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Biochar characteristics relate to its utility as an alternative soil inoculum carrier to peat and vermiculite

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ABSTRACT

Biochar materials have greatly variable physical and chemical properties, which will affect their abilities to serve as carriers for introducing bacteria into soils. Here we examined biochars made from 5 feedstocks produced at 2 highest treatment temperatures (HTT's) by slow pyrolysis. Peat and vermiculite, which are traditional inoculum carriers, and liquid inoculum with no carrier, were included for comparison. All of the carriers were inoculated with a liquid suspension of the plant growth promoting rhizobacterial (PGPR) strain, *Enterobacter cloacae* UW5, carrying a green fluorescent protein (GFP) marker. Inoculum survival was determined using quantitative PCR to enumerate the GFP markers in DNA extracted from non-sterilized soils directly after incorporation of the inoculated carriers and after a 4-week incubation. The biochars were characterized with respect to carbon and nitrogen content, specific surface area, pH, electrical conductivity, water holding capacity, pore opening diameters, and hydrophobicity to identify specific attributes that influence the survival of the inoculant after introduction into soil. The results indicated that chemical properties of biochar, particularly nitrogen and pH, were among the most important characteristics affecting initial inoculum survival and hence likely the shelf life. However, once incorporated into soil, physical features, including surface area, pore opening diameters, and water-filled pore spaces, were more closely associated with inoculum survival. All biochars tested performed as well as vermiculite and none demonstrated detrimental effects on the UW5 population. The best biochar was that made from pinewood at a HTT of 600 °C (Pine600), which performed as well as peat and sustained higher population densities than vermiculite. The Pine600 biochar was further tested to assess its effect on the expression of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase and auxin synthesis, both of which were unaffected by the presence of biochar at 2% or 5% (w/v) concentrations.

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1. Introduction

Plant growth promoting rhizobacteria (PGPR) are currently being developed for use as biofertilizers to improve agronomic productivity (Kloepper et al., 1989; Vessey, 2003; Compant et al., 2010). One of the major challenges in the development of commercial biofertilizers is assuring consistent survival rates of the inoculum, particularly non-spore forming bacteria. Carrier materials can influence inoculum success by providing protective pore spaces and also by modifying the soil structure, perhaps making it more conducive for microbial colonization (Van Elsas et al., 1992). Peat moss has served well as a carrier for rhizobium and often alternative carriers are assayed in comparison with peat (Smith, 1992; Albareda et al., 2008). Vermiculite is another well-studied carrier and has been used for several decades (Thompson, 1980; Sangeetha, 2012). Nonetheless, the use of peat and vermiculite is limited by the expense of mining the materials and by lack of availability of the materials in regions where they are not naturally present. Hence, sustainable, widely-available materials are desirable as alternative inoculum carriers.

One of the most appealing new materials that could function as an inoculum carrier is biochar, which is being advocated as a soil amendment for mitigating climate change and improving soil fertility (Woolf et al., 2010). When used as a soil amendment,

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biochar has been shown to condition soils, effectively decreasing the bulk density and improving aggregate formation, soil water holding capacity, and nutrient retention (Lehmann et al., 2003; Chan et al., 2008; Karhu et al., 2011; Major et al., 2012). Meta-analyses of publications containing biochar field trials and greenhouse studies reveal that biochar application resulted in average increased above ground biomass ranging from a conservative 10% (Jeffery et al., 2011) to 30% (Biederman and Harpole, 2013). To date, the economic costs associated with biochar production, transportation, and application are major factors that limit its widespread use (Brown et al., 2011; Mukherjee and Lal, 2014). If biochar is used as an inoculum carrier, this can potentially facilitate the development of many different biotechnology products for agriculture, including biofertilizers using PGPR and plant-disease suppressive bacteria or soil inoculants that can be used for remediation of contaminated soils.

While biochar can be produced from many different feedstocks, the physical and chemical properties of biochar will vary depending on the type of feedstock and the pyrolysis process used to produce the material (Novak et al., 2009; Enders et al., 2012; Kloss et al., 2012). Pyrolysis temperature and time are both important variables in determining the properties of the final product. Pyrolysis temperature refers to the highest treatment temperature (HTT) achieved during the pyrolysis process and can range between 200 and 1000 °C (Sohi et al., 2010). Additionally, different biochars show divergent effects on soil microbial activity, transport, and diversity, likely due to indirect changes to the soil's chemical properties (Steinbeiss et al., 2009; Abit et al., 2012; Muhammad et al., 2014). While not yet well investigated, both the feedstock and HTT will likely affect the suitability of different biochars as carrier materials.

