

Application of biochar reduces *Ralstonia solanacearum* infection via effects on pathogen chemotaxis, swarming motility, and root exudate adsorption

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Received: 22 July 2016 / Accepted: 19 December 2016 / Published online: 29 December 2016
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Abstract

Aims We evaluated the efficacy of biochar application for suppressing bacterial wilt of tomato and identified the potential underlying mechanisms involved in the disease control.

Methods We measured the impact of two different sized biochar (53–120 µm and 380–830 µm) on bacterial wilt incidence in a greenhouse experiment. The efficiency of different sized biochar for the adsorption of tomato root exudates and the pathogen was further examined in vitro. We also quantified the effects of biochar and tomato root exudates on two pathogen virulence factors, chemotaxis, swarming motility and examined the effect of biochar on pathogen root colonization.

Results Fine biochar application (3%; w:w) significantly decreased the bacterial wilt incidence by 19.9%. Biochar with different particle size had similar adsorption capacity for root exudates, while fine biochar was efficient (91%) in pathogen adsorption. Root exudates and fine biochar increased the chemotaxis ability of pathogen, while fine biochar reduced pathogen swarming motility and rhizosphere colonization.

Conclusions Application of fine biochar can significantly decreased bacterial wilt incidence. This was mechanistically explained by biochar ability to 1) adsorb pathogen directly and indirectly via adsorption of root exudates (based on pathogen chemotaxis) and to 2)

Responsible Editor: Birgit Mitter.

Electronic supplementary material The online version of this article (doi:10.1007/s11104-016-3159-8) contains supplementary material, which is available to authorized users.

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directly suppress pathogen swarming motility and subsequent root colonization.

Keywords Adsorption · Bacterial wilt · Biochar · Disease control · Root exudate

Introduction

Bacterial wilt is an important plant disease in the tropical and subtropical regions of the world (Salanoubat et al. 2002). The *Ralstonia solanacearum* bacterium, the causal agent of bacterial wilt, has an unusually broad host range and is able to infect over 200 host species belonging to more than 50 botanical families (Hayward 1991), including economically important crops such as tomato and potato (Genin 2010). *R. solanacearum* is also metabolically versatile and survives prolonged periods not only in the soil but also in irrigation water (Xue et al. 2011). High *R. solanacearum* density in the rhizosphere, which can reach up to 10^8 colony forming units (CFU) per gram of soil, is the key factor onsetting the bacterial wilt disease epidemics (Wei et al. 2011). The CFU level for disease development depends on various abiotic and biotic factors including host cultivar, temperature and root wounding (Ishihara et al. 2012; Jacobs et al. 2012; Wei et al. 2015a). *R. solanacearum* can multiply in the rhizosphere and cause disease even at low initial abundance in the bulk soil (e.g., 10^4 CFU g^{-1} soil) once the environmental conditions become suitable (Wei et al. 2011). Generally, pathogen abundance is positively correlated with disease incidence (Kempe and Sequeira 1983; van Overbeek et al. 2004). However, recent evidence suggests that complete pathogen eradication is not necessary for considerable decreases in crop losses (Wei et al. 2015a). Considering that the traditional methods of disease control, such as chemical bactericides, have proven to cause environmental pollution (Fujiwara et al. 2011; Tan et al. 2015), alternative methods need to be developed to reduce the bacterial wilt incidence.

Plants secrete a high diversity of low molecular weight compounds (i.e., root exudates) into the rhizosphere that are consumed by the soil microbes (Bais et al. 2006; Mendes et al. 2011). Previous studies have demonstrated that *R. solanacearum* is specifically attracted to root exudates of the tomato and *R. solanacearum* can utilize various compounds present in tomato root exudates, such as sugars, amino acids and

organic acids (Yao and Allen 2006). Additionally, the competition for the root exudates with the commensal rhizosphere bacteria is important factor in determining the pathogen invasion success (Wei et al. 2015b). Sensing and responding to root exudates is thus a crucial step for successful root invasion by *R. solanacearum*, and as a result, disease control mechanisms targeting plant-pathogen cross talk through root exudates could be a potential way to control bacterial wilt disease epidemics (Masiello et al. 2013).

Adsorbents specific to low molecular weight compounds in root exudates could be efficient in constraining pathogen invasions (Masiello et al. 2013). For example, biochar, which is the product of the thermal degradation of organic material under low oxygen conditions, has proven to be an effective adsorbent for removing organic pollutants and heavy metal ions from the soil (Ahmad et al. 2014; Jiang et al. 2012). The key physical properties of biochar are their large surface area and porous structure (Atkinson et al. 2010). The surface properties of biochar depends on the feedstock and pyrolysis conditions (Kinney et al. 2012; Tong et al. 2011) and biochars obtained from animal litter and solid waste feedstocks show lower surface areas compared to biochars produced from agricultural crop residues and wood biomass (Ahmad et al. 2014). Generally, biochars produced under high temperature exhibit high surface area, high microporosity, high hydrophobicity and low polarity (Ahmad et al. 2014; Zhang et al. 2011). It has been previously shown that charcoal has a significant capacity to adsorb root exudates (Bais et al. 2005; Callaway 2000). On the other hand, porous structure of biochar is also important by providing niches for diverse organisms such as mycorrhizae and bacteria that can form active multicellular biofilms in the biochar pores (Gibert et al. 2013; Warnock et al. 2007). Moreover, biochar efficiency to adsorb *Escherichia coli* in water systems has been linked to the particle size of the biochar (Mohanty and Boehm 2014). Biochar particle size has a great influence on its surface area, pore volume and organics adsorption (Sun et al. 2012; Zheng et al. 2010). Additionally, fine biochar particles can increase the compactness of soil by filling the gaps between soil particles and thus influence bacterial adsorption (Liu et al. 2016; Mohanty and Boehm 2014). Biochar is distinguished from activated charcoal by its use as soil amendment, its cheaper cost and the production process, while both of them are carbon-rich surface sorbents (Inyang and Dickenson 2015; Lehmann et al. 2011; Oleszczuk et al.

