

# Earthworm and Arbuscular Mycorrhizal Fungi Improve Plant Hormones and Antioxidant Enzymes to Alleviate Simulated Acid Rain Stress

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## Research Article

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## Abstract

**Aims** Acid rain is considered one of the three most serious environmental disasters worldwide, disrupting the normal physiological metabolism of plants and inhibiting their growth. As important parts of soil biota community, both earthworms and arbuscular mycorrhizal fungi can promote the growth of plants under adverse conditions. However, whether they can improve the stress tolerance of plants under simulated acid rain stress remains to be explored.

**Methods** Illumina high-throughput sequencing was used to conduct a relevant redundancy analysis (RDA) of soil microbial community structure and plant growth factors, and a structural equation model (SEM) was constructed for maize biomass and a biological index to study the mechanisms by which earthworms and mycorrhizal fungi affect maize stress resistance under simulated acid rain stress.

**Results** Earthworms promoted the absorption of soil organic matter by maize; promoted the growth of the root system; and increased the hormone levels of GA<sub>3</sub>, ABA and IAA; ultimately improving the stress resistance of maize. Mycorrhizal fungi increased the relative abundance of plant growth-promoting rhizosphere bacteria, increased the levels of plant hormones and antioxidant enzymes, and improved the stress resistance of maize. Earthworms promoted infection by mycorrhizal fungi, and the interaction between earthworms and mycorrhizal fungi increased the root IAA content and the Shannon index of rhizosphere bacteria.

**Conclusions** Both earthworms and mycorrhizal fungi can improve the stress resistance of maize through underground regulation. They interacted in increasing the root IAA content and the Shannon index of rhizosphere bacteria and alleviated the simulated acid rain stress of the aboveground part of the maize.

## Introduction

Acid rain is defined as acidic precipitation with a pH value less than 5.6, and its main source is sulfur dioxide (SO<sub>2</sub>) and nitrogen oxides (NO<sub>x</sub>) discharged into the atmosphere (Yun et al., 2019). As a major component of terrestrial ecosystems, plants are the major taxon affected by acid rain (Ramlall et al., 2015). Acid rain can affect plants directly or indirectly and disrupt their normal physiology and inhibit their growth (Liang et al., 2020, Hu et al., 2016). Acid rain directly inhibits leaf function by eroding the surface wax and cuticle of mesophyll cells and leaching alkali ions from mesophyll cells (Dong et al., 2017, Wu and Liang, 2017). Acid rain inhibits plant photosynthesis by decreasing the photosynthetic rate, stomatal conductance and chlorophyll content (Du et al., 2017). Acid rain leads to an increase in reactive oxygen species (ROS) in plants, which induces the peroxidation of cell membranes and causes significant damage to plants. At present, most studies have been limited to the adaptability of plants to simulated acid rain stress, and there are few studies on the effects of the plant external environment, particularly underground processes, on the improvement of plant stress resistance.

As an important sensory organ, the plant root system plays a role in regulating resistance to abiotic stress (Arsova et al., 2020). First, ensuring normal nutrient absorption by roots is important for plants to maintain their growth in stressful environments; nutrients are also signaling molecules that regulate plant growth and development. Second, water, minerals and some small molecules within the plant are in constant circulation. Long distance transport between the root and the aerial portion of the plant involves complex information transmission and mutual feedback (Zhang et al., 2008). Therefore, the regulation of root processes may play an important role in alleviating simulated acid rain stress in plants.

Soil fauna are the largest group of organisms in terrestrial ecosystems and participate in almost all subsurface ecological processes (Anslan et al., 2018). Earthworms are one of the most important soil macrofauna in terrestrial ecosystems. Due to their feeding and burrowing habits, earthworms play an important role in shaping soil structure and recycling nutrients and are known as "ecosystem engineers" (Blouin et al., 2013). Earthworms can promote plant growth by burrowing, drainage and feeding methods that form stable soil aggregates and larger soil pore structures and increase the contact area of plant roots and soil, which is conducive to plant nutrient uptake (Hallam and Hodson, 2020a). Some studies have shown that earthworms can improve plant stress resistance. In a meta-analysis, Xiao et al. (2018) found that the presence of earthworms increased chemical defenses by 31% and caused an 81% increase in resistance when plants were attacked by cell-feeding predators. The effect of earthworms on plant stress resistance may be accomplished by regulating the soil microbial community. Many studies have shown that earthworms provide good protection against plant diseases such as eyespot disease in winter wheat (Bertrand et al., 2015) and take-all disease of wheat (Puga-Freitas et al., 2016). In addition, it has been reported that earthworms can secrete signaling molecules similar to plant hormones, and these signaling molecules play a role in promoting plant growth and development (Scheu, 2003).

Arbuscular mycorrhizal fungi (AMF) are nonobligate organisms that require host plants to complete their life cycle and form mycorrhizae through infestation of the host plant roots. These fungi are ubiquitous in terrestrial ecosystems (Smith et al., 2011). Many studies have shown that mycorrhizae can significantly improve plant stress resistance to drought, salinity, herbivory, temperature, metals, and diseases due to fungal symbiosis (Zhang et al., 2020b, Evelin et al., 2019). AMF inoculation can promote dry matter accumulation and enhance water absorption, thus improving the tolerance of plants to drought and salt stress (Chitarra et al., 2016). AMF mainly improve plant stress resistance by regulating root processes such as stimulating the absorption of nutrients by the plant to increase the concentration of various macronutrients and micronutrients, improving the level of secondary metabolites and thus improving the antioxidant capacity of plants, regulating the soil microbial community, recruiting plant rhizosphere growth-promoting bacteria, and promoting plant growth. Earthworms and mycorrhizal fungi are both important components of soil ecosystems at different trophic levels, and they promote plant nutrient uptake and enhance plant stress resistance.