When evaluating new materials as carriers for PGPR, the ability to support high population densities of the inoculant after incorporation into soil is a primary criterion. The carrier also should not detrimentally affect the activity of the introduced bacteria, for example by adsorbing signal compounds, antibiotics, and plant growth hormones that are excreted by the cells. Many PGPR have the capacity to produce exogenous plant growth hormones, an activity which has been correlated to increased total root length, branching, and root hair formation (Patten and Glick, 2002; Spaepen et al., 2008). Here, we specifically assess an enzyme that is involved in the production of the auxin compound, indole-3-acetic acid (IAA). *Enterobacter cloacae* UW5 serves as a well-studied strain for IAA production by the indole pyruvate pathway (Patten and Glick, 2002). Indole-3-pyruvate-decarboxylase (IpdC) is an enzyme essential for IAA generation via this pathway and the expression of the *ipdC* gene is induced by tryptophan (Trp) (Spaepen et al., 2007; Ryu and Patten, 2008). Another important PGPR trait is the ability of some microorganisms to produce 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase. Plants generate high levels of ethylene under conditions of abiotic stress, which can accumulate in the rhizosphere and in turn inhibit root elongation, thereby reducing water and nutrient uptake and plant yields. Many soil bacteria can produce ACC deaminase, which degrades ACC, the precursor to ethylene (Blaha et al., 2006). PGPR with ACC deaminase activity have been shown to improve plant growth during flooding and drought conditions and in soils affected by salinity or heavy metals (Glick et al., 2007). Bacterial ACC deaminase has been best studied in *Pseudomonas putida* UW4, and the expression of the gene encoding this enzyme is positively regulated by the ACC compound (Cheng et al., 2008). The activities of each of these enzymes were determined to be essential to plant growth promotion by the given PGPR strains (Li et al., 2000; Patten and Glick, 2002). Hence, any interference of biochar with the expression of the enzyme genes could result in loss of benefits associated with these microorganisms.

2. Materials and methods

2.1. Carrier materials

Sunshine® sphagnum peat moss was purchased from Fisons Horticulture Inc. (Ontario, Canada) and vermiculite was manufactured by Therm-O-Rock West, Inc (Chandler, AZ, USA). All biochars materials were prepared via slow pyrolysis in a 2128 cm^3 steel cylinder within a 42 × 19 × 14 cm^3 muffle furnace fitted with an inlet for N_2 gas (flow rate 0.5 LPM) and were left at the highest treatment temperature for 2–2.5 h (300 °C) or 1–1.5 h (600 °C). The raw feedstocks used in this study were generated as waste products. Feedstocks included palm fronds (yard waste from Riverside, CA), pine wood (Lowes, Riverside, CA), coconut shells (Coconut King, Hobe Sound, FL), pistachio nut shells (Fiddyment Farms, Roseville, CA), and stone fruit pits (Wawona Frozen Foods, Clovis, CA).

2.2. Biochar characterization

Carbon and nitrogen analysis was performed on a FlashEA 1112 Elemental Analyzer (Thermo Electron). Permanganate oxidizable carbon was determined using the method described by Weil et al. (2003). Biochar pH and electrical conductivity (EC) were determined using previously described methods (Thompson et al., 2001; Rajkovich et al., 2012). Briefly, 1 g of biochar was suspended in 20 ml deionized water and shaken at 180 rpm for 1.5 h. The pH was measured using an Accumet® basic AB15 pH meter and electrical conductivity (EC) readings were determined using an Accumet® model 20 pH/conductivity meter (Fisher Scientific). Biochar surface hydrophobicity was determined for dry, fresh biochar sieved through a 0.5 mm mesh using the molarity-ethanol-drop (MED) test (Doerr, 1998; Kinney et al., 2012). MED values from 1 to 2 indicate hydrophilic samples, whereas values of 3–4 are slightly to moderately hydrophobic, and 5–7 is indicative of extremely hydrophobic materials.

As recommended by the International Biochar Initiative ("IBI Certification Program Manual: Requirements and Procedures for Biochar Certification," 2013), specific surface areas were determined using the Brunauer, Emmett, and Teller (BET) N_2 method on an ASAP 2020 Physisorption Analyzer (Micromeritics) as outlined in the Active Standard ASTM D6556 (D24 Committee, 2010). The % water holding capacity (WHC) for the carriers were determined after the materials were saturated in water for 24 h, then allowed to air dry for 1 h. Values for %WHC were calculated using the mass of water retained in the material per g dry material × 100. The physical structure and surface pore opening diameters for the 300 °C biochars were visualized using a Hitachi TM 1000 tabletop environmental scanning electron microscope (ESEM). Pore opening diameters were measured using TM-1000 software (Hitachi High-Technologies Corporation, Tokyo, Japan).