2012). The surface areas and pore structures of activated carbon are usually enhanced due to activation process post carbonization (Zheng et al. 2010). Activated charcoal has been reported to be efficient in the removal of bacteria from drinking water and the bacterial adsorption capacity of activated charcoal is positively associated with metallic oxides of mineral matter, surface hydrophobicity and macropore volume (Rivera-Utrilla et al. 2001). Additionally, activated charcoal shows different adsorption capacities to different bacterial strains (Naka et al. 2001). It is still however unclear whether biochar affects the adsorption of invading pathogen, the root exudate availability for the pathogen, and if these effects are dependent on biochar particle size.

R. solanacearum uses flagella-based motility and chemotaxis to detect various compounds in the root exudate (e.g., sugars, amino acids and organic acids), or environmental conditions (e.g., pH and temperature) when colonizing the rhizosphere (Broek and Vanderleyden 1995; Tans-Kersten et al. 2004). These traits are important for bacterial wilt epidemiology as it has been shown that *R. solanacearum* mutants that are nonmotile or nonchemotactic have often significantly lower virulence (Tans-Kersten et al. 2001; Yao and Allen 2006). We hypothesize that biochar can affect these virulence factors indirectly by trapping root exudates, which in turn increased the pathogen adsorption capacity of biochar.

Here we tested these ideas experimentally in series of laboratory assays and greenhouse experiment. We hypothesize that biochar can directly adsorb and attract pathogen potentially leading to a reduced root colonization of the pathogen. Alternatively or additionally, it is possible that biochar could also adsorb tomato root exudates and indirectly affect the pathogen growth by decreasing the availability of growth-promoting nutrients. Lastly, biochar could adsorb both the root exudates and pathogen having interactive effects on pathogen attraction via effects on bacterial chemotaxis. All these potential outcomes could be affected by the particle size of the biochar. To test these hypotheses, we first quantified the biochar effects for bacterial wilt disease epidemics in a greenhouse experiment with tomato. We then examined the adsorption of root exudates and the pathogen by biochar in the laboratory assays. Lastly, we specifically looked the biochar effects on two important *R. solanacearum* virulence factors, chemotaxis and swarming motility, and tested the *R. solanacearum* ability to colonize tomato roots in the absence and presence of biochar.

Materials and methods

Bacterial strains and biochar

The bacterial pathogen *R. solanacearum* strain QL-Rs1115 (Wei et al. 2011) was cultivated at 30 °C on semi-selective medium (SMSA) (Elphinstone et al. 1996). Red fluorescent protein-labeled *R. solanacearum* QL-Rs1115 strain QL-RFP (Tan et al. 2015) was routinely maintained on SMSA medium supplied with 30 µg ml⁻¹ gentamicin to ensure the maintenance of fluorescent and antibiotic resistance tagged plasmid. Before the experiment, bacteria grown overnight were harvested by centrifugation, washed twice with sterile saline solution (0.9% NaCl), and diluted to the appropriate concentration based on optical density (OD₆₀₀, ~10⁷ CFU ml⁻¹).

We used pinewood-derived biochar in this study, which is commercially produced by the Institute of Chemical Industry of Forest Products, Chinese Academy of Forestry (Nanjing, Jiangsu, China). Pinewood was pyrolyzed at 700 °C and the pyrolysis temperature was based on previous study investigating the production of biochar for *E. coli* adsorption (Abit et al. 2012). The biochar was ground and sieved to obtain biochar with particle sizes of 53–120 µm (fine biochar) and 380–830 µm (coarse biochar). These criteria were based on previous studies with minor modification (Liu et al. 2016; Mohanty and Boehm 2014; Zheng et al. 2010). Both biochars had similar chemical properties: pH 6.6, total C of 805 g kg⁻¹, total N of 3 g kg⁻¹, Fe of 190 mg kg⁻¹, Zn of 244 mg kg⁻¹, Al of 294 mg kg⁻¹, Ca of 759 mg kg⁻¹, Cu of 11 mg kg⁻¹, Mn of 10 mg kg⁻¹, Pb of 6 mg kg⁻¹, Ni of 2 mg kg⁻¹, Cd of 0.1 mg kg⁻¹, Cr of 3 mg kg⁻¹, ash content of 3.1%, and an electric conductivity of 233 µs cm⁻¹. The fine and coarse biochars exhibited different BET (N₂) surface areas, 516 m² g⁻¹ and 444 m² g⁻¹, respectively.