Earthworms and mycorrhizal fungi play a synergistic role in promoting many aspects of plant growth such as nutrient uptake (Cao et al., 2015, Li et al., 2012), improving the physical and chemical properties of the soil (Zhang et al., 2016, Li et al., 2012), degrading organic pollutants (Cao et al., 2016) and inhibiting soil pathogens (Li et al., 2019). However, it remains to be studied whether earthworms and mycorrhizal fungi can improve the stress resistance of maize plants under simulated acid rain stress aboveground. We proposed the following hypothesis (Fig. 1): earthworms and AM fungi promote the growth of maize under acid rain stress through the regulation of underground processes, including (1) regulating the soil microbial community structure, increasing the abundance of

soil nitrogen cycling bacteria, and accelerating soil nutrient mineralization; (2) promoting plant root nutrient absorption, changing root physiological conditions, and improving maize root stress resistance; and (3) enhancing photosynthesis and improving the antioxidant capacity of the aboveground part of maize plants to alleviate simulated acid rain stress when hormone signals are sent to the aboveground parts. This research is of great significance for exploring above- and belowground feedback regulation and the soil remediation functions of earthworms and mycorrhizae.

## Materials And Methods

### Materials

The maize seeds (*Zea mays* L.) were Zheng Dan 958 and were surface sterilized with a 10% (v/v) solution of H<sub>2</sub>O<sub>2</sub> for 10 min and incubated at 25 °C for 24 h. The earthworms (*Eisenia fetida*) were washed with distilled water and kept in sterilized glass vessels for 24 h to reduce the occurrence of naturally occurring mycorrhizal propagules. The AM inoculum (*Funneliformis mosseae*, BGC HEB02) contained approximately 1000 infectious AM spores per 10 g of soil. The propagule infectivity was tested according to the method of Sharma et al.

### Experimental design

There were four treatments, each consisting of four replicates as follows: CK, no addition of earthworms or sterilized AM fungi; EW, addition of earthworms with a sterilized AM fungi inoculation; AM, no addition of earthworms but with AM fungi inoculation; and AE, addition of both earthworms and AM fungi. Plastic pots (23 cm in diameter and 20 cm in height) were filled with 2.7 kg soil (dry weight) mixed with 8.0 g of oven-dried wheat straw. One germinated maize seed was sown in each pot. The mycorrhizal inoculum was placed approximately 2 cm under the soil surface at 2.5 g kg<sup>-1</sup>, and ten earthworms of similar fresh weights were added when the third leaf emerged. Each pot received a 10 ml soil suspension filtered through 0.45 μm filters (to eliminate mycorrhizal spores) to reintroduce fresh microorganisms. The pots were placed randomly in a greenhouse and incubated at 20/30 °C (day/night) with a photoperiod of 12/12 h of light/dark. Throughout the incubation period, deionized water was added to maintain the soil moisture content at 60% of the soil water holding capacity.

Simulated acid rain treatment: Concentrated sulfuric acid and concentrated nitric acid were mixed at a volume ratio of 3:1, distilled water was adjusted to pH 1.0 as the mother liquor, and then the mother liquor was diluted to pH 3 as the simulated acid rain. The simulated acid rain spraying period was during the seedling stage of the maize. Twenty days after the seeds were sown, acid rain stress was simulated for each treatment at 4 PM every day. The entire plant was sprayed with a spray pot to simulate acid rain. During spraying, the soil surface was covered with kraft paper to prevent the simulated acid rain solution from entering the soil. Deionized water was replenished every two days during the leaf drop limited water test to maintain the water content of the pot soil at 20%. The stress treatment lasted for 15 d, and samples were collected for measurement.

### Sampling and analysis

The maize shoots were harvested by cutting at the soil surface. Maize shoot and root biomass were recorded for the different treatments. Aliquots were stored at -80 °C for physiological analysis and root colonization or oven-dried at 65 °C for 72 h for nutrient concentration measurements. The collected earthworms were washed and weighed. The root was carefully removed from each pot, and 200 g undisturbed soil was stored separately in a rigid box and air-dried for soil aggregate analyses. The remaining aliquots were mixed and sieved through 2 mm mesh and were stored at -80 °C for DNA analysis or air-dried for nutrient concentration measurements.

### Assays for soil physical and chemical properties

The pH of the soil was determined in a 5:1 water-soil ratio. The soil organic matter was determined using the concentrated sulfuric acid-potassium dichromate oxidation method and titrated with a ferrous sulfate standard solution. The soil Olsen-P was extracted with 0.5 mol L<sup>-1</sup> NaHCO<sub>3</sub> and determined using the molybdenum-antimony colorimetric method. The soil available potassium was extracted with 1 mol L<sup>-1</sup> NH<sub>4</sub>OAc and determined using flame photometry.

### Assays for mycorrhizal colonization rate and mycorrhizal colonization density

The roots of each sample were cut into root segments of 1 cm and digested for 60 min in a water bath of 10% KOH at 90 °C. After cooling, the KOH solution was poured off, and the roots were washed with water and acidified with 2% hydrochloric acid for 5 min. After the hydrochloric acid was poured off, a lactic acid glycerin solution containing 0.05% trypan blue was directly added to the water bath for 30 min; the dye was poured off and the solution of lactate glycerin was added to decolorize the roots. Finally, 30 root segments were randomly selected from the dyed root segments, and the infection rate was measured under a microscope. The calculation method was conducted according to Trouvelot et al (Trouvelot et al., 1986).

