

Effects of sodium sulfide application on the growth of *Robinia pseudoacacia*, heavy metal immobilization, and soil microbial activity in Pb–Zn polluted soil



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ABSTRACT

Sodium sulfide (Na₂S) is usually used as an amendment in industrial sewage treatment. To evaluate the effects of Na₂S on the growth of *Robinia pseudoacacia* (black locust), heavy metal immobilization, and soil microbial activity, the *R. pseudoacacia* biomass and nutrient content and the soil heavy metal bioavailability, enzyme activity, and arbuscular mycorrhizal (AM) fungal community were measured by a single-factor pot experiment. The Pb–Zn-contaminated soil was collected from a Pb–Zn mine that had been remediated by *R. pseudoacacia* for five years. Three pollution levels (unpolluted, mildly polluted, and severely polluted) were evaluated by the pollution load index. Na₂S application increased the shoot biomass under severe and mild contamination. In soil, Na₂S application decreased the bioavailable Pb and Zn contents under severe and mild contamination, which resulted in a decrease in the Pb and Zn content in *R. pseudoacacia*. However, Na₂S application did not affect the total Pb content per plant and enhanced the total Zn content per plant because of the higher biomass of the plants under Na₂S application. Increased phosphatase activity and increased available phosphorous content may promote the uptake of phosphorus in *R. pseudoacacia*. Moreover, Na₂S application is beneficial to the diversity of AM fungi under mild and severe pollution. Overall, Na₂S application has great potential for enhancing soil heavy metal immobilization, enhancing soil microbial activity, and improving the growth of *R. pseudoacacia* in polluted soils. Therefore, Na₂S is suitable for use in Pb–Zn remediation to ameliorate environmental heavy metal pollution.

1. Introduction

The development of the mining industry has made a significant contribution to local and national economies. However, the level of heavy metal pollution around the lead-zinc mining areas in China is quite serious (Yang et al., 2015; Hu et al., 2018). Large amounts of wastewater and tailings are produced during mining that threaten groundwater and endanger the soil ecological environment and the health of local residents (Du et al., 2019). For example, excessive zinc (Zn) intake alters lymphocyte function in the human body (Plum et al., 2010), and the nervous system can be damaged after long-term exposure to lead (Pb) (Mason et al., 2014). Once heavy metals are introduced into the soils, they persist for a long time and are difficult to

remove from soils. To alleviate the negative effects of heavy metal contamination on humans and the environment, the strategy of immobilizing or removing heavy metals from the soil by woody plants and microorganisms has received extensive attention.

Black locust (*Robinia pseudoacacia* L.) is a woody legume with fast growth, a deep root system, and strong adaptability. *R. pseudoacacia* is widely cultivated as a soil remediation plant in Pb–Zn polluted areas due to its strong resistance to heavy metals (Yang et al., 2015; Fan et al., 2018). *R. pseudoacacia* can form symbiotic relationships with arbuscular mycorrhizal (AM) fungi to obtain a better growth environment and stronger resistance to heavy metals (Shi et al., 2019). However, heavy metal pollution has a negative impact on the growth of *R. pseudoacacia* and the diversity of AM fungi, thus affecting the soil

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remediation effect (Yang et al., 2015; Dezhban et al., 2015). To improve the growth of black locust and the diversity of AM fungi, the simultaneous use of both remediation plants and chemical amendments to protect *R. pseudoacacia* from severe contamination should be considered (Qiao et al., 2015).

Sodium sulfide (Na_2S) is usually used to recover heavy metals from industrial waste (Kuchar et al., 2007). Recently, Na_2S has been considered as a chemical amendment for soil remediation (Mahar et al., 2016; Lu et al., 2017). Na_2S amendment prevents leaching and dissolution of heavy metals caused by acid rain or acidic wastewater by buffering soil pH value (Gu and Yeung, 2011; Zheng et al., 2012), which may be suitable for soil remediation of Pb–Zn mining area in northwestern China. Therefore, the combined use of *R. pseudoacacia* and Na_2S may be effective for soil remediation. However, the mechanism of Na_2S on phytoremediation, especially the effect of Na_2S on soil enzyme and AM fungi, has not been fully understood.

We hypothesized that Na_2S application improves *R. pseudoacacia* growth and enhances the immobilization of heavy metals in the soil. Therefore, the aim of this study was to evaluate whether Na_2S is suitable for improving the growth of *R. pseudoacacia* and enhancing heavy metal immobilization in Pb–Zn contaminated soils using a pot experiment. The biomass and nutrient status of *R. pseudoacacia* were used to evaluate the plant growth. The heavy metal content of *R. pseudoacacia*, the bioavailability of heavy metals in the soil, the soil enzyme activity, and the diversity of AM fungi were used to assess the ability of Na_2S to decrease heavy metal toxicity to plant and the impacts on AM fungal communities. This study provides new evidence for the role of Na_2S in heavy metal remediation.

2. Materials and methods

2.1. Experimental design

The pot experiment was performed using one Na_2S treatment (0% and 0.25% w/w Na_2S application per pot) as a single-factor experiment under three Pb–Zn pollution levels (unpolluted, mildly polluted, and severely polluted). The *R. pseudoacacia* seed was bought from the seed market in Yangling. The black locust seed was sterilized with 1% NaClO for 10 min and then cultured in Petri dishes with 0.8% water agar at 28 °C in the dark. Uniform germinated seeds were selected and transplanted into plastic pots (25 cm height, 20 cm diameter). The sand and soil were mixed thoroughly (1:1; w:w) as the culture substrate for the three pollution levels.

The sand was sieved through a 2 mm soil sieve, washed with tap water and sterilized at 170 °C for 3 h in an oven to prevent interference in the creation of the soil microbial community. The unpolluted and polluted soils were collected from Liuguan Town (33°82'51" N, 106°64'96" E), Feng County, Shaanxi Province, China. The sampling site has been remediated by black locust for five years. The primary vegetation was black locust and weeds. The soil type was yellow-brown soil, and the soil texture was clay. At each pollution site, the top 5 cm was removed to eliminate part of the leaf litter in each direction. The soils at 5–20 cm depth were collected and sieved by 2 mm mesh to remove stones, coarse roots, and other litter. The Pb and Zn contents of the collected soils were determined by flame atomic absorption spectrometry (AA-7003 A spectrometer, China). The pollution index (PI) of the collected soils was evaluated by the method of Chen et al. (2005) as described in Supplementary Table S1.