Greenhouse assay

Tomato seeds (*Solanum lycopersicum* cv. ‘Hezuo 903’) were surface-sterilized with NaClO (3%; v:v) for 5 min. After four successive rinses in sterile distilled water, seeds were placed on petri dishes covered with sterile, moist filter paper and germinated in the dark at 30 °C for two days. Germinated seeds were then sown in pots (6 cm × 6 cm × 6 cm) containing field soil. The soil was collected from a tomato field naturally infected with

R. solanacearum (Qilin, Nanjing, China; 118°57'E, 32°03'N) (Wei et al. 2011). The soil was first cleared of plant debris, sieved (< 2 mm) and homogenized thoroughly. The soil was characterized as yellow-brown earth (Udic Argosol) with pH 5.4 (1:5), organic matter (OM) content of 24.6 g kg⁻¹, total N of 6.3 g kg⁻¹, available P of 172.9 mg kg⁻¹, and available K of 178 mg kg⁻¹. Fine or coarse biochar was mixed with soil (3%; w:w) before sowing. The concentration of biochar (i.e., 3%) was based on previous studies (Bais et al. 2005; Rivera-Utrilla et al. 2001) and our preliminary dose-response pathogen adsorption experiment (Fig. S1; see method below). *R. solanacearum* QL-Rs1115 was applied at a cell density of 3 × 10⁶ CFU g⁻¹ soil at two weeks post-sowing. Tomato plants were maintained under greenhouse conditions at temperatures ranging between 22 and 32 °C during the day and 20–25 °C at night. The disease index was recorded based on a scale of 0–4 (0 = no wilting, 1 = 1–25% leaf area wilting, 2 = 26–50% wilting, 3 = 51–75% wilting, 4 = 76–100% wilted or dead). Disease incidence = [∑ (number of diseased plants in this index × disease index) × (total number of plants × highest disease index)⁻¹] × 100% (Chen et al. 2013). Each treatment was replicated three times and each replicate contained 18 tomato plants. Greenhouse experiment was repeated three times with similar results. Hence, only one representative experiment is shown.

Collection of root exudates

Tomato root exudates were collected by following the method described by Badri et al. (2013) with minor modifications. Briefly, tomato seeds (cv. 'Hezuo 903') were surface-sterilized, germinated and transferred to 6-well culture plates (Corning, CA, USA) with each well containing 3 ml of Murashige and Skoog liquid medium (Murashige and Skoog 1962) supplemented with 1% sucrose (each independent well containing three seeds). Seedlings were incubated on an orbital shaker at 90 rpm and were illuminated under cool white fluorescent light with 16 h daylight cycle (25 ± 2 °C). After ten days, the tomato seedlings were gently washed with sterile distilled water, transferred to new wells on 6-well plates containing 3 ml of sterile distilled water and incubated for additional 24 h. Root exudates of 180 tomato plants (60 wells) were then pooled and filter sterilized (0.45 µm). Sterile exudates were then lyophilized to

powder to improve storage and dissolved in 10 ml sterile distilled water before the start of the experiments.

Assessment of the adsorption of *R. solanacearum* onto biochar

Bacterial adsorption experiments were conducted as described by Rivera-Utrilla et al. (2001). Briefly, 5 ml of cell suspension (~10⁷ CFU ml⁻¹) was added to tubes containing 0.15 g fine or coarse biochar and a cell suspension without biochar was included as a control. The tubes were incubated on an orbital shaker for 60 min (at 30 °C with 90 rpm shaking) and then allowed to stand for additional 60 min. We then defined changes in the density of *R. solanacearum* populations with a serial dilution method on SMSA medium. Colony forming units (CFU) were counted after incubation at 30 °C for two days. Each treatment consisted of three replicates and the adsorption experiment was repeated three times with similar results. Only one representative experiment is shown.

Assessment of the adsorption of root exudates onto biochar

Root exudates were mixed with fine or coarse biochar (3%; w:v), incubated on an orbital shaker at 30 °C with 90 rpm shaking for 60 min and sterilized by filtering with 0.45 µm nylon filters. Filtered root exudates were then used as a medium for the cultivation of *R. solanacearum* QL-Rs1115 on 96-well culture plates (Corning, CA, USA). Non biochar-treated root exudates were used as a control treatment. All microplate wells were inoculated with 198 µl of root exudates and 2 µl of bacterial suspension (~10⁷ CFU ml⁻¹). The plates were incubated at 30 °C under aeration at 170 rpm. Each treatment had six replicates. Bacterial growth (OD₆₀₀) was determined using a SpectraMax M5 (Molecular Devices, CA, USA).

Chemotaxis assay

We used a capillary assay to measure bacterial chemotaxis towards tomato exudates and biochar by following a method described by Rudrappa et al. (2008) with small modifications. Briefly, a 200 µl pipette tip containing 100 µl of *R. solanacearum* QL-Rs1115 suspension (~10⁷ CFU ml⁻¹) was attached to 1 ml syringes with a 4 cm 25 gauge needle (Becton-Dickinson, Franklin

Lakes, NJ, USA). The 1 ml syringes were then filled with 100 μl of a) sterile distilled water (control), b) tomato exudates, c) an aqueous suspension of fine biochar (3%; w:v) or d) an aqueous suspension of root exudate-treated fine biochar (3%; w:v). After 2 h incubation at 30 °C in the dark, the amount of migrated bacteria were measured by serially diluting the contents of the syringe on SMSA medium. Root exudate-treated fine biochar was prepared by mixing root exudate with fine biochar (3%; w:v) and incubating on an orbital shaker for 60 min (at 30 °C with 90 rpm shaking). Fine biochar was then filter sterilized (0.45 μm) and dried at room temperature. Each treatment consisted of three replicates and the adsorption experiment was repeated independently three times with similar results. Only one representative experiment is shown.

