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Combined Effect and Mechanism of Acidity and Lead Ion on Soybean Biomass

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Abstract Heavy metal pollution and soil acidification are serious global environmental issues. The combined pollution from acidification and heavy metal has become a new environmental issue in regions where the two issues simultaneously occur. However, studies on combined pollution are still limited. In the current study, we investigated the combined effect and mechanism of acidity and heavy metal [lead ion (Pb^{2+})] on soybean biomass as well as on growth, nitrogen nutrition, and antioxidant system in soybean roots. Results showed that the combined treatment with acidity and Pb^{2+} decreased the soybean biomass. At pH 4.5, the soybean biomass in the combined treatment with acidity and 0.9 mmol L^{-1} Pb^{2+} was lower than that in the combined treatment with acidity and Pb^{2+} at 0.3 or 1.5 mmol L^{-1}. This result was also observed at pH 3.5 and 3.0. The combined treatment with acidity and Pb^{2+} also resulted in the following consequences: root growth inhibition; decrease in nitrate, ammonium, and malondialdehyde contents; increase in nitrite reductase activity; and decrease in peroxidase activity. The extent at which the test indexes decreased/increased in the combined treatment was higher than that in the single acidity treatment. The correlation analysis results indicated that the decrease in the soybean biomass in the combined treatment with acidity and Pb^{2+} resulted from the decrease in the root growth, nitrate–nitrogen assimilation, and peroxidase activity.

Keywords Lead · Acidity · Combined Effect · Soybean · Biomass

Introduction

Heavy metal pollution has become a major environmental issue because of its high persistence in the environment and its serious impact on human health [1–3]. Lead (Pb) is one of the most common natural and hazardous heavy metals in soil [4]. Pb in the soil originates from various sources such as ore smelting, effluents from storage battery industries, industrial wastewater irrigation, urban solid waste, additive in pigments, and gasoline [1, 5–7]. Pb in the soil can then be absorbed by plant roots and influence the mineral nutrient absorption and metabolism, water metabolism, and photosynthesis in plants, ultimately affecting the growth, morphogenesis, and biomass of plants [1, 8, 9].

Soil acidification induced by acid rain is a serious global environmental problem that can deteriorate the natural ecosystem and soil property [10, 11]. Soil acidification has caused deleterious effects on the physiological characteristics, growth, and plant biomass of various plants [12–14]. These deleterious effects change the population structure of plants, thereby affecting the function of community [15].

Plant biomass depends on two important physiological processes: the carbon reduction-based air nutrition and the moisture/mineral nutrient metabolism-based soil nutrition. Plant roots absorb the moisture/mineral nutrients from the soil, and translocate them to aboveground organs. The inorganic nutrient absorbed by roots and the organic nutrients produced from aboveground photosynthesis contribute to the

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crop biomass [16, 17]. Herein, some problems should be paid attention to, including whether and how the combined pollution of acidification and Pb^{2+} affect the biomass and root growth of plants. Up to now, little information is available regarding these questions. Soybean (*Glycine max*) is one of the world's major economic crops and has been recommended for the use in phytotoxicity studies by the US EPA [18]. In this study, the crop biomass was used to evaluate the combined effects of acidity and Pb^{2+}, in which soybean (*G. max*), an important economic crop, was used as experimental plant [19]. The indexes of root growth, nitrogen nutrition, and antioxidant enzyme activities were assayed under combined pollution to elucidate its mechanism on crop biomass. This study aimed to investigate the combined effect and mechanism of acidity and Pb^{2+} on plant biomass.

Materials and Methods

Plant Culture and Treatments

Sterilized soybean seeds (Zhonghuang 25, Wuxi Seed Co., Ltd., China) were germinated and grown in a plastic case (32 × 21.5 × 10 cm) at 25±5 °C, 300 μmol m^{-2} s^{-1} light intensity, and 16/8-h day/night cycles in a greenhouse. The soybeans were cultured in the plastic case, in which vermiculite (3 L) and 500 mL of distilled water were added. The cases were shaken vigorously to ensure thorough mixing of contents. Fifteen-day-old seedlings were irrigated with modified Hoagland's solution at pH 7.0, and phosphate and sulfate were replaced by chloride and nitrate to avoid the precipitation of Pb^{2+} nutrient media. The modified nutrient solution was supplied every 3 days to provide enough nutrients. At the seed-filling stage, the soybean plants were subjected to four treatments. (1) For the control treatment, the soybean plants were irrigated with approximately 500 mL of modified Hoagland's solution at pH 7.0. (2) For the Pb^{2+} treatment, the soybean plants were irrigated with approximately 500 mL of modified Hoagland's solution (pH 7.0) with $(CH3COO)_2Pb$ at different concentrations (0.3, 0.9, and 1.5 mmol L^{-1}). (3) For the simulated acidification treatment/acidity treatment, the soybean plants were irrigated with approximately 500 mL of modified Hoagland's solution at different pH (3.0, 3.5, and 4.5). (4) For the Pb^{2+} and acidity treatment, the soybean plants were irrigated with approximately 500 mL of modified Hoagland's solution with Pb^{2+} at different concentrations (0.3, 0.9, and 1.5 mmol L^{-1}) and pH values (3.0, 3.5, and 4.5). All of the treatments were performed in three replicates. The soybean plants were sprayed with phosphate and sulfate every other day to provide the required phosphate and sulfate for plant growth. The solution was supplied every 3 days and the pH value of nutrient solution was adjusted daily with 1-M KOH or HCl. The soybean plants that were treated with Pb^{2+} and/or acidity for 7 days were collected to determine the test indexes.