The pollution load index (PLI) of the culture substrates (soil: sand = 1:1; w/w) was calculated by the method of Chen et al. (2015). The PLI values of the three Pb–Zn pollution levels were classified as unpolluted ($\text{PI} \leq 1$), mildly polluted ($1 < \text{PI} \leq 4$), and severely polluted ($\text{PI} > 4$) conditions as described in Supplementary Table S2. In the Na_2S treatment, 0.25% (w/w) Na_2S was mixed into the culture substrate before transplanting. Each pot contained 1.5 kg mixed substrate and one black locust seed. Each treatment contained three

seedlings as three biological replicates. The seedlings were grown in a greenhouse at 28 °C/24 °C day/night temperatures under 14 h daylight and 40–60% humidity. Tap water was supplied every day throughout plant growth to maintain the soil moisture.

2.2. Plant sampling and biomass measurement

The plants were harvested after 10 months of cultivation. The rhizosphere soil was sampled by slight shaking and brushing. The roots were washed with tap water. The fresh shoots and roots were weighed. The shoot and root dry weights of each plant were calculated according to the fresh weight and fresh-to-dry mass ratio (Ma et al., 2014). Part of the fresh root was used to measure AM colonization. Parts of the root and shoot were dried to determine the heavy metal and nutrient content in black locust tissue.

2.3. AM fungal colonization

Part of the black locust root segment was washed under flowing water for 5 min, cleared for 30 min in 10% KOH at 90 °C, acidified in 1% HCl for 5 min, and stained in trypan blue (0.12%) at 90 °C (Koske and Gemma, 1989). AM colonization was evaluated following the modified method of McGonigle et al. (1990). The decolorized root segments were placed parallel to the long axis of the slide and then covered with a transparent coverslip. Five slides were used for each sample. Another coverslip with a vertical line was placed over the transparent coverslip. All intersections between roots and the vertical line were counted. AM colonization (%) = number of intersections colonized (hyphae, arbuscules, vesicles, and hyphal coils)/total number of intersections examined \times 100%.

2.4. Soil chemical properties and plant element content

The soil pH was evaluated by measuring the pH value of the soil solution (soil: water = 1:2.5 w/v) (Sheng et al., 2017). The available phosphorus (AP) was extracted by NaHCO_3 solution and was evaluated using the molybdenum antimony colorimetric method (Sheng et al., 2017). The available potassium (AK) was extracted by $\text{CH}_3\text{COONH}_4$ (pH 7.0) solution and was determined by flame atomic absorption spectrometry (AA-7003 A spectrometer, China). The available nitrate-nitrogen (AN) was extracted by CaCl_2 solution and was determined using an AA3 continuous flow analytical system (Bran + Luebbe AA3 autoanalyzer, Germany) (Hu et al., 2016). The diethylenetriaminepentaacetic acid (DTPA)-extractable Pb and Zn contents were extracted by DTPA solution and were determined by flame atomic absorption spectrometry (Yang et al., 2015).

Plant samples were digested by HNO_3 and HClO_4 (4:1 v/v) solution at 260 °C for 3 h. The digested solutions were used to determine the total potassium (TK), total calcium (TCa), total magnesium (TMg), total lead (TPb), and total zinc (TZn) contents using flame atomic absorption spectrometry. The digested solutions were used to determine the total phosphorus (TP) content using the molybdenum antimony colorimetric method. The total nitrogen (TN) was digested by concentrated H_2SO_4 and H_2O_2 and was determined using an AA3 continuous flow analytical system. The total amount of Pb or Zn removed per plant was calculated as follows: total amount of Pb/Zn removed per plant = shoot dry weight per plant \times shoot Pb/Zn content + root dry weight per plant \times root Pb/Zn content.

2.5. Soil enzyme activity

The soil urease activity was measured by the method of Błońska et al. (2016). The alkaline phosphatase activity was measured by the method of Dick et al. (2000). The soil sucrase activity was measured by the method of Ge et al. (2010).

2.6. DNA extraction, PCR, DGGE, and DGGE band sequencing

The soil DNA was extracted from 5.0 g of the fresh rhizosphere soil sample using SDS-CTAB (Sagar et al., 2014). The sediment was purified using a column centrifugation method (Kathiravan et al., 2015).

The diversity of AM fungi was assessed by denaturing gradient gel electrophoresis (DGGE). The 18 S rRNA genes of AM fungi were amplified by nest PCR. The universal primers NS1/NS4 were used for the first amplification of the AM fungi 18 S rRNA gene (Lankau and Nodurft, 2013). The universal primers AMV4.5NF/AMDGR were used for the second amplification of the AM fungi 18 S rRNA gene (Dai et al., 2013). A GC clamp was added to the 5' end of the forward AM fungi (AMV4.5NF) primers to stabilize the melting behavior of the DNA fragments. PCR was conducted using the parameters in the respective references at a final volume of 50 μ l. Negative controls (containing no soil DNA) were included in each PCR experiment.

DGGE was carried out on a DCode™ universal mutation detection system (Bio-Rad, Hemel Hempstead, UK) using 8% polyacrylamide gels with denaturant urea-formamide gradients of 15%–30% for AM fungi. Twenty-five microliters of the PCR products were loaded on denaturing gradient gel. Electrophoreses were run at 60 °C 120 V for 6 h. DGGE gels were stained for 30 min in 250 mL of TAE 1 \times buffer containing 25 μ l of DuRed stain (BioLinker, Shanghai). Bands were analyzed by Quantity One 4.6 (BioRad Laboratories, Hercules, CA, USA). Richness (*S*), evenness index (*E*), Shannon-Weaver index (*H*), and Simpson index (*D*) were calculated by the method of Agnolucci et al. (2015). The interesting bands were amplified with specific DGGE bands of AM fungi through the primer of AMV4.5NF/AMDGR without GC clamps. The amplified PCR product was used for sequencing. Sequences were analyzed using BLAST on the NCBI web site (<https://www.ncbi.nlm.nih.gov/>). The AM fungal sequences were phylogenetically analyzed using MEGA5.1 (<http://www.megasoftware.net/>). The DGGE band sequences of the AM fungi were submitted to GenBank (SUB5592969).

