

# Effects of biochar on the growth, physiology and heavy metal uptake of *Trifolium pratense* L. in Pb-contaminated soil

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## Article

**Keywords:** Biochar, Lead pollution, Red clover, Photosynthetic pigment, Antioxidant enzyme, Random Forest analysis

**Posted Date:** July 29th, 2024

**DOI:** <https://doi.org/10.21203/rs.3.rs-4679541/v1>

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**Additional Declarations:** No competing interests reported.

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# Abstract

Biochar is an inexpensive and effective material that can immobilize heavy metals. However, the impact of varying levels of biochar application on red clover cultivated in soil contaminated with Pb remains unclear. In this study, five different doses of biochar (0, 2.5, 5, 10, and 15%) were applied to soils containing two levels of Pb (LPb, 1000 mg/kg; HPb, 5000 mg/kg) to assess the growth, physiological characteristics, and heavy metal enrichment capacity of red clover. Pb stress dramatically slowed down plant development, decreased the amount of photosynthetic pigment, and caused oxidative damage, according to the results of pot tests. However, the addition of biochar mitigated Pb toxicity in red clover by effectively adsorbing Pb from soil and reducing Pb translocation to plant tissues. Specifically, red clover growth was significantly enhanced by the addition of 5% biochar to soil contaminated with either low- or high-level Pb. This resulted in an increase in plant biomass, stimulated the upregulation of *LHCA1* and *CCS* genes, and facilitated the production of photosynthetic pigments. Furthermore, it increased antioxidant enzyme activity while reducing proline accumulation. This work offers a theoretical foundation for the remediation of Pb-contaminated soils using plants and biochar.

## 1 Introduction

Heavy metal pollutants are frequently found in atmospheric precipitation and sewage due to the quick development of industries, mining operations, and excessive pesticide usage <sup>1</sup>. Lead (Pb), the second most hazardous heavy metal after arsenic (As), persists in the environment and affects humans, especially young children, due to its high toxicity and mobility in soil, plants, and human systems <sup>2</sup>. Pb is absorbed by plant roots in heavy metal-contaminated soil, where it then diffuses and builds up in various tissues and organs. This process disrupts plant growth and development of various parts, including leaves, stems and roots, generating toxicity in plants. In extreme instances, this can lead to plant death <sup>3</sup>. Moreover, similar to other biological and nonbiological stressors, elevated levels of Pb can interfere with plant photosynthesis by destroying or inhibiting the development of photosynthetic pigments like chlorophyll <sup>4</sup>. In order to counteract the negative effects of exposure to heavy metals, plants have developed a variety of defensive mechanisms, including the generation of various antioxidants and substances, both enzymatic and nonenzymatic. These substances include proline (Pro), catalase (CAT), peroxidase (POD) and superoxide dismutase (SOD), all of which contribute to preserving the integrity of cells by the reduction and management of reactive oxygen species <sup>5</sup>. Thus, it's critical to create soil remediation methods that are affordable, effective, sustainable, and kind to the environment.

Recently, bioremediation approaches such as bioleaching, biofiltration, biostimulation, and phytoremediation have been employed for the removal of the environmental contaminants <sup>6,7</sup>. Phytoremediation is a biologically mediated technique aimed at detoxifying soils polluted with heavy metals; this method is cost-effective, environmental friendly, and highly efficient <sup>8</sup>. Red clover, or *Trifolium pratense* L., is a perennial herb of the legume family that was originally found in Asia Minor and southeastern Europe and is now widely cultivated in temperate regions <sup>9</sup>. Red clover has a variety of uses because of its high nutritional value, high protein content, and large and soft leaves, and it can be used as a

grazing grass or as silage <sup>10</sup>. Additionally, red clover serves as a green manure crop and a plant for soil and water conservation, improving soil fertility <sup>11</sup>. Research has indicated that red clover, which may exhibit resistance to heavy metal toxicity, not only aid in rapidly detoxifying polluted areas but are also utilized as indicator plants for heavy metals-contaminated soil; the resilience of red clover is specifically reflected in low heavy metal root mobility, excellent antioxidant activity and robust photosynthetic capacity <sup>12-14</sup>.

Presently, to improve plant tolerance and boost phytoremediation efficiency for heavy metal-contaminated soils, a variety of techniques are currently being used, including the use of external plant growth regulators <sup>15</sup>, appropriate mineral fertilization <sup>16</sup>, the addition of chelating agents <sup>17</sup>, suitable application of biochar <sup>18</sup> and inoculation of beneficial microorganisms <sup>19</sup>. Biochar is a durable substance made from biomass pyrolysis with a high carbon content that is used to improve soil fertility and minimize heavy metal toxicity to plants <sup>20</sup>. Phytoremediation-assisted biochar remediation is an innovative technique that has attracted significant interest recently and has been demonstrated to be an effective strategy for addressing environmental contamination <sup>21,22</sup>. Biochar has many functional groups and a large specific surface area, enabling it to efficiently immobilize heavy metals within soil, thus reducing the heavy metal toxicity to plants <sup>23,24</sup>. In addition, the application of biochar can improve soil fertility and structure, increase soil bacterial diversity, and stimulate plant growth <sup>25</sup>. Some studies have revealed that compared to biochar derived from wood, biochar produced through the pyrolysis of manure or crop straw is more alkaline and has superior heavy metal fixation performance <sup>26,27</sup>. According to a specific study by Xu, et al. <sup>28</sup>, corn straw biochar (CSB) has the capacity to increase soil pH and change the speciation of Cd and Pb from acid soluble forms to reducible and further to residual and oxidizable forms, which significantly decreases the bioavailability of these heavy metals, effectively preventing their uptake by plants and thereby mitigating potential adverse effects. In addition to biochar type, the amount of biochar applied is the key factor to determine the soil remediation effect of biochar. Ai, et al. <sup>29</sup> reported that, compared with other doses (1%, 4% and 10%), the incorporation of 2% biochar into soil was more effective in enhancing plant photosynthesis and antioxidant capabilities, alleviating the stress induced by heavy metals. Therefore, it is important to determine the dose of biochar that is best for remediating soil contaminated with Pb.

However, given the complexity of Pb-polluted soil environments, the ability of biochar to uptake Pb and the mechanisms that sustain stable plant growth remain unclear. We speculate that with the addition of biochar, the physiological defence mechanisms in red clover are improved, and heavy metal enrichment decreases, alleviating the toxicity caused by Pb. In this study, different Pb concentrations (0, 1000 and 5000 mg/kg) were used to simulate different Pb pollution levels, and red clover pot experiments were used to investigate the effects of five doses of biochar on red clover growth performance, physiological response, and heavy metals absorption. Specifically, we aimed to determine (a) the impact of Pb toxicity and biochar application on the plant growth, photosynthetic pigment content and antioxidant system status of red clover, (b) how biochar regulates the Pb migration and distribution in red clover, and (c) the optimal dose of biochar to reduce Pb toxicity in plants. In summary, the findings of this study shed light on the effective management of Pb-contaminated soils, as well as the capacity of biochar to reduce plant absorption of heavy metals.

## 2 Materials and methods

### 2.1 Biochar characterization

Biochar was purchased from Henan Lize Environmental Protection Technology Co., Ltd. This material was produced by pyrolyzing corn straw in a furnace (LZ-LS-600\*800 – 420, China) at 500°C. The following were the fundamental chemical and physical characteristics of the biochar: pH 9.4, total phosphorus content 2.32 g/kg, total nitrogen content 8.43 g/kg and total potassium content 15.59 g/kg. The Pb concentration was 8.12 mg/kg. A scanning electron microscope (SEM) (Hitachi s-4800, Japan; Fig. S1) was used to analyse the surface morphology of biochar.

### 2.2 Soil collection and preparation

The samples of soil were taken from the surface soil (0–20 cm) of the experimental field at Northeast Agricultural University, Harbin city, China (126°14'E, 45°05'N). The soil samples were pulverized, air-dried, and sieved through a 2 mm mesh screen for basic soil analysis and pot experiment. Table S1 provides a full description of physicochemical properties of the initial soil.

