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2021-12-21

Fu , Y , Jia , M , Wang , F , Wang , Z , Mei , Z , Bian , Y , Jiang , X , Virta , M & Tiedje , J M 2021 , ' Strategy for Mitigating Antibiotic Resistance by Biochar and Hyperaccumulators in Cadmium and Oxytetracycline Co-contaminated Soil ' , Environmental Science and Technology , vol. 55 , no. 24 , pp. 16369-16378 . https://doi.org/10.1021/acs.est.1c03434

http://hdl.handle.net/10138/341380
https://doi.org/10.1021/acs.est.1c03434

Environmental Science & Technology

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Article

Strategy for Mitigating Antibiotic Resistance by Biochar and Hyperaccumulators in Cadmium and Oxytetracycline Co-contaminated Soil

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Cite This: *Environ. Sci. Technol.* 2021, 55, 16369−16378

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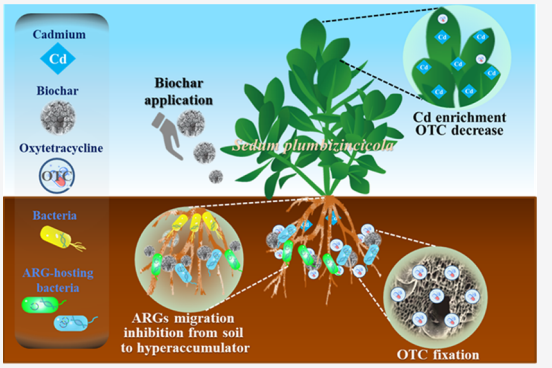
ABSTRACT: The global prevalence of antibiotic resistance genes (ARGs) is of increasing concern as a serious threat to ecological security and human health. Irrigation with sewage and farmland application of manure or biosolids in agricultural practices introduce substantial selective agents such as antibiotics and toxic metals, aggravating the transfer of ARGs from the soil environment to humans via the food chain. To address this issue, a hyperaccumulator (*Sedum plumbizincicola*) combined with biochar amendment was first used to investigate the mitigation of the prevalence of ARGs in cadmium and oxytetracycline co-contaminated soil by conducting a pot experiment. The addition of biochar affected the distribution of ARGs in soil and plants differently by enhancing their prevalence in the soil but restraining transmission from the soil to *S. plumbizincicola*. The planting of *S. plumbizincicola* resulted in an increase in ARGs in the soil environment. A structural equation model illustrated that mobile genetic elements played a dominant role in shaping the profile of ARGs. Taken together, these findings provide a practical understanding for mitigating the prevalence of ARGs in this soil system with complex contamination and can have profound significance for agricultural management in regard to ARG dissemination control.



KEYWORDS: *antibiotic resistance genes, soil remediation, biochar, hyperaccumulator, co-contamination, metal*

INTRODUCTION

Continuing increase in antimicrobial resistance is a global concern as indicated by the rapid emergence and proliferation of multidrug-resistant strains[1,2] and is regarded as one of the most significant emerging environmental and global health challenges in the 21st century.[3] The misuse and/or overuse of antibiotics in humans and animals[4,5] and agricultural practices[6] significantly increase the worldwide prevalence of antibiotic resistance genes (ARGs) via sustained selection pressure. Heavy metals released from human activities also accelerate the proliferation of ARGs due to co-selective stress.[7,8] Furthermore, horizontal gene transfer mediated by mobile genetic elements (MGEs) can amplify the potential risk by increasing the transmission frequency of ARGs via conjugation, transformation, and transduction among microbial populations.[9] Substantial ARGs and ARG-encoding pathogenic and non-pathogenic bacteria originating from animal and human waste can spread into arable soil, which may then enter the food chain via contaminated crops.[6,10] Due to the capacity of ARGs to transfer in diverse environments and integrate into human pathogens, ARGs are gaining more attention as emerging biological pollutants.[11] Therefore, strategies to mitigate the proliferation and transmission of ARGs in the soil environment are of great importance for ecological security and public health.

Irrigation by sewage and farmland application of manure or biosolids for nutrient recycling are the main sources of the introduction of antibiotics into agricultural land, frequently coexisting with abundant heavy metals.[8,12] An exacerbating factor is that soil exposed to these selective agents (e.g., antibiotics, toxic metals, and disinfectants) provides a hospitable environment for antibiotic-resistant bacteria colonization and ARG propagation.[13] Phytoremediation, an eco-friendly and cost-effective approach, can enable plants to stabilize, extract, volatilize, or degrade the organic and inorganic pollutants, thereby facilitating the removal or harmlessness of the contaminants and *in situ* soil remediation,[14] which may be feasible for reducing selection pressure and mitigating the prevalence of ARGs. Hyperaccumulators

Received: May 27, 2021
Revised: August 26, 2021
Accepted: October 13, 2021
Published: October 25, 2021

https://doi.org/10.1021/acs.est.1c03434
Environ. Sci. Technol. 2021, 55, 16369−16378

with high capacities of bioconcentration and endurance to pollutants have practical significance in soil remediation, and their application has been widely investigated in alleviating the pollution of organics and/or toxic metals in soil.[15,16] However, to our knowledge, investigations of hyperaccumulators in the remediation of co-contaminated soil and the impacts of ARG profiles in this soil−plant system have yet to be documented.

