Effects of Biochar and AM Fungi on Growth, Mineral Elements and Cadmium Uptake of Mulberry under Cadmium Stress

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Abstract: In order to explore the synergistic effect of biochar and arbuscular mycorrhizal (AM) fungi on plant growth under cadmium (Cd) stress, a pot experiment was conducted to investigate the effects of biochar application and AM fungal inoculation on mulberry (Morus alba) seedling growth, mineral element absorption, Cd uptake, soil pH and Cd availability under Cd contamination at 2 levels (0 and 4 mg·kg⁻¹). The results showed that biochar application could increase the mycorrhizal infection rate of AM fungi. Biochar application and AM fungal inoculation alone or their combination could all increase plant height, biomass, mineral element content and soil pH, while reduce Cd bioavailability in soil and Cd uptake by mulberry seedlings. The effects of biochar application in increasing soil pH and N and K uptake were better compared with AM fungal inoculation. However, AM fungal inoculation presented better effects in promoting mulberry seedling growth, improving P, Ca and Mg absorption by mulberry seedlings, and reducing Cd availability in soil and Cd absorption by mulberry seedlings. The combination of biochar application and AM fungal inoculation obtained the best effect. Under Cd level of 4 mg·kg⁻¹, the combination of biochar application and AM fungal inoculation increased plant height and N, P, K, Ca and Mg contents in mulberry leaves by 43.28%, 125.56%, 178.48%, 3.63%, 181.04% and 128.04%, respectively. While, Cd concentration in the roots, stems and leaves of mulberry seedlings decreased by 46.48%, 67.86% and 58.97%, respectively. Moreover, pH increased by 5.33%, while Cd availability in soil decreased by 69.53%. In conclusion, biochar and AM fungi alone or their combination can all reduce Cd stress, and promote plant growth and mineral element absorption. The combination of biochar and AM fungi presents the best effect. Biochar combined with AM fungi can be used as an effective measure for ecological restoration of degraded soil and agricultural production safety.

1. Introduction
With the acceleration of industrialization and urbanization, mining and smelting of mines, discharge of considerable industrial “three wastes”, sewage irrigation and application of pesticides and fertilizers, soil heavy metal contamination is becoming increasingly serious. Cadmium (Cd) is one of the soil heavy metal contamination-related elements with high toxicity to plants [1]. Cd can damage chloroplast structure and function in plants, which further damages plant antioxidant system, inhibits plant photosynthesis, and hinders the transportation and distribution of photosynthetic products [2]. Eventually, it results in slow growth of crops, decline in yield and quality [3], and entering the human body through the food chain, thus causing huge harm to human health [4]. Therefore, it is very urgent
to explore an economic and effective measure to restore contaminated and degraded soil and ensure the safety of agricultural products in Cd-contaminated farmlands.

Mulberry is a deciduous tree or shrub belonging to Moraceae, characterized by rich resources, wide distribution and high adaptability, which is one of the important economic crops in China. Some studies have shown that mulberry has strong tolerance and enrichment ability to heavy metals. For example, Zhou et al. [5] demonstrated that mulberry had a strong potential to extract Cd from plants, and could enhance Cd tolerance by depositing Cd on cell wall and vacuolar compartmentalization. Moreover, Zeng et al. [6] found that mulberry could effectively enrich Cd and Zn in contaminated soil. Therefore, mulberry presents a certain potential for restoration of heavy metal-contaminated soil, and can be used as a species for phytoremediation.

Arbuscular mycorrhizal (AM) fungi are a group of rhizosphere microorganisms that can mutualize with 80% plant roots. They can not only promote the absorption of mineral elements by plants [7], but also improve the tolerance of host plants to heavy metal Cd [8]. In addition, AM fungi can not only directly fix Cd by infecting host plants to form external hyphae and secrete glocalin, but also improve microbial community structure and physicochemical properties of the rhizosphere soil of host plants, thus reducing the availability of heavy metals and alleviating Cd stress [9]. It has been demonstrated that under heavy metal contamination, AM fungi can inhibit the mobility of heavy metals in mulberry by avoiding heavy metals [10], mycelial fixation [11], chelating heavy metals and spore immobilization, so as to reduce the toxicity of heavy metals to mulberry.

In recent years, biochar has become a hot material in environmental restoration. Biochar, as a modifier for Cd contamination, can passivate soil Cd in situ [12] and reduce Cd concentration [13]. Biochar can reduce the content of exchangeable Cd in soil and increase the content of residual Cd, so as to effectively fix Cd in soil and reduce the toxicity of Cd, as well as reduce the absorption and accumulation of Cd by crops [14,15]. Therefore, biochar has attracted wide attention in restoration of Cd-contaminated soil.