### Determination of high-throughput microbial community structure

Bacterial 16S rDNA V3-V4 regions were selected for microbial diversity detection, and the DNA samples were sent to Beijing Baimaike Gene Technology Co., Ltd. for sequencing using the Illumina MiSeq PE2500 high-throughput sequencing platform. A PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) was used to extract the DNA. The primers for the amplification of bacterial 16S rDNA V3-V4 were 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3'). The PCR system included 12.5 μL 2×Taq PCR MasterMix, 3 μL BSA (2 ng/L), 2 μL primer (5 μM), 2 μL primer, and 5.5 μL ddH<sub>2</sub>O. The reaction parameters were predenaturation at 95 °C for 5 min; denaturation at 95 °C for 45 s, annealing at 55 °C for 50 s, and elongation at 72 °C for 45 s, repeated for 32 cycles. The original sequence was uploaded to the NCBI SRA database after 10 min extension at 72 °C.

### Photosynthesis and transpiration assays

The photosynthetic and transpiration rates of the maize leaves were measured using a portable photosynthesis system (model 6400; Li-Cor, Lincoln, NE) at a light intensity of 1800  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PAR and a constant 350  $\mu\text{bar CO}_2$  partial pressure in the sample chamber. All measurements were performed between 08:00 and 11:00 h. During the measurements, the relative humidity of the air was approximately 75%, the leaf temperature ranged from 25 to 27 °C, and the ambient  $\text{CO}_2$  concentration was approximately 355 mmol.

### Plant hormones and antioxidant enzymes assays

Enzyme-linked immunosorbent assays (ELISAs) were used to quantify IAA, ABA, GA3 and ZR. A 0.50 g sample was weighed and ground into a homogenate in an ice bath with phosphate buffer. The sample was extracted at 4 °C for 4 h and centrifuged at 3500 r for 8 min, and the supernatant was collected. One milliliter of the extract was added for repeated extraction for 1 h and centrifuged. The supernatants were combined and purified using a  $\text{C}_{18}$  solid phase extraction column. The product was dried under nitrogen to a constant volume. Standards, samples, and antibodies were added and quantified using an enzyme-linked plaque spectral photometer (490 nm). The POD and SOD activities of plant antioxidant enzymes were measured with an antioxidant enzyme kit (Jancheng, Nanjing).

### Data analysis

SPSS 17.0 software (SPSS Institute, Inc., Cary, NC, USA) was used to test the homogeneity of variance for all data (Levene's test). Differences between treatments were compared using a one-way ANOVA ( $P < 0.05$ ). The significance of earthworm and mycorrhizal influences on each measurement index was compared using a two-factor analysis of variance.

Paired-end sequencing was performed on an Illumina MiSeq platform. The debarking data were filtered, spliced, and chimeras were removed using QIIME (V1.8.0) software; paired sequences with scores less than 20, base ambiguity, primer mismatch, or a sequencing length less than 150 bp were removed. Based on barcodes, the sequence information from each treatment was grouped into OTUs (operational taxonomic units) for species classification, and OTU similarity was set at 97%. The species classification information corresponding to each OTU was obtained by comparison with the SILVA database.

AMOS 21.0 was used to construct a structural equation model (SEM) to analyze the causal relationship among biological or nonbiological indicators and biomass.

## Results

### Soil physicochemical properties

In this experiment, the addition of earthworms significantly ( $p < 0.05$ ) improved the rate and density of mycorrhizal infection (Table 1). Both the EW and AM treatments significantly ( $p < 0.05$ ) reduced soil organic matter, total N and available P and significantly increased soil pH (Table 1). The AE treatments significantly ( $p < 0.05$ ) reduced soil organic matter, total N, exchangeable K and available P and significantly increased soil pH (Table 1). Earthworms and mycorrhizae had significant ( $p < 0.05$ ) interactions in reducing soil organic matter and total N and increasing soil pH.

### Changes in the rhizosphere microbial community

A total of 1,086,110 clean tags were generated after double-end reads were spliced and filtered. At least 56,042 clean tags were generated for each sample, with an average of 67,882 clean tags. EW and AM processing significantly ( $p < 0.05$ ) increased the number of clean tags compared with the control (Table 1). AE processing significantly ( $p < 0.05$ ) increased the number of observed OTUs and increased the Shannon index and Chao 1 index. Earthworms and mycorrhizae had a significant ( $p < 0.05$ ) interaction in increasing the number of observed OTUs and the Chao1 index and had an extremely significant ( $p < 0.001$ ) interaction in increasing the Shannon index (Table 1). Based on the sample abundance and principal component analysis, the horizontal clustering of dominant phyla in the maize rhizosphere soil was Proteobacteria (69.93-56.21%), Bacteroidetes (15.18-9.55%), Actinobacteria (7.34-4.18%), Verrucomicrobia (6.29-4.56%) and Firmicutes (3.97-3.37%) (Fig. 2a). The addition of earthworms and mycorrhizal fungi changed the rhizosphere microbial community structure (Fig. 2b). Treatment with EW significantly ( $p < 0.05$ ) increased the relative abundance of *Devosia* and *Ensifer*. Treatment with AM significantly ( $p < 0.05$ ) increased the relative abundance of *Gemmatimonas*, and treatment with AE significantly ( $p < 0.05$ ) increased the relative abundance of *Sphingomonas* (Table 2).

### Root physiology and nutrient uptake

The addition of earthworm and mycorrhizal fungi significantly ( $p < 0.05$ ) affected the activity of growth hormones and antioxidant enzymes in maize roots under acid rain stress (Table 3). Compared with the CK, the EW treatment significantly ( $p < 0.05$ ) increased the gibberellin A3 (GA3) content and peroxidase (POD) activity in maize roots. The AM treatment significantly ( $p < 0.05$ ) increased the abscisic acid (ABA) content and POD activity in maize roots. The AE treatment significantly ( $p < 0.05$ ) increased the POD and superoxide dismutase (SOD) activity of maize roots as well as the content of GA3 and IAA. Earthworms and mycorrhizal fungi had significant ( $p < 0.05$ ) interactions in increasing indole-3-acetic acid (IAA) content and POD enzyme activity in the roots. The treatments with AM and AE all significantly ( $p < 0.05$ ) increased the biomass and N, P and K uptake by maize roots (Table 3). The root biomasses and N, P and K nutrient uptakes in the AM and AE treatments were significantly ( $p < 0.05$ ) higher than those in EW.