Yield Parameters

The number of soybean legumes at the seed-filling stage was counted. The dry weight (DW) of total legumes and per legume was also determined.

Root Growth Parameters

The root growth parameters were determined using a root automatism scan apparatus (Perfection V700 Photo, Seiko Epson Corp, Japan) equipped with WinRHIZO software (Version 2009a, Regent Instruments, Quebec, Canada) according to previously described procedures [20]. In brief, the root segments were placed on the scan apparatus in a transparent plastic tray filled with distilled water. A white plastic plate was used as the image background. Image recording was performed at a resolution of 800 dpi and the images were saved in the tagged image file format. The phenotypic traits of the roots, including root length, root surface area, root volume, and average root diameter were evaluated using the WinRHIZO 2009a software. Three roots of soybean plants were analyzed for each replicate.

Measurement of the Content of Nitrate

Root samples (1 g) and polyvinylpolypyrrolidone (50 mg) were homogenized with 1 mL of saturated borax and 5 mL of double-distilled water. The homogenized mixture was then heated for 15 min in a boiling water bath. After the mixture was cooled, 2 mL of potassium ferrocyanide (0.25 M) and 2 mL of zinc acetate (1 M) were added and the mixture was centrifuged at room temperature for 10 min (10,000×*g*). Nitrate was obtained by reducing nitrate with vanadium (III) and detecting the acidic Griess reaction [21]. Nitrate was expressed as microgram per gram fresh weight.

Measurement of the Content of Ammonium

The ammonium content was determined spectrophotometrically according to Nessler's reagent method [22]. Root samples (500 mg) from the control and treated plants were homogenized in 0.3 mmol L^{-1} H_2SO_4 and centrifuged at 20,000×*g* for 20 min. The supernatant was used to estimate the ammonium content. The reaction mixture (2.7 mL) contained 0.1 mL of the extract, 0.1 mL of 10 % (*w*/*v*) potassium sodium tartrate, 2.4 mL of double-distilled water, and 0.1 mL of Nessler's reagent. After 5 min of incubation, the absorbance of the reaction mixtures was recorded at 425 nm. The ammonium content was calculated using the standard curve prepared with NH_4Cl.

Assay of the Activities of Nitrate Reductase and Nitrite Reductase

The soybean roots were homogenized in three volumes of extraction buffer according to a previously described method [23]. The extraction buffer contained 50 mmol L^{-1} Tris–HCl (pH 7.9), 5 mmol L^{-1} cysteine, and 2 mmol L^{-1} EDTA. The homogenate was centrifuged at 10,000×*g* for 20 min and the supernatant (500 μL) was concentrated with Microcon 10 (Amicon, USA) to reduce the number of nitrate ions. The concentrated supernatant was diluted by adding 500 μL of buffer [50 mmol L^{-1} Tris–HCl (pH 7.5), 1 mmol L^{-1} cysteine, and 2 mmol L^{-1} EDTA]. The nitrate reductase (NR) and nitrite reductase (NiR) activities were then assayed. The procedures described above were performed at 4 °C. Enzyme activity was assayed by using an in vitro method. The assay mixture for NR contained 25 mmol L^{-1} potassium phosphate buffer (pH 7.5), 10 mmol L^{-1} KNO_3, 0.2 mmol L^{-1} NADH, 5 mmol L^{-1} $NaHCO_3$, and 5 μL of extract in a final volume of 0.5 mL. The assays were conducted at 30 °C for 15 min. The reaction was terminated by adding 50 μL of 0.5-M $Zn(CH_3COO)_2$ and the excess NADH was oxidized by adding 50 μL of 0.15 mmol L^{-1} phenazine methosulfate [24]. The mixture was centrifuged at 10,000×*g* for 5 min. The amount of produced NO_2^- was measured by combining 500 μL of the supernatant with 250 μL of 1 % sulfanilamide, which was prepared in 1.5-N HCl and 250 μL of 0.02 % N-(1-naphtyl)ethylene-diamine dihydrochloride, and read at 540 nm with a spectrophotometer [25].

The NiR activity was assayed by observing the reduction of NO_2^- in the assay mixture [26], which contained 50 mmol L^{-1} Tris–HCl (pH 7.5), 0.5 mmol L^{-1} $NaNO_2$, 1 mmol L^{-1} methyl viologen, and 50 μL of the extract in a final volume of 0.9 mL. The reaction was initiated by adding 100 μL of 0.12-M $Na_2S_2O_4$ dissolved in 0.2-M $NaHCO_3$, and then incubated at 30 °C for 60 min. The reaction was terminated by vigorously vortexing until the color of the methyl viologen disappeared completely. After 100 μL of 1-M $Zn(CH_3COO)_2$ was added, the mixture was centrifuged at 10,000×*g* for 10 min. The residual NO_2^- in the supernatant was determined based on the same procedure used in the NR activity assay.