Subsequently, a Pb(II) solution was then made by dissolving  $\text{Pb}(\text{NO}_3)_2$  in deionized water. All of the analytical-grade chemicals used in this investigation were purchased from Sinopharm Chemical Reagent Co., Ltd. Prior to the pot experiment, the soil samples were evenly combined with varying levels of  $\text{Pb}(\text{NO}_3)_2$  to produce two different levels of Pb polluted soil, namely LPb and HPb treatment, indicating that the final soil Pb concentration was 1000mg/kg and 5000mg/kg, respectively. Soils without added Pb were utilized as controls. The Pb-contaminated soils were subsequently stored in an area shaded from direct sunlight and were regularly stirred for two weeks to ensure a consistent distribution of Pb in the soil. Then, corn straw biochar at concentrations of 0%, 2.5%, 5%, 10%, and 15% was added to the soil in powder form and passivated in the dark for one week.

### 2.3 Pot experiment

The *T. pratense* seeds of the MaoEr mountain variety were obtained from Northeast Agricultural University. Healthy seeds were first surface-sterilized for 30 minutes with 2% sodium hypochlorite (NaOCl), then thoroughly washed multiple times with deionized water, and subsequently allowed to germinate on petri dishes at a temperature of 25°C in the dark. After that, seedlings exhibiting uniform growth were transferred to pots containing 1 kg of soil. Throughout the cultivation period, the temperature was kept at  $25 \pm 5^\circ\text{C}$ , and the red clover were grown on a 16-hour light/8-hour dark cycle. Additionally, to keep the soil at a 70% moisture level, additional distilled water was added. Every two days, the leachate from the pots was collected and returned to the soil using impermeable plastic containers placed beneath them to prevent the loss of Pb from water. In this study, we used 11 treatments, a control in which plants were grown without Pb and biochar (CK), 1000 mg/kg Pb without biochar (LPb0), 2.5% biochar + 1000 mg/kg Pb (LPb2.5), 5% biochar + 1000 mg/kg Pb (LPb5), 10% biochar + 1000 mg/kg Pb (LPb10), 15% biochar + 1000 mg/kg Pb (LPb15), 5000 mg/kg Pb without biochar (HPb0), 2.5% biochar + 5000 mg/kg Pb (HPb2.5), 5% biochar + 5000 mg/kg Pb (HPb5), 10% biochar + 5000 mg/kg Pb (HPb10) and 15% biochar + 5000 mg/kg Pb (HPb15) (Fig. S2). All treatments were tested in triplicate in accordance with a fully randomized factorial design.

## 2.4 Plant harvest and growth measurement

After a 45-day growth period, the plant height, which is the distance between the neck of the root and the top of plant, was measured with a ruler. Next, the plants were gingerly taken out of the pots and cleaned with distilled water to get rid of any dirt that could have clung to the roots. Root length was determined with a ruler. Plant biomass indicators, such as shoot weight and root weight, were determined with an analytical balance. Each treatment consists of three replicates, each replicate containing five plants.

## 2.5 Determination of photosynthetic pigment content in plants

According to the methods of Lichtenthaler and Wellburn<sup>30</sup>, the following parameters were measured: total chlorophyll, carotenoids, chlorophyll a, and chlorophyll b. In brief, a mortar and pestle were used to crush and homogenize 0.1 g of newly gathered leaves in 10 ml of 80% acetone. The samples were maintained in the dark for a full day to allow the chlorophyll pigments to fully digest and prevent light-induced deterioration. The quantification of chlorophyll a, chlorophyll b and carotenoids were then performed on the filtrate using a spectrophotometer set to operate at wavelengths of 663, 645, and 470 nm, respectively. The contents of photosynthetic pigments were calculated using Eq. (1–4).

$$\text{Chlorophyll a (mg/g fresh wt. )} = \frac{(12.71A_{663} - 2.59A_{645}) \times V}{W \times 1000} \quad (1)$$

$$\text{Chlorophyll b (mg/g fresh wt. )} = \frac{(22.88A_{645} - 4.67A_{663}) \times V}{W \times 1000} \quad (2)$$

$$\text{Total Chlorophyll (mg/g fresh wt. )} = \frac{(20.29A_{645} + 8.04A_{663}) \times V}{W \times 1000} \quad (3)$$

$$\text{Carotenoids (mg/g fresh wt. )} = \frac{(1000A_{470} - 3.27A_{645} - 104A_{663}) \times V}{W \times 1000 \times 229} \quad (4)$$

## 2.6 Oxidative damage estimation

Liquid nitrogen was used to homogenize frozen tissue samples (0.5 g) in order to measure the activity of the antioxidant enzymes catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD). As described in the kit (Comin, Suzhou, China), the ground leaf samples were homogenized with 900  $\mu$ L of phosphate buffer (pH 7.4, 100 mM), and the homogenized sample was subsequently centrifuged (for 10 min at 8000 g at 4°C). The supernatant was then transferred to fresh Falcon tubes for subsequent analysis. After adding the reagents in accordance with the kit instructions ([www.cominbio.com](http://www.cominbio.com)), CAT, POD and SOD were measured at 240 nm, 470 nm, and 560 nm, respectively.

The acid ninhydrin colorimetric method was used to measure the proline (Pro) content<sup>31</sup>. After homogenizing a 0.1 g leaf sample with 5% sulphosalicylic acid, the sample was filtered using filter paper. Next, 2 ml of acid ninhydrin and 2 ml of glacial acetic acid were added to the mixture. After one hour of

heating the combination in a water bath at 100°C, the reaction is stopped by submerging the mixture in an ice bath. The toluene mixture was extracted, and absorbance was measured at 520 nm using an ELISA reader (K6600-A) from Beijing Kaiao Technology Development Company in China.

## 2.7 Determination of Pb in plants and soil

To determine the Pb content in the root and shoot tissues of red clover, a crucible containing 0.5 g (dry weight) of pulverized sample was digested on a hot plate at 150°C for 15 mL of HNO<sub>3</sub>: HCl combination (3:1, v/v) until the solution became clear <sup>32</sup>. Samples of soil were dried in an oven for 48 hours at 70°C, and then they were crushed into a fine powder. Next, Then, using a microwave digestion device, 0.1 g of dried soil sample was digested for 25 minutes at 180°C with a 6 ml combination of HNO<sub>3</sub> and HF (2:1, v/v) <sup>33</sup>. The digested contents were filtered, then diluted with distilled water to make 50 mL in a volumetric flask. Inductively coupled plasma–mass spectrometry (ICP–MS) was used for heavy metal content analysis (Agilent, 7800 ICP–MS, USA).

The transport factor (TF) was determined using Eq. (5), while Eq. (6) and Eq. (7) were employed to calculate root bioconcentration factor (BCF<sub>root</sub>) and shoot bioconcentration factor (BCF<sub>shoot</sub>), respectively.

$$TF = \text{Pb concentration in shoots (mg/kg)} / \text{Pb concentration in roots (mg/kg)} \quad (5)$$

$$BCF_{\text{root}} = \text{Pb concentration in roots (mg/kg)} / \text{Pb concentration in soil (mg/kg)} \quad (6)$$

$$BCF_{\text{shoot}} = \text{Pb concentration in shoots (mg/kg)} / \text{Pb concentration in soil (mg/kg)} \quad (7)$$

## 2.8 Quantitative polymerase chain reaction experimental design

TRIzol reagent was used to extract total RNA, and a Qubit® RNA Assay Kit and Qubit® 2.0 Fluorometer (Life Technologies, CA, USA) were used to determine the concentrations of the extracted RNA. Quantitative polymerase chain reaction (qRT–PCR) was performed in 10 µl of solution containing 0.5 µl of diluted cDNA, 0.2 µl of forward primer, 0.2 µl of reverse primer, and 1×SYBR Premix Ex Taq II (TaKaRa) under the following conditions: 95°C for 180 s; 40 cycles of 95°C for 15 s, 59°C for 15 s, and 72°C for 15 s. The formula  $2^{-\Delta\Delta Ct}$  was used to compute the changes in the relative expression levels values. The specifics of the primers utilized in this investigation are listed in Table S2.

## 2.9 Statistical analysis

The obtained data was subjected to one-way analysis of variance (ANOVA), with the average of five replicates ± the standard error of each replication being the outcome. To assess the variations in means across treatments statistically, Duncan's multiple range test ( $p < 0.05$ ) was utilized. SPSS (ver. 24, SPSS, Inc., Chicago, IL, USA) was used for performing the statistical analyses. Data visualization was used for data visualization.