### Shoot physiology and growth of maize

The addition of earthworms and mycorrhizal fungi had a significant effect on the biomass of maize shoots (Table 3). The EW, AM and AE treatments significantly increased the biomass of maize shoots, and the biomass of the AE treatment was significantly higher than those of the EW and AM treatments.

Regarding nutrient uptake, the uptakes of N, P and K in the AM treatment were significantly higher than those in the CK and EW treatments. The addition of earthworms and mycorrhizae significantly ( $p < 0.05$ ) affected the activities of growth hormones and antioxidant enzymes in maize shoots under acid rain stress (Table 3). The EW treatment significantly increased ( $p < 0.05$ ) ABA and IAA contents and SOD activity in maize shoots compared with the CK treatment. The AM treatment significantly ( $p < 0.05$ ) increased ABA content and POD activity in the maize shoots. The AE treatment significantly increased ( $p < 0.05$ ) POD and SOD activities in the maize shoots. Earthworms and mycorrhizal fungi had significant ( $p < 0.05$ ) interactions in decreasing the ZR and ABA contents in maize shoots. The photosynthetic rates of the EW, AM and AE treatments were significantly ( $p < 0.05$ ) higher than those of the CK treatment. The stomatal conductance and transpiration coefficient of the AE treatment were significantly lower ( $p < 0.05$ ) than those of the CK treatment (Fig. 3).

### Relationships between plant growth factors and microbial communities

The distribution of plant growth factors in different quadrants indicated that plant growth was significantly ( $p < 0.05$ ) affected by soil bacterial structure. Plant biomass, photosynthetic rate and soil pH were significantly ( $p < 0.05$ ) positively correlated with AE and AM and negatively correlated with CK. The plant Zr and ABA hormone contents were significantly ( $p < 0.05$ ) positively correlated with EW and AM (Fig. 4).

## Discussion

### Effects of earthworms and mycorrhizal fungi on root growth

The feeding and burrowing habits of earthworms promote litter decomposition, nitrogen mineralization and water infiltration in the soil, which have profound effects on soil properties (Hallam and Hodson, 2020b). In this experiment, the EW treatment significantly ( $p < 0.05$ ) reduced the soil organic matter and total N contents and significantly ( $p < 0.05$ ) increased the N content of maize roots (Table 3), indicating that earthworms can promote the transfer of nitrogen from soil to the maize roots. In addition, high-throughput sequencing results of soil bacterial abundance showed that the EW treatment significantly ( $p < 0.05$ ) improved the relative bacterial abundance of *Devosia* and *Ensifer* in the soil (Table 3). Rivas (Rivas et al., 2002) identified *Devosia*, as a nodule-forming and nitrogen-fixing bacterial species with leguminous plants, and mapped the *nodD* and *nifH* genes involved in node formation and nitrogen fixation on plasmids. Studies have shown that *Ensifer* fixes nitrogen, which promotes the expression of denitrifying enzymes via nitrate respiration (Torres et al., 2014, Le Quere et al., 2017). Here, we hypothesize that earthworms promote soil N mineralization mainly through feeding habits and burrowing and increase the abundance of nitrogen-fixing bacteria in the rhizosphere by regulating the soil microbial community, thus improving the availability of soil nitrogen and promoting the uptake of nitrogen by plant roots.

Mycorrhizal fungi can promote nutrient absorption by plants through the activation of insoluble soil nutrients and the transfer of hyphae (Xu et al., 2019). We found that the addition of mycorrhizal fungi significantly ( $p < 0.05$ ) reduced the organic matter, total N and exchangeable K contents in the soil and significantly increased the maize root biomass and N, P, and K contents (Table 1). Our high-throughput sequencing results showed that the addition of mycorrhizae significantly increased the relative abundance of *Gemmatimonas* and significantly decreased the dominant genus *Massilia* in the rhizosphere soil (Table 3). *Gemmatimonas* are beneficial bacteria that play important roles in phosphate dissolution, microbial nitrogen metabolism and soil respiration (Liu et al., 2020, Lu et al., 2020). Therefore, we speculated that the main reasons why mycorrhizal fungi change soil physical and chemical properties and promote maize root nutrient absorption and biomass accumulation are as follows: 1. Mycorrhizal fungi activated insoluble nutrients in the soil through secretions, including plasmids and organic acids, making them more conducive to plant absorption. 2. The hyphae increased the area for nutrient absorption by roots and increased the absorption of soil nutrients by plants, particularly those nutrients with poor mobility. 3. Mycorrhizal fungi affected the composition of the microbial community in rhizosphere soil, increased the relative abundance of the beneficial bacterium *Gemmatimonas*, and promoted the growth of plant roots.

### Effects of earthworms and mycorrhizal fungi on root resistance

Plant hormones are essential endogenous organic substances that regulate plant growth and yield and play an important role in inducing plant tolerance to various biological and abiotic stresses (Khan et al., 2020). Antioxidant enzymes can effectively remove reactive oxygen species to reduce oxidative stress in cells and maintain homeostasis (Perkins et al., 2015). To explore the effects of earthworms and mycorrhizae on the stress resistance of maize roots, we measured the activity of plant growth regulators and two antioxidant enzymes (Table 3). ABA is an important signaling substance that regulates stomatal closure and enhances plant stress tolerance (Nakashima and Yamaguchi-Shinozaki, 2013). GA3 can promote the growth and development of crops, early maturity, and can increase yield (Hedden and Sponsel, 2015). IAA in the appropriate concentration range can promote plant growth, including effects on plant photosynthetic characteristics and antioxidant enzyme activity (Li et al., 2020). Our results showed that the addition of earthworms and mycorrhizal fungi significantly ( $p < 0.05$ ) increased the contents of plant hormones and the activities of antioxidant enzymes in maize roots (Table 3). We hypothesized that earthworms and mycorrhizae could improve the stress resistance of maize roots by regulating the activities of plant hormones and POD enzymes in roots and could induce the physiological response of maize branches by regulating the transport of plant hormones to the ground.