Biochar, pyrolyzed from carbonaceous-rich biomass under anoxic environments, is different from active charcoal through serviceability as a soil amendment.[17,18] Biochar has been widely used for soil amelioration and to increase crop yields by modifying soil properties (e.g., pH, moisture, and pore structure),[19] improving soil nutrient retention,[20] and changing the soil biological community structure.[21] Therefore, the addition of biochar may enhance phytoremediation efficiency by promoting plant growth.[16] Additionally, biochar amendment has been demonstrated to decrease the bioavailability of heavy metals and antibiotics via adsorption, which thus may inhibit the proliferation of ARGs.[10] This indicates that the application of biochar combined with hyperaccumulators has the potential to enhance the phytoremediation efficiency and mitigate the migration of ARGs in the soil environment. However, whether biochar amendment with hyperaccumulators can mitigate the prevalence of ARGs remains unexplored, especially in soil that is complexly co-contaminated with antibiotics and heavy metals, which requires detailed investigation.

China is the largest user of antibiotics in the world, and more than 50% of the antibiotics are applied in animal husbandry for disease control and growth improvement.[22,23] Up to 90% of the consumed antibiotics are unable to be metabolized completely in animal guts, which is followed by being excreted in urine and feces, and finally, being discharged into the receiving soil via wastewater irrigation and manure/biosolids.[24] Exogenous pollutants such as antibiotics and toxic metals may drive selection pressure on soil microorganisms and accelerate the diffusion of ARGs into various environments.[7] However, strategies to mitigate the prevalence of ARGs in agricultural land are mostly scarce, which is of great importance from the viewpoint of food security and public health. Herein, a hyperaccumulator with biochar amendment was first used to investigate the mitigation of the ARGs in cadmium and oxytetracycline co-contaminated soil. The objectives of this study are as follows: (1) to quantify the diversity and abundance of ARGs and MGEs in antibiotic- and/or toxic metal-contaminated soil and hyperaccumulators, respectively, and (2) to investigate the underlying mechanisms of the alleviation of ARGs by biochar and hyperaccumulators combination. This research will provide insight into the combination of biochar and hyperaccumulators as a remediation strategy for the abatement of the prevalence of antimicrobial resistance.

MATERIALS AND METHODS

Materials. Soil (Agri-Udic Ferrosol) was collected from the arable layer (0−20 cm depth) of a peanut field in Xuancheng, Anhui Province, China, that had not previously been treated with biochar. Soil samples comprising 10.08% sand, 56.04% silt, and 33.88% clay were air-dried and ground through a 2.0 mm sieve. Biochar, using a maize straw as the feedstock due to its abundance in agricultural waste, was fabricated through a stepwise heating procedure at a final temperature of 600 °C under limited oxygen conditions.[25,26] The basic properties of the soil with or without biochar and maize straw biochar are presented in Tables S1 and S2, respectively. Cadmium (Cd), with the property of universality and persistence in contaminated agricultural soil,[27] was chosen as the target pollutant and purchased from the National Analysis Center for Iron and Steel (Shanghai, China). Oxytetracycline (OTC, >99% purity), which is extensively used in infectious therapy as well as an additive in animal feed for growth promotion and has a concentration typically up to several mg/kg in the soil environment,[28] was selected as the model and obtained from Sigma Chemical Company (New Jersey). Furthermore, the background concentrations of Cd and OTC were <0.10 mg/kg and not detected in the soil, respectively. The Cd hyperaccumulator *Sedum plumbizincicola*, which was used as the representative plant based on its remarkable phytoremediation capability,[29] was obtained from Fuyang, Zhejiang Province, China.

Pot Experiment and Sample Collection. Soil was spiked with Cd solution to reach a final concentration of 5 mg/kg and then aged at 25 °C and moderate soil moisture (70% of maximum water-holding capacity) for 8 weeks to simulate heavy Cd-contaminated soil as previously described.[30] Aliquots of original and Cd-contaminated soil were spiked with OTC solution to obtain a concentration of 10 mg/kg and subsequently aged at 25 °C and 70% of the maximum water-holding capacity for 1 week. Soil with four treatments was used for pot experiments, including original soil (CK), Cd-contaminated soil (M), OTC-contaminated soil (A), and Cd- and OTC-contaminated soil (A + M).

Soil with four treatments was divided into two portions: one served as the control, and the other was homogenously mixed with biochar at a mass ratio of 5% on a soil dry weight basis. The control soil or amended soil (1.2 kg) was transferred to a plastic pot (6 cm bottom diameter, 10 cm upper diameter, and 10 cm height) and aged at 25 °C and 70% of the maximum water-holding capacity for 1 week. Then, the obtained soils were planted with or without *S. plumbizincicola* (Figure S1). Triplicate samples were conducted in each treatment. Uniform-sized seedlings of *S. plumbizincicola* were selected, and three plants were transferred into each designated plastic pot. Pots were randomly cultivated in the greenhouse with natural humidity and illumination at a moderate daily temperature of 25 °C and watered with deionized water to maintain 70% of the maximum water-holding capacity.[31] After 60 days, the aboveground parts (leaf and stem) of *S. plumbizincicola* were harvested as previously described,[29] and the bulk soil from each pot was manually ground and passed through a 2.0 mm sieve. All soil and plant samples were stored at −20 °C pending analysis.