2.3. Bacterial strains, culture conditions, and transformation

E. cloacae UW5 was generously provided by Dr. Cheryl Patten (University of New Brunswick, Canada). *P. putida* UW4 was kindly donated by Dr. Bernard Glick (University of Waterloo, Canada). Microbial cultures were grown at 30 °C, on an orbital shaker at 170 r min^{-1}, in Luria–Bertani (LB) medium (Difco), unless otherwise specified. Electrocompetent UW5 cells were prepared using methods described by Conte et al. (2013). The UW5 cells were transformed with a rhizosphere stable plasmid, pSMC21, carrying a bright mutant of green fluorescent protein (GFP) (Hale et al., 2014). Expression of GFP, plasmid stability, and normal growth were verified previously (Hale et al., 2014).

2.4. Survival study

An Arlington sandy loam, collected from a field with previous agricultural history from the University of California, Riverside (Riverside, CA), was passed through a 4 mm sieve and used for all treatments. To prepare the liquid inoculum, UW5-pSMC21 cultures were grown overnight to late log phase in LB + kanamycin. Cultures were washed twice with sterile 0.85% NaCl using 30 min centrifuge steps at 4000, 4 °C. Washed cell pellets were brought to ½ initial culture volume with sterile 0.85% NaCl. This constituted the liquid inoculum, which had a final cell density of $5.6 \times 10^9 \pm 0.3$ CFU ml^{-1} that was used for all treatments. Twenty milliliters of liquid inoculum were shaken at 25 °C for 24 h with 2 g of carrier material in 125 ml flasks. Treatments were prepared by thoroughly mixing inoculated carriers with 20 g soil or by mixing 20 ml liquid inoculum directly into soil, providing a final carrier application rate of 1% (w/w). Four replicate microcosms were prepared from each treatment soil in 200 ml plastic cups with drainage holes and foam tops to allow water and air flow. DNA was extracted from each replicate after the initial inoculation. Microcosms were weighed daily and watered with DI to maintain microcosms at 60% field capacity. After four weeks, a second round of DNA extractions were performed for all replicate microcosms. This DNA served as template for qPCR of GFP genes.

2.5. Expression study

The effect of biochar on the expression of *ipdC* in *E. cloacae* UW5 or *acdS* in *P. putida* UW4 was determined in broth cultures using Dworkin and Foster (DF) minimal salts media, prepared with $(NH_4)_2SO_4$ nitrogen source as described by Penrose and Glick (2003). For this, 25 ml DF broth cultures were added to 125 ml flasks to which 0%, 2%, or 5% (w/v) Pine600 biochar was added. Non-induced cultures were used to inoculate inducing cultures. To induce the expression of *ipdC*, 0.5 μM filter-sterilized Trp was added to each UW5 culture. Cultures of UW4 were amended with 3 μM filter sterilized ACC to induce *acdS*. Cultures were shaken at 170 rpm at 30 °C, after which RNA was extracted from cell pellets after 6 h of growth. RNA extracted from non-induced cultures, lacking Trp or ACC, served as negative controls. For each treatment, 4 RNA extractions were obtained from 4 individual flask cultures.

2.6. DNA and RNA extractions

DNA was extracted from 0.25 g of soil using the PowerSoil® DNA isolation kit from MoBio Laboratories (Carlsbad, CA, USA) with the following modifications to increase yield and purity; 200 μl of bead solution was removed from each tube and replaced with 25:24:1 phenol:chloroform:isoamyl alcohol, pH 7–8. Initial cell lysis was performed using a FastPrep® FP120 cell disrupter, speed setting 5 for 45 s (Qbiogene, Carlsbad, CA, USA). Two additional wash steps were included using solution C4 and 100% ethanol, and final, 60 μl DNA elutions were treated with 1 μl 10 μg ml^{-1} RNase. Total RNA extractions were obtained from bacterial cultures using the Aurum™ Total RNA Mini Kit and were subsequently treated with the iScript™ cDNA synthesis kit, following manufacturer's instructions (Bio-Rad Laboratories, Inc., Hercules, CA). All extractions were tested for purity and concentration using a NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, DE, USA).