### Aboveground-underground feedback-induced shoot stress resistance

The direct harm of acid rain to plants is mainly seen in aboveground growth inhibition caused by ion toxicity and secondary stress such as oxidative stress (Zhang et al., 2020a, Ju et al., 2020). Roots play an important role in the process of plant growth and stress resistance. As a bridge between the aboveground and underground parts of the plant, plant roots transport the carbon fixed by the plant to the soil and obtain the nutrients needed for their own growth from the soil. In addition, as an interface between plants and soil organisms, the root system relays the effects of soil organisms to the aboveground portion of the plant. In this study, we found that the addition of earthworms and mycorrhizal fungi could increase the biomass of maize seedlings, particularly mycorrhizal fungi, significantly increasing the nutrient absorption of maize shoots. The addition of earthworms and mycorrhizal fungi also affected aboveground physiological changes (Table 3). We established a structural equation model to analyze the relationship between maize biomass and its physiological indices

(Fig. 5). The overall effect of standardization showed that shoot N ( $\lambda = 0.67$ ) and root IAA ( $\lambda = 0.65$ ) were the most important factors affecting maize biomass, followed by root K ( $\lambda = 0.56$ ), shoot POD ( $\lambda = 0.35$ ), root POD ( $\lambda = 0.31$ ) and root ABA ( $\lambda = 0.28$ ), which indicated that plant hormones and enzymes played important roles in promoting the growth of maize seedlings and the absorption soil nutrients. We hypothesized that earthworms and AM fungi regulated stomatal closure of mesophyll cells by increasing the ABA content in maize branches, and earthworms significantly increased the IAA content in maize branches, thus affecting plant photosynthesis and antioxidant enzyme activities. In addition, both earthworms and mycorrhizal fungi increased the activities of POD and SOD in the shoots and roots of maize. The stomatal movement of plants is affected by a variety of environmental stimuli such as the plant hormone abscisic acid, which regulates stomatal closure and reduces water loss under drought stress (Zhu et al., 2020). In this experiment, we hypothesized that maize seedlings would reduce the inflow of acid rain by closing stomata. Earthworms and mycorrhizal fungi, through underground regulation, first changed the physiological status of the maize root system, and plant hormones were transferred aboveground as signal molecules, thus enhancing the stress resistance of the aboveground portion of the plant and significantly reducing the stomatal conductance and transpiration rate of maize leaves (Fig. 3), effectively preventing acid rain from entering leaf tissues and impacting the homeostasis of tissue cells, so that the maize leaves had a higher photosynthetic rate.

## Conclusion

Under simulated acid rain stress, earthworms and mycorrhizal fungi changed the rhizosphere soil community structure by increasing the abundance of functional nitrogen-fixing bacteria, activating soil nutrients and promoting transfer to the root system. The rhizosphere growth-promoting bacteria *Ensifer*, *Gemmatimonas* and *Sphingomonas* were induced by earthworms and mycorrhizal fungi to increase their abundance, thus improving root stress resistance. Earthworms and mycorrhizal fungi increased GA3, ABA and IAA; increased POD and SOD enzyme activities in maize; and eventually slowed the damage of acid rain on corn growth.

## Declarations

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## Tables

Table 1. Mycorrhizal colonization, soil chemistry and microbial properties.

Treatment	Mycorrhizal colonization			Soil chemistry properties				Soil microbial properties	
	Mycorrhizal infection rate (%)	Mycorrhizal infection density (%)	Organic matter (g kg <sup>-1</sup> )	Total N (g kg <sup>-1</sup> )	Exchangeable K (mg kg <sup>-1</sup> )	Available P (mg kg <sup>-1</sup> )	pH	Clean tags	Observed OTU
CK	-	-	25.15±0.11a	1.15±0.06b	210.03±1.10a	8.21±0.75a	7.39±0.01c	59817.65±152a	745.00±35
EW	-	-	23.42±0.11c	0.98±0.01a	210.03±1.10a	8.15±0.11a	7.44±0.01b	75681.35±133b	746.00±16
AM	88.33±1.67b	23.53±1.85b	24.49±0.27b	1.00±0.01a	200.47±3.12b	7.14±0.14a	7.53±0.01a	72777.65±377b	755.50±18
AE	92.50±1.60a	30.66±1.89a	24.17±0.07b	0.98±0.02a	199.51±2.87b	8.55±0.83a	7.54±0.01a	62604.45±585c	857.50±15
Significance <sup>a</sup> due to									
EW	NS	NS	41.6***	*	NS	NS	9.33*	***	*
AM	6142***	418***	NS	*	19.7***	NS	134***	***	*
EW*AM	NS	7.25*	19.6***	*	NS	NS	5.39*	***	*

Data are the mean ± SE based on four replicates and were compared using Duncan's multiple range test. Values with the same lowercase letter in each column are not significantly different. CK, treatment without earthworms or AM fungi; EW, treatment with the addition of earthworms; AM, inoculation of soil with AM fungi; and AE (AM + EW), inoculation of soil with both earthworms and AM fungi. NS, not significant. \*\*\* P < 0.001, \*\* P < 0.01, \* P < 0.05.

Table 2. Relative abundance of rhizosphere bacteria.