## 3 Results

### 3.1 Changes in the growth characteristics of red clover seedlings

Growth characteristics such as plant height, root length, shoot and root weight of red clover were investigated by adding biochar to Pb contaminated soil (Table 1). The 1000 mg/kg Pb treatment significantly decreased biomass accumulation in red clover by reducing plant height by 32.78%, root length by 27.21%, shoot weight by 54.94%, and root weight by 57.50% in comparison to control. However, different doses of biochar alleviated the Pb toxicity to red clover to different degrees at different growth levels. In particular, the plant height, root length, shoot and root weights in LPb5 were 1.45, 1.31, 2.55, and 1.74 times greater than those in LPb0, respectively. Significant reductions in plant height, root length, shoot and root weight were seen at high Pb concentration treatment (5000 mg/kg), with increases of 64.48%, 35.14%, 59.29%, and 50.83%, respectively. Similarly, the application of 5% biochar effectively alleviated this inhibition, and compared with those in the Pb-only treatment (HPb0), the plant height, root length, shoot and root weight in the HPb5 increased by 73.88%, 44.69%, 29.40% and 83.69%, respectively. These findings demonstrated that biochar might reduce the growth-inhibiting effects of Pb stress on plants, with a dosage of 5% showing the most benefit (Fig. S3).

Table 1

Effects of different concentrations of biochar on growth of red clover under Pb stress (means  $\pm$  SD, n = 3).

| Treatment                                                                                                                                                                                                                             |     | Plant height (cm)  | Root length (cm)   | Shoot fresh weight (g) | Root fresh weight(g) |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|--------------------|--------------------|------------------------|----------------------|
| CK                                                                                                                                                                                                                                    |     | 29.53 $\pm$ 1.07a  | 13.49 $\pm$ 1.32a  | 11.03 $\pm$ 0.56ab     | 1.20 $\pm$ 0.09a     |
| LPb                                                                                                                                                                                                                                   | 0   | 19.85 $\pm$ 1.55c  | 9.82 $\pm$ 0.98bc  | 4.97 $\pm$ 0.16c       | 0.51 $\pm$ 0.11d     |
|                                                                                                                                                                                                                                       | 2.5 | 25.33 $\pm$ 1.42b  | 10.98 $\pm$ 0.67b  | 9.10 $\pm$ 2.37b       | 0.66 $\pm$ 0.26c     |
|                                                                                                                                                                                                                                       | 5   | 28.72 $\pm$ 0.81a  | 12.84 $\pm$ 0.62ab | 12.68 $\pm$ 1.04a      | 0.89 $\pm$ 0.39b     |
|                                                                                                                                                                                                                                       | 10  | 24.06 $\pm$ 1.33b  | 10.19 $\pm$ 0.76b  | 9.26 $\pm$ 0.31b       | 0.62 $\pm$ 0.10c     |
|                                                                                                                                                                                                                                       | 15  | 19.69 $\pm$ 1.70c  | 9.88 $\pm$ 0.44bc  | 8.58 $\pm$ 0.57bc      | 0.52 $\pm$ 0.05d     |
| HPb                                                                                                                                                                                                                                   | 0   | 10.49 $\pm$ 0.38d  | 8.75 $\pm$ 1.15c   | 4.49 $\pm$ 1.87bc      | 0.39 $\pm$ 0.034e    |
|                                                                                                                                                                                                                                       | 2.5 | 14.71 $\pm$ 1.44c  | 10.84 $\pm$ 0.27b  | 5.44 $\pm$ 0.32b       | 0.57 $\pm$ 0.11c     |
|                                                                                                                                                                                                                                       | 5   | 18.24 $\pm$ 3.56b  | 12.66 $\pm$ 0.65ab | 5.81 $\pm$ 1.03b       | 0.75 $\pm$ 0.28b     |
|                                                                                                                                                                                                                                       | 10  | 12.94 $\pm$ 0.43cd | 10.33 $\pm$ 0.56bc | 3.46 $\pm$ 0.25c       | 0.40 $\pm$ 0.08d     |
|                                                                                                                                                                                                                                       | 15  | 12.52 $\pm$ 2.46cd | 9.35 $\pm$ 1.05bc  | 1.82 $\pm$ 0.31d       | 0.42 $\pm$ 0.21d     |
| Note: LPb, 1000 mg/kg Pb; HPb, 5000 mg/kg Pb; 0, 2.5, 5, 10 and 15 represent the dosage of biochar added. Different letters in the same column indicate significance of different treatment at $p < 0.05$ according to Duncan's test. |     |                    |                    |                        |                      |

### 3.2 Changes of physiological and biochemical in red clover

### 3.2.1 Photosynthetic pigments contents in red clover leaves

In this study, the photosynthetic pigment contents of red clover cultivated in soils treated with varying doses of biochar under different Pb stress (Fig. 1a-d). Compared with the control, two Pb treatments (1000 and 5000 mg/kg) significantly decreased the contents of chlorophyll a (24.00%, 31.47%), chlorophyll b (4.42%, 11.22%), total chlorophyll (16.89%, 24.12%) and carotenoids (28.70%, 35.66%). Under Pb pollution, the photosynthetic pigments tended to increase first and then decrease with increasing biochar dose. When Pb dosage was 1000 mg/kg, 2.5–15% biochar all had positive effects on the photosynthetic pigment content of red clover. In particular, 5% biochar had the greatest promoting effect, increasing the contents of carotenoids, chlorophyll a, chlorophyll b and total chlorophyll by 68.08%, 92.08%, 33.74% and 67.73%, respectively. Notably, the impact of biochar on photosynthetic pigments exhibited a "low promotion and high inhibition" phenomenon in soils polluted by high Pb concentrations. Moreover, biochar addition at the 5% dose significantly increased the contents of carotenoids (7.25%), chlorophyll a (31.47%), chlorophyll b (21.94%) and total chlorophyll (51.66%). Overall, the benefit of 5% biochar on photosynthetic pigment content was much greater than that of other concentrations of biochar, which was consistent with the growth characteristics of red clover.

### 3.2.2 Oxidative damage and antioxidant mechanism in red clover leaves

Oxidative damage parameters, such as Pro content and the activities of antioxidant enzymes, including CAT, POD and SOD, were studied by adding biochar under two levels of soil Pb. Compared with that in CK, SOD activity in LPb0 and HPb0 treatments significantly decreased by 37.49% and 53.55%, respectively (Fig. 2a). SOD activity first increased and subsequently fell as the dosage of biochar increased. In comparison to those in the two treatments where only Pb was administered, the SOD activity increased significantly, by 36.76% and 77.96%, when 5% biochar was applied. POD enzyme activity in red clover leaves increased in response to both Pb concentrations. Although POD activity did not significantly change with the addition of biochar at low Pb concentrations, POD activity increased significantly at high Pb concentrations, with the greatest effect occurring at 5% biochar (Fig. 2b). Under 5000 mg/kg Pb stress, the change of CAT activity was consistent with that of SOD. However, soil Pb at the 1000 mg/kg level stimulated an increase in CAT activity, while biochar concentrations ranging from 2.5–10% inhibited CAT activity (Fig. 2c). In addition, Pb stress led to a large accumulation of Pro in red clover, which was reduced by the application of various dosages of biochar (Fig. 2d). To summarise, the findings revealed that adding varying amounts of biochar to Pb-contaminated soils could improve plant defence systems by increasing antioxidant enzyme activities and reducing proline accumulation. In particular, 5% biochar had a greater effect than all the other treatments in improving SOD and POD enzyme activity.

### 3.3 Pb bioconcentration and translocation in red clover tissues

Under 1000 mg/kg Pb stress, red clover shoots showed a reduction in Pb concentration with increasing biochar dose. Specifically, compared with those in the LPb0 treatment, the Pb contents in the shoots in the

LPb2.5, LPb5, LPb10 and LPb15 treatments decreased by 18.38%, 32.98%, 52.95% and 67.11%, respectively. A similar trend was observed under 5000 mg/kg Pb, in which the Pb contents in shoots of the HPb2.5, HPb5, HPb10 and HPb15 treatments was 43.52%, 44.82%, 56.25% and 66.91%, respectively, lower than that in the shoots of the HPb0 treatment (Fig. 3a). In addition, under both low and high Pb concentrations, when the biochar amount was 10%, the Pb content in roots reached its lowest level, which was 36.13% and 35.37% lower than that in LPb0 and HPb0, respectively (Fig. 3b). In contrast, adding biochar increased the content of Pb in the soil. Specifically, under high Pb stress, soil Pb content in HPb10 treatment was the highest, at 3597.22 mg/kg, which was 38.93% greater than that in the HPb0 treatment (Fig. 3c).