At present, there are many studies on biochar and AM fungi alone in improving the properties of contaminated soil and promoting plant growth [16,17]. However, the effects of their combination in promoting plant growth and enhancing heavy metal tolerance are rarely reported. It has been shown that biochar application can promote spore germination, hyphal branching and growth of AM fungi [18]. Mulberry is a typical AM plant, and has certain tolerance to heavy metals. Therefore, the effects of biochar application combined with AM fungal inoculation on the growth, physiological properties, mineral element absorption and Cd tolerance of mulberry in Cd-contaminated soil need to be further explored.

In this study, mulberry seedlings were used as host plants and subjected to biochar application and AM fungal inoculation. Moreover, the change rules of the growth, mineral element absorption, photosynthetic pigment content, Cd enrichment, rhizosphere pH and Cd availability of mulberry seedlings under different Cd levels were compared. It provides a theoretical basis for evaluating the mycorrhizal effect of biochar on AM fungi in heavy metal-contaminated soil and the effect of their combined application on plant growth, and also provides a new idea for the establishment of “biochar-mycorrhiza-plant”, a quick-acting model of collaborative improvement, and the restoration of heavy metal-contaminated soil.

2. Materials and methods

2.1. Experimental materials

The experimental soil was clay loam taken from the topsoil (0-20 cm) of Zhou Shan by China West Normal University. The soil was air-dried after passing through a 2-mm sieve for next use. The soil (pH 7.5) contained 13.24 g·kg⁻¹ organic matter, 0.27 g·kg⁻¹ total nitrogen, 50.23 mg·kg⁻¹ alkali-hydrolyzable nitrogen, 0.26 g·kg⁻¹ total phosphorus, 15.69 mg·kg⁻¹ available phosphorus, 14.92 g·kg⁻¹ total potassium and 0.069 mg·kg⁻¹ total Cd. “Chuansang 98-1” was used as the experimental mulberry seedling.
AM fungi (Glomus intraradices BEG 168) were provided by Nanjing Institute of Soil Science, Chinese Academy of Sciences. Before the use of mycorrhizal fungi, corn was used as the host for propagation, with river sand as the substrate. Biochar was purchased from Shangqiu Sanli New Energy Co., Ltd. (Henan, China). Wheat straw was prepared by oxygen-limited pyrolysis at 450 °C, with the basic physicochemical properties including pH 10.2, specific surface area of 8.73 m²·g⁻¹, 51.2% organic carbon, 5.6 g·kg⁻¹ total nitrogen, 0.89 g·kg⁻¹ total phosphorus and 23.2 g·kg⁻¹ total potassium.

2.2. Experimental design
In this experiment, Cd stress at 2 levels were designed: 0 mg·kg⁻¹ Cd and 4 mg·kg⁻¹ Cd. Additionally, 4 treatments were performed: control (CK), biochar application (B), AM fungal inoculation (M), and biochar application combined with AM fungal inoculation (BM). Each treatment was repeated 8 times, with a total of 64 pots.

The soil was sterilized firstly. The soil treated with biochar or AM fungi were mixed well and put into a pot (diameter of pot edge, 32 cm; height, 44 cm), with 20 g·kg⁻¹ biochar and 20 g·kg⁻¹ AM fungi. The weight of each pot was maintained the same in each treatment, with the net weight of each pot of 7 kg. Cd was well mixed with soil in the form of CdSO₄ solution. After adsorption and precipitation in soil for about 2 months, 2 mulberry seedlings (height, 10 cm) with similar size and growth were transplanted in each pot. After sprout emergence, 1 seedling was planted in each pot. The water was balanced with deionized water regularly, during which no fertilizer was applied. Three months after transplanting, 4 replicates of each treatment were selected to determine the chlorophyll content in leaves of mulberry seedlings under Cd stress. In the 5th month, plant height was measured before harvest. After the mulberry seedlings were harvested, the aboveground parts and roots were sampled separately, washed and dried. The fibrous roots of mulberry seedlings were used to determine the mycorrhizal infection rate. Soil samples (0.5 kg) from the plant rhizosphere were collected, air-dried naturally and sieved for soil pH determination.

2.3. Measurement items and methods
Measurement of plant height and biomass: The height of mulberry seedling was determined before harvest using a scale. After washing, the aboveground parts and roots were dried with filter paper, and the fresh weight of the whole plants was weighed. Afterwards, they were put into an oven to remove water at 105 °C for 30mins, and then dried at 80 °C for 48 h until the constant weight of the samples. The biomass of dry matter was weighed and the dry weight was calculated.