Heavy Metal and Antibiotic Detection. The bioavailability of heavy metals is crucial to the assessment of toxic effects in the environment rather than the total concentration.[32] The bioavailable Cd in soil samples was extracted using $CaCl_2$ (0.01 M) at a solid-to-liquid ratio of 1:10 (w/v)[33] and quantified by an atomic absorption spectrophotometer (AAS, Varian Spectra AA 220 FS, Heraeus, Germany). The Cd concentration in plants was also measured using AAS by digesting 0.25 g of samples (dry weight) with 10 mL of $HNO_3/HClO_4$ (3:2, v/v).[34] The accuracy of Cd extraction from soil and plants was determined using a certified reference material (GBW07401, Institute of Geophysical and Geochemical Exploration, Hebei, China) for quality control.

https://doi.org/10.1021/acs.est.1c03434
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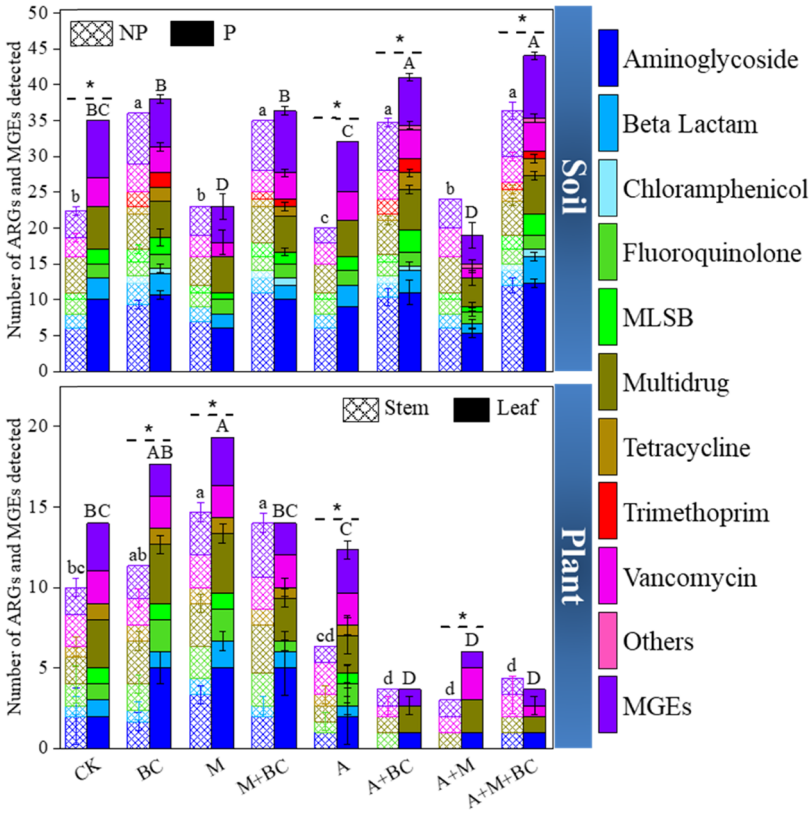

Figure 1. Detected number of antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs) of both soil (NP, nonplanted soil; P, planted soil) and plant samples among different treatments. Values with lowercase letters in the same column or black stars indicate significant differences at $P < 0.05$ (LSD). MLSB, macrolide−lincosamide−streptogramin B; CK, control treatment; M, application with cadmium (Cd); A, application with oxytetracycline (OTC); and BC, biochar.

The procedure for antibiotic detection of soil and plant samples followed a previous method.[35] Briefly, the freeze-dried samples (2.0 g of soil or 0.2 g of plant) were weighed into 50 mL Teflon tubes and then 20 mL of extraction solution (acetonitrile/$Mg(NO_3)_2$-$NH_3{\cdot}H_2O$ in 3:1 (v/v)) was added into the tubes, followed by dark treatment overnight. After vortexing for 1.5 min, the tubes were shaken at 200 rpm by a horizontal shaker for 30 min, sonicated for 15 min, and centrifuged for 10 min (4 °C, 3000 rpm). After two repeated extractions, the incorporated supernatants were concentrated using Oasis HLB extraction cartridges (Waters), rinsed with 10 mL of acetone solution (containing 0.1% formic acid), and dried to approximately 100 μL under nitrogen. Then, the analytes were redissolved in the mobile phase (methanol: 0.1% formic acid in 3:2) to obtain a final volume of 1.0 mL before high-performance liquid chromatography tandem mass spectrometry (HPLC-MS-MS, Waters Acquity UPLC system) analysis. Recovery tests of OTC in the extraction of samples of soil and plants were also conducted with recovery rates of 92.3 ± 4.2 and 83.9 ± 3.4%, respectively.

DNA Extraction and Quantification of ARGs. Total DNA was extracted from 0.50 g of soil sample using a PowerSoil DNA Isolation Kit (MO BIO, Carlsbad, CA) following standard protocols. For the genomic DNA of *S. plumbizincicola* (stem and leaf), 50 mg of fresh tissue was weighed into PowerBead tubes to extract the DNA using a PowerPlant Pro DNA Isolation Kit (MO BIO, Carlsbad, CA). The concentration and quality of the obtained DNA were assessed using a Qubit 3.0 fluorometer (Life Technologies, Eugene, OR).