2.7. Quantitative PCR

All qPCR protocols and data analysis were performed within the standards outlined by the MIQE guidelines (Bustin et al., 2009). Reactions were set up using the SsoAdvanced universal SYBR® Green Supermix and were run on a MyiQ® Thermal Cycler (BioRad Laboratories, Hercules, CA). All primers were designed using Beacon Designer 7.8 (PREMIER Biosoft International, Palo Alto, CA). For the survival study, primers targeted an 89 bp region of mutGFP. Their sequences were; GFP1.F (5′- GAAGATGGAAGCGTTCAA) and GFP1.R (5′- AGGTAATGGTTGTCTGGTA). The cycle conditions for qPCR detection of GFP included a 15 min initial denaturing step, 40 cycles using 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 15 s. For expression analysis of ACC deaminase, primers were designed to target an 87 bp region of the *acdS* gene in UW4, which was previously sequenced and functionally verified (Accession number: AY823987). They were AcdS.F (5′- CTACGAGGGCAAATCCAT) and AcdS. R (5′- AGGTGGGCATAAAGAACT). The *ipdC* gene in UW5 has been previously sequenced and verified (Accession number: AF285632.2) (Patten and Glick, 2002). The following primers were used to target a 76 bp region of the *ipdC* gene sequence, IpdcF (5′- AATGAACCAGGCGATTGA) and IpdcR (5′- GGATGATGACGACATAAGG). Both the *acdS* and *ipdC* target oglios were amplified using a protocol which used a 15 min initial denaturing step and 30 cycles of 94 °C for 15 s, 57 °C for 30 s, and 72 °C for 15 s. Melt curve analyses were performed after all qPCR runs using the following conditions; 55 °C–95 °C with 0.5 °C temperature increases with 30 sec dwell times. To ensure that residual DNA did not affect expression analysis, RNA templates served as negative controls for all RNA samples. No amplification was observed for RNA samples without reverse transcriptase treatment. Amplicons obtained from PCR products derived from UW5 (*ipdC*) or UW4 (*acdS*) chromosomal DNA or pSMC21 plasmid (GFP) DNA were ligated into pGEM T vectors using the kit protocol (Promega Coorporation, Madison, WI). These plasmids were isolated and used to develop DNA standard curves, which included triplicate reactions and 4 plasmid-based standards, diluted 10 fold, ranging from 10^4 to 10^7 gene copies ul^{-1}, respectively. All qPCR reactions involving sample DNA or control DNA or RNA templates were prepared in duplicate.

2.8. Statistical analysis

SigmaPlot 11.0 was used to generate plots and to perform all ANOVA and pairwise multiple comparison procedures (Systat Software, San Jose, CA, USA). Principle components, Gaussian peak, and linear regression analyses were performed using JMP 11 (SAS Institute, Cary, NC, USA). Peat and vermiculate treatments were excluded from analyses that focused on identifying biochar characteristics related to differences in cell survival after introduction into non-sterile soil.

3. Results

3.1. Survival study

Fig. 1 depicts the results of the qPCR strategy to determine inoculum cell density based on GFP copy numbers immediately after inoculation and after a 4-week incubation in non-sterile soils. The Pine300 biochar consistently demonstrated detrimental effects on cell density while the peat showed positive effects, but these differences were not statistically significant (Kruskal–Wallis ANOVA on Ranks, $P < 0.05$). When Pine600 was used as a carrier, significantly greater UW5 populations were detected in soil after 4 weeks as compared to direct soil inoculation with liquid inoculum (Student–Newman–Keuls Method, $P < 0.05$). With respect to week 4 population densities, Pine600 performed as well as peat, as these values were not significantly different (Tukey Test, $P < 0.05$). There were significantly greater week 4 population densities when Pine600 was used as a carrier as compared to all other biochar

