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Effect of biochar amendment on the alleviation of antibiotic resistance in soil and phyllosphere of *Brassica chinensis* L.

Qing-Lin Chen[a,b], Xiao-Ting Fan[a,b], Dong Zhu[a,b], Xin-Li An[a,b], Jian-Qiang Su[a], Li Cui[a,*]

[a] Key Laboratory of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, No. 1799 Jimei Road, Xiamen 361021, China
[b] University of Chinese Academy of Sciences, 19A Yuquan Road, Beijing 100049, China

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ABSTRACT

The increasing prevalence of antibiotic resistance is a global threat to public health. Antibiotic resistance genes (ARGs) in soil have been demonstrated to be able to enter food chain. Strategies to mitigate the spread of antibiotic resistance from soil to crops are of great importance for food safety and human health. Soil amendment with biochar is a widely used approach to improve soil fertility. However, the impact of biochar on mitigating antibiotic resistance of soil and organically produced vegetables that are eaten raw is largely unknown. To gain insights into this impact, pot experiments planting *Brassica chinensis* L. in soil amended with biochar were conducted and antibiotic resistance genes (ARGs) were characterized by HT-qPCR targeting almost all major classes of ARGs and 10 mobile genetic elements (MGEs) marker genes. A total of 131 ARGs and 9 MGEs in soil and phyllosphere samples were identified. After biochar amendment, the abundance of ARGs was significantly decreased in non-planted soil. By comparison, no significant decrease of ARGs was found in rhizosphere and phyllosphere, indicating that vegetable planting affected antibiotic resistome in biochar-amended soil. To understand this effect, bacterial phylogeny structures within soil and phyllosphere were analyzed and found to correlate with their respective resistome, indicating that planting can influence the effect of biochar on soil antibiotic resistome by altering microbial community compositions. Structure equation models further revealed that a higher bacterial diversity corresponded to a decreased ARGs content. These results suggested that biochar amendment alone was not sufficient enough to alleviate ARGs level in planted soil and crops, instead, maintaining or increasing soil microbial diversity is potentially more useful in mitigating ARG spread and accumulation.

1. Introduction

Antibiotic resistance is ancient and naturally present in environmental bacteria (D'Costa et al., 2011). Several studies, based on metagenomics and functional metagenomics, have revealed diverse homologues of known resistance genes widely distributed across environmental locales — from soil and human gut to extreme environments (Sommer et al., 2009; Allen et al., 2010; D'Costa et al., 2011; Forsberg et al., 2012, 2014; Hu et al., 2013). These studies have shown that all environments have a base level of resistance. However, over the past decades, a growing body of direct and indirect evidences suggested that medical and veterinary antibiotic use facilitated the occurrence and fixation of antibiotic resistance genes (ARGs) in bacterial genomes (Bush et al., 2011; Heuer et al., 2011; Zhu et al., 2013). More seriously, the global spread of ARGs and the acquisition of ARGs by clinically relevant pathogens, have led to lack of effective antibiotic therapies available for life-threatening infections in many areas of the world (World Health Organization, 2014). The pace of developing novel antibiotics is alarmingly low (Hu et al., 2017); an alternative top priority is to preserve efficacy of antibiotics, which requires concerted effort to track and control the emergence and dissemination of antibiotic resistance (Kesselheim and Outterson, 2010; Pruden et al., 2013; World Health Organization, 2014).

With the increasing pursuing for a healthy lifestyle, the demand for organically-produced food is rapidly rising. However, agricultural practices, especially organic farming practices, such as direct application of organic fertilizer (animal manures, sewage sludge, struvite etc.), can directly introduce antibiotic resistant bacteria (ARB) and ARGs in soil, and indirectly increase the resistance level of indigenous bacteria in agricultural soils through a positive selection (Chen et al., 2016; Heuer et al., 2008; Kyselková et al., 2015). What makes the situation even worse is that ARGs from the soil antibiotic resistome (a collection of all ARGs and their precursors in both pathogenic and non-pathogenic bacteria) can enter the food chain via contaminated crops (Marti et al.,

* Corresponding author.
E-mail address: lcui@iue.ac.cn (L. Cui).

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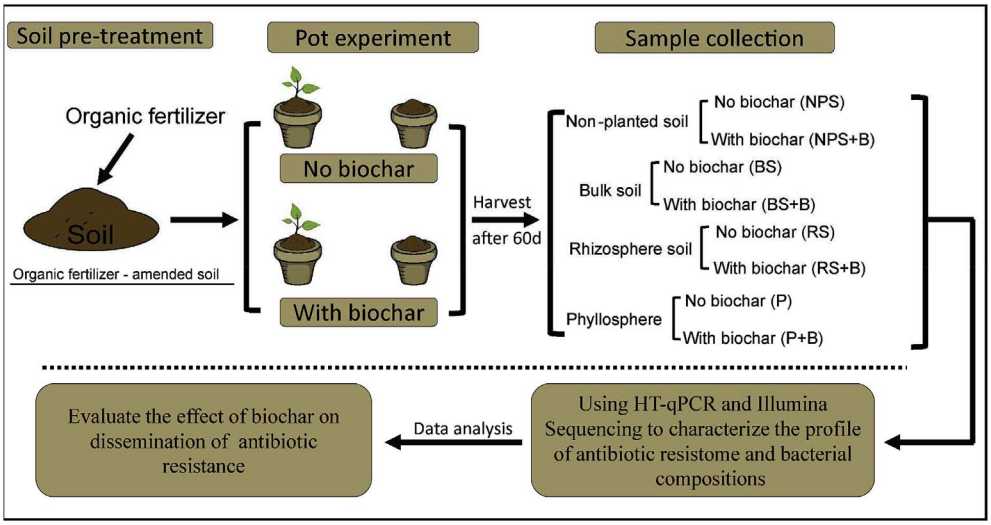

Fig. 1. Schematic of experimental design, methods and objective.