The Wafergen Smartchip real-time PCR system (Fremont, CA) was used to investigate the overall profile of ARGs and MGEs. To achieve substantive progress in analytical throughput, a preliminary test was conducted using extracted DNA from pooled soil samples to select a subset of the 384 primer sets. Consequently, 80 validated primer sets (Table S3) were employed to investigate the ARG profile.[36,37] Samples and primers were allocated into a 5184-nanowell Smartchip (100 nL reaction system per well) by the Multisample Nanodispenser. Each 100 nL qPCR mixture contained 50 nL of 2 × LightCycler 480 SYBR Green I Master, 10 nL of 2 ng/μL DNA template, 5 nL of 0.5 μM each primer, and 30 nL of nuclease-free PCR-grade water. Each sample was analyzed with three technical replicates, and a negative control without DNA template was included. The thermal cycle included initial enzyme activation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 34 s, annealing at 60 °C for 64 s, and finally an autogenerated melting curve using Wafergen software. Positive amplification with a threshold cycle value <30 and more than two detected replicates was retained.[6,37] The gene copy number was calculated according to the following equation[38,39]

$$\text{gene copy number} = 10^{(30-C_t)/(10/3)} \quad (1)$$

where C_t is the threshold cycle.

Relative abundance (copies/16S rRNA) was standardized using each gene's copy number relative to that of the 16S rRNA.[40] The absolute abundance (copies/g) of ARGs and MGEs was calculated by multiplying the absolute number of

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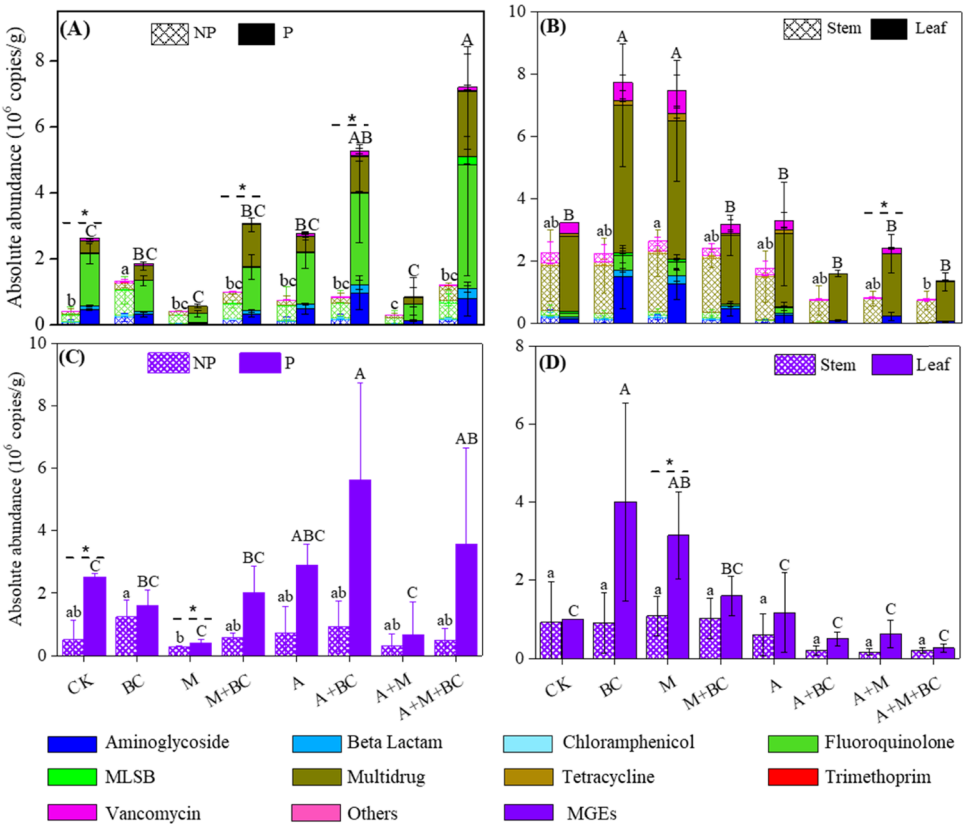

Figure 2. Absolute abundance of antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs) in soil and plants among different treatments. (A) Absolute abundance of ARGs in nonplanted (NP) and planted (P) soil. (B) Absolute abundance of ARGs in steam and leaf. (C) Absolute abundance of MGEs in nonplanted (NP) and planted (P) soil. (D) Absolute abundance of MGEs in steam and leaf. Values with lowercase letters in the same column or black stars indicate significant differences at $P < 0.05$ (LSD). MLSB, macrolide−lincosamide−streptogramin B. Sample code and abbreviations are the same as in Figure 1.

gene copies of 16S rRNA by the relative abundance in the same sample.[41]

Statistical Analysis. The raw data were processed in Microsoft Excel 2013 (Microsoft) in the form of the mean ± standard deviation and diagramed using OriginPro 9.1 (OriginLab, Northampton, MA). One-way analysis of variance (ANOVA) was conducted to analyze the statistical significance ($P < 0.05$) using SPSS 22.0 (SPSS Inc., Chicago). A structural equation model (SEM) was used to evaluate the direct and indirect effects of the biochar, hyperaccumulators, Cd concentration, OTC concentration, and MGEs on the ARG pattern. All variances in the matrices were imported into AMOS 21.0 (SPSS Inc., Chicago) for SEM construction according to the maximum-likelihood estimation.[9,10] The network analysis of the co-occurrence of ARG subtypes with MGEs based on a strong ($\rho > 0.8$) and significant ($P < 0.01$) Spearman's correlation was visualized using the Gephi platform (0.9.2). The heatmap displaying the distribution of the absolute abundance of each ARG and MGE was generated in R software (version 3.4.1) with the "pheatmap" package.[42] A Venn diagram was produced using the Bioinformatics & Systems Biology online tool (http://bioinformatics.psb.ugent.be/webtools/Venn/).