Measurement of mycorrhizal infection rate: Fresh fibrous roots were collected and cut into 1-cm root segments. After decocting with 10% KOH, they were stained with acid fuchsin. The AM infection was observed under a microscope (100 ×), and the mycorrhizal infection rate was calculated using the square cross method [19].

Measurement of Cd and Pb contents in mulberry leaves: The dried mulberry seedling leaves were crushed, and 0.5 g ± 0.01 g mulberry leaf samples were weighed and put into a special digestion bottle, which was added with 15 mL mixed acids (concentrated HNO₃ : HClO₄, 4 : 1) for soaking overnight and then put on an electric heating plate for digestion at 180°C ± 5°C. After complete acid driving till nearly dry, the samples were taken down and cooled to room temperature. Subsequently, the volume was constant using deionized water to 50 mL and labeled, and Cd content was determined by ICE-3400 atomic absorption spectrophotometer (Thermo, USA).

Measurement of pH value and Cd availability: PH value was measured using a pH meter (Shanghai Precision Scientific Instrument Co., Ltd., Thunderstorm pHS-3c), with water-soil ratio of 5 : 1. Soil available Cd was extracted by DTPA and determined by ICE-3400 atomic absorption spectrophotometer (Thermo, USA).
2.4. Data processing and analysis
The data were processed using Excel 2017, and analysis of variance was conducted using SPSS 19.0. Multiple comparisons were performed using LSD, and drawing was carried out using Origin 9.0.

3. Results and analysis

3.1. Mycorrhizal infection rate and growth of mulberry
As shown in Figure 1, compared with 0 mg·kg\(^{-1}\) Cd (without Cd stress), the mycorrhizal infection rate of all treatments decreased under 4 mg·kg\(^{-1}\) Cd stress. Under 4 mg·kg\(^{-1}\) Cd stress, the mycorrhizal infection rates of B and BM treatments were 19.37\% and 4.84\% lower than those under 0 mg·kg\(^{-1}\) Cd. Under 0 mg·kg\(^{-1}\) Cd and 4 mg·kg\(^{-1}\) Cd stress, the mycorrhizal infection rates of BM treatment was 11.14\% and 31.16\% higher than those of M treatment, respectively (Fig. 1A). Compared with 0 mg·kg\(^{-1}\) Cd stress, the aboveground biomass, underground biomass and plant height of mulberry seedlings in each treatment decreased under 4 mg·kg\(^{-1}\) Cd stress. No matter under Cd stress or not, B, M and BM treatments all significantly increased the aboveground biomass, underground biomass and plant height of mulberry seedlings (P < 0.05). The increase of aboveground biomass, underground biomass and plant height of mulberry seedlings in M treatment was more obvious than that in B treatment, and BM treatment showed the most significant effect in increasing the biomass and plant height of mulberry seedlings. Under 0 mg·kg\(^{-1}\) Cd, the aboveground biomass, underground biomass and plant height of mulberry in BM treatment increased by 74.77\%, 86.11\% and 33.31\%, respectively, compared with CK. Under 4 mg·kg\(^{-1}\) Cd stress, the aboveground biomass, underground biomass and plant height in BM treatment increased by 75.51\%, 62.50\% and 43.28\%, respectively, compared with CK (Fig. 1A, 1B, 1C).
3.2. Concentration of mineral elements in mulberry leaves

Compared with 0 mg·kg⁻¹ Cd stress, the contents of N, P, K, Ca and Mg in mulberry leaves of each treatment decreased under 4 mg·kg⁻¹ Cd stress. The contents of N, P, K, Ca and Mg in mulberry leaves were significantly increased by B, M and BM treatments no matter under Cd stress or not (P < 0.05), and the increase of P, Ca and Mg contents in mulberry leaves of M treatment was more obvious than that of B treatment. However, the increase of N and K contents in mulberry leaves in B treatment was more obvious than that in M treatment, and BM treatment showed the most significant effect in increasing all mineral elements. Except for K, the contents of mineral elements in mulberry leaves under BM treatment were significantly different from those under CK (P < 0.05). Under 0 mg·kg⁻¹ Cd, the contents of N, P, K, Ca and Mg in mulberry leaves of BM treatment increased by 110.19%, 226.35%, 37.22%, 159.29% and 152.85%, respectively, compared with CK. Under 4 mg·kg⁻¹ Cd stress, the contents of N, P, K, Ca and Mg in mulberry leaves of BM treatment increased by 125.56%, 178.48%, 3.63%, 181.04% and 128.04%, respectively, compared with CK.