The effects of exudates and biochar on swarming motility of *R. solanacearum*

The effects of root exudates and biochar on *R. solanacearum* swarming motility was assayed as described previously by Park et al. (2008) with small modifications. Briefly, four different *R. solanacearum* suspensions (20 μl , $\sim 10^7$ CFU ml^{-1}) were inoculated into the center of semisolid SMSA plates (diluted 1:5; 0.05% agar), including the following a) an aqueous suspension of *R. solanacearum* (control); b) a root exudate suspension of *R. solanacearum*; c) an aqueous suspension of fine biochar and *R. solanacearum* (biochar:water =3:100; w:v); and d) a root exudate suspension of fine biochar and *R. solanacearum* (biochar:root exudate =3:100; w:v). The different suspensions were prepared by mixing the corresponding constituents together and were incubated on an orbital shaker at 30 °C and 90 rpm for 60 min. Four replicates of colony diameters were measured in three directions on each plate after incubation for 24 h at 30 °C.

Root colonization assay

Tomato seeds (cv. ‘Hezuo 903’) were surface-sterilized and germinated as described above. Germinated seeds were then sown in pots (6 cm \times 6 cm \times 6 cm) with nursery substrate (soil:vermiculite =1:1; v:v; 130 g). The soil was the same as mentioned in greenhouse assay. Fine biochar was added to the nursery substrate by mixing it with water (1.5 g in 5 ml water per 50 g of nursery substrate) (Bais et al. 2005). As a result, half of

the replicate plants were grown in the absence and half in the presence of biochar. Plants were illuminated under cool white fluorescent light with 16 h daylight (25 \pm 2 °C). All the pots were weighed at least once a week to maintain the same soil moisture content among the pots. *R. solanacearum* QL-RFP was applied at a cell density of 7×10^6 CFU g^{-1} soil at two weeks post-sowing. After five days post-inoculation (dpi) of the pathogen, all plants were sampled and pathogen density was determined. To this end, excess soil was first gently shaken from the plant roots and the remaining soil that was left attached to the roots was defined as rhizospheric soil (Panke-Buisse et al. 2015; Schreiner et al. 2010; Shi et al. 2015). The nursery substrate (soil:vermiculite =1:1; v:v) used in this study protects plant roots from mechanical damage when collecting rhizospheric soil. Plant exudates make the rhizospheric soil attach to the roots, while the bulk soil will be easily shaken off due to high vermiculite concentration. Rhizospheric soil and bulk soil from four nursery pots were pooled as one replicate to minimize the bias of soil collection method and each treatment consisted of three replicates. To estimate pathogen densities, soil suspensions of 100 mg of rhizospheric soil and 10 g of bulk soil from each replicate were serially diluted in sterile water and plated on SMSA medium. Pathogen CFUs were counted after incubation at 30 °C for two days. The experiment was replicated independently three times with similar results and only one representative experiment is shown.

Confocal laser scanning microscopy (CLSM) of colonized roots

To observe pathogen colonization on the root surfaces after 7 days post pathogen inoculation, four asymptomatic plants with or without fine biochar application were harvested and gently washed with sterile water to remove nursery substrate and unattached pathogens. Adhered pathogens were observed using a confocal laser scanning microscope (Carl Zeiss LSM 700, Oberkochen, Germany) with emission wavelengths of 610 nm. The experiment was repeated three times and representative images are presented.

Statistical analysis

We used analysis of variance (ANOVA; Tukey’s test for pairwise comparisons) and Student’s t test to compare differences between different treatments. Proportional data

was arsin transformed before the analysis and all analyses were performed with SPSS (v 19) where a p -value of <0.05 was considered statistically significant different.

Results

Biochar application decreases the bacterial wilt disease incidence

We conducted a greenhouse experiment to measure biochar efficiency in controlling bacterial wilt disease incidence. Bacterial wilt disease progression followed the ‘S’ pattern in time (Fig. 1). Compared with the control, fine biochar treated soils had 19.9% lower disease incidence after four weeks post-inoculation ($p < 0.01$, Student’s t test). However, no significant difference in disease incidence was observed between coarse biochar treated and control soils.

Biochar has the ability to adsorb *R. solanacearum* cells

To determine whether the biochar has the ability to adsorb *R. solanacearum* cells, and if this is affected by biochar particle size, we compared the adsorption capability of fine (53–120 μm) and coarse (380–830 μm) biochars originating from the same biochar source. Our results showed that 91% of the pathogenic cells were adsorbed by the fine biochar compared to the control

treatments ($p < 0.001$, Student’s t test), while coarse biochar had no effect on pathogen adsorption (Fig. 2a). These results suggest that only fine biochar has the ability to adsorb *R. solanacearum*.