In addition, Pb transport capacity and enrichment in red clover treated with different concentrations of biochar were shown in Table 2. With the addition of biochar, the transport capacity of Pb in red clover decreased. The TF value of LPb15 was 68.14% lower than that of LPb0, and the TF value of HPb15 was 53.89% lower than that of HPb0. The plant bioconcentration factor (BCF) reflects the enrichment of heavy metals within a certain part of the plant. With increasing biochar concentration, the BCF in the shoot tended to decrease. In addition, the BCF of roots decreased significantly after the addition of biochar, but it was still greater than that in the shoots. In conclusion, the addition of biochar could fix Pb in soil, significantly inhibit Pb transport from roots to shoots, and reduce Pb enrichment in plant tissues, thus alleviating Pb toxicity to plants.

Table 2  
The transport factor and bioconcentration factor of Pb in red clover with different concentrations of biochar (means ± SD, n = 3).

| Pb  | Biochar | TF              | BCF <sub>Shoot</sub> | BCF <sub>Root</sub> |
|-----|---------|-----------------|----------------------|---------------------|
| LPb | 0       | 0.113 ± 0.008ab | 0.024 ± 0.002a       | 0.213 ± 0.019a      |
|     | 2.5%    | 0.121 ± 0.019a  | 0.019 ± 0.0026b      | 0.157 ± 0.006d      |
|     | 5%      | 0.087 ± 0.013c  | 0.015 ± 0.0037c      | 0.169 ± 0.025c      |
|     | 10%     | 0.083 ± 0.007c  | 0.010 ± 0.0004d      | 0.121 ± 0.004e      |
|     | 15%     | 0.036 ± 0.008d  | 0.007 ± 0.0002e      | 0.189 ± 0.009b      |
| HPb | 0       | 0.167 ± 0.007a  | 0.126 ± 0.004a       | 0.754 ± 0.016a      |
|     | 2.5%    | 0.101 ± 0.009b  | 0.064 ± 0.004b       | 0.636 ± 0.044b      |
|     | 5%      | 0.110 ± 0.003b  | 0.058 ± 0.003c       | 0.530 ± 0.014c      |
|     | 10%     | 0.111 ± 0.017b  | 0.040 ± 0.007d       | 0.361 ± 0.042e      |
|     | 15%     | 0.077 ± 0.020c  | 0.032 ± 0.100e       | 0.419 ± 0.013d      |

Note: TF, transport factor; BCF<sub>Shoot</sub>, bioconcentration factor in shoot; BCF<sub>Shoot</sub>, bioconcentration factor in root. LPb, 1000 mg/kg Pb; HPb, 5000 mg/kg Pb; 0, 2.5, 5, 10 and 15 represent the dosage of biochar added. Different letters in the same column indicate significance of different treatment at *p* < 0.05 according to Duncan's test.

### 3.4 Changes of soil pH

The present results demonstrated that toxicity caused by different concentrations of Pb had varying impacts on soil pH (Fig. 4). In comparison to the CK treatment, soil pH significantly increased in the LPb0 treatment, whereas in the HPb0 treatment, it remained mostly unchanged. However, adding varying amounts of biochar significantly increased the soil pH. Under 1000 mg/kg Pb, compared with that in LPb0, soil pH in LPb2.5, LPb5, LPb10 and LPb15 treatments increased by 2.72%, 3.80%, 6.06% and 14.56%, respectively. When Pb concentration was 5000mg/kg, soil pH under the HPb2.5, HPb5, HPb10 and HPb15 treatments increased by 5.70%, 9.44%, 14.90% and 18.54%, respectively, compared with that under HPb0. The above results showed that soil pH increases with increasing biochar content under both low- and high-Pb stress.

### 3.5 Variable importance in random forest analysis

Randomized forest analysis (RFA) was conducted to pinpoint crucial predictors from among various plant and soil parameters that influence shoot and root biomass accumulation in red clover under Pb stress and varying doses of biochar. Among the plant-related parameters, SH, Car, Chla and Chlb had significant effects on the accumulation of shoot biomass, while RL, SH, SOD and Chla were significantly correlated with root biomass (Fig. 5a, b). On the other hand, Pb-related factors such as PbS and PbSt were significantly correlated with the growth of red clover, while PbS, PbRt, PbSt and TF were strongly correlated with root growth (Fig. 5c, d). In addition, there was no significant correlation between soil pH and plant development characteristics, such as shoot and root biomass, when different doses of biochar were added under Pb stress.

### 3.6 Biochar alleviates Pb toxicity by regulating the expression of red clover functional genes

To explore the mechanism underlying the response of red clover chloroplast development and antioxidant activity to biochar under Pb stress, the expression levels of several chloroplast development-related genes (*VIPP1*, *LHCA1*, *HCF*, *THF*, and *rbcS*) and antioxidant-related genes (*FLS2*, *CCS*, *STI1*, and *PCS*) were evaluated via qRT-PCR (Fig. 6). The expression levels of *VIPP1*, *LHCA1*, *HCF*, *THF*, *CCS*, *STI1* and *PCS* in red clover leaves decreased after high Pb exposure. The upregulation of *FLS2*, *CCS* and *STI1* suggested that a low Pb concentration might enhance the activity of antioxidant enzyme. Furthermore, the *LHCA1*, *HCF*, *THF*, *rbcS* and *CCS* genes all showed higher expression levels when biochar was added; in particular, 5% biochar significantly increased the expression levels of *LHCA1* and *CCS* under Pb stress.

The correlations between the above genes and plant physiological indices, including those for photosynthetic pigments and antioxidants, were further analysed (Fig. S4). The results showed that the contents of Chla, Chlb, and carotenoids were positively correlated with the chloroplast development-related gene *LHCA1*. Furthermore, the antioxidant-related gene *CCS* showed a positive correlation with CAT, POD and SOD activities. In general, the addition of 5% biochar promoted the synthesis of photosynthetic pigments, accelerated the elimination of ROS by increasing the expression levels of *LHCA1* and *CCS* and improved the resistance of red clover to Pb stress.

## 4 Discussion

In the current study, red clover plants planted in Pb-polluted soil had worse growth, yield, and physiological performance than plants cultivated in unpolluted soil. As the soil Pb contents increased, there was a notable reduction in growth, yield, and physiological performance. These results aligned with the research conducted by Zeng, et al. <sup>34</sup>, Saleem, et al. <sup>35</sup> and Shah, et al. <sup>36</sup>. The biomass reduction and growth inhibition induced by Pb exposure may be attributed to the inhibition of nutrient absorption by Pb, which disrupts the permeability of the cell membrane and damages plants photosynthetic activity <sup>4</sup>. Plant cells may produce a variety of reactive oxygen species (ROS) in response to elevated Pb concentrations in the soil. This surge in ROS leads to severe oxidative damage, adversely affecting plant growth <sup>37</sup>. In summary, high Pb levels in soil often cause plant poisoning as Pb accumulates in plant tissues, limiting plant growth and reducing yield.

However, a number of studies have demonstrated that applying biochar can improve plant growth, physiological performance, and yield while reducing the harmful impacts of heavy metals <sup>38–40</sup>. On the one hand, corn straw biochar has relatively high concentrations of trace elements and inorganic nutrients, improves soil fertility, and provides the necessary elements (Na, P, K, Ca, Mg, etc.) for plant growth <sup>41</sup>. On the other hand, the surface of biochar possesses various functional groups that boost its cation exchange capacity. These groups can be substituted by heavy metals, thereby decreasing damage to plants from these heavy metals <sup>42</sup>. Our results showed that adding biochar to Pb-contaminated soil reversed the toxic effects of Pb, leading to improved growth, physiological performance, and yield in red clover compared to plant cultivated in Pb- polluted soil without adding biochar. It is possible that the transfer and fixation of Pb in biochar reduced the migration of Pb to plant roots and shoots, thus reducing Pb toxicity and increasing plant development. In addition, the auxiliary effect of biochar varies with the dose of biochar <sup>43</sup>. Ma, et al. <sup>44</sup> found that 2% corn stalk biochar application significantly decreased Pb accumulation in various tissues of tomato plants, thus promoting their growth. In this study, 5% biochar addition had the greatest pronounced impact on plant height, root length, and biomass of red clover.