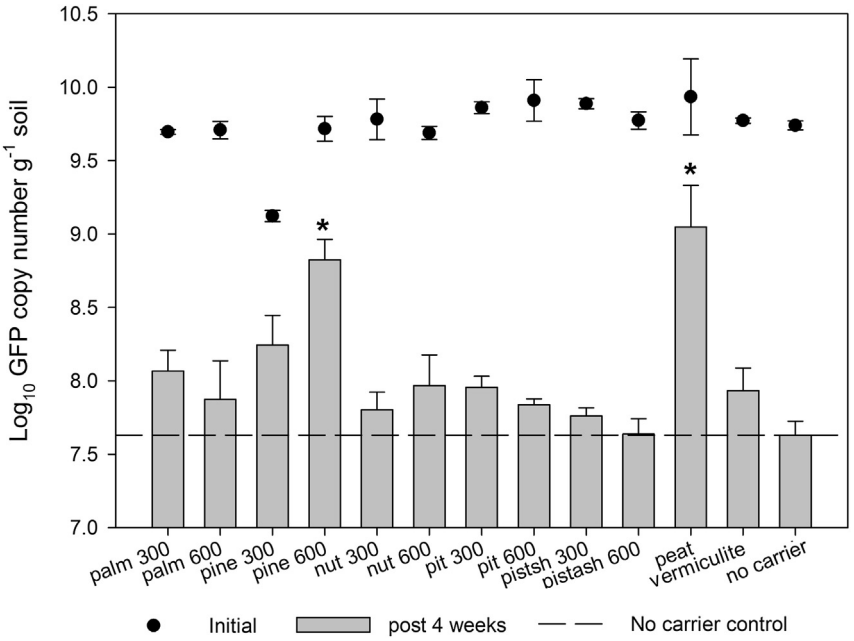

Fig. 1. Data derived from soil survival studies based on Log_{10} GFP copy number g^{-1} soil. Initial GFP copy number means and standard errors (determined from soil DNA extractions taken immediately after soil inoculation) are depicted with dots. The mean GFP copy numbers and standard errors determined from the same soils after the 4 week incubation are depicted by bars. Values were obtained from the means of duplicate qPCR reactions prepared with DNA templates from 4 replicate microcosms. Asterisks reflect treatments in which week 4 GFP copy numbers are significantly different than that of the no-carrier control (Student–Newman–Keuls Method, $P < 0.05$).

carriers and vermiculite (Tukey Test, $P < 0.05$). All other biochar materials sustained population densities at levels similar to the vermiculite and none showed reduced population densities as compared to the soil-only control (Tukey Test and Student-Newman-Keuls Method, $P < 0.05$) (Fig. 1).

3.2. Biochar physico-chemical characteristics relating to cell survival

The 10 biochars assessed here showed a high level of variability in all of physical and chemical parameter tested (Tables 1 and 2). The biochar pH values and SSAs were not significantly different with respect to feedstock (ANOVA, $P < 0.05$), but there were significant differences in pH and SSA values for biochars made at the two pyrolysis temperatures (Mann–Whitney Rank Sum Test, $P < 0.001$). Whereas the different feedstocks had significantly different % WHC's (ANOVA, $P < 0.05$), there was not a significant difference in %WHC between biochars made at the same HTT's (Mann–Whitney Rank Sum Test, $P < 0.05$). The % WHC and hydrophobicity of the biochars did not correlate (linear regression analysis).

There was no significant difference in cell densities based on GFP copy numbers immediately after inoculation (ANOVA, $P < 0.001$). However, we did see a consistent trend that Pine300 had a negative impact and peat had a positive impact on bacterial population density during the inoculation procedure. Different characteristics were correlated to inoculum population densities post inoculation and after the 4-week incubation period. Linear regression analyses and Gaussian peak models were used to assess the fit of the GFP copy number data to all biochar characteristics tested. Table 3 reflects those properties that were significantly correlated ($R^2 > 0.30$). Of the biochar physico-chemical characteristics tested here, pH, C:N ratio, and %N, correlated with initial inoculum density. In contrast, at the end of the 4-week incubation in soil, biochar C:N ratio, SSA, % WHC, and pore-opening diameters were significantly correlated with GFP copy number (Table 3). Inoculum survival after 4 weeks in soil was related to biochar feedstock, but not the pyrolysis temperature (2-way ANOVA,

Table 1
Chemical characteristics of biochars and traditional carriers.

Material	%C	%N	C:N ratio	Oxidizable C[a] (mg kg^{-1})	pH[a]	EC[a] (dS m-1)	Hydrophobicity (MED index)
Palm300	59.22	1.33	45	740 ± 12	6.47 ± 0.08	1.33 ± 0.07	4
Palm600	64.32	0.67	96	409 ± 71	10.13 ± 0.01	2.36 ± 0.1	1
Pine300	71.90	0.25	285	459 ± 5	4.63 ± 0.05	0.07 ± 0.01	1
Pine600	72.35	0.21	350	83 ± 23	6.75 ± 0.10	0.06 ± 0.01	4
Nut300	60.60	0.35	174	150 ± 10	6.88 ± 0.14	0.72 ± 0.12	1
Nut600	71.27	0.66	108	142 ± 12	9.85 ± 0.02	1.60 ± 0.0 9	1
Pit300	78.94	3.02	26	69 ± 27	7.39 ± 0.01	0.17 ± 0.01	6
Pit600	72.41	1.96	37	194 ± 3	8.26 ± 0.13	0.78 ± 0.12	1
Shell300	69.63	1.79	39	200 ± 9	6.11 ± 0.04	0.31 ± 0.07	1
Shell600	79.77	0.62	128	250 ± 5	9.06 ± 0.44	1.01 ± 0.15	1
Peat	41.21	1.24	33	1019 ± 16	4.27 ± 0.02	0.54 ± 0.01	7
Vermiculite	N/A[b]	N/A	N/A	N/A	8.19 ± 0.32	0.04 ± 0.01	1

[a] Values reflect mean ± standard error of the mean.
[b] Not Applicable (N/A).