| Cd levels (mg·kg⁻¹) | Treatment | N concentration (g·kg⁻¹) | P concentration (g·kg⁻¹) | K concentration (g·kg⁻¹) | Ca concentration (g·kg⁻¹) | Mg concentration (g·kg⁻¹) |
|---------------------|-----------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| 0                   | CK        | 4.12±0.43b               | 1.67±0.12c               | 28.32±3.50b              | 2.53±0.05c               | 3.69±0.19c               |
|                     | B         | 8.41±0.73a               | 3.14±0.30b               | 39.14±2.61a              | 5.47±0.36b               | 7.36±0.12b               |
|                     | M         | 8.39±0.83a               | 3.94±0.54ab              | 25.48±3.1b               | 6.21±0.04a               | 9.32±1.62a               |
|                     | BM        | 8.66±1.12a               | 5.45±0.07a               | 38.86±4.11a              | 6.56±0.21a               | 9.33±0.09a               |
| 4                   | CK        | 3.56±0.42b               | 1.58±0.21c               | 25.63±3.06b              | 2.11±0.06c               | 3.21±0.18c               |
|                     | B         | 7.93±0.83a               | 2.25±0.11b               | 32.63±5.02a              | 4.68±0.13b               | 6.56±0.86b               |
|                     | M         | 7.63±0.91a               | 3.54±0.13ab              | 23.35±4.01b              | 5.37±0.19b               | 8.21±1.01a               |
|                     | BM        | 8.03±0.63a               | 4.40±0.19a               | 26.56±2.12b              | 5.93±0.44ab              | 7.32±1.16b               |

3.3. Cd concentration in mulberry plants

Fig. 2 shows that Cd concentration in roots, stems and leaves of mulberry seedlings was low under 0 mg·kg⁻¹ Cd. Under 4 mg·kg⁻¹ Cd stress, Cd concentration in roots, stems and leaves of mulberry seedlings increased significantly (P < 0.05). No matter under Cd stress or not, B, M and BM treatments reduced Cd concentration in roots, stems and leaves of mulberry seedlings, and the reducing effect was BM > M > B. Under 0 mg·kg⁻¹ Cd, there were no significant differences among the treatments (P > 0.05). Under 4 mg·kg⁻¹ Cd, significant differences were found between B, M and
BM treatments and CK (P < 0.05). Compared with CK, Cd concentration in roots decreased by 9.86%, 19.72% and 46.48% (Fig. 2A), Cd concentration in stems decreased by 17.86%, 39.29% and 67.86% (Fig. 2B), Cd concentration in leaves decreased by 28.21%, 58.97% and 58.97% (Fig. 2C), respectively. The effects of M treatment in reducing Cd concentration in mulberry roots, stems and leaves were better than those of B treatment, and Cd concentration in mulberry roots was higher than that in stems and leaves.

![Graph showing Cd concentration in roots, stems, and leaves under different treatments.](image)

Figure 2. Effects of AMF inoculation, biochar amendment on Cd concentrations in roots (A), stems (B) and leaves (C) of mulberry leaves under Cd stress.