Excessive heavy metals in plant tissues can impair plant physiology, primarily by decreasing photosynthetic activity <sup>45</sup>. Chlorophyll a, which serves as the primary pigment for light capture, functions as the reaction centre for photosynthesis in leaves and plays a pivotal role in this process. Chlorophyll b acts as an accessory pigment with a photoprotective effect, aiding chlorophyll a in the photosynthetic process within leaves <sup>46</sup>. Additionally, carotenoids, auxiliary pigments that aid in light harvesting, play a crucial role in photoprotection through nonphotochemical quenching of excess light energy, safeguarding the plant's photosynthetic system <sup>47</sup>. According to a study conducted by Nelissen, et al. <sup>48</sup>, biochar can increase the chlorophyll contents by promoting nitrogen mineralization, increasing the amount of nitrogen available for absorption by plants. This study showed that when biochar was added, particularly at a 5% dose, significantly elevated the levels of carotenoids, chlorophyll a and chlorophyll b in red clover leaves exposed to Pb. This improvement might be attributed to the ability of biochar to mitigate heavy metal toxicity through the immobilizing heavy metals and enhancing nutrient uptake by plants. This helps to maintain chloroplast

structural stability, safeguards photosynthetic pigments, boosts photosynthesis, and enhances the growth of red clover.

Under heavy metal stress, the ROS content increases sharply, which causes lipid peroxidation in cell membranes, damaging nucleic acids, lipids and proteins in plant cells and ultimately inhibiting plant growth and yield if these substances cannot be removed in time <sup>49</sup>. However, plants can enhance antioxidant defence mechanisms through upregulating crucial antioxidant enzymes activities, which play vital roles in the detoxification of ROS <sup>50,51</sup>. SOD converts  $O_2^{\cdot-}$  into  $H_2O_2$  and  $O_2$ , acting as the first line of defence in the reactive oxygen scavenging and plant antioxidant enzyme systems <sup>52</sup>. Subsequently,  $H_2O_2$  is broken down by CAT and POD into  $H_2O$  and  $O_2$ , further preventing ROS damage in plant <sup>53</sup>. Our study found that incorporating biochar significantly boosted activities of antioxidant enzymes in red clover grown in Pb-contaminated soil, indicating an enhanced defence response to oxidative stress. In particular, the 5% biochar treatment had better effects than did all the other treatments in improving SOD and POD activities. Numerous studies have demonstrated that by stimulating the antioxidant enzymes, biochar can strengthen the antioxidant defence <sup>54–56</sup>. In addition to ROS scavenging, osmotic regulation is also necessary for plants growth <sup>57</sup>. Proline regulates the osmotic pressure in plants by acting as a free radical scavenger, metal chelator, and protein stabilize <sup>58</sup>. In this study, when plants were exposed to Pb stress, large quantities of proline was produced, which is in line with the results of Mu et al. <sup>59</sup>. After the addition of biochar, there was a noticeable reduction in proline accumulation, which could be explained by the ability of biochar to decrease heavy metal toxicity to plant leaves and decrease cellular damage. As a result, the proline content decreased, indicating the mitigation of stress-induced responses in red clover.

Our study suggested that the addition of biochar substantially decreased the Pb content in shoots and roots compared to that of plants cultivated in biochar-free Pb-contaminated soil, mirroring the findings presented by Wang, et al. <sup>40</sup>. The addition of biochar to Pb-polluted soils resulted in a decrease in Pb contents in the roots and shoots, possibly because the addition of biochar increased the soil pH, which is crucial for the dissolution and absorption of heavy metal <sup>60</sup>. When biochar with high pH is used to remediate soils, the oxygen-containing functional groups and mineral components on the surface of biochar react with heavy metals via complex precipitation, which fixes and passivates heavy metals, thus reducing the bioavailability of heavy metals in soil as well as their absorption by plants <sup>61,62</sup>. In this study, the addition of biochar hindered Pb transfer from roots to shoots and reduced Pb enrichment in plant, similar with previous findings by Zulqurnain Haider, et al. <sup>63</sup> and Medyńska-Juraszek, et al. <sup>64</sup>. It is possible to restrict the migration of heavy metals to plants by adding a suitable amount of biochar to the soil. This is because heavy metals in the soil may be changed from reactive to more inert forms by using biochar, leading to a decrease in the transport and bioaccumulation of these metals <sup>65,66</sup>.

Through random forest analysis, various important predictors of root and shoot biomass in red clover treated with different doses of biochar were evaluated. The results showed that stem biomass was closely related to plant height, chlorophyll a, chlorophyll b and carotenoid contents, while root biomass was significantly related to root length, plant height, SOD activity and chlorophyll a content. Therefore, the ability of biochar to promote biomass accumulation may be an indirect result of increased photosynthetic pigment

content. Studies have shown that exposure to Pb can harm the intricate structures present within chloroplasts, causing the breakdown of thylakoids, which hampers gas exchange and the production of photosynthetic pigments.<sup>67,68</sup> However, the addition of biochar can alleviate this phenomenon and improve the photosynthetic activity of plants, thus promoting plant growth and biomass<sup>69</sup>. On the other hand, shoot biomass was significantly correlated with Pb-related parameters, such as shoot and soil Pb contents, while root biomass was significantly correlated with soil Pb contents, root and shoot and transport factors. These results can be connected to the research of Ma, et al.<sup>44</sup> that biochar can adsorb Pb from soil, thereby reducing lead transport and accumulation in plants to mitigate Pb toxicity.

## 5 Conclusions

The findings of this study revealed that Pb was toxic to red clover at 1000 mg/kg and 5000 mg/kg, stunted plant growth, decreased photosynthetic pigment levels, and triggered oxidative damage. By applying biochar to Pb-contaminated soil, the migration of Pb from soil to plants and the enrichment of Pb in plant tissues were significantly reduced, thus reducing the toxicity of heavy metals to red clover. In addition, the chloroplast development-related gene *LHCA1* and the antioxidant-related gene *CCS* were upregulated by 5% biochar, thus promoting the synthesis of pigments involved in photosynthetic processes, such as carotenoids and chlorophyll a, b; increasing the antioxidant enzymes activities (SOD, POD); accelerating the elimination of ROS; and further improving the resistance of red clover to Pb stress. Overall, at the 5% level, biochar had the greatest potential for improving plant growth and physiological characteristics compared to other doses, providing a viable option for remediation in areas with Pb-contaminated soil. Therefore, more extensive field studies are required to evaluate the possible physiological and biochemical mechanisms by which biochar alleviates Pb toxicity in red clover and other plants.

## Declarations

## Competing interests

The authors declare no competing interests.

## Funding

This work was supported by the National Center of Pratacultural Technology Innovation (under preparation) Special fund for innovation platform construction [grant number CCPTZX2023N04.]; the National Key Research and Development Program of China [grant number 2023YFD1200302]; the Department of Education, Heilongjiang Province [grant number LJGXCG2023-071]; and the Heilongjiang Province modern agricultural dairy industry technology collaborative innovation and promotion system [grant number 230000237613110000244].

## Author Contribution

M.Q.M: Conceptualization, Methodology, Software, Visualization, Writing - original draft. W.T.N: Formal analysis, Visualization. S.W.Z: Data curation, Visualization. J.W.J: Data curation, Methodology. Z.C.W: Software, Validation. Y.C.W: Visualization. L.N.Z: Supervision, Funding acquisition. Z.M.W: Funding acquisition. G.W.C: Writing-review & editing, Supervision. X.J.Y\*: Conceptualization, Methodology, Supervision, Funding acquisition. All authors reviewed the manuscript.

## Data Availability

Experimental data can be available from corresponding authors on reasonable request.