RESULTS

Growth of *S. plumbizincicola* and Distribution of Cd and Oxytetracycline Concentrations. Compared with the control (CK treatment), the biomass of *S. plumbizincicola* was reduced by 44.2% via the application of biochar (BC treatment) but was increased by 33.6% in the highly polluted soil with Cd (M + BC treatment) (Figure S2). However, the addition of OTC (A treatment) had little impact on the growth of *S. plumbizincicola* ($P > 0.05$; Figure S2). With the application of biochar, many basic properties of the soil were significantly increased, including pH, total organic carbon, total phosphorus, total potassium, available phosphorus, and available potassium ($P < 0.05$), while the available nitrogen was reduced from 69.8 ± 6.7 to 52.5 ± 6.4 mg/kg (Table S1).

Without the planting of *S. plumbizincicola*, the application of biochar in Cd-contaminated soil (M + BC and M + A + BC treatments) could significantly decrease the bioavailable Cd content by 22.7−26.7% ($P < 0.05$), where the bioavailable Cd in soil was as high as 4.10 mg/kg (Figure S3A). Plant cultivation prompted the alleviation of bioavailable Cd contamination in soil, decreasing by 14.3, 48.3, 15.2, and 42.8% compared with the corresponding treatments of M, M + BC, A + M, and A + M + BC without planting (Figure S3A), respectively. In addition, biochar amendment significantly enhanced the accumulation capacity of stem and leaf Cd in the above treatments, reaching a maximum of 354.84 mg/kg (Figure S3B). There was no significant impact on the reduction of OTC in the soil with the planting of *S. plumbizincicola* ($P > 0.05$; Figure S3C); however, adding biochar to co-contaminated soil effectively reduced the migration of OTC to plants (Figure S3D).

https://doi.org/10.1021/acs.est.1c03434
Environ. Sci. Technol. 2021, 55, 16369−16378

Diversity and Abundance of ARGs and MGEs. Diverse ARGs and MGEs were detected in both soil and plant samples, which covered all major classes of antibiotics, including aminoglycoside, β-lactam, chloramphenicol, fluoroquinolone, macrolide−lincosamide−streptogramin B (MLSB), multidrug, tetracycline, trimethoprim, vancomycin, and others (Figure 1). The number of detected ARG subtypes in the soil ranged from 19 to 44, much higher than that in *S. plumbizincicola*, which ranged from 3 to 19. The addition of biochar significantly increased the detected number among all treatments in the soil, whereas the opposite effect occurred in stems and leaves polluted with Cd and/or OTC (Figure 1).

The absolute abundance of ARGs in soil (from 2.89×10^5 to 7.20×10^6 copies/g, dry weight) was slightly lower than that in the plants (from 7.12×10^5 to 7.74×10^7 copies/g, dry weight) (Figure 2A,B). In addition, in the presence of pollutants (OTC and/or Cd), biochar amendment increased the ARG abundance in the soil, whereas a decrease occurred in the tissues of *S. plumbizincicola*. Among all treatments, the abundance of ARGs in planted soil was universally higher than that in nonplanted soil (Figure 2A). Furthermore, compared with the stem, a higher absolute abundance of ARGs was detected in the leaf (Figure 2B). Diverse ARGs targeted resistance to multiple kinds of antibiotics, of which the absolute abundance of multidrug resistance genes accounted for the majority of constituents in the soil and plants (Figure 2A,B). Two genes (*czcA* and *trbC*) with high absolute abundance were shared among all treatments, which belonged to multidrug resistance genes and MGEs, respectively (Figure S4). The behavior of MGEs was similar to that of ARGs with the application of contaminants or biochar (Figure 2C,D).

Co-occurrence Patterns and Correlations among ARG Subtypes. Most shared ARGs occurred in planted and nonplanted soil, and the addition of OTC and Cd was favored to induce the production of unique ARG subtypes in planted soil (Figure S5). Additionally, most ARG subtypes that emerged in stems and leaves were in line with those in the soil environment (Figure S5), indicating the internal pathway of ARG transmission from soil to plants via plant tissue. The absolute abundance of total ARGs was linearly and positively correlated with that of MGEs ($P < 0.01$, $R^2 = 0.70$) based on the ordinary least-squares regression model (Figure 3), especially for resistance genes of fluoroquinolone ($P < 0.01$), MLSB ($P < 0.01$), multidrug ($P < 0.01$), trimethoprim ($P < 0.01$), and vancomycin ($P < 0.01$) (Table S4). Network analysis was further adopted to represent the co-occurrence patterns among ARG subtypes. The same numbers of nodes (22) were shown in the network of soil amended with/without biochar; however, biochar amendment decreased the link of edges from 154 to 66, as well as the node connectivity from 12.50 to 5.18 (Figure 4A,B; Table S5). In contrast to the weak changes in the nodes of ARG subtypes via biochar amendment, planting of *S. plumbizincicola* significantly increased the nodes and edges of ARGs in the soil environment (Figure 4C,D and Table S5). Furthermore, the network analysis of the holistic soil−plant system consisted of 31 nodes and 293 edges with a high node connectivity of 16.26 and a clustering coefficient of 0.83 (Figure S6) and exhibited a complicated interactive relationship among ARGs in soil and on soil-grown hyperaccumulators.

