that they extracted from earthworm casts, in our experiment there was a lack of differential expression of genes uniquely related to nitrogen nutrition (Table S5) between treatments suggesting that nitrate availability and nutrition was not directly responsible for the earthworm enhanced plant growth. Although the nitrate reductase gene TraesCS6A02G326200 was down regulated in the droughted earthworm-absent treatment and is potentially associated with nitrate assimilation, the gene is also potentially associated with resistance to both abiotic and biotic stress (Fu et al., 2018). Also, TraesCS6A02G326200 was most highly expressed when earthworms were absent from watered pots. This treatment also had the highest levels of available nitrate. Similarly TraesCS4A02G099000 was observed to show different responses to drought depending on the presence of earthworms. It has previously been found to be downregulated under nitrogen stress in wheat (Sultana et al., 2020) but is also important in defence against fungal pathogens (Guo et al., 2020). This may reflect the well-known trade-offs between growth and defence, mediated by cross talk between hormonal mechanisms (e.g. Coley et al., 1985; Stepanova et al., 2007). When earthworms were absent, this gene was downregulated under the drought condition, consistent with the plants responding to nitrogen stress induced by drought. When earthworms were present, this gene was slightly upregulated under the drought condition suggesting that nitrogen stress may be somewhat alleviated by the presence of earthworms. Evidence suggests that moderate increases in N availability can lead to improved drought tolerance (e.g. Song et al., 2019). Despite the lack of increased available nitrate in the earthworm-present treatments the microbiome associated with these treatments has more organisms involved in the nitrogen cycle associated with it than the earthworm-absent treatments (Tables S11 and S12) consistent with, for example Xue et al. (2022) who found that typically endogeic earthworms increase nitrification, denitrification and nitrogen mineralisation rates. This may relate to earthworm enhanced break down of organic matter (e.g. Haimi and Huhta, 1990) though recent work has also highlighted earthworm mucus as an important source of nutrients (Shutenko et al., 2022). Earthworms themselves are also a rich source of N (Curry et al., 1995) and in the droughted earthworm-present treatments the missing earthworms at the end of the experiment may have died and released their N. The above indicates that plant responses to nitrate availability cannot fully explain the observed earthworm enhanced plant growth in our experiments. As the soil that we used was sampled from an agricultural field it may be that this contrast to other studies (van Groenigen et al., 2014) is because of previous fertiliser applications such that nitrate is not limiting; the increase in total plant N matching increases in plant biomass is consistent with this interpretation. Furthermore, the sixteen or more years of

arable crop production experienced by the soil may have reduced organic matter residues such that there is limited additional N to be released from such material by earthworm processing.

In addition to N, it has been suggested that earthworms may promote plant biomass via their impact on other nutrients. Van Groenigen et al. (2014) found little evidence for earthworms having a role in P mobilisation despite elevated concentrations of P being regularly reported in earthworm cast material (e.g. Vos et al., 2019; Kuczak et al., 2006). Similar to previous studies (e.g. Ros et al., 2017; Haimi and Huhta, 1990) and despite microbiota previously identified as phosphate solubilising bacteria (PSB) or as being able to bring P into solution (Nouioui et al., 2022; Wang et al., 2020; Farhat et al., 2015; Altaf et al., 2018) being more associated with the earthworm-present treatments (Tables S11 and S12) we found no difference in available P between treatments (Fig. 5c). Ros et al. (2017) ascribed increased P availability in casts in part to increased pH; whilst we did not examine casts, bulk soil pH decreased very slightly in the presence of earthworms in our experiments (Fig. 5a). None of the gene expression differences between plants in the different earthworm treatments are explicitly linked with P uptake (Table S5 and S6). These data, together with the plant %P and plant total P data suggest that P limitations were not responsible for reduced plant growth in the absence of earthworms. The increases in PSB in the earthworm-present treatments may have contributed to P supply keeping pace with plant growth.

The Si data show similar trends to the P data, with little difference in the available Si between treatments (Fig. 5d) or %Si in the plants (Fig. 3h) and with plant total Si reflecting biomass (Fig. 3i), suggesting earthworms did not enhance plant growth due to changes in Si availability. Bacteria previously identified as being silicate solubilising (SSB) (e.g. Hu et al., 2018; Huang et al., 2014) are more associated with the earthworm-present treatments (Table S11), as are bacteria and fungi identified as (PSB) (see above) which are often able to solubilise Si (Etesami et al., 2021). Although plant growth does not seem to have been limited by Si availability in the earthworm-absent treatments, just as with P, changes in the soil microbiome due to earthworm activity may have allowed Si supply to keep pace with demand in the earthworm-present treatments. The increased above ground biomass in the droughted earthworm-present treatments compared to the earthworm-absent treatments may reflect the increased available Si in the soil in these treatments (Fig. 5d) due to the increased relative abundance of SSB (Tables S11 and S12) leading to an enhanced response of the plants to drought stress.

However, the relationships between Si additions, shoot Si concentrations and plant growth under drought are complex (Eneji et al., 2008; Cooke et al., 2016; Thorne et al., 2020; Wade et al., 2022): Si uptake can be reduced under drought, but yields maintained or even improved. Furthermore, Si uptake is influenced by the availability of other soil nutrients, such as nitrogen (de Tombeur et al., 2022). None of the gene expression differences between plants in the different earthworm treatments are explicitly linked with Si uptake (Table S5 and S6), though genes associated with stress responses can be upregulated when soil Si levels are increased. For example, increased Si in solution can increase activity of aquaporins, potentially enhancing water uptake (Manivannan and Ahn, 2017).