Table 2
Physical properties of biochars and traditional carriers.

Material	BET surface area[a] ($m^2\ g^{-1}$)	% WHC (w/w)	Pore opening diameter[a] (μm)
Palm300	3.1 ± 0.3	258 ± 40	25 ± 13
Palm600	196.4 ± 16.3	333 ± 11	ND[b]
Pine300	9.6 ± 0.8	154 ± 40	32 ± 6
Pine600	112.9 ± 10.1	80 ± 9	ND
Nut300	0.04 ± 0.02	25 ± 7	30 ± 7
Nut600	59.5 ± 5.44	33 ± 3	ND
Pit300	0.02 ± 0.004	20 ± 4	62 ± 50
Pit600	221.5 ± 18.4	37 ± 10	ND
Shell300	1.7 ± 0.3	25 ± 4	2 ± 1
Shell600	261.6 ± 20.9	50 ± 2	ND
Peat	ND	602 ± 79	ND
Vermiculite	ND	144 ± 20	ND

[a] Values reflect mean ± standard error of the mean.
[b] No Data (ND).

$P < 0.001$). The pinewood feedstock had a significant impact on the survival outcome (Holm-Sidak method for pair-wise multiple comparison, $P < 0.05$). A principal component analysis on correlations was applied to the data for all biochar chemical and physical characteristics and the Log_{10} GFP copy numbers (survival) (Fig. 2). The results reflected that week 4 survival and C:N ratio had similar effects on variance within the dataset.

3.3. Expression study

When used as template for qPCR, cDNA from UW5 cultures grown in the absence of tryptophan produced small quantities of *ipdc* transcripts, which were negligible in comparison to the transcript copies present in the induced cultures. The cDNA from non-induced UW4 cells, grown with no ACC, resulted in no amplification of *acdS* during qPCR. For all RNA samples, templates with no reverse transcriptase treatment served as negative controls and resulted in no qPCR amplification. Fig. 3 displays the results of the RT-qPCR assays for gene expression in cultures with and without biochar. Statistics were performed on Log_2 transcripts of *acdS* or *ipdC* per ng RNA extracted. There was no significant difference in transcript numbers between cultures with 0%, 2%, or 5% biochar for either gene assayed (ANOVA, $P < 0.05$).

4. Discussion

All of the biochar materials tested here were shown to be useful as inoculum carriers for the PGPR strain *E. cloacae* UW5, but also varied in their efficacy. This appeared to be based on differences in the chemical and physical properties of the individual biochars. Among the different materials, Pine600 was identified as the best biochar for use as an inoculum carrier. It performed as well as the standard carrier, peat moss, and its use resulted in higher sustained cell densities than did vermiculite. All biochars tested performed as well as vermiculite and none demonstrated detrimental effects on the UW5 population. Peat moss supported the highest cell density, measured by GFP copy number, in samples analyzed after inoculation and also promoted the best survival after 4 weeks in non-sterile soil. This was associated with high availability of labile carbon and high nitrogen content of the peat. To identify specific characteristics that related to the survival outcomes, the biochars were assessed based on several chemical and physical parameters. The degree of variability in the biochars made from different feedstocks and at high and low pyrolysis temperatures is consistent with previously reported findings (Uchimiya et al., 2011; Mukome et al., 2013). The pyrolysis temperature had the greatest effects on pH and SSA, whereas feedstock type largely determined the % WHC of the individual biochars.

Table 3
Biochar characteristics that relate to inoculum population density.

	Correlation (R^2)	Model peak	95% range
Post inoculation			
pH	Gaussian (0.837)	8.08	7.9–8.3
C:N	Negative, linear (0.37)	N/A[a]	N/A
%N	Positive, linear (0.29)	N/A	N/A
Post 4-week soil incubation			
C:N	Positive, linear (0.59)	N/A	N/A
SSA	Gaussian (0.61)	112 $m^2\ g^{-1}$	94–129 $m^2\ g^{-1}$
Pore opening diameter	Gaussian (0.46)	36 μm	26–46 μm
%WHC	Gaussian (0.35)	184%	138–230%

Significant correlations between GFP copy numbers in DNA extracted from soils treated with inoculated biochars and characteristics tested.
[a] Not applicable (N/A).