3.4. Soil pH and Cd availability

Fig. 3 displays that B, M and BM treatments all increased the pH value of rhizosphere soil of mulberry seedlings at two Cd levels. The differences between B and BM treatments and CK were significant (P < 0.05). Under 0 mg kg⁻¹ Cd, the pH value of rhizosphere soil in B and BM treatments increased by 9.33% and 5.33%, respectively, compared with CK (Fig. 3A). Under 4 mg kg⁻¹ Cd, the pH value of rhizosphere soil in B and BM treatments increased by 10.81% and 4.05%, respectively, compared with CK (Fig. 3A), but no significant difference was found between M treatment and CK (P > 0.05). Under the two Cd levels, all treatments reduced the available Cd content in rhizosphere soil. Under 0 mg kg⁻¹ Cd, the soil available Cd content of B, M and BM treatments decreased by 40.00%, 44.00% and 62.00%, respectively, compared with CK (Fig. 3B). When Cd was 4 mg kg⁻¹, it reduced by 50.78%, 39.84% and 69.53%, respectively (Fig. 3B). The effect of B treatment in reducing available Cd content in mulberry rhizosphere soil was better than that of M treatment, and the difference between BM treatment and CK was the most significant (P < 0.05). These results demonstrated that biochar could increase soil pH, but AM fungi had no obvious effect in changing soil pH. Additionally, they both significantly reduced Cd availability, and their combination presented the best effect.
4. Discussion
Cd stress has a great influence in changing the growth and physiological characteristics of mulberry. It has been shown that mulberry leaves in Cd-contaminated soil present physiological stress response and blocked mineral element absorption [20], which is consistent with the results of our study. It has been demonstrated that AM fungi or biochar alone can both promote the growth and mineral element absorption of mulberry under Cd stress. Our study also showed that under Cd stress, both biochar application and AM fungal inoculation promoted the growth of mulberry seedlings and the absorption of mineral elements. However, the effects of biochar application combined with AM fungal inoculation in increasing mulberry seedling height, biomass and mineral elements uptake were superior to those of biochar or AM fungi alone. Plant height and biomass are the apparent characteristics of mulberry seedling growth. Photosynthesis is the main pathway of organic matter synthesis in plants and one of the key factors affecting the growth of mulberry seedlings [21]. Cd stress induces plants to produce massive reactive oxygen species (ROS), which leads to the peroxidation of plant leaf cells, thus causing damage to the submicrostructure of chloroplasts. Consequently, photosynthetic pigment synthesis is affected, which results in the disorder of physiological processes such as photosynthesis [22], thereby reducing the plant height and biomass of plant organs and inhibiting plant growth [23]. The combination of biochar application and AM fungal inoculation presented the best effect in promoting the growth, photosynthetic pigment synthesis and mineral element absorption of mulberry seedlings under Cd stress. This may be caused by that the porous structure and rich mineral nutrients of biochar can not only provide good soil environment for water and fertilizer conservation of mulberry seedlings [24], but also promote the propagation of AM fungi [25]. Moreover, AM fungi can improve soil environment, absorb available mineral nutrients (especially P) into plants, and alleviate chlorophyll decomposition [26], which was confirmed by that the mycorrhizal infection rate and mineral absorption of mulberry seedlings treated with biochar combined with AM fungi were higher than those treated with AM fungi alone in this study. Further, biochar increases AM fungal infection and promotes the expansion of ectomycorrhizal hyphae. Extensive ectomycorrhizal hyphae network promotes mycorrhizal plants to occupy more soil space, which is conducive to the absorption of nutrients by plants [27].

In this experiment, both AM fungal inoculation and biochar application increased soil pH, but the effect of biochar application on soil pH was greater than that of AM fungal inoculation. This is resulted from that biochar has a large surface area and may bind to different functional groups of cations, so it can reduce the bioavailability of heavy metals in soil [28]. In addition, the increase in soil pH will lead to the decrease in heavy metal availability [29]. The concentration of Cd in roots, stems and leaves of mulberry seedlings was significantly reduced by AM fungal inoculation and biochar application. Its mechanism is that biochar can reduce soil Cd activity, and its special structure adsorbs Cd [30]. On the other hand, the secretions of AM fungi, including polyphosphate, organic acids and
glomalin, can bind to Cd ions at the plant-hypha interface to fix heavy metals [31]. Moreover, mycorrhization of plant roots can produce chelating molecules that form complexes with Cd, thus binding Cd in rhizosphere soil [32]. Further, biochar and mycorrhiza have significant growth-promoting effects on mulberry seedlings, and the “dilution effect” caused by the increase in biomass is also an important cause for the decrease of Cd concentration in plants. This study demonstrated that biochar and AM fungi had synergistic effects on plant growth, photosynthetic characteristics and resistance to heavy metal stress.

5. Conclusion
In conclusion, Cd stress reduced plant height, biomass and hindered mineral element absorption of corn, indicating that Cd stress interfered with the normal development of mulberry seedlings. Application of biochar and AM fungi could both increase the plant height, biomass and mineral element contents and soil pH of corn, while reduce Cd bioavailability in soil and Cd uptake by mulberry seedlings. Biochar was superior to AM fungi in reducing Cd availability and increasing N and K contents in mulberry seedling leaves. However, the effects of biochar in promoting the growth of mulberry seedlings reducing the Cd content of mulberry seedlings and enhancing the contents of P, Ca and Mg in mulberry seedling leaves were not as good as those of AM fungi. The combination of biochar application and AM fungal inoculation presented synergistic effects in increasing plant growth mycorrhizal infection rate and soil pH while reducing Cd uptake compared with biochar application and AM fungal inoculation alone. However, the effect of biochar application combined with AM fungal inoculation on Cd content in mulberry seedlings as well as its mechanism and effects on field production and restoration need to be further explored.

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