Treatment	<i>Massilia</i>	<i>Pseudomonas</i>	<i>Lysobacter</i>	<i>Ramlibacter</i>	<i>Phenyllobacterium</i>	<i>Sphingomonas</i>	<i>Pedobacter</i>	<i>Devosia</i>	<i>Ensifer</i>
CK	18.20±1.43b	7.33±1.27b	6.84±1.11a	3.52±0.32b	3.48±0.43c	2.87±0.53b	1.99±0.62ab	1.76±0.07a	1.51±0
EW	18.22±1.66b	8.18±0.53b	4.24±0.27a	2.80±0.29ab	3.07±0.29bc	1.72±0.12a	2.71±0.39b	3.21±0.34b	2.15±0
AM	9.33±1.17a	5.54±1.06ab	5.67±1.16a	2.59±0.47ab	2.02±0.44ab	2.08±0.28ab	2.18±0.06ab	1.83±0.42a	0.93±0
AE	7.22±0.67a	4.25±0.34a	7.04±1.04a	2.43±0.07a	1.59±0.15a	10.13±0.30c	1.14±0.13a	1.55±0.29a	1.51±0
Significant <sup>a</sup> due to									
EW	NS	NS	NS	NS	NS	***	NS	NS	***
AM	***	**	NS	NS	***	***	NS	*	***
EW*AM	NS	NS	NS	NS	NS	***	*	*	NS

Data are the mean ± SE based on four replicates and were compared using Duncan's multiple range test. Values with the same lowercase letter in each column are not significantly different. CK, treatment without earthworms or AM fungi; EW, treatment with the addition of earthworms; AM, inoculation of soil with AM fungi; and AE (AM + EW), inoculation of soil with both earthworms and AM fungi. NS, not significant. \*\*\* P < 0.001, \*\* P < 0.01, \* P < 0.05.

Table 3. Nutrient contents and physiological indices of the root and shoot systems of maize seedlings.

Plant parts	Treatment	Biomass (g plant <sup>-1</sup> )	N content (mg plant <sup>-1</sup> )	P content (mg plant <sup>-1</sup> )	K content (mg plant <sup>-1</sup> )	ZR (ng/g FW)	GA3 (ng/g FW)	ABA (ng/g FW)	IAA (ng/g FW)	PC (U)
Root	CK	0.59±0.02b	6.72±0.35c	1.31±0.11b	2.97±0.21c	3.95±0.17a	3.97±0.26c	27.53±1.21b	29.31±2.26b	32
	EW	0.81±0.08b	11.97±1.11b	2.28±0.24b	3.81±0.53c	3.99±0.13a	5.05±0.26b	27.89±1.98b	27.87±1.11b	41
	AM	1.17±0.13a	13.87±0.57ab	3.77±0.24a	6.91±0.54b	3.55±0.18a	3.91±0.29c	32.84±0.85a	31.32±0.90b	48
	AE	1.47±0.09a	16.88±1.80a	4.44±0.52a	8.52±0.07a	3.97±0.18a	6.08±0.28a	28.14±1.53b	48.20±2.97a	43
Significance <sup>a</sup> due to										
	EW	7.74**	13.9**	6.54*	9.73*	NS	36.45***	NS	14.9**	NS
	AM	44.4***	29.4***	52.1***	121***	NS	NS	NS	31.3***	11
	EW*AM	NS	NS	NS	NS	NS	NS	NS	21**	6.1
Shoot	CK	1.39±0.09d	38.35±2.25c	3.21±0.16b	40.32±3.44b	7.83±0.24a	6.44±0.19ab	75.38±4.00d	39.10±1.05bc	44
	EW	2.10±0.11c	72.43±4.69c	6.34±1.21b	58.47±4.24b	8.57±0.29a	6.91±0.31a	130.34±5.12a	54.35±3.97a	49
	AM	3.87±0.31b	127.74±5.08b	20.61±1.03a	118.90±5.21a	8.29±0.34a	5.80±0.36b	112.28±4.30b	34.15±1.12c	58
	AE	5.09±0.31a	175.70±8.08a	24.21±2.39a	145.12±14.9a	7.76±0.16a	5.97±0.17b	89.93±2.53c	44.32±1.79b	55
Significance <sup>a</sup> due to										
	EW	17.4**	14.2**	5.48*	7.05*	NS	NS	15.9**	30.10***	NS
	AM	138***	78.1***	150***	97.7***	NS	8.42*	NS	10.4*	24
	EW*AM	NS	NS	NS	NS	5.71*	NS	89.1***	NS	NS

Data are the mean ± SE based on four replicates and were compared using Duncan's multiple range test. Values with the same lowercase letter in each column are not significantly different. CK, treatment without earthworms or AM fungi; EW, treatment with the addition of earthworms; AM, inoculation of soil with AM fungi; and AE (AM + EW), inoculation of soil with both earthworms and AM fungi. NS, not significant. \*\*\* P < 0.001, \*\* P < 0.01, \* P < 0.05.