Biochar pH had the greatest effect on initial GFP copy numbers, which were determined immediately after soil inoculation and reflected the direct effect of the carrier on the inoculum during preparation. The population density was fit to pH via a Gaussian distribution, which identified an optimal pH range for use of biochar as an inoculum carrier for the test strain. After inoculation, the Pine300 biochar, which had a pH of 4.63, the lowest of the biochars, also supported the lowest starting cell density. However, after 4 weeks in soil, the Pine300 biochar supported cell densities that were similar or improved as compared to those supported by the other biochars and vermiculite. Also, when cell densities were compared after the 4 week incubation, there was no correlation with the biochar pH. Hence, while the pH may have been initially influential, after application to the soil, this effect was no longer detected.

Although not tested here, biochar products, particularly those prepared at low pyrolysis temperatures, commonly contain a large number of adsorbed volatile organic compounds (VOCs) that may affect microbial growth (Spokas et al., 2011). The Pine300 may have had VOC residues, which reduced the initial UW5 populations, but after an incubation period, they may have been degraded and had reduced toxicity.

Other variables associated with higher initial population densities were related to nitrogen in the biochar, lower C:N ratios and higher N contents. Saranya et al. (2011) also observed a positive influence of N when testing the shelf life of *Azospriliium lipoferum* soil inoculants with various biochars. However, in the present study, there was no relationship between biochar N contents and cell densities after the 4-week duration in soil. Survival of the introduced strain after 4 weeks in non-sterile soil was strongly correlated with the C:N ratios of the different biochar materials. Soil C:N ratios can influence soil microbial community composition and in particular have shown positive correlations with total phospholipid fatty acids (PLFA'S) (Högberg et al., 2007). In agreement with this finding, another recent study demonstrated a positive relationship between the C:N ratio of biochar amended soils and soil total PLFA's and bacterial PLFA's, in particular (Muhammad et al., 2014). However, Jindo et al. (2012) report a negative correlation between C:N ratio and bacterial biomass in biochar-compost mixtures. Altogether these findings indicate that biochar application will influence soil C:N ratios, and that C:N ratio will have an important effect on soil bacteria, but that this effect may be inconsistent across different soil types.

We also noted that the top performing carriers, Pine600 and peat, were moderately to strongly hydrophobic when tested as a dry materials, yet they have high % WHCs. The hydrophobicity was assayed on dry materials, but % WHC values were obtained after 24 h of saturation. Hence, the hydrophobicity of the dry biochar

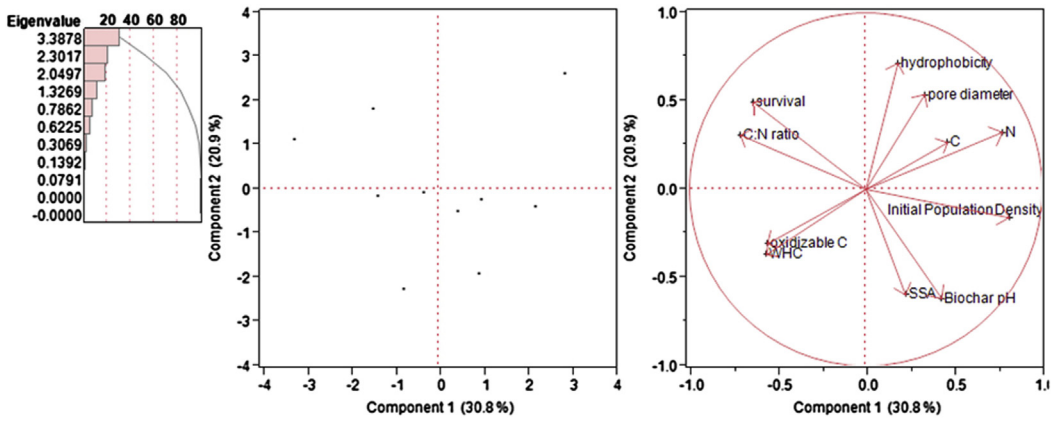

Fig. 2. Principal components analysis of biochar physio-chemical characteristics and survival.

does not appear to be a key concern when evaluating the utility of biochar as an inoculum carrier. This also indicates the importance of sufficient inoculation periods to ensure infiltration of the material if using liquid inoculum.