Biochar has the ability to adsorb root exudates

The ability of biochar to adsorb root exudates was studied indirectly by measuring the *R. solanacearum* growth with non-treated and biochar-treated root exudates (coarse and fine biochar). We found that pathogen growth was significantly decreased with biochar-treated root exudates compared to non-treated root exudates ($p < 0.01$, Student’s t test), and that both fine and coarse biochar had similar effect at single dose of 3% (Fig. 2b).

Root exudates and biochar attract *R. solanacearum* pathogen via chemotaxis

According to our chemotaxis assays, tomato root exudates attracted pathogen more efficiently compared to water control ($p < 0.001$, Student’s t test) and that the highest attraction was found in fine biochar-root exudate treatment (4.9-fold compared to root exudate-only treatment, $p = 0.001$, Student’s t test; Fig. 3a). These results show that biochar and root exudates increase *R. solanacearum* chemotaxis synergistically.

Biochar inhibits the swarming motility of *R. solanacearum*

A swarming assay was conducted to determine whether the addition of biochar affects the movement of *R. solanacearum*. We found that biochar significantly decreased the diameter of the *R. solanacearum* swarming area compared to the control ($p < 0.001$, Student’s t test; Fig. 3b). In contrast, the tomato root exudate strongly induced the swarming motility of *R. solanacearum*, and as a result, intermediate effect was observed in biochar-root exudate treatment (no difference to control). These results suggest that root exudates and biochar have contrasting (positive and negative, respectively) effects on *R. solanacearum* swarming motility.

Pathogen adsorption in the biochar-treated rhizosphere

We used *in vivo* experiments to test the biochar efficiency to adsorb pathogen in the rhizosphere and the

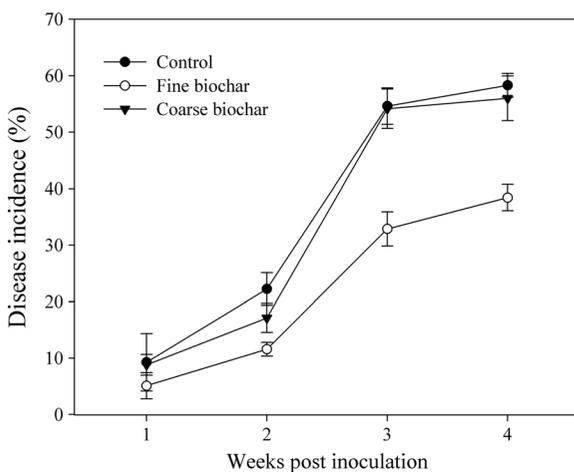


Fig. 1 Effect of fine and coarse biochar on the progression of bacterial wilt in tomato. Disease incidence is represented by the percentage of tomato seedlings with wilt symptoms (mean value \pm SE, $N = 3$). Each replicate contained 18 seedlings

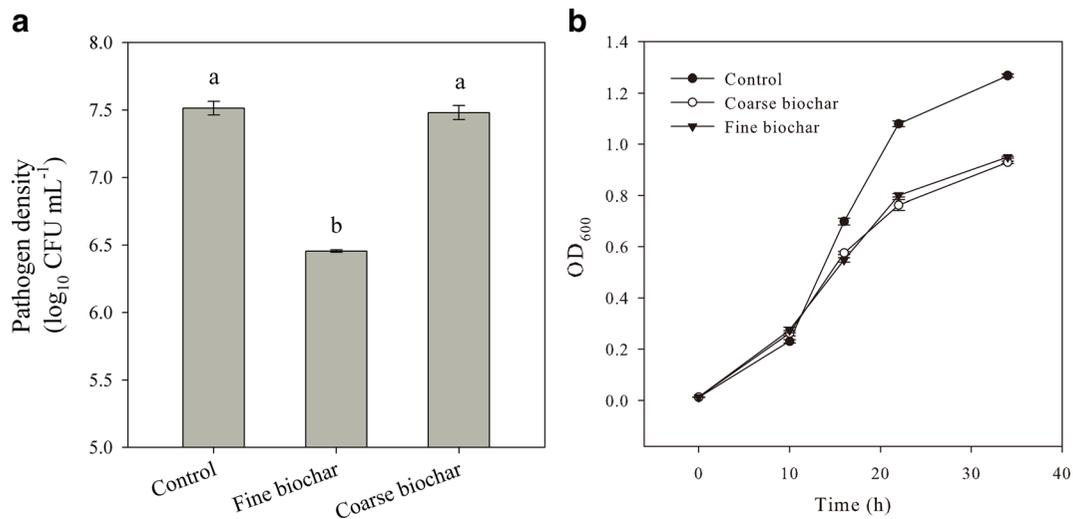


Fig. 2 Adsorption of **a** *R. solanacearum* QL-Rs1115 by different sized biochar and **b** *R. solanacearum* growth in root exudates treated with fine or coarse biochar. Error bars indicate the standard error of the mean. Different letters indicate significant differences

pathogen ability to colonize tomato roots in the presence and absence of biochar with CLSM. We found that pathogen densities were generally higher in the rhizosphere compared to the bulk soil ($p < 0.05$, Student's *t* test; Fig. 4a). After five days post-inoculation, a significantly higher (2.4-fold, $p < 0.05$, Student's *t* test) population of the pathogen was observed in the rhizosphere of the control plants compared to the biochar-treated plants (Fig. 4a). The addition of biochar did not alter the *R. solanacearum* densities in the bulk soil. Similarly, the CLSM analysis showed reduced pathogen colonization on tomato roots in the presence of fine biochar (Fig. 4b and c).