## Figures

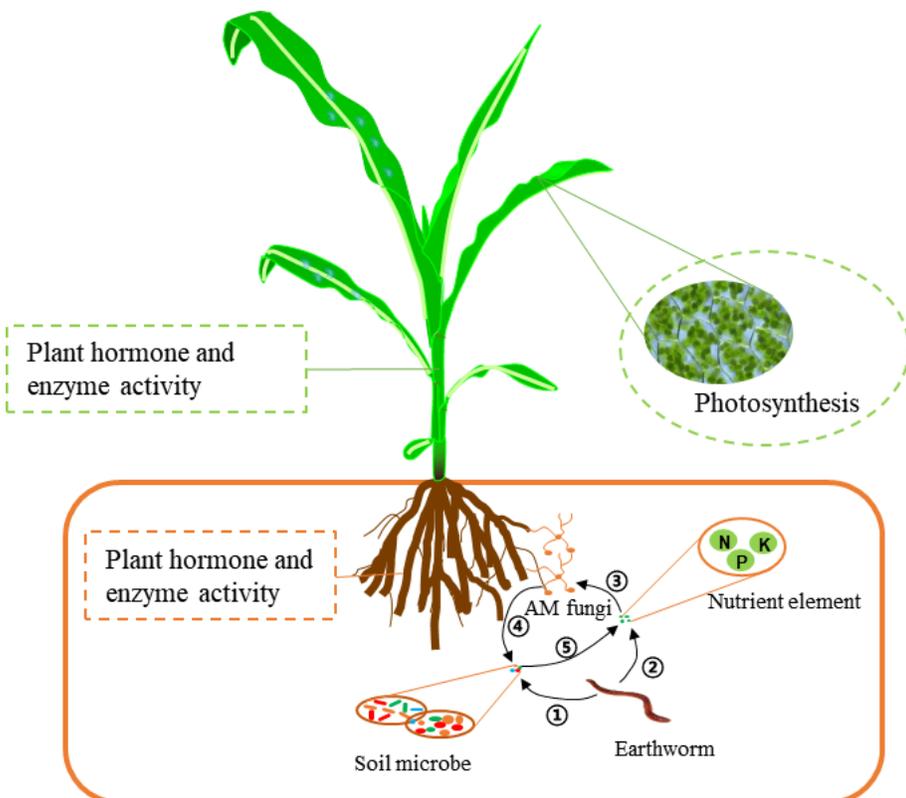


Figure 1

Underground regulation of earthworm and arbuscular mycorrhizal fungi on maize growth under acid rain stress. (1) Regulating the soil microbial community structure, increasing the abundance of soil nitrogen cycling bacteria, and accelerating soil nutrient mineralization; (2) promoting plant root nutrient absorption, changing root physiological conditions, and improving maize root stress resistance; and (3) enhancing photosynthesis and improving the antioxidant capacity of the aboveground part of maize to alleviate simulated acid rain stress when hormone signals were fed back to the aboveground parts.

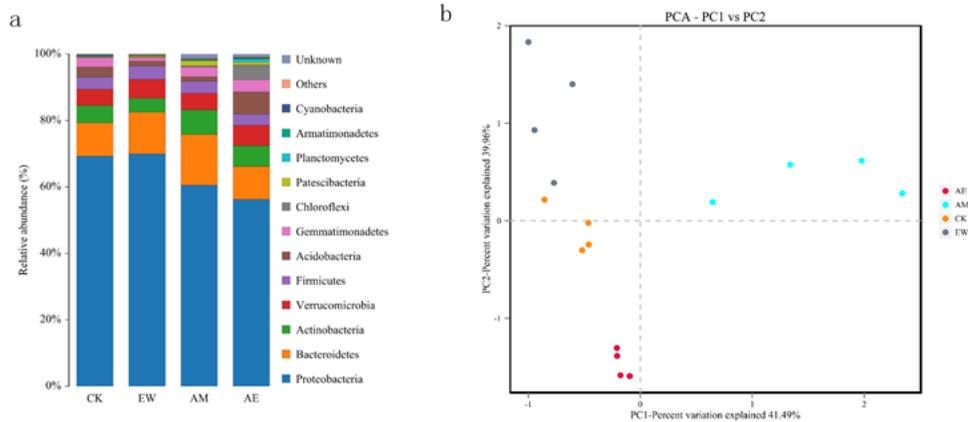


Figure 2

Sample abundance and principal component analysis. a, Distribution and comparison of Illumina-based 16S rRNA gene metagenomic profiling; and b, PCA based on OTU composition of different treatments. All OTUs were selected based on an  $R > 0.80$ , a  $p < 0.05$ , and a relative abundance  $> 0.1\%$ . CK is saline soil without the addition of earthworms or AM fungi; EW is saline soil with the addition of earthworms; AM is saline soil with the addition of AM fungi; AE or AM + EW is saline soil with the addition of both earthworms and AM fungi.

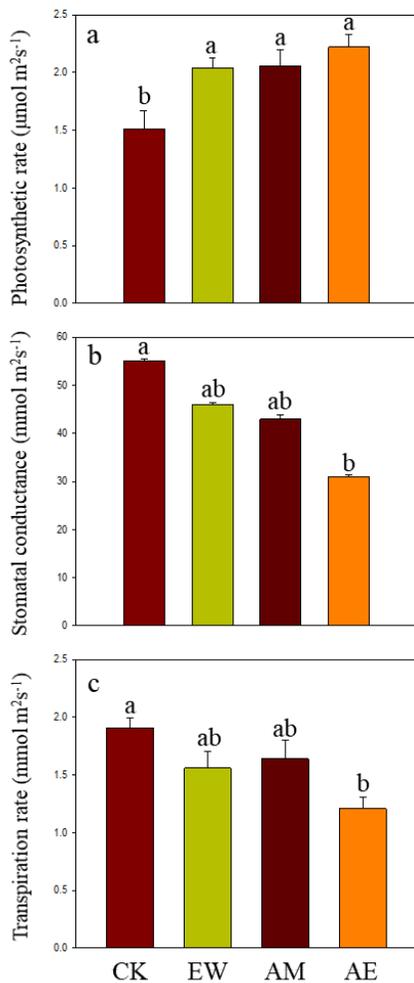


Figure 3

Effects of earthworms and mycorrhizae on photosynthetic rate (a), stomatal conductance (b), and transpiration rate (c) of maize under simulated acid rain stress. CK, treatment without earthworms or AM fungi; EW, treatment with the addition of earthworms; AM, inoculation of soil with AM fungi; and AE (AM + EW), inoculation of soil with both earthworms and AM fungi. Data represent the mean value  $\pm$  standard deviation from four replicates. Different lowercase letters indicate treatments that are significantly different ( $p < 0.05$ ).