Lipid Peroxidation

The level of lipid peroxidation was expressed as the content of malondialdehyde (MDA) [27]. The samples were repeatedly extracted with 4:1 (*v*/*v*) ethanol/water that contained 1 mg L^{-1} of butylated hydroxytoluene (BHT) by sonication. After the samples were centrifuged, the supernatant was pooled and an aliquot of appropriately diluted sample was placed in a test tube at an equal volume of either (1) −thiobarbituric acid (TBA) solution with 20 % (*w*/*v*) trichloroacetic acid and 0.01 % (*w*/*v*) BHT or (2) +TBA solution with the same content plus 0.65 % TBA. The sample was heated at 95 °C for 25 min. After cooling, the absorbance was determined at 440, 532, and 600 nm. MDA is expressed as follows:

$$MDA\left(nmolml^{-1}\right) = 10^6 \times (A-B)/157,000$$

$$\mathrm{A} = \left(Ab_{\mathrm{S532}\ +TBA} - Ab_{\mathrm{S600}+TBA}\right) / \left(Ab_{\mathrm{S532}-TBA} - Ab_{\mathrm{S600}-TBA}\right)$$

$$\mathrm{B} = \left(Ab_{\mathrm{S440}+TBA} - Ab_{\mathrm{S600}+TBA}\right) \times 0.0571$$

Here, 157,000 was the molar extinction coefficient for MDA. The molar absorbance of 1–10 mmol L^{-1} sucrose at 532 and 440 nm was 8.4 and 147, respectively, giving a ratio of 0.0571.

Activities of Peroxidase and Calatase

The roots were homogenized in 50 mmol L^{-1} potassium phosphate buffer (pH 7.8) with 5 mmol L^{-1} ascorbic acid, 5 mmol L^{-1} dithiothreitol, 5 mmol L^{-1} EDTA, and 2 % (*v*/*v*) polyvinylpyrrolidone. The homogenates were centrifuged at 15,000×*g* for 15 min and the supernatants were used for the enzyme activity assay. The catalase (CAT) activity was determined by following the consumption of H_2O_2 at 240 nm according to Beers and Sizer [28]. Each 3 mL of the reaction mixture contained 100 mmol L^{-1} potassium phosphate buffer at pH 7.0 and 50 μL of the enzyme extract. The reaction was initiated by adding 15 mmol L^{-1} H_2O_2. The peroxidase (POD) activity was determined according to a previously described method [29]. The reaction mixture contained phosphate buffer (pH 7.0, 25 mmol L^{-1}), guaiacol (0.05 %), H_2O_2 (10 mmol L^{-1}), and crude peroxidase. The CAT activity was determined based on the increase per minute in the absorbance at 470 nm by guaiacol oxidation (E =26.6 mmol L^{-1} cm^{-1}).

Membrane Permeability

Membrane permeability was measured according to the relative conductivity method described in a previous study [30]. In brief, the soybean roots were rinsed, placed in 40 mL of deionized water, and gently tumbled at ambient room temperature. Conductance of the deionized water was determined after 15 min (C_1), 2 h (C_2), and 2 h after a freeze–thaw treatment (C_{total}). The rate of electrolyte leakage was expressed as $^{\%}/_{h} = 100 \times (C1–C2)/(1.75C_{total})$.

Statistical Analysis

Significant differences between different treatments were analyzed by ANOVA on SPSS 16. Student's *t* test was used to determine the significance between different treatments ($p \leq 0.05$ or $p \leq 0.01$).

Results

Combined Effects of Acidity and Pb^{2+} on Soybean Biomass

Table 1 shows the biomass indexes in the soybean plants that were subjected to acidity and treated with different Pb^{2+} concentrations. At pH 4.5, 3.5, and 3.0, the number of legumes decreased by 8.33, 20.83, and 29.17 %, respectively, compared with that in the control treatment. At the same pH levels, the DW of total legumes decreased by −14.76, 9.39, and 29.87 %, and the DW per legume also increased by 25, 14.58, and −6.25 %, respectively, compared with that in the control treatment. At Pb^{2+} concentrations of 0.3, 0.9, and 1.5 mmol L^{-1}, the number of legumes decreased by 4.17, 20.83, and 25 %, respectively, in comparison with the control. At the same Pb^{2+} concentrations, the DW of total legumes also decreased by 22.77, 29.57, and 51.3 %, respectively, by contrast, the DW per legume decreased by 19.79, 10.42, and 38.66, respectively, compared with the control treatment.

The combined treatment with acidity and Pb^{2+} decreased the number of legumes by 4.17 to 62.5 %. At pH 4.5, the DW of total legumes in soybean treated with 0.3, 0.9, and 1.5 mmol L^{-1} Pb^{2+} increased by 9.83, −56.02, and −0.35 %, respectively, compared with the control treatment; meanwhile, the DW per legume also increased by 39.58, -33.59, and 26.1 %, respectively. At pH 3.5, the DW of total legumes decreased by 50.39, 24.98, and −7.88 % in soybean treated with 0.3, 0.9, and 1.5 mmol L^{-1} Pb^{2+}, and the DW per legume decreased by 20.14, −29.17, and12.5 %, respectively, compared with the control treatment. At pH 3.0, the DW of total legumes in soybean treated with 0.3, 0.9, and 1.5 mmol L^{-1} Pb^{2+} decreased by 8.00, 86.62, and 51.39 %, respectively; meanwhile, the DW per legume also decreased by 0, 99.84, and 37.5 %, respectively. Thus, the soybean biomass in the combined treatment with acidity and 0.9 mmol L^{-1} Pb^{2+} was the lowest among the combined treatments with acidity at the same pH and other Pb^{2+} concentrations (0.3 and 1.5 mmol L^{-1}). The crop biomass increased when the soybean plants were subjected to acidity (4.0, 3.5, and 3.0) and treated with 1.5 mmol L^{-1} Pb^{2+} compared with the single treatment of 1.5 mmol L^{-1} Pb^{2+}.