2.7. Statistical analysis

The statistical analysis was performed using the SPSS 22.0 statistical program (SPSS Inc., Chicago, IL, USA). The data used for ANOVA complied with the assumption of a normal distribution, and the variance equality was also tested. The data were analyzed using a one-way ANOVA followed by Duncan's test when the ANOVA was significant. Principal component analysis (PCA) was performed using SPSS 22.0. The parameters of biomass, soil available nutrient content, plant nutrient content, soil enzyme activity, and AM fungal diversity were used for the PCA. Canonical correlation analysis (CCA) was performed using Canoco 5.0. The soil available nutrient content, soil enzyme activity, and soil available heavy metal content were used for environmental factors. The AM fungal genus composition were used for species variant. Pearson's correlation analysis was performed to evaluate the relationship between soil chemical properties, soil enzyme activity, and the AM fungal diversity index (*S*, *H*, *D*, and *E*).

3. Results

3.1. The biomass and AM colonization

The plants in severely polluted soil had lower root and shoot dry weights compared with the plants in the mildly polluted and unpolluted soils (Fig. 1). In the severely polluted soil, Na₂S application significantly increased the shoot and root dry weight, by 34% and 11%, respectively, compared with the dry weight without Na₂S application. In the mildly polluted soil, Na₂S application increased shoot dry weight compared with the shoot dry weight without Na₂S.

The plants in the severely polluted soil had the lowest AM colonization, at 49% (Fig. 1c). The plants in the mildly polluted and unpolluted soils had the higher AM colonization, compared with severely

polluted soil. Na₂S application did not affect AM colonization in mildly and severely polluted soils.

3.2. The soil properties

Na₂S application significantly increased the pH value among the two pollution levels (Table 1). The severely polluted soil with Na₂S application had the highest pH value among six treatments, up to 8.72. Na₂S application did not affect the AK and AN contents. The content of AK decreased with increasing pollution levels. The AP content was significantly increased by Na₂S application by 60% and 76% under severe and mild pollution, respectively.

The DTPA-extractable Zn and Pb contents increased with increasing pollution levels (Table 1). Na₂S application significantly decreased the DTPA-extractable Zn and Pb contents under severe and mild pollution.

3.3. Nutrient content and heavy metal content

Na₂S application did not affect the TK content in black locust with or without pollution (Table 2). Na₂S application increased the TP content by 23%–39% and 34%–55% in shoots and roots, respectively, under mild and severe pollution compared with the TP content in plants without Na₂S. Na₂S application increased the TN content in shoots by 22% under severely polluted conditions and increased the TN content in shoots by 15% under mildly polluted conditions. However, the TN content in roots was not influenced by Na₂S application at any of the three pollution levels. The TCa content in shoots was greatly increased by Na₂S application under severe and mild pollution, but Na₂S application decreased the TCa content in roots under severe and mild pollution.

Na₂S application decreased the TPb and TZn contents in roots under severe pollution (Table 2). Na₂S application did not affect the TPb and TZn contents in shoots. Under mild pollution, Na₂S application did not affect the TPb or TZn contents in roots or shoots. Under unpolluted conditions, Na₂S application increased the TZn content in roots and increased the TPb content in shoots. The total amount of Pb removed was not affected by Na₂S application under severe and mild pollution (Fig. 2a). The total amount of Zn removed under severe pollution was increased by 39% by Na₂S application (Fig. 2b). However, under mild pollution, Na₂S application did not affect the total amount of Zn removed.

3.4. The soil enzyme activity

The sucrase activity decreased by 31%–32% and 43%–47% under mild and severe pollution conditions, respectively, compared with that under unpolluted conditions (Fig. 3a). Na₂S application increased the sucrase activity by 22%–31% compared with that under no Na₂S application.

Uncontaminated soil had the highest urease activity of the three soil conditions (Fig. 3b). However, no difference was observed in urease activity between the soils with severe and mild pollution. Na₂S application increased the urease activity by 20% under severe pollution conditions.

Na₂S application decreased the alkaline phosphatase activity under unpolluted conditions by 23% but increased the alkaline phosphatase activity under severe pollution by 56% (Fig. 3c). Na₂S application did not affect the alkaline phosphatase activity under mild pollution.

3.5. The AM fungal community

Na₂S application increased the Shannon-Weaver index of AM fungi under severe pollution (Fig. 4). Under unpolluted conditions, Na₂S application had a negative effect on the Shannon-Weaver and Simpson indices of AM fungi. Na₂S application had a positive effect on the Shannon-Weaver and Simpson indices of AM fungi under mild