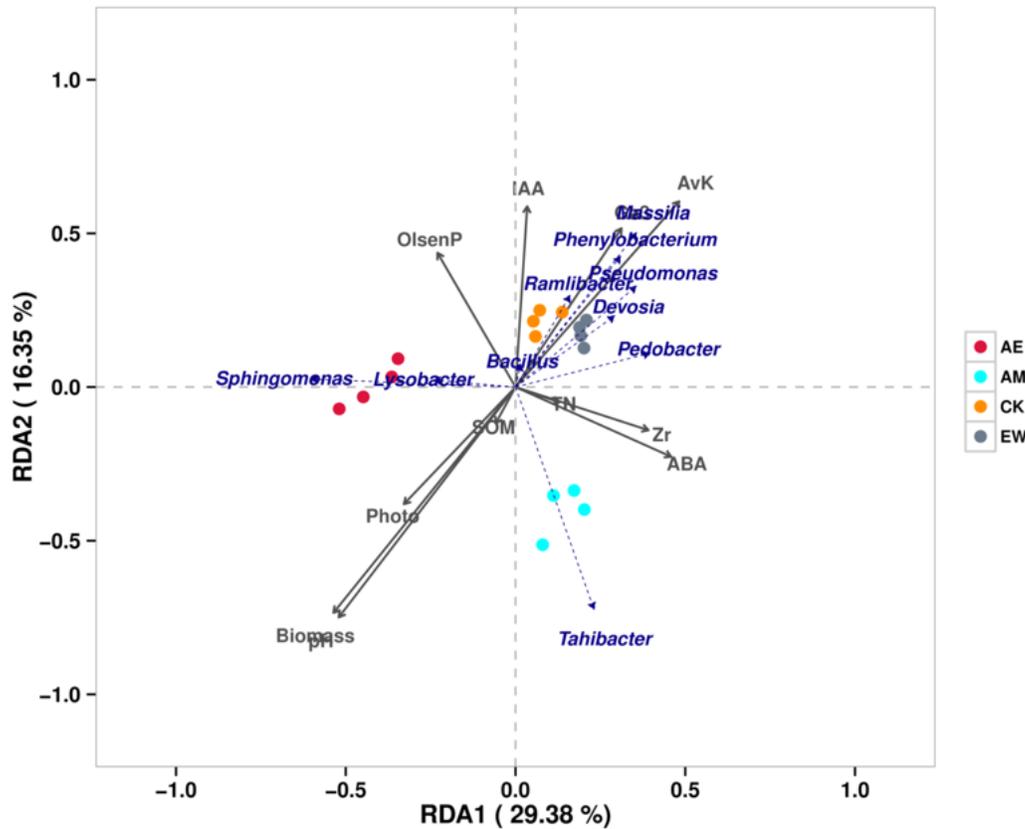
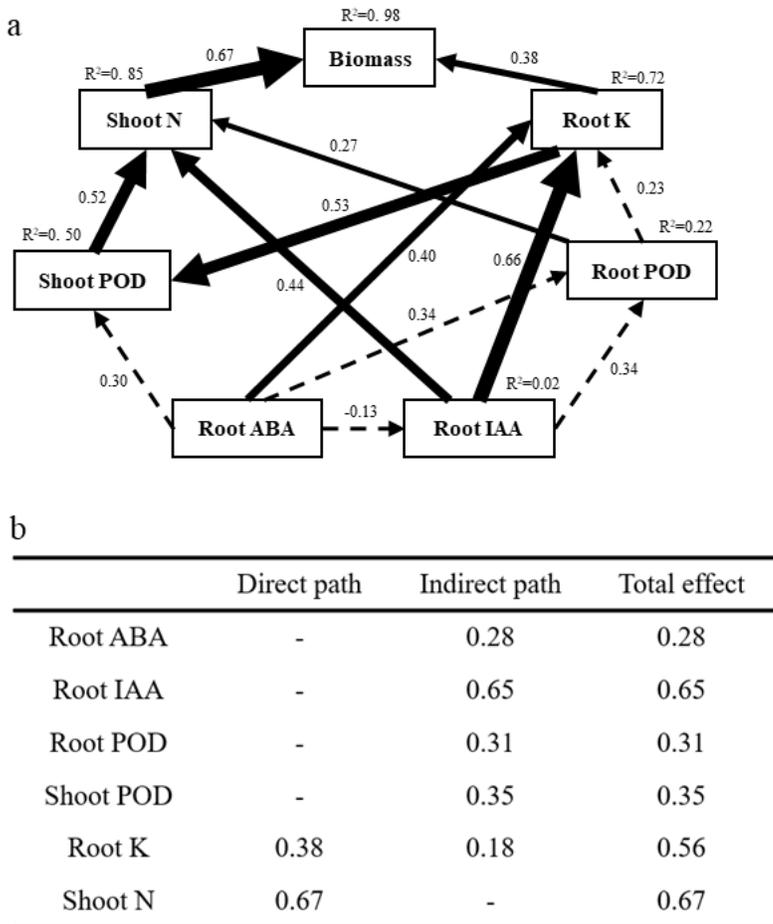


Figure 4

Combined correlation redundancy analysis of the OTU distribution of bacteria and the horizontal distribution of bacterial genera and plant growth factors. All OTUs were selected based on an  $R > 0.80$ , a  $p < 0.05$ , and a relative abundance  $> 0.1\%$ . CK is saline soil without the addition of earthworms and AM fungi, EW is saline soil with the addition of earthworms, AM is saline soil with the addition of AM fungi, and AE or AM + EW is saline soil with the addition of both earthworms and AM fungi.



**Figure 5**  
 Structural equation model simulating the relationship between physiological indices and biomass of maize under acid rain stress. a The relationships among root ABA, root IAA, root POD, shoot POD, root K, shoot N and maize biomass explained by a structural equation model. The path coefficients are located above the arrows. A thicker line indicates a stronger effect, and a dashed line indicates a nonsignificant effect at  $p < 0.05$ . The  $R^2$  values above each variable indicate the proportion of the variance explained. b The contribution of each variable to maize biomass via direct, indirect, and total effects.