Combined Effects of Acidity and Pb^{2+} on the Growth of Soybean Roots

At different pH levels (4.5, 3.5, and 3.0), a decrease was observed in the main root length (−7.74, 13.07, and 22.67 %), root surface area (9.63, 22.76, and 30.2 %), root volume (36.27, 19.79, and 39.97 %), and average root diameter (2.57, −3.04, and −0.47 %) compared with the control treatment (Table 2). The single treatment with different Pb^{2+} concentrations (0.3, 0.9, and 1.5 mmol L^{-1}) also decreased the main root length (1.75, 21.11, and 18.88 %), root surface area (4.39, 22.22, and 27.60 %), root volume (13.33, 18.66, and 42.22 %), and average root diameter (−13.55, 0.47, and −0.23 %) compared with the control treatment (Table 2).

The combined treatments with acidity at pH 4.5 and different Pb^{2+} concentrations (0.3, 0.9, and 1.5 mmol L^{-1}) caused the growth parameters to decrease compared with the control treatment: main root length (−1.66, 4.44, and 27.44 %); root surface area (16.46, 18.76, and 40.23 %); root volume (36.44, 36.17, and 37.65 %); and average root diameter (3.97, 1.87, and 4.22 %) (Table 2). The combined treatments with acidity at pH 3.5 and different Pb^{2+} concentrations (0.3, 0.9, and

Table 1 Effects of acidity and Pb^{2+} on the biomass of soybean

Treatment	TLN (legume)	DWTL (g)	DWPL (g)
pH 7.0	24±0.7a (100.00)	2.310±0.060c (100.00)	0.096±0.002f (100.00)
pH 4.5	22±0.6d (91.67)	2.651±0.067a (114.76)	0.120±0.003b (125.00)
pH 3.5	19±0.6c (79.17)	2.093±0.053d (90.61)	0.110±0.003d (114.58)
pH 3.0	17±0.5b (70.83)	1.620±0.041gh (70.13)	0.095±0.002g (93.75)
Pb 0.3	23±0.7b (95.83)	1.784±0.045ef (77.23)	0.077±0.002i (80.21)
Pb 0.9	19±0.6b (79.17)	1.627±0.041g (70.43)	0.086±0.002h (89.58)
Pb 1.5	18±0.5ab (75.00)	1.125±0.028i (48.70)	0.059±0.002k (61.34)
pH 4.5+Pb 0.3	19±0.6de (79.17)	2.537±0.064b (109.83)	0.134±0.003a (139.58)
pH 4.5+Pb 0.9	16±0.5e (66.67)	1.016±0.026i (43.98)	0.064±0.002k (66.41)
pH 4.5+Pb 1.5	19±0.5f (79.17)	2.302±0.058b (99.65)	0.121±0.003b (126.10)
pH 3.5+Pb 0.3	17±0.5c (89.47)	1.146±0.029f (49.61)	0.115±0.002b (120.14)
pH 3.5+Pb 0.9	15±0.4cd (62.50)	1.733±0.044h (75.02)	0.077±0.003i (80.21)
pH 3.5+Pb 1.5	23±0.7d (95.83)	2.492±0.063b (107.88)	0.108±0.002b (112.50)
pH 3.0+Pb 0.3	22±0.6c (91.67)	2.125±0.054d (92.00)	0.096±0.003f (100.00)
pH 3.0+Pb 0.9	9±0.3c (37.50)	0.309±0.008j (13.38)	0.004±0.001l (4.16)
pH 3.0+Pb 1.5	19±0.6b (79.17)	1.123±0.028h (48.61)	0.060±0.002e (62.50)

Data are presented as means ± standard deviation

Significantly differences at $p < 0.05$ were showed with different letter in the same column

pH 4.5+Pb 0.3 represents the combined treatment with acidity at pH 4.5 and 0.3 mmol L^{-1} Pb^{2+}

TLN total legume number, *DWTL* dry weight of total legumes, *DWPL* dry weight per legume

Table 2 Effects of acidity and Pb^{2+}on root growth of soybean

Treatment	RL (cm)	RSA (cm^2)	RV (cm^3)	ARD (mm)
pH 7.0	1,808.38±122.18ab (100.00)	284.05±35.46a (100.00)	3.32±0.28a (100.0)	0.42±0.03bc (100)
pH 4.5	1,947.51±65.16a (107.74)	256.70±26.69b (90.37)	2.11±0.32cd (63.73)	0.41±0.03bc (97.43)
pH 3.5	1,572.11±174.58cd (86.93)	219.00±28.54d (77.24)	2.66±0.57b (80.21)	0.44±0.01ab (103.04)
pH 3.0	1,397.27±79.98de (77.33)	198.29±18.32e (69.8)	1.99±0.03cde (60.03)	0.42±0.02bc (99.53)
Pb 0.3	1,776.83±55.02b (98.25)	271.58±16.57ab (95.61)	2.88±0.23ab (86.67)	0.48±0.02a (113.55)
Pb 0.9	1,426.81+139.81de (78.89)	201.04±24.81d (77.78)	2.70±0.30b (81.34)	0.42±0.02bc (99.53)
Pb 1.5	1,466.25±117.47d (81.12)	205.62±23.86ad (72.40)	1.92±0.24def (57.78)	0.43±0.02bc (100.23)
pH 4.5+Pb 0.3	1,838.50±76.74a (101.66)	237.30±13.12c (83.54)	2.11±0.21cd (63.56)	0.41±0.02bc (96.03)
pH 4.5+Pb 0.9	1,728.18±136.71b (95.56)	232.91±19.52cd (81.24)	2.12±0.23cd (63.83)	0.43±0.01bc (98.13)
pH 4.5+Pb 1.5	1,312.39±140.75e (72.56)	169.77±19.05f (59.77)	2.07±0.36cde (62.35)	0.40±0.01c (95.78)
pH 3.5+Pb 0.3	1,563.15±273.18c (86.52)	226.96±53.25cd (79.90)	2.47±0.19bc (74.48)	0.44±0.03b (100.45)
pH 3.5+Pb 0.9	1,569.08±101.19c (86.77)	239.13±32.19c (84.19)	1.48±0.24fg (44.55)	0.47±0.03a (111.45)
pH 3.5+Pb 1.5	1,485.78±84.90cd (82.12)	191.91±16.12e (67.56)	1.62±0.27efg (48.89)	0.41±0.01c (93.45)
pH 3.0+Pb 0.3	1,712.35±106.74b (94.74)	256.32±17.28b (90.24)	1.77±0.19defg (53.25)	0.48±0.02a (111.92)
pH 3.0+Pb 0.9	1,439.72±72.78d (79.61)	190.55±9.34e (67.10)	1.35±0.07g (40.72)	0.42±0.01a (98.60)
pH 3.0+Pb 1.5	1,320.62±257.54e (73.03)	188.66±46.47e (66.40)	1.41±0.37g (64.04)	0.44±0.03a (102.10)