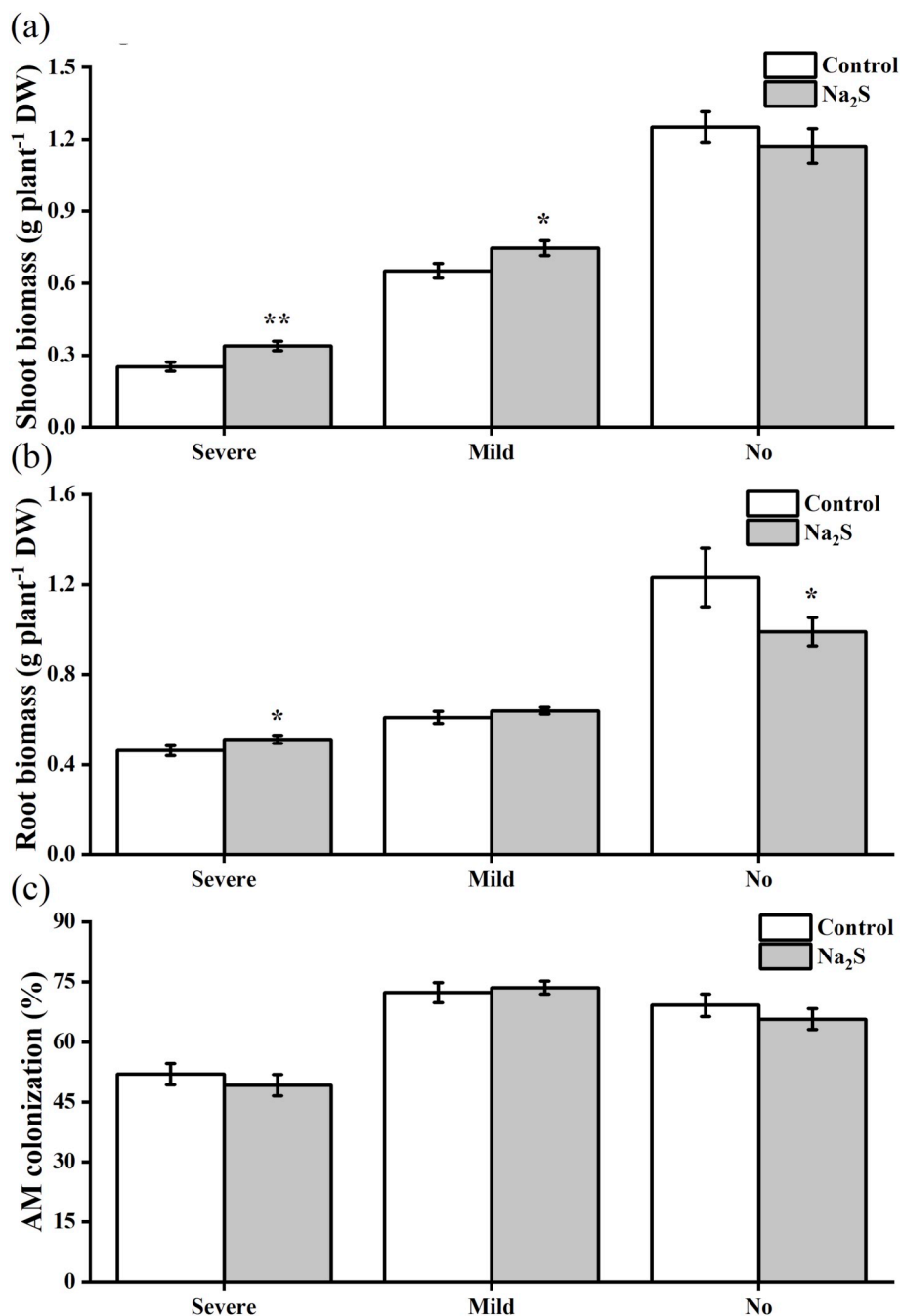


Fig. 1. (a–b) The shoot and root biomass of black locust under Na₂S application in three contaminate levels. (c) The AM colonization of black locust under Na₂S application in three contaminate levels. The data are the means \pm standard deviation ($n = 3$). Different asterisk above the columns indicate significant differences between the means by Duncan's test ($P < 0.05$). * $P < 0.05$, ** $P < 0.01$. Control = No Na₂S application; Na₂S = Na₂S application. Severe = Severe contamination; Mild = Mild contamination; No = No contamination.

pollution.

Na₂S application significantly increased the richness of AM fungi under severe pollution (Fig. 4). However, no significant effect of Na₂S application on the richness of AM fungi was found under mild pollution conditions. Na₂S application decreased the richness of AM fungi under unpolluted conditions. Na₂S application did not affect the evenness of AM fungi with or without pollution.

A total of twelve 18 S sequences of AM fungi were obtained, including three families: Glomeraceae, Claroideoglomeraceae, and Acaulosporaceae (Supplementary Fig. S1). A total of 10 species in Glomeraceae were found, belonging to 3 genera (*Glomus*, *Rhizophagus*, and *Funneliformis*). Glomeraceae is the dominant family in the black

locust rhizosphere (Supplementary Fig. S1). Under severe pollution, *Rhizophagus* had the highest relative abundance (Supplementary Fig. S2). Na₂S application greatly decreased the relative abundance of *Rhizophagus*, from 79.4% to 38.3%, but increased the relative abundance of *Claroideoglomus* under severe pollution. As the contamination levels increased, the relative abundance of *Glomus* increased. No significant difference in *Glomus* abundance was found between the soils with and without Na₂S. Na₂S application did not affect the composition of the AM fungal community under mildly polluted and unpolluted conditions.

Table 1
The soil properties of different contamination under Na₂S application.

Soil properties	S_Control	S_Na ₂ S	M_Control	M_Na ₂ S	N_Control	N_Na ₂ S
pH	8.48 ± 0.05	8.72 ± 0.07*	8.22 ± 0.09	8.42 ± 0.03*	8.13 ± 0.03	8.35 ± 0.01**
AK(mg/kg)	11.24 ± 1.26	13.91 ± 3.74	23.46 ± 5.34	22.57 ± 2.87	50.24 ± 8.33	49.59 ± 6.43
AP(mg/kg)	0.43 ± 0.05	0.69 ± 0.05*	0.95 ± 0.11	1.67 ± 0.16*	0.91 ± 0.13	0.78 ± 0.08
AN(mg/kg)	40.28 ± 4.95	47.99 ± 5.11	52.56 ± 5.92	61.48 ± 7.27	58.45 ± 4.08	52.96 ± 3.52
DTPA-Zn	33.63 ± 2.71	27.25 ± 1.72*	18.69 ± 2.18	13.27 ± 1.31*	4.2 ± 2.39	3.4 ± 2.36
DTPA-Pb	109.78 ± 16.40	75.56 ± 12.41*	65.48 ± 10.78	37.59 ± 5.43*	10.94 ± 0.56	11.41 ± 1.23

The data are the means ± standard deviation (n = 3). The asterisk within each column indicate significant differences between Na₂S and non-Na₂S applied soil by Duncan's test ($P < 0.05$). * $P < 0.05$, ** $P < 0.01$. S_Control = Severe contamination without Na₂S application; M_Control = Mild contamination without Na₂S application; N_Control = No contamination without Na₂S application; S_Na₂S = Severe contamination with Na₂S application; M_Na₂S = Mild contamination with Na₂S application; N_Na₂S = No contamination with Na₂S application.