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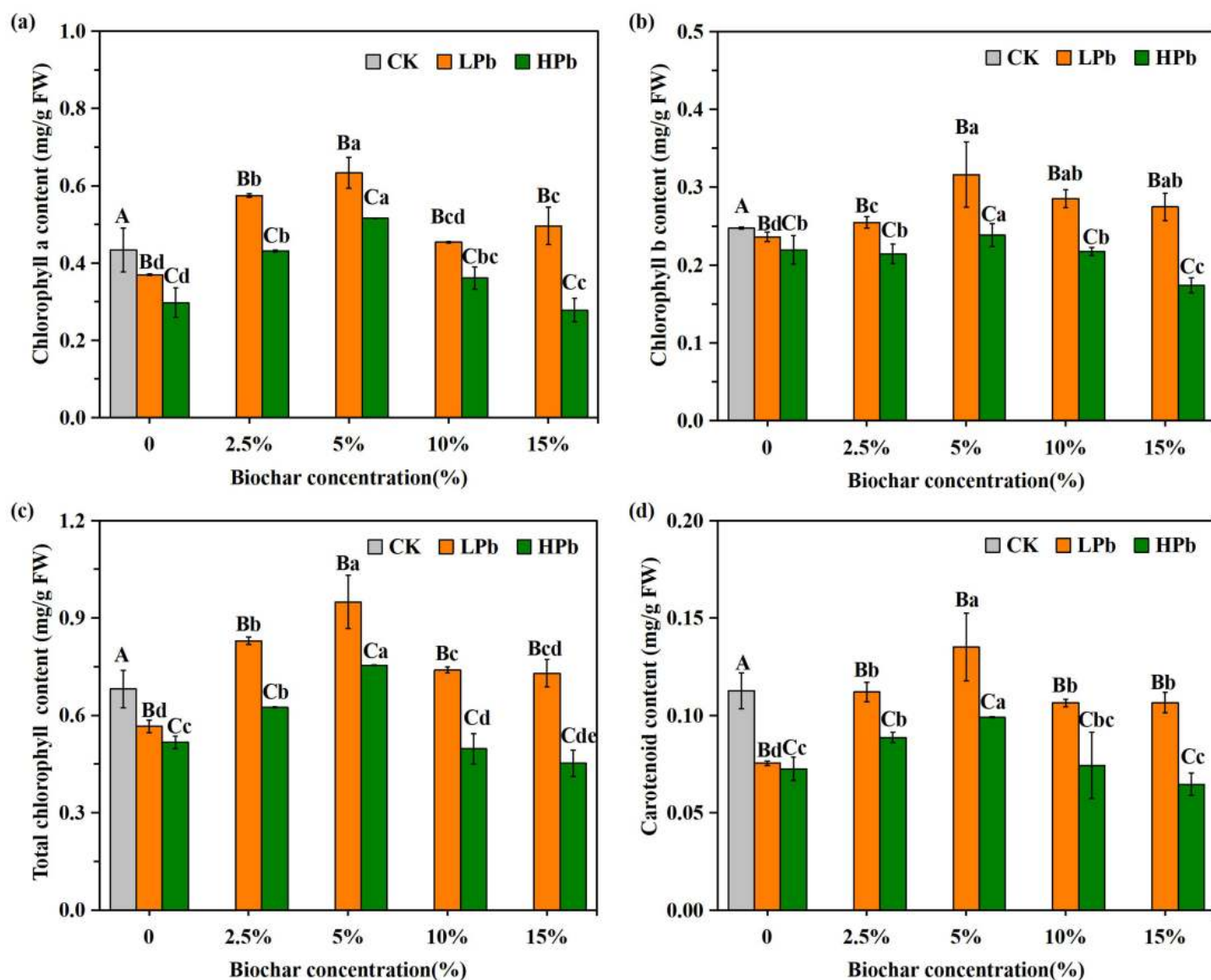
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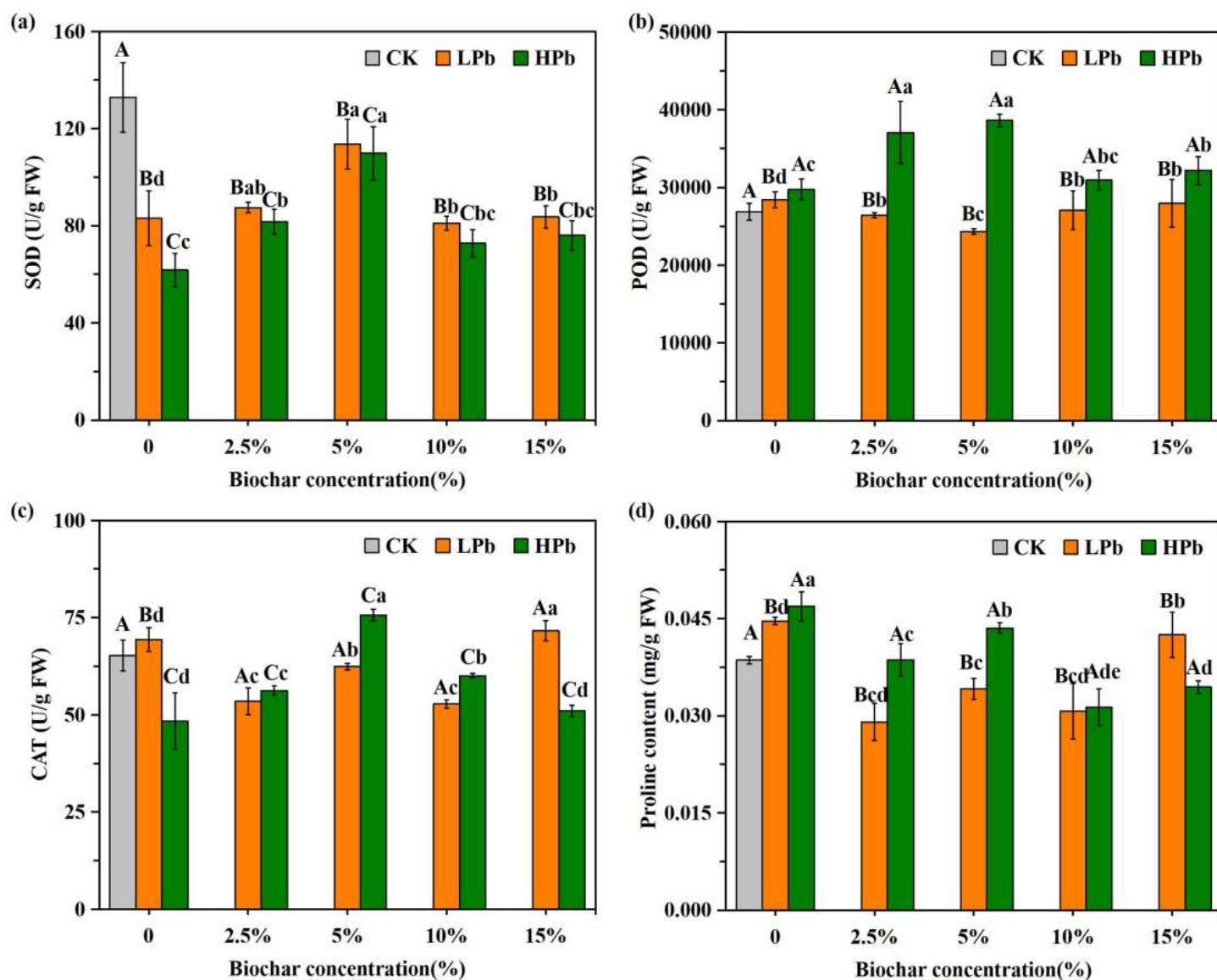
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## Figures