Discussion

Biochar has been recognized as an effective material for the removal of organic and inorganic contaminants from the soil (Ahmad et al. 2014; Zheng et al. 2010). However, only a few studies have examined the influence of biochar for the control of plant disease (Elad et al. 2010; Elmer and Pignatello 2011; Wardle et al. 1998). Here we studied directly the effects of biochar of two size classes on the adsorption of tomato root exudates and the *R. solanacearum* pathogen. We found that biochar was effective in adsorbing both root exudates and the pathogen cells (Fig. 2) leading to lower pathogen densities in the rhizosphere (Fig. 4) and a reduced bacterial wilt incidence (Fig. 1). While biochar particle

size had no effect on the adsorption of root exudates, only the fine biochar was effective in pathogen adsorption. Mechanistically, this effect was due to biochar-mediated loss of swarming motility (Fig. 3b; Fig. 5, mode a), which is known to be important factor for successful colonization of tomatoes by *R. solanacearum* (Addy et al. 2012; Tans-Kersten et al. 2001). Additionally, the pathogen exerted strongest chemotactic response to root exudate-treated biochar (Fig. 3a), which led to increased pathogen adsorption on the biochar (Fig. 5; mode b). Together these results suggest that biochar application could be a potential way to reduce bacterial wilt disease incidence.

Our data demonstrate that small biochar particle size plays an important role in bacterial adsorption (Fig. 2a) and the suppression of bacterial wilt (Fig. 1). This result is similar with a previous study where the removal of fine ($< 125 \mu\text{m}$) biochar particles from a biochar biofilter decreased the removal efficiency of *E. coli* from 95% to 62% in a water system (Mohanty and Boehm 2014). Decreased pathogen adsorption capacity of the coarse biochar (Fig. 2a) could be attributed to decrease in specific surface area for pathogen adsorption. The fine sized biochar used in this study exhibited a larger BET (N_2) surface area than coarse biochar. However, the surface area determined by gas adsorption was mainly influenced by micropores (nm scale) (Sohi et al. 2010; Sun et al. 2012) and could not reliably reflect bacterial adsorption. The pore size distribution of biochar is highly variable, including nanopores ($< 0.9 \text{ nm}$),

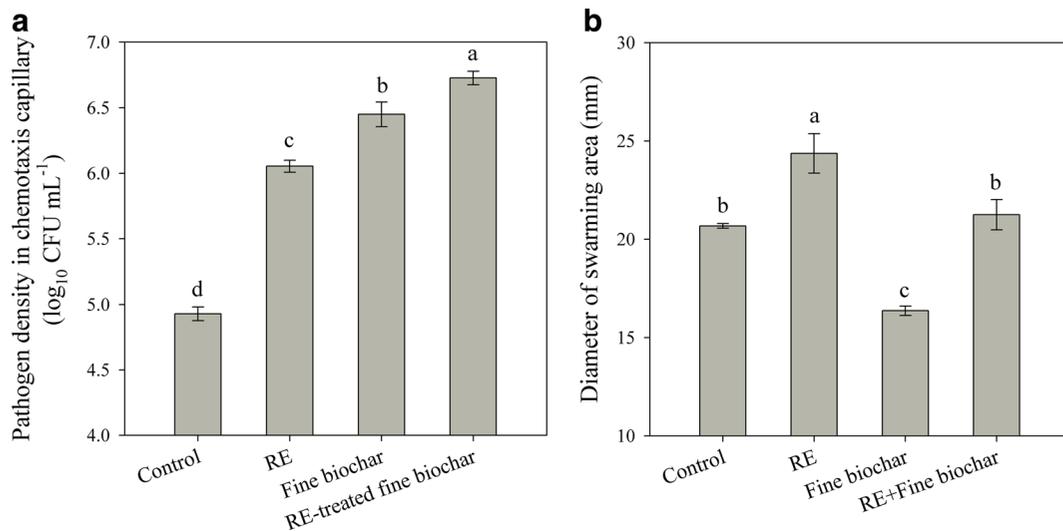


Fig. 3 Effects of fine biochar on chemotaxis **a** and swarming motility **b** of *R. solanacearum* QL-Rs1115. **(a)** Chemotaxis of *R. solanacearum* QL-Rs1115 towards sterile distilled water (Control), tomato root exudates (RE), an aqueous suspension of fine biochar (Fine biochar) or an aqueous suspension of root exudate-treated fine biochar (RE-treated fine biochar). **(b)** Four different suspensions were inoculated on the center of semisolid SMSA