3.6. PCA and CCA results

The PCA revealed the effects of Na₂S application both on the growth of black locust and on the soil conditions (Fig. 5a). PC1 accounted for 60% of the variance, and PC2 accounted for 21% of the variance. Under the three pollution levels, the Na₂S application and non-Na₂S application treatments were separated. The sample points for the three pollution levels were also separated.

The CCA revealed the relationship between environmental factors and AM genus composition (Fig. 5b). The length of the arrows indicates the relative importance of each environmental factor in explaining variation of AM community composition, while the angle between the arrows or axis indicates the degree to which they are correlated. More than 86.6% of the variance in AM fungal community composition could be explained by two canonical axes. The first canonical axis explained 68.6% of the AM community composition and was positively correlated with soil DTPA-extractable Pb and Zn content. The second canonical axis explained 18.0% of the AM community composition and was positively correlated with soil alkaline phosphatase activity.

4. Discussion

4.1. Na₂S application decreased the bioavailability of Pb and Zn under pollution

For heavy metal remediation plants such as black locust and maize, although they have strong heavy metal resistance, excessive heavy metals inhibit their due to heavy metal toxicity and nutrient uptake limitation, especially under severe pollution conditions (Vigliotta et al., 2016; Huang et al., 2017). In remediation, black locust as a xylophyta is suitable for phytostabilization, and maize as an industrial crop is suitable for phytoextraction. To improve the growth of black locust, Na₂S

was used as a chemical amendment to decrease heavy metal toxicity in soils. In this study, Na₂S application inhibited the release of bioavailable Pb and Zn under mild and severe pollution, which suggests that Na₂S application could immobilize Pb and Zn in the soil to prevent heavy metal toxicity to plant growth. The addition of Na₂S may decrease the release of heavy metal by forming lead or zinc sulfide precipitates (Manahan, 2017). Mahar et al. (2016) suggested that Na₂S application influences heavy metal solubility by increasing soil pH value. Na₂S in soil may induce NaOH through dissolution process ($\text{Na}_2\text{S} + \text{H}_2\text{O} \rightarrow \text{NaHS} + \text{NaOH}$), which results in the tendency to raise soil pH value (Mahar et al., 2016). Na₂S application also inhibited the content of Pb and Zn in black locust under mild and severe pollution, which supports the hypothesis that Na₂S application promotes black locust growth by decreasing the bioavailability of Pb and Zn and inhibiting the uptake of Pb and Zn. The immobilizing ability of Na₂S application to soil Pb is higher than Zn, indicating that Na₂S is more suitable for Pb phytostabilization. However, the immobilization of Pb and Zn by Na₂S application is not suitable for the phytoextraction of heavy metal from the soil. The total content of Zn per plant was enhanced by Na₂S application due to the higher biomass of Na₂S applied plants, suggesting Na₂S application enhancing the ability of Zn phytoextraction. Therefore, we considered that Na₂S application not only promotes the immobilization of Pb and Zn but also improves the total amount of Zn taken up by black locust.

The application of phosphate compounds, phosphogypsum, or phosphate rock increased the availability of phosphorus and then promoted the immobilization of heavy metals such as Pb and Zn, which indicates that soil with more available phosphorus is beneficial for heavy metal immobilization (Seshadri et al., 2017; Zhu et al., 2015; Mahmoud and Abd El-Kader, 2015). Increased available phosphorus content may also account for the enhancement of heavy metal immobilization. Under severe pollution, Na₂S application stimulated

Table 2
The nutrient content and heavy metal content in black locust under Na₂S application.

Plant element	Position	S_Control	S_Na ₂ S	M_Control	M_Na ₂ S	N_Control	N_Na ₂ S
TK (mg/g)	shoot	3.2 ± 0.32	2.9 ± 0.11	3.4 ± 0.095	3.3 ± 0.42	4.4 ± 0.52	4.2 ± 0.29
	root	3.0 ± 0.52	3.3 ± 0.63	6.0 ± 1.4	5.3 ± 0.83	6.5 ± 0.69	7.2 ± 0.91
TP (mg/g)	shoot	0.61 ± 0.044	0.72 ± 0.048*	0.77 ± 0.088	1.1 ± 0.091*	0.94 ± 0.077	0.77 ± 0.065*
	root	0.53 ± 0.079	0.79 ± 0.073*	0.93 ± 0.12	1.5 ± 0.20*	1.4 ± 0.21	0.80 ± 0.089*
TN (mg/g)	shoot	8.8 ± 0.65	11 ± 0.61*	12 ± 0.61	14 ± 0.53*	12 ± 1.1	12 ± 0.59
	root	12 ± 0.55	13 ± 0.53	15 ± 0.32	16 ± 0.53	16 ± 0.57	15 ± 0.88
TCa (100 mg/g)	shoot	0.26 ± 0.049	0.44 ± 0.085*	0.12 ± 0.018	0.42 ± 0.095**	0.18 ± 0.016	0.14 ± 0.0095*
	root	1.1 ± 0.086	0.31 ± 0.069**	1.5 ± 0.16	0.80 ± 0.019**	0.26 ± 0.079	0.35 ± 0.09
TMg (mg/g)	shoot	88 ± 10	72 ± 4.1	62 ± 5.0	52 ± 5.1	54 ± 4.7	47 ± 8.1
	root	34 ± 3.1	30 ± 1.7	24 ± 3.0	30 ± 6.7	24 ± 4.3	48 ± 4.6**
TZn (100 µg/g)	shoot	0.39 ± 0.048	0.37 ± 0.038	0.19 ± 0.047	22 ± 0.063	11 ± 0.019	10 ± 0.021
	root	1.6 ± 0.12	1.3 ± 0.13*	1.0 ± 0.15	0.82 ± 0.15	0.20 ± 0.016	0.31 ± 0.036**
TPb (100 µg/g)	shoot	2.4 ± 0.24	2.2 ± 0.28	1.4 ± 0.11	1.3 ± 0.18	0.31 ± 0.079	0.60 ± 0.063**
	root	9.8 ± 0.83	7.9 ± 0.69*	6.4 ± 0.56	5.6 ± 0.66	1.5 ± 0.13	1.8 ± 0.35

The data are the means ± standard deviation (n = 3). The asterisk within each column indicate significant differences between Na₂S and non-Na₂S applied soil by Duncan's test ($P < 0.05$). * $P < 0.05$, ** $P < 0.01$. The abbreviation is consistent with the above.