Data are presented as means ± standard deviation

Significantly differences at $p<0.05$ were showed with different letter in the same column

pH 4.5+Pb 0.3 represents the combined treatment with acidity at pH 4.5 and 0.3 mmol L^{-1} Pb^{2+}

RL total root length, *RSA* root surface area, *RV* root volume, *ARD* average root diameter

1.5 mmol L^{-1}) also caused the growth parameters to decrease compared with the control treatment (Table 2): main root length (13.48, 13.23, and 17.88 %), root surface area (20.1, 15.81, and 32.44 %), root volume (25.52, 55.45, and 51.11 %), and average root diameter (−0.45, −11.45, and 6.55 %). At pH 3.0 and different Pb^{2+} concentrations (0.3, 0.9, and 1.5 mmol

Table 3 Effects of acidity and Pb^{2+}on nitrogen nutrition of roots in soybean

Treatment	Nitrate content (μg/100 g)	NR activity ([μg/(g h)])	NiR activity ([μg/(g h)])	Ammonium content (μg/g)
pH 7.0	390.37±11.27a (100.00)	0.39±0.10f (100.00)	24.15±0.01h (100.00)	49.32±2.41a (100.00)
pH 4.5	292.52±8.44bc (74.93)	3.52±0.34c (902.56)	25.51±0.01fg (105.63)	44.48±2.01ab (90.19)
pH 3.5	233.81±6.75bcd (59.89)	7.50±0.59b (1928.21)	25.60±0.03f (106.00)	42.86±1.44bc (86.90)
pH 3.0	194.67±5.62bc (49.87)	0.29±0.00f (74.36)	25.86±0.07de (107.08)	42.10±1.74bcd (85.50)
Pb 0.3	312.09±9.01ab (79.94)	0.70±0.10ef (200.00)	25.35±0.13g (104.97)	46.55±1.28ab (94.38)
Pb 0.9	269.86±7.79bc (69.13)	1.20±0.10de (325.64)	25.36±0.04g (105.01)	33.88±1.83ef (68.69)
Pb 1.5	214.24±6.18cde (54.88)	0.29±0.00f (74.36)	25.69±0.05ef (106.38)	37.56±0.83de (76.16)
pH 4.5+Pb 0.3	222.60±6.43bcd (58.05)	3.61±0.42c (925.64)	26.56±0.02b (109.98)	38.00±0.80cde (77.09)
pH 4.5+Pb 0.9	213.21±6.15cde (54.62)	0.39±0.10f (100.00)	26.24±0.04c (108.65)	30.88±1.22fg (62.61)
pH 4.5+Pb 1.5	212.18±6.13cde (54.09)	2.05±0.45d (525.64)	25.97±0.02d (107.54)	30.88±1.28fg (62.61)
pH 3.5+Pb 0.3	156.56±4.52bc (40.11)	0.70±0.10ef (200.00)	26.32±0.17c (108.99)	36.18±0.61e (73.36)
pH 3.5+Pb 0.9	198.79±5.74bc (50.92)	2.05±0.34d (525.64)	26.80±0.07a (110.97)	26.96±1.06g (54.66)
pH 3.5+Pb 1.5	132.87±3.84efg (34.04)	11.00±0.59a (2879.49)	26.95±0.02a (111.59)	26.96±1.60g (54.66)
pH 3.0+Pb 0.3	136.99±3.95efg (35.09)	0.40±0.10ef (125.64)	25.59±0.03f (105.96)	38.40±1.00cde (78.04)
pH 3.0+Pb 0.9	120.51±3.48fg (30.87)	0.40±0.20ef (125.64)	25.67±0.04ef (106.29)	34.11±3.10ef (69.16)
pH 3.0+Pb 1.5	104.00±3.00g (21.10)	0.29±0.00f (74.36)	25.91±0.09d (107.29)	36.64±3.01e (74.29)

Data are presented as means ± standard deviation

Significantly differences at $p<0.05$ were showed with different letter in the same column

NR nitrate reductase, *NiR* nitrite reductase

pH 4.5+Pb 0.3 represents the combined treatment with acidity at pH 4.5 and 0.3 mmol L^{-1} Pb^{2+}

L^{-1}), a decrease was also observed in the main root length (5.26, 20.39, and 26.97 %), root surface area (9.76, 32.9, and 33.6 %), root volume (46.75, 59.28, and 35.96 %), and average root diameter (−11.92, 1.4, and −2.1 %) of the soybeans compared with the control treatment (Table 2).