Influence of Multiple Factors on ARG Patterns. A structural equation model was employed to estimate the effect of biochar, *S. plumbizincicola*, Cd, and OTC contamination, and MGEs on the profile of ARGs (Figure 5). Planting of *S. plumbizincicola* directly impacted the abundance of ARGs (λ = 0.37) and indirectly impacted ARG abundance by affecting the Cd contamination (λ = 0.35), the OTC contamination (λ = −0.32), and the abundance of MGEs (λ = 0.19) (Figure 5A). In contrast to the positive effect on ARG abundance via the Cd (λ = 0.07) and OTC (λ = 0.06) contamination, biochar application negatively impacted the ARG abundance in a direct way (λ = −0.04) and the MGE abundance in an indirect way (λ = −0.09). Strikingly, the abundance of MGEs exhibited a significantly positive correlation with ARG abundance (λ = 0.84), indicating its key role in the diffusion of ARGs (Figure 5A).

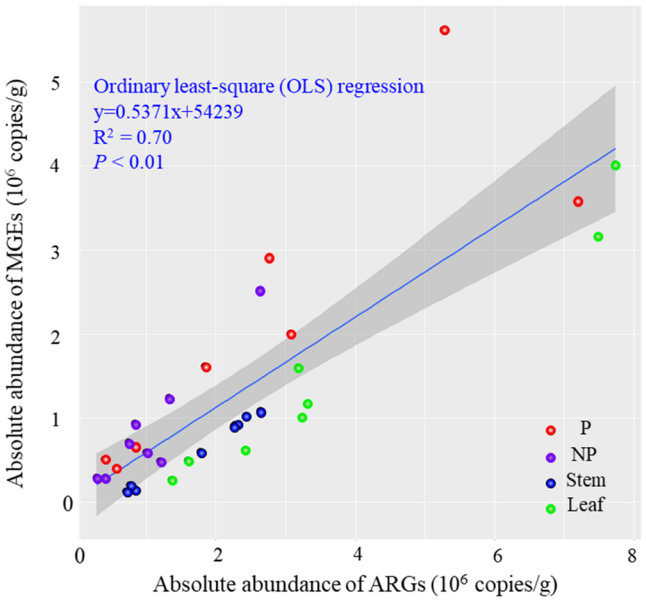

Figure 3. Correlation between the absolute abundance of mobile genetic elements (MGEs) and antibiotic resistance genes (ARGs) in all samples. P, planted soil; and NP, nonplanted soil. The shaded area represents the 95% confidence interval.

Another major feature of the SEM was used to evaluate the intensity of multiple drivers shaping ARGs based on standardized direct and indirect effects (Figure 5B). For the total effects (direct effect plus indirect effect), MGEs were the most dominant and positive factor in shaping ARG patterns, followed by the impact of *S. plumbizincicola* planting. In contrast, biochar amendment exhibited a negative effect on ARG abundance (Figure 5B). The contaminants Cd and OTC exerted opposite effects on the abundance of ARGs, of which OTC had a slightly stronger promotional effect on ARG proliferation (Figure 5B).

DISCUSSION

Effects of Biochar Amendment on the ARGs. Biochar amendment increased the detected number and absolute abundance of ARGs and MGEs in the co-contaminated soil while decreasing the corresponding number and absolute abundance in plant tissues (Figures 1 and 2). The potential mechanisms might be due to (1) biochar application aiding the carbon sequestration and improving nutrient and water retention, as well as soil porosity structures, thereby shifting the soil microbial community,[18,43] which was closely related to ARG distribution; (2) adsorption on contaminants by the biochar decreasing the bioavailability, which may have formed

https://doi.org/10.1021/acs.est.1c03434
Environ. Sci. Technol. 2021, 55, 16369−16378