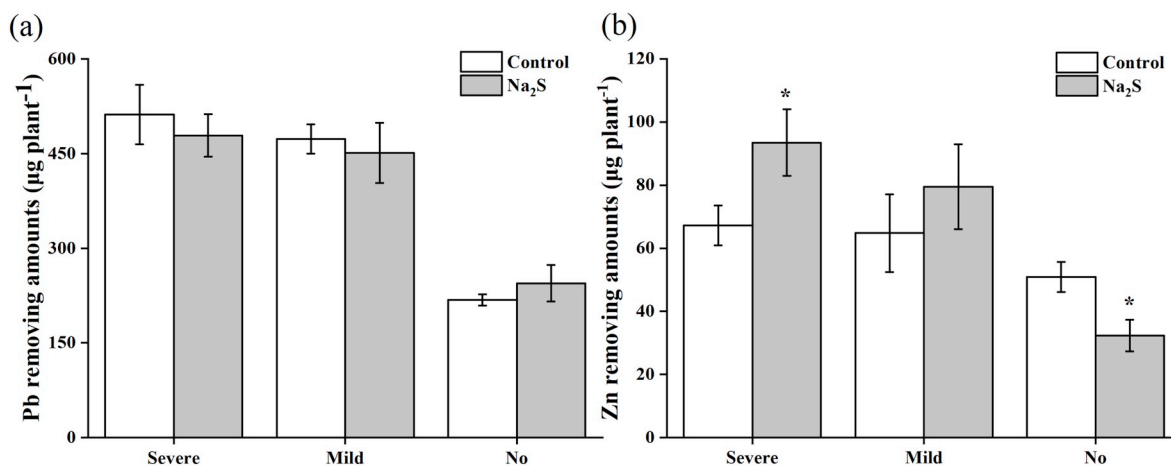


Fig. 2. (a–b) The Pb and Zn removing amount of black locust under Na₂S application. The data are the means ± standard deviation (n = 3). Different asterisk above the columns indicate significant differences among the means by Duncan's test ($P < 0.05$). * $P < 0.05$, ** $P < 0.01$. The abbreviation is consistent with the above.

alkaline phosphatase activity, which supports the finding that Na₂S application improved phosphorus activation in soils. Therefore, Na₂S application may also improve black locust growth by stimulating the release of available phosphorus. However, Na₂S application had a negative effect on the growth of black locust in unpolluted soil. Although Na₂S application did not affect the available phosphorus content in the soil, it reduced the activity of alkaline phosphatase and reduced the phosphorus content in shoots and roots, thereby inhibiting the growth of black locust in unpolluted soil. Moreover, Xu et al. (2016) indicated that an increase in pH leads to a decrease in the solubility of heavy metals in the soil, thereby reducing the bioavailability of heavy metals. Because Pb and Zn movement is restricted in a high-pH environment, the higher pH value in the Na₂S-applied soil is beneficial for heavy metal immobilization. Taken together, we suggest that Na₂S application may enhance heavy metal immobilization by precipitation, improving phosphorus release, and increasing soil pH value. Using Na₂S and black locust simultaneously to remediate Pb–Zn polluted soils has application potential.

4.2. Na₂S application improved soil microbial activity under severe pollution

Soil microbes also make an essential contribution to helping remediation plants survive in environments polluted with heavy metals (de Mora et al., 2005). Carbon, nitrogen, and phosphorus are the basic elements required for microbial metabolism. Therefore, the activities of urease, sucrase, and alkaline phosphatase were used to reflect soil microbial activity under the three pollution levels. Na₂S application improved soil microbial activity by increasing urease, sucrase, and alkaline phosphatase activities in severely polluted soils. The increased soil enzyme activity in Na₂S-treated soil may be due to the decreased heavy metal bioavailability and the increased available phosphorus content. Huang et al. (2017) indicated that the decreased extractable fraction of heavy metals resulted in the induction of urease activity because heavy metal toxicity inhibits soil microbial activity; this was consistent with our finding that the bioavailability of both Pb and Zn was negatively related to the activities of the three soil enzymes (Supplementary Table S3). Moreover, the soil enzyme activity was positively related to the soil available nitrogen and potassium content (Supplementary Table S3). Bowles et al. (2014) indicated that soil nutrients interact with soil metabolic enzymes; for example, an increase in carbon metabolism enzyme activity is accompanied by increased nitrogen availability in the field. Xie et al. (2016) considered that soil nutrients are positively related to soil enzyme activity in coastal saline soils. Taken together, these results indicate that soil microbes prefer to live in areas rich in

nutrients and low in heavy metal toxicity. Polluted soil with Na₂S is more suitable for microbial growth than polluted soil without Na₂S. Higher microbial activity creates positive feedbacks with the growth of black locust its heavy metal remediation abilities (Bezemer et al., 2006; Ke et al., 2015).

AM fungi are beneficial soil microbes that help host plant growth and improve stress tolerance. Many studies have demonstrated that AM fungal diversity decreases with increasing levels of heavy metal toxicity (Yang et al., 2015; del Mar Montiel-Rozas et al., 2016). Remediated soils have better AM fungal diversity than polluted soils due to the decreased heavy metal mobility (Alguacil et al., 2011; Ahmad et al., 2018). Correlation analysis also supports the hypothesis that Na₂S application may enhance AM fungal diversity under mild and severe pollution by decreasing Pb and Zn bioavailability (Supplementary Table S3). Therefore, we considered that Na₂S application is beneficial for the improvement of AM fungal diversity under Pb–Zn pollution. However, Na₂S negatively affected AM fungal diversity in the unpolluted soil. The available nutrient content was positively related to the AM fungal diversity, which suggests that AM fungal diversity is positively regulated by soil nutrients such as available phosphorus and nitrogen (Ohtomo and Saito, 2005; Zarei et al., 2010). Therefore, the negative effect of Na₂S on the AM fungal diversity of unpolluted soil may result from the decreased available phosphorus content.