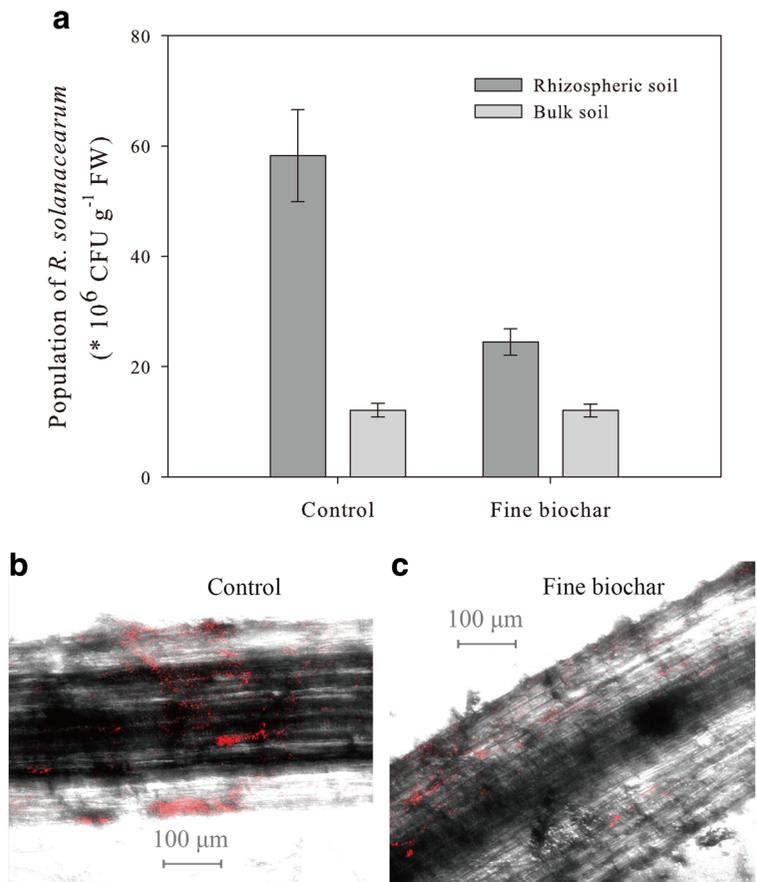
plates including an aqueous suspension of *R. solanacearum* (Control), a root exudate suspension of *R. solanacearum* (RE), an aqueous water suspension of fine biochar and *R. solanacearum* (Fine biochar), and a root exudate suspension of fine biochar and *R. solanacearum* (RE + Fine biochar). Error bars indicate the standard error of the mean. In both panels, different letters indicate significant differences

micropores (< 2 nm), mesopores (< 50 nm) and macropores (> 50 nm) (Atkinson et al. 2010). As the size of *Ralstonia* sp. is measured in microns (Miyake-Nakayama et al. 2006), it is likely that only charcoal pores with a dimension larger than the size of the bacteria (i.e. macropores) would be able to adsorb bacteria (Mohanty et al. 2014; Rivera-Utrilla et al. 2001; Sun et al. 2012; Warnock et al. 2007). Low specific area of biochar for pathogen may result in the release of the pathogen (Fig. 5; mode C), which could explain why coarse biochar had no effect on pathogen adsorption in the laboratory and greenhouse experiments. Biochar micropore volume is negatively correlated with particle size (Sun et al. 2012). Unfortunately, the relevance of macropore volume (especially pores for bacteria) and particle size under highly controlled production condition is largely unknown. Mercury porosimetry, optical microscopy and three-dimensional (3-D) reconstruction techniques could be used to characterize macropores of biochar (Rivera-Utrilla et al. 2001; Sun et al. 2012; Zygourakis et al. 2013). However these methods show many drawbacks including inability to distinguish between inter-particle and intra-particle porosity for powdered samples (mercury porosimetry) and a lack of method for quantifying macropore volume (optical microscopy and 3-D reconstruction techniques) (Brewer

et al. 2014). Therefore, effective biochar porosity characterization methods are still needed.

To shed light on the potential mechanism, we found that biochar affected the root exudate adsorption and two important *R. solanacearum* virulence factors: chemotaxis and swarming motility. Treating root exudates with biochar led to efficient removal of nutrients in the root exudates and clearly reduced pathogen growth (Fig. 2b). Adsorption of root exudates could intensify the resource competition with the ‘untrapped’ pathogens having negative secondary effect on pathogen invasion success in multibacterial rhizosphere communities (Raaijmakers et al. 2008; Wei et al. 2015b). Moreover, we found that *R. solanacearum* showed strong chemotactic response towards both root exudates and biochar. Crucially, root exudate-treated biochar had the highest attraction with the pathogen (Fig. 3a). This suggests that biochar was able to attract both root exudates and the pathogen directly, and that treating biochar with root exudates increased the adsorption of the biochar via bacterial chemotaxis. We also found that root exudates had a positive, and biochar had a negative, effect on the *R. solanacearum* swarming motility (Fig. 3b), which is important for *R. solanacearum* virulence and root colonization (Tans-Kersten et al. 2001). Prevention of swarming could have restricted *R. solanacearum* from

Fig. 4 The effect of fine biochar on the root colonization by *R. solanacearum*. The size of the *R. solanacearum* population colonizing the tomato roots was determined by serial dilution (a) and CLSM (b and c). CLSM images of *R. solanacearum* QL-RFP on untreated (b) and fine biochar-treated (c) tomato roots are shown. Error bars indicate the standard error of the mean ($N = 3$)