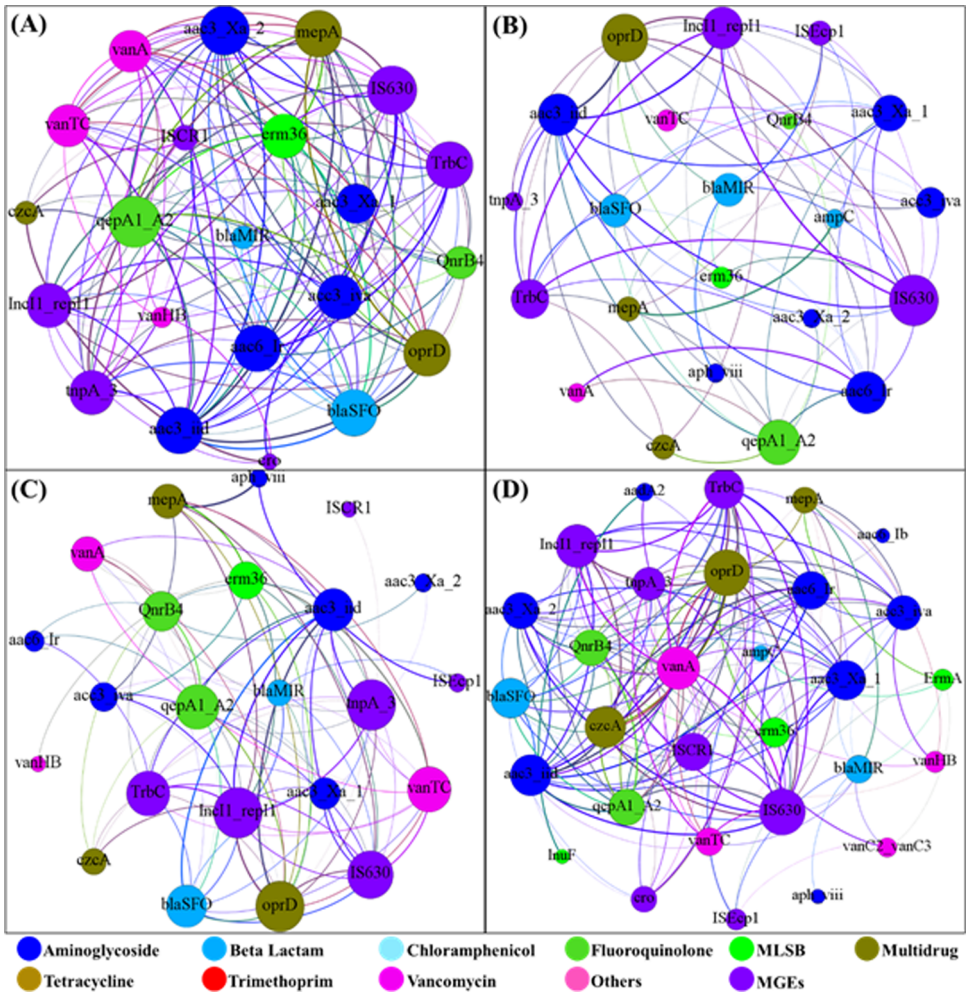

Figure 4. Network analysis of the co-occurrence patterns between antibiotic resistance gene (ARG) subtypes and mobile genetic elements (MGEs) in the soil (A) without the addition of biochar, (B) with the addition of biochar, (C) without *S. plumbizincicola*, and (D) with *S. plumbizincicola*. A connection represents a strong (Spearman's correlation coefficient $\rho > 0.8$) and significant ($P < 0.01$) correlation. The edge weight is according to the correlation coefficient, and the node size weight is proportional to the number of connections. MLSB, macrolide−lincosamide−streptogramin B.

subinhibitory concentrations of Cd and OTC in the soil (Figure S3), promoting the development of antibiotic resistance between bacteria;[44] and (3) biochar amendment inhibiting the transport of OTC from soil to *S. plumbizincicola* through adsorption and fixation effects (Figure S3), which reduced the selective pressure on the endophytes of *S. plumbizincicola*. Previous studies have reported that pathogens and ARGs can be completely removed during biochar fabrication when the pyrolysis temperature is >400 °C.[45,46] Exogenous ARGs introduced by biochar are expected to be negligible under the present condition of high pyrolysis temperature (600 °C) and low dosage (5%). Some related studies have reported that biochar is an effective farmland amendment for reducing the abundance of antibiotics and ARGs.[47,48] However, biochar properties (e.g., pH, pore size, specific surface area, cation-exchange capacity, and organic and inorganic composition) can vary significantly due to different pyrolysis conditions and feedstock types,[49] and thus may impact the dynamics of ARGs in soil. Biochar amendment has shown an increase in ARG abundance in planted soil and crops.[10] The existing results are insufficient to establish the relationship between biochar physicochemical properties and their impacts on ARG variation, which requires more detailed study in the future.

In contrast to the decrease in OTC after biochar application, the Cd concentration in plants was significantly increased ($P < 0.05$; Figure S3B,D), which was inconsistent with some reports that biochar amendment inhibited the metal uptake in crops and hyperaccumulators.[16,50,51] Despite the decrease in available Cd in acidic soil introduced with biochar, the remaining available Cd at high concentrations may not be a limiting factor for plant uptake (Figure S3A,B). Furthermore, biochar addition, improving nutrient supply and soil conditions (Table S1), could promote root proliferation and biomass,[52] which may enhance the hyperaccumulator in regard to Cd phytoextraction in contaminated soil. Interestingly, biochar amendment significantly decreased the detected number and absolute abundance of ARGs in the leaves of Cd-contaminated treatments (M and A + M), while those indexes remained relatively stable in the stems (Figures 1 and 2B,D). In a certain range, elevated Cd concentrations in *S. plumbizincicola* would not induce more severe antibiotic resistance, which might be due to the noticeable capacity of endophytic bacteria to bear metal stress.[53] However, the cumulative Cd concentration in leaves beyond the endurance capacity of indigenous

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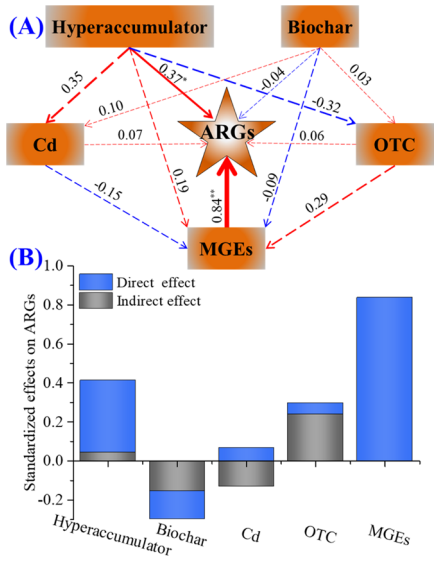

Figure 5. (A) Structural equation models showing the direct and indirect effects of biochar, hyperaccumulators, cadmium (Cd), oxytetracycline (OTC), and mobile genetic elements (MGEs) on the antibiotic resistance gene (ARGs) profile. (B) Standardized effects (direct and indirect effects) were derived from the structural equation models. Red and blue arrows indicate positive and negative relationships, respectively. Continuous and dashed arrows indicate significant and nonsignificant relationships, respectively (*P < 0.05 and **P < 0.01). Numbers adjacent to the arrows are path coefficients (λ), and the width of the arrows is proportional to the strength of the path coefficients. Sample code and abbreviations are the same as in Figure 1.

endophytes could decrease the diversity and abundance of the microbial community,[54] which may decrease the prevalence of ARGs in plants. Furthermore, the sustained decrease in antibiotic content from stem to leaf may account for the decrease in the diversity and abundance of ARGs.