Rhizophagus usually appears in severely polluted conditions to help host plants survive heavy metal toxicity by enhancing the accumulation of protective substances (Merlos et al., 2016) and decreasing the formation of reactive oxygen species (Ferreira et al., 2015). Correlation and CCA results also indicated that *Rhizophagus* prefers to exist in environments with high heavy metal toxicity (Fig. 5b; Supplementary Table S3). Due to the rapid extension of *Rhizophagus* in the root, most of the root spaces are occupied by *Rhizophagus*, which inhibits the toxicity of Pb and Zn with its hyphal barrier. However, the rapid extension of *Rhizophagus* also fills up the living space of AM fungi, which grow more slowly in the roots; this process also negatively affects AM fungal diversity (Supplementary Table S3). Therefore, Na₂S application to the soil may have been beneficial for AM community development under Pb–Zn pollution because it decreased the abundant percentage of *Rhizophagus* under severe pollution. Na₂S application in the used concentration is toxic to black locust and soil microorganisms, causing slow black locust growth, inhibition of soil enzyme activity, and a decrease in the AM fungal diversity in unpolluted soils.

5. Conclusion

To our knowledge, this is the first study evaluating the effects of

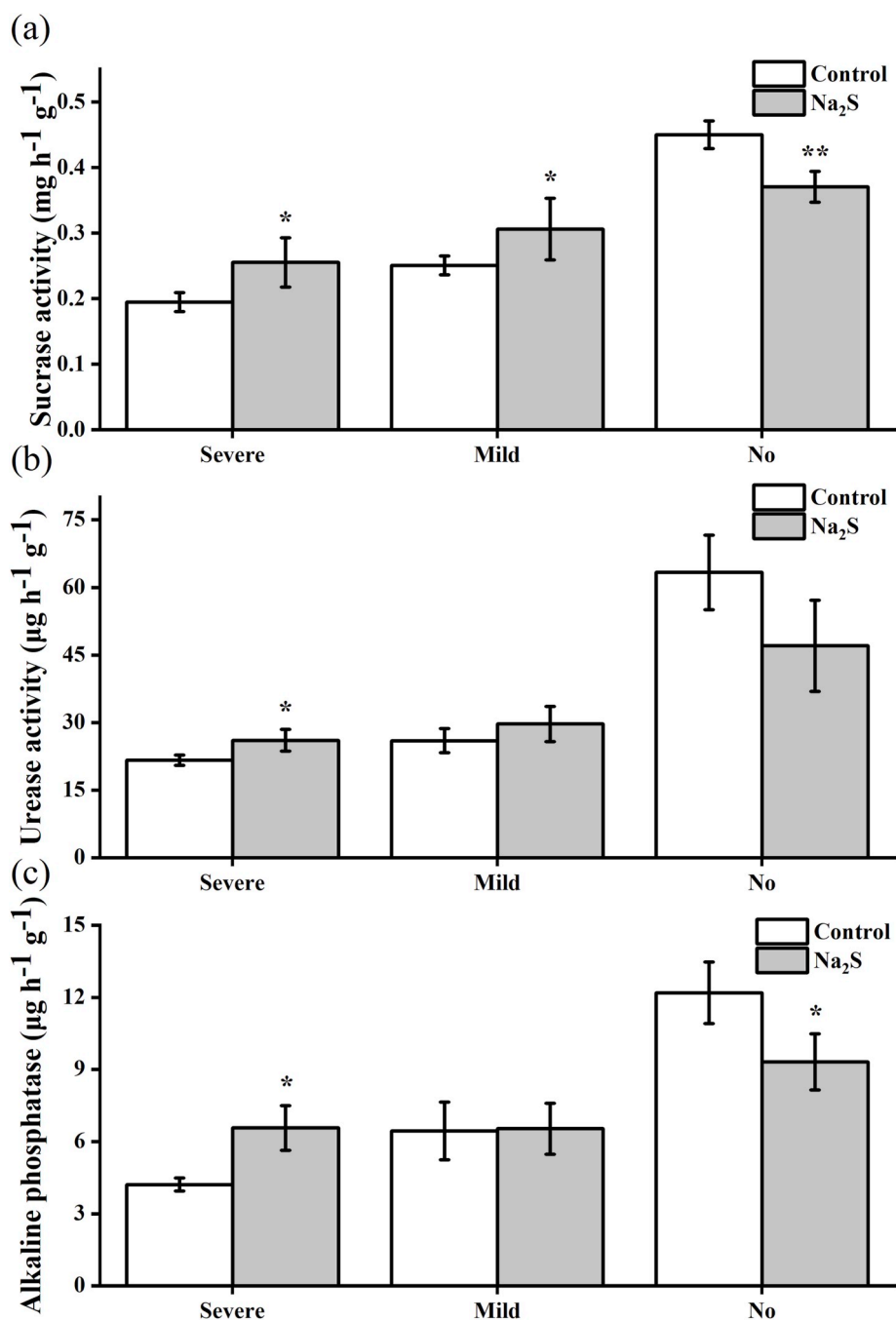


Fig. 3. (a–c) The soil enzyme activity of three contaminated soils under Na_2S application. The data are the means \pm standard deviation ($n = 3$). Different asterisk above the columns indicate significant differences among the means by Duncan's test ($P < 0.05$). * $P < 0.05$, ** $P < 0.01$. The abbreviation is consistent with the above.

Na_2S application on the growth of remediation plants, their capacity for heavy metal immobilization and soil enzyme activity in Pb–Zn polluted soil, and this study provides new evidence for the role of Na_2S in soil remediation. Na_2S application had different remediation effects in polluted and unpolluted soil. In unpolluted soil, Na_2S application negatively affects black locust growth by decreasing the available phosphorus content, inhibiting soil enzyme activity, and decreasing the AM fungal diversity. Na_2S application has a greater immobilization effect on Pb than Zn in polluted soil. In polluted soil, Na_2S application positively affects black locust growth by decreasing the bioavailability of Pb and Zn, increasing the available phosphorus content, stimulating soil enzyme activity, and improving the AM fungal diversity. The PCA result also supports the hypothesis that Na_2S application is beneficial in

polluted soil but is not suitable for unpolluted soil. Overall, Na_2S has potential for use as a chemical amendment in the remediation of Pb–Zn polluted soil. Field experiment is needed to verify the beneficial effect of Na_2S on phytoremediation.