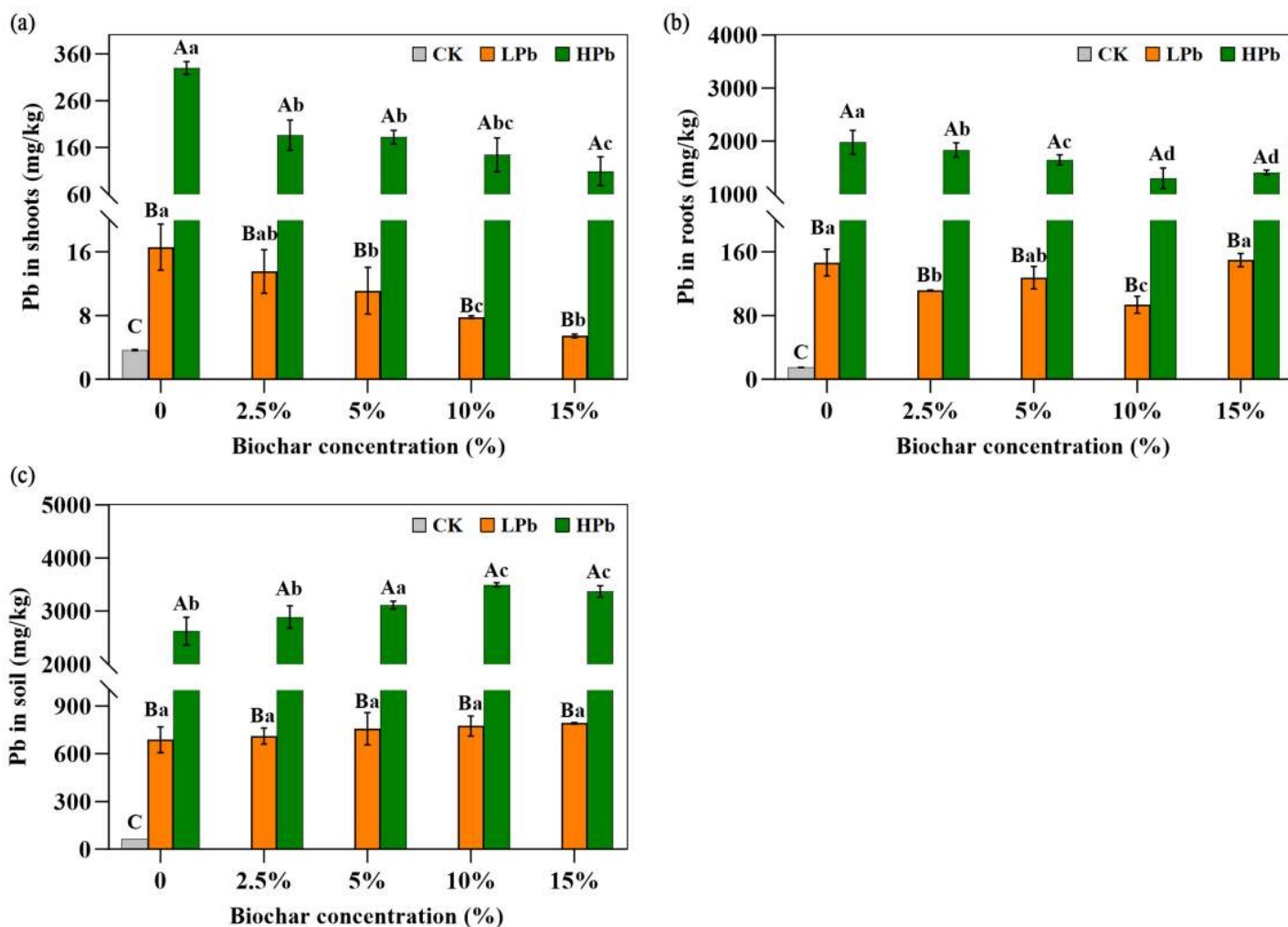
**Figure 1**

Effects of different doses of biochar on contents of chlorophyll a (a), chlorophyll b (b), total chlorophyll (c) and carotenoid (d) in red clover under low or high Pb stress. Different capital letters represent the significant difference between low Pb and high Pb stress under the same biochar concentration ( $P < 0.05$ ). Different lowercase letters represent the significant difference between different biochar concentrations under low or high Pb stress ( $P < 0.05$ ). Error bars represent standard deviation of triplicate replications.



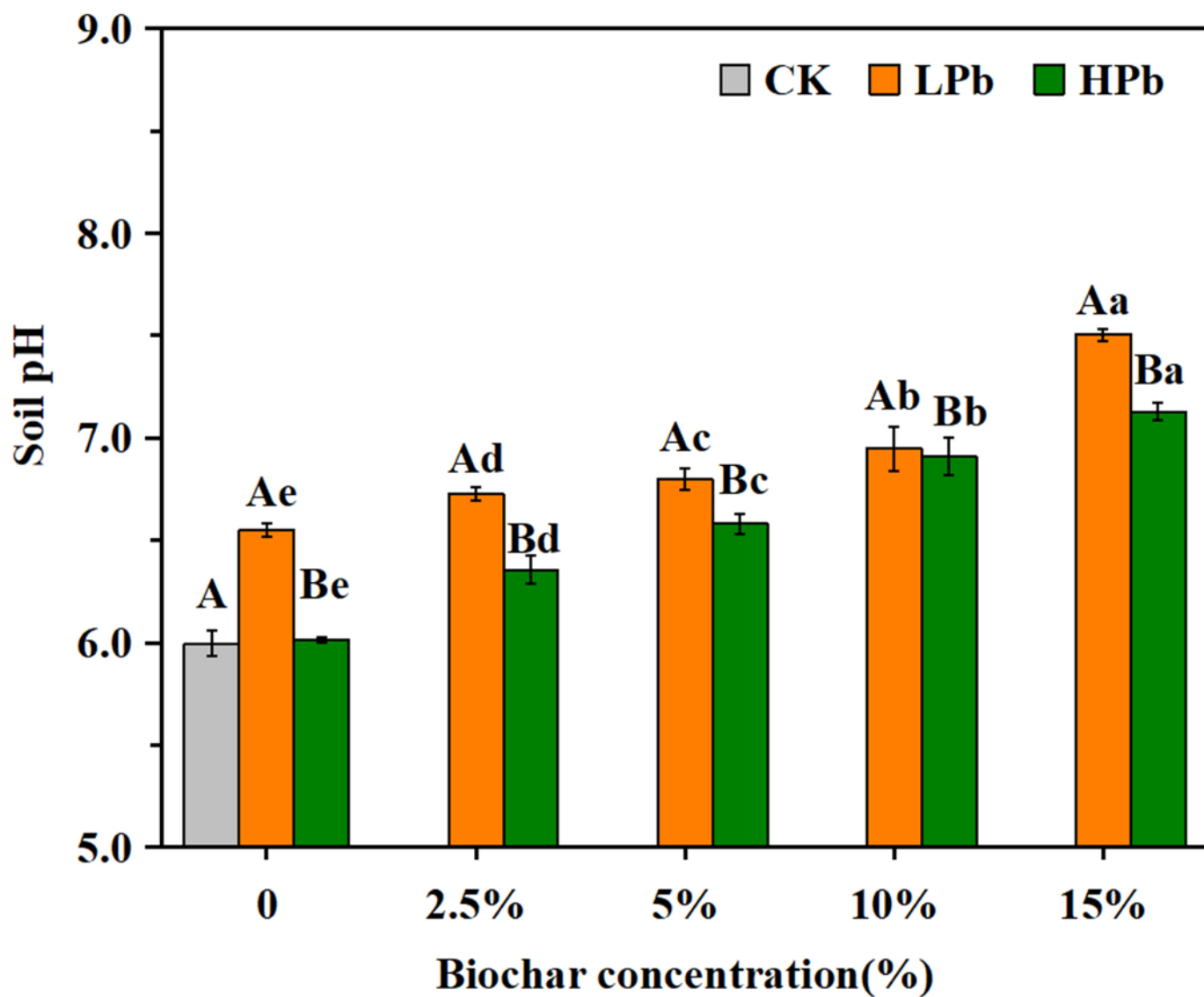
**Figure 2**

Effects of different doses of biochar on SOD (a), POD (b) and CAT (c) activities and Pro (d) content of red clover under low or high Pb stress. Different capital letters represent the significant difference between low Pb and high Pb stress under the same biochar concentration ( $P < 0.05$ ). Different lowercase letters represent the significant difference between different biochar concentrations under low or high Pb stress ( $P < 0.05$ ). Error bars represent standard deviation of triplicate replications.