To understand the relationship between the biomass and root growth in soybean plants that were treated with Pb^{2+} and acidity, the correlation coefficients (r) between the growth and biomass indexes were calculated. The linear regression equation and r of the total number of legumes and the main root length (root surface area, root volume, average root diameter) were $y=0.359x+59.241$, $r=0.306$, $p<0.05$ ($y=0.407x+61.282$, $r=0.154$, $p>0.05$; $y=0.633x+52.901$, $r=0.302$, $p<0.05$; $y=-0.176x+110.662$, $r=0.046$, $p>0.05$). The linear regression equation and r of the DW of total legumes and the main root length (root surface area, root volume, root average diameter) were $y=0.151x+0.05$, $r=0.407$, $p<0.05$ ($y=0.117x+0.054$, $r=0.304$, $p<0.05$; $y=0.193x+0.072$, $r=0.369$, $p<0.05$; $y=-0.038x+0.034$, $r=0.162$, $p>0.05$). The results indicated that the total number of legumes was positively related to the main root length and root volume ($p<0.05$). The DW of total legumes was positively related to the main root length, the root volume and root surface area ($p<0.05$).

Combined Effects of Acidity and Pb^{2+} on Nitrogen Nutrition in Soybean Roots

Table 3 shows the nitrogen nutrition indexes of soybean subjected to acidity at different pH levels and treated with different concentrations of Pb^{2+}. In the soybean roots that were subjected to acidity at pH 4.5, 3.5, and 3.0, the nitrate/ammonium contents decreased by 25.07/50.13, 40.11/13.1, and 9.81/14.5 %, respectively, compared with those in the control treatment. The NR activity initially increased sharply and subsequently decreased. At the same pH levels, the NiR activities in the soybean roots increased by approximately 5.63, 6.0, and 7.08 % compared with that in the control treatment, respectively. In the soybean roots that were treated with Pb^{2+} at 0.3, 0.9, and 1.5 mmol L^{-1}, the nitrate/ammonium contents decreased by 20.06/5.62, 30.87/31.31, and 45.12/23.84 %, respectively, compared with those in the control treatment. At the same Pb^{2+} concentrations, the NiR activities in the soybean roots increased by 4.97, 5.01, and 6.38 %, respectively, compared with that in the control treatment. The NR activity initially increased sharply and subsequently decreased.

For the combined treatment with acidity and Pb^{2+}, the nitrate and ammonium contents decreased compared with those in the control treatment and the single treatment of acidity, respectively. The NiR activity increased by 5.86 to 11.59 % compared with that of the control treatment. The changes in the NR activity in the soybean roots subjected to acidity and Pb^{2+} ($pH+Pb^{2+}$) were as follows: 825.64 % (0, 425.64 %; pH 4.5+Pb^{2+}), 100 % (425.64, 2,779.49 %; pH 3.5+Pb^{2+}), and 25.64 % (25.64, −25.64 %; pH 3.0+Pb^{2+}) compared with the control treatment, respectively.

The linear regression equation and r of the main root length (root surface area, root volume) and the ammonium content was $y=-0.147x+103.168$, $r=0.116$, $p>0.05$ ($y=0.433x+45.536$, $r=0.502$, $p<0.01$; $y=0.627x+16.715$, $r=0.578$, $p<0.01$). The correlation analysis indicated that the root surface area and the root volume were positively correlated with the ammonium content ($p<0.01$).

Combined Effects of Acidity and Pb^{2+} on the Antioxidant System of Soybean Roots

Table 4 shows the antioxidant indexes in soybeans subjected to acidity and Pb^{2+}. At different pH levels (4.5, 3.5, and 3.0), the following changes in the MDA content and membrane permeability as well as POD and CAT activities in the soybean roots that were subjected to acidity were respectively observed compared with the control treatment as follows: −13.11, 2.91, and −22.61 %; −12.65, 6.29, and 0.30 %; −14.74, −25.67, and −3.40 %; and 69.76, 58.13, and 68.02 %. At different Pb^{2+} concentrations (0.3, 0.9, and 1.5 mmol L^{-1}), the following changes in the MDA content and membrane permeability as well as POD and CAT activities in the soybean roots were respectively observed compared with those of the control treatment as follows: −47.55, −59.21, and −62.47 %; 62.68, 45.73, and 77.09 %; −92.08, −88.83, and −90.98 %; and 22.09, −31.98, and −18.03 %.

For the combined treatment at pH 4.5 and different Pb^{2+} concentrations (0.3, 0.9, and 1.5 mmol L^{-1}), the changes in the MDA content and membrane permeability as well as POD and CAT activities were respectively observed as follows: −42.66, −43.01, and −45.69 %; −15.7, −19.94, and 0.47 %; −91.46, −84.69, and −90.25 %; and 47.38, 73.25, and 38.37 %. At pH 3.5 and different Pb^{2+} concentrations (0.3, 0.9, and 1.5 mmol L^{-1}), the changes in the MDA content and membrane permeability as well as POD and CAT activities were observed respectively as follows: −37.18, −42.31, and −1.98 %; 4.86, 39.39, and 57.57 %; −88.46, −93.37, and −94.88; and 59.88, 35.17, and 127.61 %. At pH 3.0 and different Pb^{2+} concentrations (0.3, 0.9, and 1.5 mmol L^{-1}), the changes in the MDA content and membrane permeability as well as POD and CAT activities were respectively observed as follows: −37.18, −29.6, and −36.13 %; 33.39, 65.76, and 82.95 %; −58.38, −65.98, and −81.49 %; and 57.84, 32.55, and 56.97 %.