Several other parameters were related to week 4 survival when fit to Gaussian models. In particular, biochars having SSA's, pore opening diameters, and %WHC's in the mid-ranges maintained greater UW5 population sizes. These physical characteristics depend on the surface structure of the biochar materials. Two of the biochars, Pit600 and Shell600, had the highest SSA's but did not result in improved inoculum survival. Previous research demonstrated that biochars prepared from the same feedstocks had increasing microporosity and SSA's with increasing HTT's (Downie et al., 2009; Yu et al., 2010; Sun et al., 2012). These materials may have a large volume of nano–micropores, which are not accessible to bacteria and thus do not reflect the functional capacity of the material as an inoculum carrier. In fact, macroporosity often makes up only a small portion of the total surface area on biochar particles (Downie et al., 2009; Hardie et al., 2014). The pore opening diameters will determine which fauna are excluded from the biochar interior pore space and whether they are accessible to bacterial inoculants. Here we only visualized the pore openings of the 300 °C biochars, based on the assumption that the higher HTT's will have a significant effect on micro– nanoporosity, which was measured by SSA, not the macropores visualized during ESEM. The materials closely resembled that of the feedstock at a cellular level, as has been reported previously (Keiluweit et al., 2010; Sun et al., 2012). The biochars with pore-opening diameters between 26 and 46 μm were ideal. Pores in this size range could play a significant role in protecting pre-established colonies from predation. Overall, pretreatment of chars can change some of their chemical properties but, unless blocked, pore openings are not easily distorted. Thus, the physical properties and surface features of a potential feedstock should be an important consideration when selecting a biochar-based carrier.

Many beneficial microbial traits involve enzymatic activity, which has been shown to be increased or decreased in the presence of biochar (Jin, 2010; Bailey et al., 2011). Enzymes and their substrates could adsorb to char surfaces or be regulated by signaling molecules that interact with biochar. In a recent study that has generated concern over this phenomenon, biochar had an effect on plant gene regulation (Viger et al., 2014) and was shown to interfere with microbial signaling (Masiello et al., 2013). In the present study, we did not see a biochar-induced change in the expression of genes involved in IAA production or the ACC deaminase structural gene (*ipdC* or *acdS*). It appears that precursor compounds, such as tryptophan, were not irreversibly adsorbed to the biochars and that bacterial gene expression was not affected by the presence of 2 or 5% biochar. This addresses the concern that biochar may interfere with PGPR activities and lower the efficacy of beneficial soil inoculants.

5. Conclusions

Altogether, the chemical properties of biochar, particularly nitrogen content and pH were identified as important characteristics to consider when focusing on initial inoculum density and shelf life. However, these factors can be fine-tuned during inoculum preparation if using a buffered inoculum medium supplemented with nitrogen, unlike the conditions in this study in which the inoculum were suspended in non-buffered, sterile saline solution. Also, once introduced into the soil, biochar pH and N content no longer had

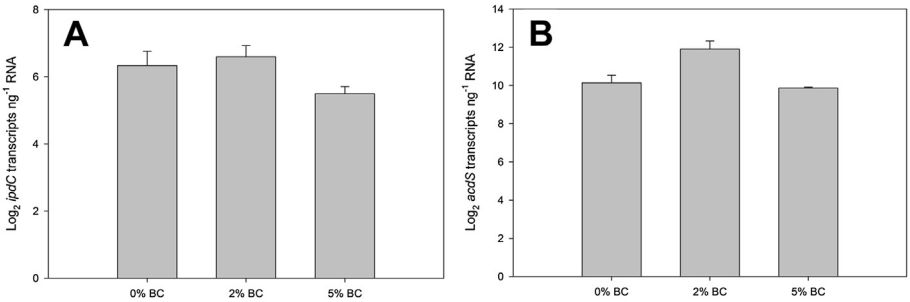

Fig. 3. Log_2 transcript ng^{-1} RNA of gene *ipdC* or *acdS* obtained from RT-qPCR. Values reflect means and SEMS from cDNA templates obtained from 4 replicate cultures of strain UW5 (*ipdC*) or UW4 (*acdS*) induced in the presence of 0%, 2%, or 5% (w/v) Pine600. There were no significant differences in gene expression among the treatments (ANOVA, $P < 0.05$).

the same effects. When incorporated into soil, characteristics related to the surface structure such as, surface area, pore openings, and water filled pore spaces, were the most important for determining inoculum survival. The C:N content of the biochar was also correlated to week 4 cell densities, which is a common association observed between soil properties and microbial populations.

Results of this study showed that many different types of biochars can potentially be used as alternative inoculum carriers to peat and vermiculite. Peat moss has nutrients available for supporting growth and survival of the inoculum, and this offers an addition variable affecting survival beyond physical protection. Yet, even with little-to-no nutritional benefit the Pine600 performed as well as the peat. If the Pine600 biochar were treated with a compost extract or supplemental nutrients, the product could possibly outperform peat moss as a carrier material. Future studies should continue to investigate biochars, particularly those produced from woody feedstocks generated a pyrolysis temperatures that ensure low-no VOC contents, and the use of compost-biochar mixtures for development of new carriers for soil inoculation.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2014.11.023.

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