2013; Wright, 2007). For example, organically produced lettuce was indicated to harbor more diverse ARGs and the absolute abundance was 8-fold higher than conventionally produced lettuce (Zhu et al., 2017a). These studies indicated that organic farming practices may potentially threaten human health because of the potential transfer of resistance genes in crops to human pathogens. Therefore, strategies to alleviate antibiotic resistance in soil and subsequent accumulation by crops are of great importance from the perspectives of food safety and human health.

Biochar, a carbon-rich solid formed by pyrolysis of biomass in the absence of air, is distinguished from charcoal by its applicability as a soil amendment (Lehmann, 2007; Lehmann et al., 2011). Biochar can be used to increase soil fertility and crop yields by changing the soil physiochemical (such as pH) and biological properties (Van Zwieten et al., 2010; Atkinson et al., 2010; Woolf et al., 2010), improving nutrient retention through cation adsorption (Liang et al., 2006), reducing soil bulk density by the macro- and micropores in biochar (Lehmann and Joseph, 2009; Major et al., 2010; Ogawa and Okimori, 2010), and increasing diversity of soil microbiota (Xu et al., 2014). Considering that bacterial community composition is the primary determinant of soil ARG content (Forsberg et al., 2014), and biochar can increase the diversity of soil microbiota, biochar application may potentially alter the profile of soil antibiotic resistome. Furthermore, biochar application can reduce the bioavailability and mobility of soil pollutants including antibiotics and heavy metal (Teixido et al., 2011; Zeng et al., 2015). These soil pollutants may otherwise drive selection or co-selection of antibiotic resistance in soil microorganisms (Stepanauskas et al., 2006; Martinez, 2008; Allen et al., 2010), and promote the spread of antibiotic resistance. It has been indicated that biochar application can reduce the level of ARG in soil (Ye et al., 2016; Duan et al., 2017), however, very limited number of (less than 10) primer sets were used. It is therefore hard to provide an integrated profile of ARGs to evaluate the impact of biochar amendment on soil ARGs.

Here, by using high-throughput qPCR (HT-qPCR) with 296 validated primer sets targeting almost all major classes of ARGs and 10 MGEs marker genes (Zhu et al., 2017a), as well as Illumina sequencing, we characterized profiles of antibiotic resistome and bacterial compositions in both biochar-amended soil and planted vegetable. We aimed to (1) compare the distribution of antibiotic resistome in non-planted soil, planted soil and phyllosphere after biochar amendment, and (2) explore the effect of biochar on the fate of antibiotic resistome and the underlying mechanisms. These findings will contribute to a more comprehensive and accurate evaluation of whether biochar amendment is an effective way to mitigate the dissemination of antibiotic resistance in soil environment.

2. Materials and methods

2.1. Soil collection and biochar preparation

Soils used for pot experiments were collected from a vegetable field, which had not been amended with organic fertilizers in recent three years. Soil properties were as follows: pH 7.0, organic matter content 45.93 g kg^{-1}, total carbon 28.56 g kg^{-1}, total nitrogen 3.34 g kg^{-1}, Cr 42.53 mg kg^{-1}, Cu 18.70 mg kg^{-1}, Zn 57.68 mg kg^{-1}, and Pb 28.57 mg kg^{-1}. After air-dried and passed through a 2 mm mesh sieve, struvite as organic fertilizer was added to soil at a ratio of 0.2% (w/w) and was fully mixed before the experiment. Biochar used in this experiment was made from rice straw. Air-dried rice stalks were charred at 500 °C for 4 h in a closed container under oxygen-limited conditions using nitrogen as the medium gas in a muffle furnace (Isotemp, Fisher Scientific, USA). Biochar was milled to pass a 2 mm sieve before analysis and application to the soils. The basic properties and element composition of the biochar are shown in Table S1.

2.2. Microcosm design and sample collection

Soils were amended with or without 0.5% biochar (w/w). Soils were then planted or not planted with *Brassica chinensis* L. (Fig. 1). Each pot contained 3000 g soil and each treatment was performed in triplicate. A rhizo-bag (nylon mesh bag, containing 500 g soil) that allowed smaller molecular substrates to penetrate but prohibited penetration by roots, the soil in or out of the bag was taken as rhizosphere soil or non-rhizosphere soil (bulk soil) (Nie et al., 2015).

The soils were thoroughly watered and pre-incubated overnight. *Brassica chinensis* L seeds were sown in the nylon bags next day. All pots were incubated in a greenhouse with natural illumination and humidity at a temperature of 25 ± 2 °C during the day and 20 ± 2 °C in the night. Deionized water was added to maintain the soil water content every two days. Plants were harvest after 60 days. Four types of samples were collected, including non-planted soil, bulk soil, rhizosphere soil and phyllosphere of *Brassica chinensis* L.

2.3. DNA extraction

Total DNA was extracted from 0.5 g of soil for each replicate microcosm from each dilution using a FastDNA® Spin Kit for soil following the supplier's manual (MP Biomedical, Santa Ana, USA). Phyllosphere DNA was extracted according to (Chen et al., 2017b). Briefly, around 5 g leaf tissue was weighed into a conical flask (250 mL) containing 100 mL of 0.01M phosphate-buffered saline (PBS). The mixture was