The linear regression equation and r of the nitrate content, NR and NiR activities, ammonium content, and POD activity (CAT) was $y=0.179x+0.058$, $r=0.424$, $p<0.01$ ($y=-0.076x+0.079$, $r=0.145$, $p>0.05$); $y=1.39x+558.804$, $r=0.054$, $p>$

Table 4 Effects of acidity and Pb^{2+} on MDA content, membrane permeability, POD activity and CAT activity of roots in soybean

Treatment	MDA content (μmol L^{-1} × 100)	Membrane permeability %	POD activity [ΔOD_{470}·(g min)$^{-1}$ × 100]	CAT activity [mg (g min)$^{-1}$ × 100]
pH 7.0	2.86±0.11ab (100.00)	37.62±2.82fgh (100.00)	529.10±15.36a (100.00)	97.47±6.68g (100.00)
pH 4.5	2.49±0.00bc (86.89)	32.87±1.79ghi (87.35)	451.10±35.62c (85.26)	165.47±6.68bc (169.76)
pH 3.5	2.94±0.09a (102.91)	39.99±0.81f (106.29)	393.30±12.99d (74.33)	154.13±11.38cd (158.13)
pH 3.0	2.21±0.13cd (77.39)	37.74±0.32fgh (100.30)	511.10±17.44b (96.60)	163.77±4.09bc (168.02)
Pb 0.3	1.50±0.06fg (52.45)	61.21±1.96bc (162.68)	41.90±3.20ij (7.92)	119.00±4.91f (122.09)
Pb 0.9	1.17±0.01gh (40.79)	54.84±2.77de (145.73)	59.10±0. 35hi (11.17)	66.30±2.60h (68.02)
Pb 1.5	1.07±0.05 h (37.53)	66.64±2.51ab (177.09)	47.70±3.12ij (9.02)	79.90±1.96h (81.97)
pH 4.5+Pb 0.3	1.64±0.03ef (57.34)	31.74±1.13hi (84.33)	45.20±4.36ij (8.54)	143.65±0.49de (147.38)
pH 4.5+Pb 0.9	1.63±0.34ef (56.99)	30.13±2.54i (80.06)	81.00±1.04gh (15.31)	168.87±3.97b (173.25)
pH 4.5+Pb 1.5	1.55±0.25fg (54.31)	37.8±0.36fg (100.47)	51.60±1.54hij (9.75)	134.87±5.04e (138.37)
pH 3.5+Pb 0.3	1.80±0.11ef (62.82)	39.46±0.01f (104.86)	61.05±1.99hi (11.54)	155.83±7.50bcd (159.88)
pH 3.5+Pb 0.9	1.65±0.08ef (57.69)	52.45±0.80e (139.39)	35.10±1.73ij (6.63)	131.75±2.45ef (135.17)
pH 3.5+Pb 1.5	2.80±0.15ab (98.02)	59.29±5.46cd (157.57)	27.10±0.78j (5.12)	221.85±0.49a (227.61)
pH 3.0+Pb 0.3	1.80±0.08ef (62.82)	50.19±0.40e (133.39)	220.20±1.39e (41.62)	153.85±0.49cd (157.84)
pH 3.0+Pb 0.9	2.01±0.13de (70.40)	62.37±0.96bc (165.76)	180.00±4.45f (34.02)	129.20±6.44ef (132.55)
pH 3.0+Pb 1.5	1.83±0.19def (63.87)	68.84±0.58a (182.95)	97.95±2.51g (18.51)	153.00±0.00cd (156.97)

Data are presented as means ± standard deviation

Significantly differences at $p<0.05$ were showed with different letter in the same column

pH 4.5+Pb 0.3 represents the combined treatment with acidity at pH 4.5 and 0.3 mmol L^{-1} Pb^{2+}

0.05 ($y=11.704x-1{,}115.039$, $r=0.558$, $p<0.01$); $y=-0.22x+108.118$, $r=0.135$, $p>0.05$ ($y=0.048x+100.45$, $r=0.369$, $p<0.01$); and $y=0.234x+67.089$, $r=0.582$, $p<0.01$ ($y=-0.03x+78.325$, $r=0.093$, $p>0.05$). The results indicated that the ammonium content and nitrate content were positively related to the POD activity ($p<0.01$). Likewise, the NR and NiR activities were positively related to the CAT activity ($p<0.01$).

Discussion

This study investigated the combined effects of acidity and Pb^{2+} on the soybean biomass. The mechanism, by which the combined treatment with acidity and Pb^{2+} affects the soybean biomass, was explained based on the root growth, nitrogen nutrition, and antioxidant system.

The combined treatment with Pb^{2+} and acidity decreased the biomass of soybean (Table 1). The combined treatment with 0.9 mmol L^{-1} Pb^{2+} and acidity caused the soybean biomass to decrease at a larger extent than the combined treatment with Pb^{2+} (0.3 and 1.5 mmol L^{-1}) and acidity at the same pH value (Table 1). The crop biomass in the acidity treatment at pH 4.0 (pH 3.5, 3.0) and 1.5 mmol L^{-1} Pb^{2+} decreased at a lower extent than that of the single treatment with 1.5 mmol L^{-1} Pb^{2+} (Table 1). The crop biomass in the acidity treatment at pH 4.0 (pH 3.5, 3.0) and 0.9 mmol L^{-1} Pb^{2+} decreased at a higher extent than that of the single treatment with 0.9 mmol L^{-1} Pb^{2+}. The results suggested that the combined pollution of acidity and Pb^{2+} should be addressed accordingly. Some combined treatments with acidity and Pb^{2+} (such as acidity at pH 4.5 and 1.5 mmol L^{-1} Pb^{2+}) increased the DW of total legumes, which may lead to the increase of the high Pb^{2+} accumulation in the legume. The reason would be further investigated in future studies.