CRediT authorship contribution statement

Xiangyu Zhang: Methodology, Conceptualization, Writing - original draft, Writing - review & editing. **Xiao Lou:** Investigation. **Haoqiang Zhang:** Resources. **Wei Ren:** Investigation. **Ming Tang:** Supervision, Funding acquisition, Project administration.

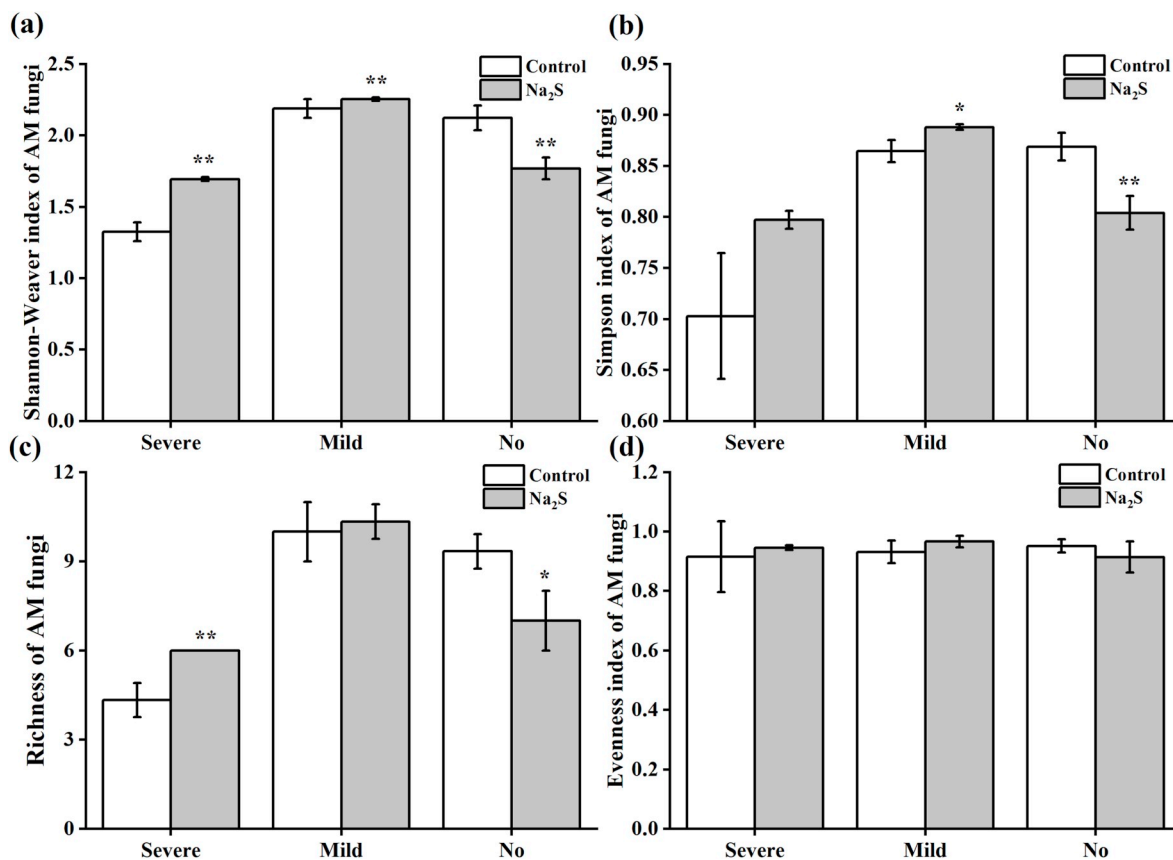


Fig. 4. (a–d) The ecological index of rhizosphere AM fungal community under Na₂S application at three contaminate levels. The data are the means ± standard deviation (n = 3). Different asterisk above the columns indicate significant differences among the means by Duncan's test (P < 0.05). *P < 0.05, **P < 0.01. The abbreviation is consistent with the above.

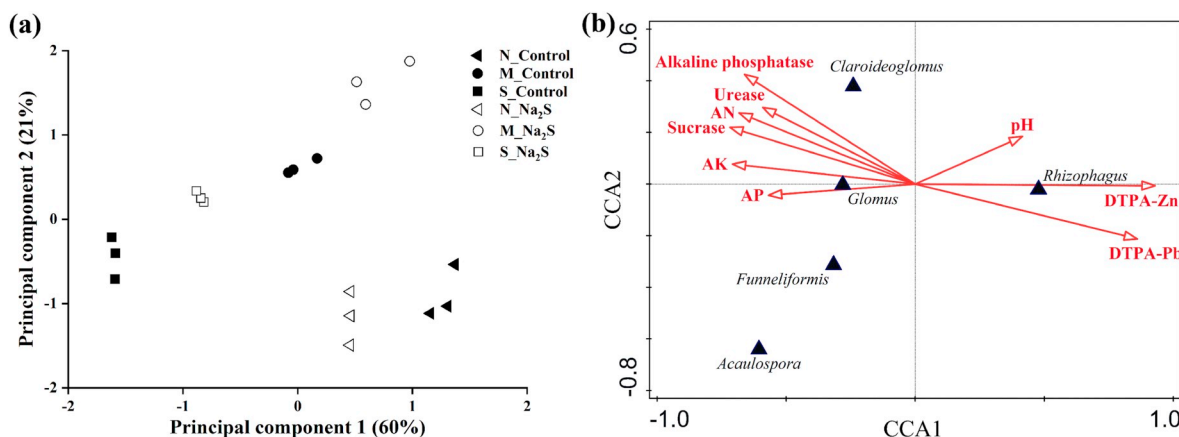


Fig. 5. (a) The PCA results of biomass, soil available nutrient content, plant nutrient content, soil enzyme activity, and AM fungal diversity; (b) The CCA results of environmental factor and species variant. The soil available nutrient content, soil enzyme activity, and soil available heavy metal content were used for environmental factors. The AM fungal genus composition were used for species variant.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ecoenv.2020.110563>.

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