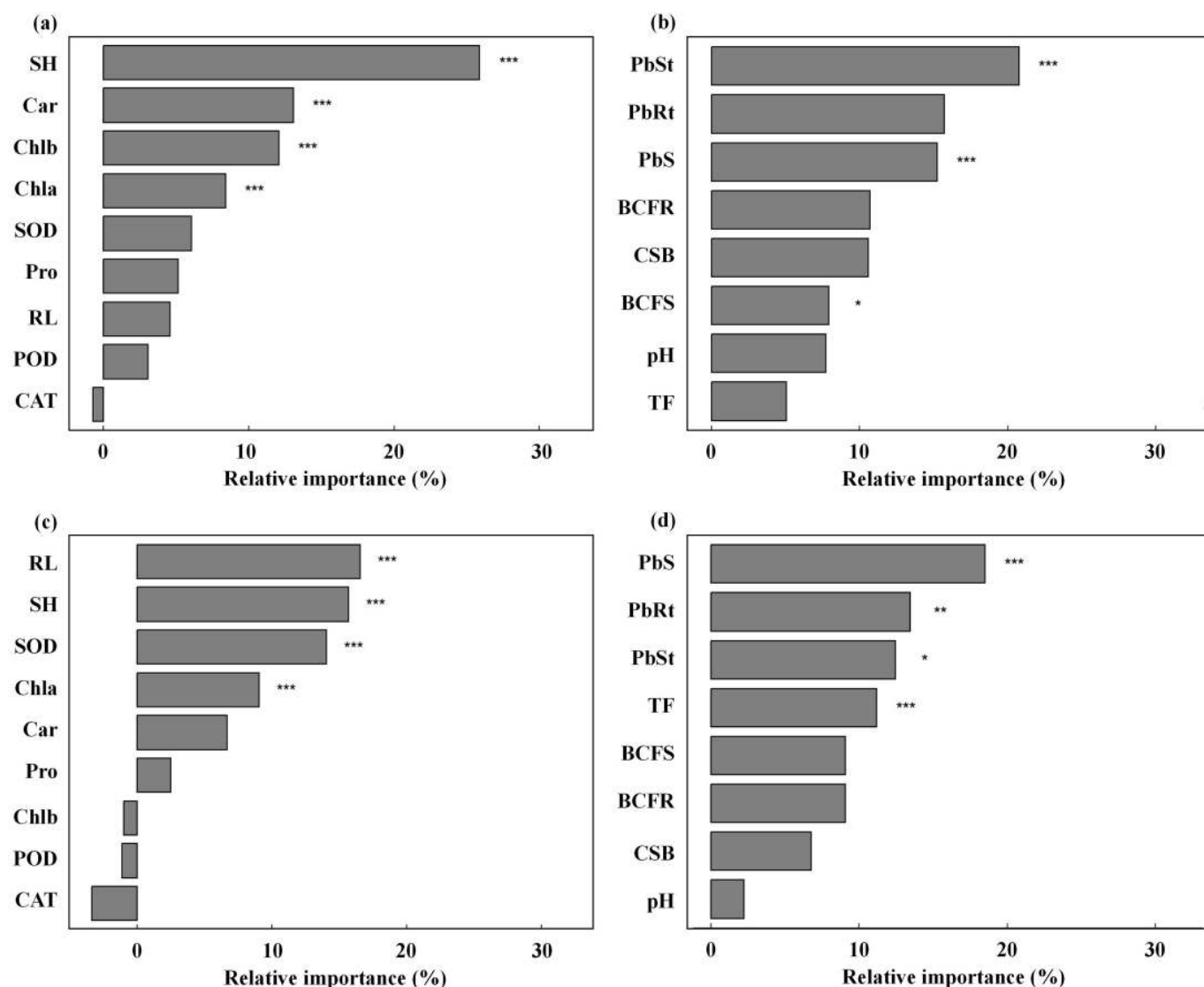
**Figure 3**

Contents of Pb in the red clover shoots (a), roots (b) and soil (c). Different capital letters represent the significant difference between low Pb and high Pb stress under the same biochar concentration ( $P < 0.05$ ). Different lowercase letters represent the significant difference between different biochar concentrations under low or high Pb stress ( $P < 0.05$ ). Error bars represent standard deviation of triplicate replications.



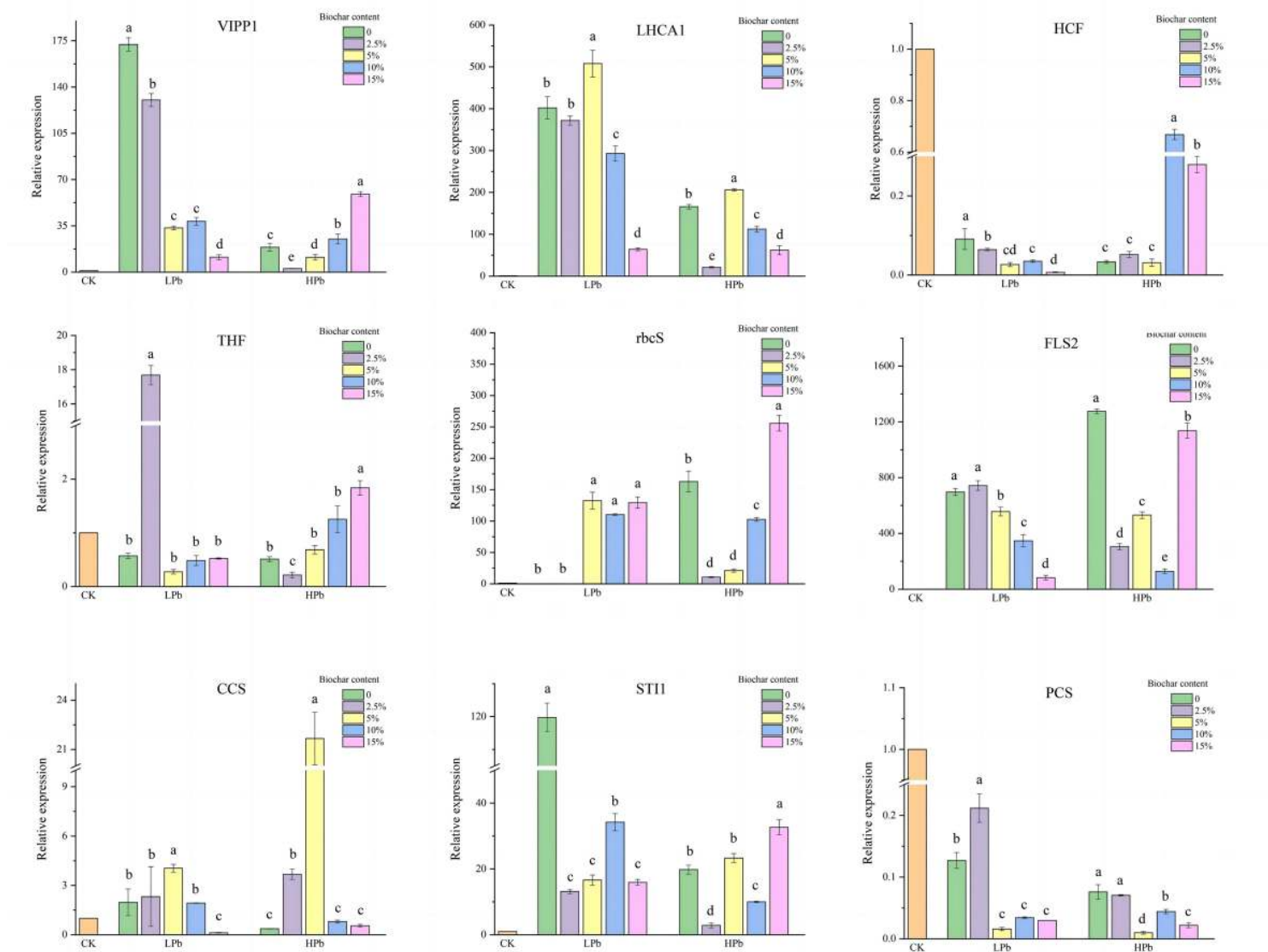
**Figure 4**

The pH values of soil contaminated with low or high Pb under different doses of biochar. Different capital letters represent the significant difference between low Pb and high Pb stress under the same biochar concentration ( $P < 0.05$ ). Different lowercase letters represent the significant difference between different biochar concentrations under low or high Pb stress ( $P < 0.05$ ). Error bars represent standard deviation of triplicate replications.



**Figure 5**

The Random Forest Analysis identifying the important plant and soil related predictors having influence on shoot (a, b) and root (c, d) biomass accumulation in Pb-stressed clover under the effect of biochar. The abbreviations are as follows: RL: root length, SH: shoot height, CAT: catalase activity, POD: peroxidase activity, SOD: superoxide dismutase activity, Pro: proline content, Chla: chlorophyll a content, Chlb: chlorophyll b content, Car: carotenoid content, PbRt: Pb content in roots, PbSt: Pb content in shoots, PbS: Pb content in soil, Tf: translocation of Pb from roots to shoots, BCFS: bioconcentration factor in shoot, BCFR: bioconcentration factor in root, CSB: dosage of corn straw biochar and pH: soil pH.



**Figure 6**

The expression levels of chloroplast development related genes (*VIPP1*, *LHCA1*, *HCF*, *THF*, *rbcS*) and antioxidant related genes (*FLS2*, *CCS*, *STI1*, *PCS*). Different lowercase letters represent the significant difference between different biochar concentrations under low or high Pb stress ( $P < 0.05$ ). Error bars represent standard deviation of triplicate replications.

## Supplementary Files

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