Root growth plays an important role in water and nutrient supply for crop biomass [17]. Root growth indexes, such as the root length, root surface area, root volume, and average root diameter represent the growth status of the root. In this study, the growth indexes were investigated; the correlation of the root growth and biomass was studied to understand the decrease in the soybean biomass in the combined treatment with acidity and Pb^{2+}. The changes in the main root length and the root volume in the combined treatment with acidity and Pb^{2+} were similar to those of the soybean biomass (Table 2). Our correlation analysis results indicated that the decrease in the soybean biomass induced by the combined treatment with acidity and Pb^{2+} was positively related to the main root length and the root volume. This result also suggested that the decrease in the main root length and the root volume was responsible for the lower nutrient absorption [17], thereby leading to the deficient nutrients that were transported to the legume, and thus decreased the soybean biomass. The deleterious effect on the main root length and root volume was most evident under the combined treatment with 0.9 mmol L^{-1} Pb^{2+}

and acidity (pH 4.5/3.0), in which the lowest soybean biomass was observed.

Nitrogen (N) is a major inorganic nutrient in plants and a major constituent of amino acids and proteins [31, 32]. N is also considered as one of the most important limiting factors of plant growth [33]. Plants easily absorb NO_3^- through their roots and integrate this compound into various amino acids and proteins by nitrogen assimilation [34, 35]. Before NO_3^- is integrated into plant proteins, nitrate is initially reduced to nitrite by NR, and then to ammonium by NiR [36–38]. In the present study, nitrite assimilation was investigated; the correlation between nitrite assimilation and root growth were analyzed. The decreasing and increasing extents of the NiR activity, nitrate, and ammonium contents in the combined treatment with acidity and Pb^{2+} were stronger than those of the single acidity or Pb^{2+} treatment. The correlation analysis suggested that the root growth inhibition in the combined treatment with acidity and Pb^{2+} was significantly related to nitrate assimilation. The combined treatment inhibited the nitrate absorption and partly resulted in the decrease in ammonium content, which affected the biosynthesis of organic substances (such as proteins) in soybeans [39], thereby leading to detrimental effects on plant growth and the decrease in the soybean biomass.

Abiotic stress can induce the excessive accumulation of active oxygen specials in plants [40–42]. The active oxygen specials oxidize the unsaturated fatty acids in the membrane lipid of the cell, thereby resulting in the peroxidation of the lipid in the cell membrane [43, 44] and affecting the physiological activities in the cell. The cell membrane is subsequently damaged and the amount of electrolytes that leak from the cytoplasm increases (the membrane permeability). Therefore, the MDA content and membrane permeability are considered as indicators that correspond to the extent of injury of the cell membrane [43, 44]. CAT and POD activities are two of the main antioxidant enzymes that can effectively decompose H_2O_2 (an active oxygen special) by redox homeostasis [41, 45]. In this study, the antioxidant indexes were investigated; the correlation of the antioxidant system and nitrogen nutrition was analyzed. Our experiment and correlation analysis results revealed that the decrease in the POD activity partly caused the active oxygen to accumulate, thereby disrupting the cell membrane, and thus decreased the absorption of nitrate. This decrease in nutrient absorption affected the root growth, and thus decreased the soybean biomass.

How did the combined treatment with acidity and Pb^{2+} affect the antioxidant system of soybean plants? We speculated that the change in the antioxidant system of soybean plants was related to the accumulation of Pb in the plant in the combined treatment with acidity and Pb^{2+}. It has been reported that the different concentrations of H^+ can regulate and increase the absorption of Pb^{2+} by plants [46–49]. In the present work, the activities of POD and CAT in soybean plants in the combined treatment of Pb^{2+} and acidity were higher than those of the single treatment of Pb^{2+} except the POD activities in the combined treatment of 0.9/1.5 Pb^{2+} and acidity at pH 3.5 (Table 4). We speculated that the more accumulated Pb in the soybean plants will lead to more excess accumulation of reactive oxygen species in the cells [50], which might activate POD and CAT to scavenge reactive oxygen species more effectively. But the biomass, growth, and nitrate assimilation in roots of soybean were finally inhibited (Tables 1–3) because the increased activities of POD and CAT could not fully scavenge the excess reactive oxygen species. The directly experimental proof would be obtained with the further investigation in the future.

In summary, the relationships among root growth, nitrogen nutrient in roots, antioxidant system in roots, and soybean biomass were discussed in detail under the combined toxic effects of acidity and Pb^{2+}. However, some questions should be considered and further investigated. For example, the MDA content in soybean under the combined treatments of acidity and Pb^{2+} was decreased and the NR activity was significant increased. The decrease in the MDA content was possibly attributed to its efficient participation in other metabolic processes in various tissues because lipid peroxides are unstable in non-enzymatic reactions and the accumulation of various free fatty acids in stressed plants can change lipid peroxidation [51]. In addition, the mechanism by which the NR activity increases under the combined treatment with acidity and Pb^{2+} remains unclear. Further studies should be conducted to tackle on these issues.

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