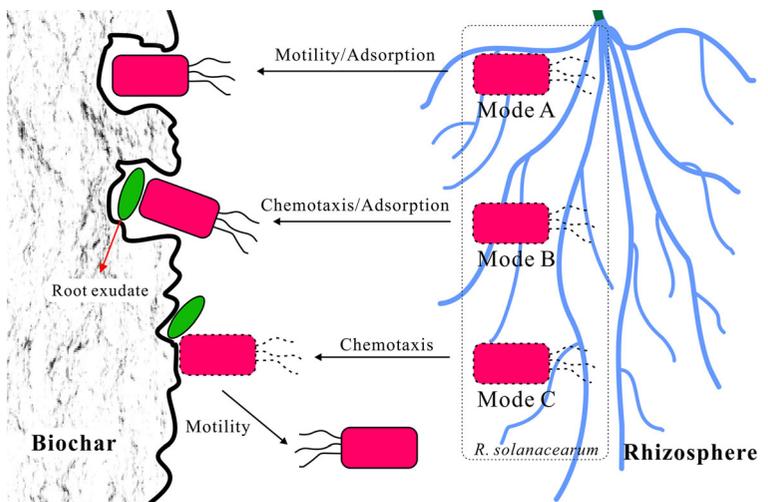


escaping the biochar surface leading to lower levels of bacterial wilt disease incidence.

In addition to functioning as nutrients, root exudates, including hormones, also aid in establishment of

symbiotic and parasitic interactions with microbes and regulate the development of root architecture (Bais et al. 2006; Boyer et al. 2014). It is thus possible that biochar may adsorb plant hormones (Boyer et al. 2014;

Fig. 5 Schematic drawing depicting the adsorption modes of biochar for *R. solanacearum*. Mode A: direct adsorption of *R. solanacearum* on biochar. Mode B: indirect adsorption of *R. solanacearum* on biochar via root exudate adsorption. Mode C: root exudates, which are adsorbed by biochar, induce chemotaxis of *R. solanacearum* to biochar. However, low adsorption ability of certain biochars may result in the release of the pathogen



Xie et al. 2013) leading to effects on pathogen invasion via changes in the balance of plant hormone concentrations. Biochar might thus have multiple effects on plant growth and health, although no clear negative effects have been observed in the previous studies (Atkinson et al. 2010; Elad et al. 2010; Elmer and Pignatello 2011; Hale et al. 2014). Application of biochar to decrease the incidence of bacterial wilt is still at an experimental stage. Long-term field experiments are thus an absolute requirement not only to determine the disease control efficacy of biochar under field condition but also to understand how often biochar needs to be re-applied in order to prevent re-infections during consecutive crop seasons. It is also possible that the porous structure of biochar provides a temporal refuge for pathogen and that the nutrients present in biochar enhance pathogen growth (Taghizadeh-Toosi et al. 2011; Warnock et al. 2007). Such concerns need to be tested to ensure the safe and wide use of biochar.

The colonization of plant roots is a critical early step in the pathogenesis of bacterial wilt prior to penetration of the root surface (Colburn-Clifford et al. 2010; Digonnet et al. 2012). We found that the fine particle sized biochar was effective in reducing *R. solanacearum* densities both in the rhizosphere and on the root surface (Fig. 4) leading to clearly reduced levels of bacterial wilt disease incidence (Fig. 1). We propose that biochar could offer a potential method to decrease bacterial wilt incidence and reduce the use of chemical agents, such as fumigants, which are often harmful for the environment and beneficial soil microbes. The strong bacterial adsorption by biochar could also provide an opportunity to use biochar as a carrier for biocontrol agents (Hale et al. 2014). Compared to other soil remediation materials, biochar is a low-cost agent with broad benefits (Ghosh et al. 2011). Hence, biochar produced from inexpensive agricultural residues may promote the broader application of charcoal technology in the future (Oleszczuk et al. 2012). More work is however still needed regarding the application. In this study, the concentration of biochar applied to the soil was quite high (3%), which might create some practical limitations for using biochar. Also, we used only two different size classes of biochar. Both the source and pyrolysis conditions influence the physical and chemical properties of biochar (Atkinson et al. 2010). Comprehensive comparison of the roles of pore size and the optimal biochar production conditions could thus considerably improve the disease control efficacy of biochar in the future. Lastly, soil

amendment with biochar needs to be tested in multibacterial communities to determine its role for the commensal and plant growth promoting bacteria.

In conclusion, here we show that biochar can significantly decrease bacterial wilt disease incidence by attracting pathogen both directly and indirectly via adsorption of root exudates that exert strong chemotactic signal towards the pathogen. Furthermore, biochar suppressed the swarming motility of the pathogen, which likely directly decreased pathogen virulence and potentially prevented the pathogen from escaping the biochar pores. Lastly, the adsorption of tomato root exudates could indirectly reduce pathogen invasion by intensifying resource competition with other bacteria in more natural settings. Biochar could thus potentially offer a cheap and novel way to decrease plant disease incidence.

Acknowledgements This research was supported by the National Key Basic Research Program of China (2015CB150503), the National Natural Science Foundation of China (41471213, 41671248), the 111 project (B12009), Jiangsu Key Technology R&D Program (BE2014340), the Young Elite Scientist Sponsorship Program by CAST (2015QNRC001), the Priority Academic Program Development (PAPD) of Jiangsu Higher Education Institutions (Qirong Shen), and the Qing Lan Project (Yangchun Xu).

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