The microbiome data appear to be at odds with the soil chemical and plant gene expression data with the former suggesting that earthworms enhance nutrient availability, which could plausibly lead to increased growth, whilst changes in nutrient availability are not detected by the chemical extractions and differential gene expression is not obviously related to nutrients. It may be the case that the chemical extractions are not sensitive enough to detect changes. However the increase in plant total N, P and Si in line with increased plant biomass, together with limited variation in tissue nutrient concentrations, suggests that enhanced nutrient availability due to earthworm activity is not responsible for the increased biomass observed in the presence of earthworms. However, it remains possible that earthworm activity allows nutrient supply to match plant demand in light of enhanced plant growth. Shifts in the microbiome from oligotrophic to copiotrophic organisms have been linked to a decrease in the decomposition of recalcitrant C and potential increases in soil carbon sequestration (Ramirez et al., 2012). These earthworm induced shifts may therefore be an important, but under-investigated component of the "earthworm-dilemma" relating to whether earthworm activity increases or decreases carbon retention in soils (Lubbers et al., 2013; Zhang et al., 2013).

4.4. Plant growth promoting hormones and pathogens

For both the bacteria and fungi, the taxa more associated with the earthworm-present treatment, either in terms of relative abundance or proximity to the treatment centroid tend to be linked to plant growth promotion and the suppression of plant pathogens whereas those associated with the earthworm-absent treatments include a variety of plant pathogens (Tables S11 and S12). This supports the plant RNA data that indicate plant responses to the presence or absence of earthworms are linked to changes in the soil microbiome and plant hormones (Tables S5 and S6).

Differences in bacterial and fungal β diversity were greater between the watering treatments than between the earthworm treatments as were numbers of differentially expressed genes supporting the link between changes in gene expression and changes in the soil microbiome. Extractable nutrients showed no consistent trends between treatments and total plant nutrient content reflected plant biomass suggesting that nutrient availability did not limit growth. This suggests that increased plant growth in the presence of earthworms more likely resulted from either indirect effects mediated by changes in the soil microbiome or direct molecular signalling between the earthworms and the plants. For example, interaction between plants and the microbiome have been linked with drought tolerance (e.g. de Vries et al., 2020). This supports the earthworm-mediated changes in the microbiome of the earthworm-present droughted treatments being linked with the increased plant growth in this treatment relative to the earthworm-absent treatments. Defendable hypotheses can be constructed around individual genes. For example a gene related to fungal pathogen detection (TraesCS4A02G483200, Jehle et al., 2013) is down regulated in the earthworm-present treatments, consistent with the earthworm-present microbiome being characterised by fewer fungal pathogens and more fungal pathogen suppressors (Table S12b). This reduction in fungal pathogens and genes associated with their detection is consistent with plants having more energy available for growth and increased biomass. However, the data set as a whole is less clear cut with up- and down-regulation of genes associated with changes in the microbiome, plant growth hormones and abiotic stress. In addition there is an increasing awareness that many signalling pathways are responsive to both abiotic and biotic stress (Ku et al., 2018) so the relationship between changes in gene regulation and the microbiome might be less clear cut than it seems.

Whilst the changes in the microbiome in the earthworm treatments are consistent with enhancing plant growth, another possibility that our data cannot exclude, is that enhanced plant growth in the presence of earthworms is a direct consequence of earthworm-produced signal molecules. In reality a combination of both is most likely responsible. In a number of studies signal molecules have been isolated from earthworm casts or earthworm-processed soil (e.g. Canellas et al., 2002; High et al., 2019; Muscolo et al., 1999; Puga-Freitas et al., 2012a, 2012b; Quaggiotti et al., 2004). However in these studies the microbiome was not characterised, nor were experiments conducted in sterile conditions to allow for a definitive separation of direct-earthworm and earthworm-mediated microbiome effects to be assessed.

5. Conclusion

It is widely established that earthworms can enhance plant growth. Meta-analysis suggests that this is frequently due to increased nitrate

availability due to the accelerated degradation of organic matter. In this experiment we explored the hypothesis that enhanced growth might be due to changes in the availability of other nutrients (P, Si) and / or changes in the soil microbiome. We also hypothesised that earthworm activity would reduce the impact of drought on plant growth and that different strains of wheat would respond to earthworms differently.

We found no evidence from soil and plant chemistry to support the hypothesis that plant growth was enhanced by earthworms due to increased nutrient availability and all 5 wheat strains responded to the earthworm and watering treatments in a similar fashion. Our results are consistent with the hypothesis that enhanced plant growth in the presence of earthworms is due to changes in the microbiome resulting in reductions in potential plant pathogens and increases in plant growth promoting taxa. However, we are unable to exclude the possibility that earthworms are directly impacting plant growth by the production of plant signalling molecules.

Enhanced plant growth in the presence of earthworms was observed in droughted treatments as well as in regularly watered treatments and this could be due to either the above described changes in the soil microbiome and / or the protective effect that increased Si availability, resulting from an increase in SSB in the presence of earthworms, has under drought conditions. However, it was also not possible to rule out either the legacy effect of earthworm processing of the soil leading to retention of higher soil moisture contents for a longer period of the drought or death of the earthworms due to the drought conditions leading to increased N availability.

Whilst the present study was only carried out on a single soil type it highlights the importance of considering not just soil chemistry but also the soil microbiome and the plant molecular response, when trying to develop a clear understanding of responses of plant growth to soil biota.

Declaration of Competing Interest

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

Data availability

Plant RNA data is in the NCBI Gene Expression Omnibus, study accession code GSE214932. Soil DNA data is available from the authors. All other data is in the SI

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.geoderma.2023.116426.

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