Effects of *S. plumbizincicola* Planting on the ARGs. Biochar application influenced the ARG distribution in soil and plants differently by increasing the diversity in the soil but inhibiting the transmission from soil to plants (Figures 1 and 2). However, the planting of *S. plumbizincicola* caused an obvious increase in the detected number and absolute abundance of ARGs among the overall treatments in the soil environment (Figures 1 and 2A,C). This might be attributed to the "rhizosphere effect", in which the production of root exudates as nutrients (e.g., carbohydrates, amino acids, and organic acids) could promote the bacterial proliferation in contaminated soil and then affect the distribution of ARGs.[11,55]

The detected number and absolute abundance of ARGs in leaves were mostly higher than those in stems (Figures 1 and 2B,D). Furthermore, the absolute abundance of enriched ARGs in leaves was 1.32−3.71-fold higher than those in stems (Figure 2B,D), indicating other possible routes for ARGs entering *S. plumbizincicola* in addition to internal transmission via plant tissue. Multiple sources of ARGs originating from the soil particles, the applied water, and air might be involved in the ARG transmission into *S. plumbizincicola*, which deserves further attention in the future.[56,57]

Multiple Factors Accounting for the ARGs. Sustained selective pressure on bacteria due to antibiotic use leads to the prevalence of antibiotic resistance.[8] Heavy metals, human activity exerted to the environment, also drive the selection or co-selection on antibiotic resistance and promote the proliferation and dissemination of ARGs.[7,58] However, we found that the introduction of OTC and/or Cd (A, M, and A + M treatments) did not cause a significant increase in ARG abundance in the soil (P > 0.05; Figure 2A,C), which may be related to available concentrations of pollutants, the period for microbial evolution, and the self-adjustment capability of complex microbial communities.[59,60] Remarkably, biochar amendment and/or planting of *S. plumbizincicola* can largely influence the ARG profiles regardless of the presence of contaminants (Figures 1 and 2). Biochar amendment and planting can directly alter the soil microenvironment, influencing the structure and composition of the microbiota and then shifting the ARGs.[10,11]

Some ARGs are naturally effective in resistance, but others can be or have been upgraded from existing genes by mutational modification and recombination, or by horizontal gene transfer.[61] They can be transferred between diverse pathogens and environmental bacteria, even distantly related species, through conjugation, transduction, and transformation mediated by MGEs.[62,63] The high positive correlation between ARGs and MGEs in the ordinary least-squares regression model (P < 0.01, R^2 = 0.70; Figure 3) confirmed the important role of MGEs in shaping the pattern of ARGs.[64] Furthermore, the correlation and network analysis between ARGs and MGEs suggested that MGEs were the primary determinants of ARG proliferation (Figure 4 and Table S5). This result was further supported by SEM analysis, which showed that ARG proliferation was significantly impacted by both direct and indirect effects of multiple factors via MGEs (Figure 5). These findings collectively strengthened the evidence of ARG propagation mediated by MGEs. Mathematical statistics based on correlation analysis might be insufficient to provide straightforward evidence in these complicated systems. A substantial number of related studies using various metagenomic surveys and analytical tools will aid in investigating the mechanisms of ARG transmission in the future.

Overall, this study was the first to reveal the feasibility of using biochar combined with hyperaccumulators for mitigating the migration of ARGs in an antibiotic and toxic metal co-contaminated system. Biochar application could increase the diversity and abundance of ARGs in polluted soils, but effectively decrease the possibility of transmission from the soil to plants. Planting was demonstrated to promote the proliferation of ARGs in the soil environment, and in addition to the internal pathway via plant tissue, ARG transmission into plants may also be contributed by aerosol particles, especially in leaves. The occurrence and fate of ARGs was affected by biochar, hyperaccumulators, contaminants, and MGEs via both direct and indirect pathways; however, the prevalence of ARGs may be dominantly mediated by MGEs. These findings contribute to filling our gap in knowledge regarding ARG dissemination control in agricultural management. To better investigate the combined effects on ARG alleviation achieved via the use of biochar and hyperaccumulators, different biochar types, hyperaccumulator species, soil conditions (e.g., biological and physicochemical properties), and contaminant classes are needed, especially in the field environment.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.1c03434.

https://doi.org/10.1021/acs.est.1c03434
Environ. Sci. Technol. 2021, 55, 16369−16378

Absolute quantification of 16S rRNA gene, physicochemical properties of soil and biochar, primer sets used in this study, Pearson's correlation of ARGs and MGEs, parameters of co-occurrence patterns, scheme of experimental design, biomass of *S. plumbizincicola*, cadmium and oxytetracycline concentrations, absolute abundance of ARGs, shared number of ARGs, and co-occurrence network of ARGs (PDF)

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (41977137, 41991333, and 21677149) and the Key Program of Frontier Sciences, Chinese Academy of Sciences (QYZDJ-SSW-DQC035), and the Center for Health Impacts of Agriculture (CHIA) of Michigan State University. F.W. was partially supported by the fellowship of Alexander von Humboldt for experienced researchers.

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