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Biochar and Plant Growth Promoting Rhizobacteria as Soil Amendments

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Environmental Sciences

by

Lauren Elizabeth Hale

December 2014

Dissertation Committee:
Dr. David E. Crowley, Chairperson
Dr. Sharon L. Walker
Dr. Marylyn V. Yates

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The Dissertation of Lauren Elizabeth Hale is approved:

Committee Chairperson

University of California, Riverside

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groups. Also, Dr. Yanbin Guo offered me the first opportunity to organize and disseminate research through our collaborations. Dr. Guo also provided invaluable guidance on working with molecular biology.

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ABSTRACT OF THE DISSERATION

Biochar and Plant Growth Promoting Rhizobacteria as Soil Amendments

by

Lauren Elizabeth Hale

Doctor of Philosophy, Graduate Program in Environmental Sciences
University of California, Riverside, December 2014
Dr. David E. Crowley, Chairperson

The notion of introducing biochar into soil for carbon sequestration provides the impetus for studying these materials as inoculum carriers. For this goal I assessed the survival of plant growth promoting rhizobacteria (PGPR) introduced into natural soils after pre-inoculation onto biochar materials. First, molecular and culture-based methods, which involved the use of a green fluorescent protein (GFP) marker, were developed to track the introduced bacterial strain. The key findings of a case study indicated that cucumber root and shoot development were improved with biochar treatment but were unresponsive to treatment with auxin-producing strain, *Enterobacter cloacae* UW. Also, UW5 survival slightly increased when biochar was used as a carrier. This indicated that there are positive benefits of using biochar as a carrier, but did not provide an optimal formulation of biochar and PGPR.

Next, 10 types of biochar were characterized and assessed as inoculum carriers. Survival of UW5 was determined by enumeration of the GFP marker by quantitative

PCR (qPCR) and was statistically related to biochar characteristics. From this study it became apparent that biochar chemical characteristics, such as C:N ratio and pH, have a strong influence on inoculum density post inoculation. However, after 4-week incubations in natural soils characteristics, like specific surface area, mean pore-opening diameter, and water holding capacity, had greater impacts on inoculum survival. Biochar made from pinewood at 600°C had a significantly greater outcome on inoculum survival compared to others and was equivalent to traditionally-used carrier, peat moss.

Lastly, the influence of biochar on PGPR activity was assessed. Specifically, I assayed mineral phosphate solubilization, auxin production, and an enzyme, 1-amino-cyclopropane-1-carboxylate (ACC) deaminase, which reduces rhizosphere ethylene concentrations that could alternatively trigger plant stunting responses. Soils from collaborative projects were used to source strains and mixed consortia to examine the effects of biochar on native soil communities. The influence of biochar on the expression of an enzyme involved in auxin production and ACC deaminase was evaluated using spectrophotometric assays, promoter-reporter systems, and reverse transcriptase qPCR. Biochar had no negative impact on PGPR activity in most cases.

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ABBREVIATIONS

ACC	1-aminocyclopropane-1-carboxylic acid
ACC deaminase	1-aminocyclopropane-1-carboxylate deaminase
AcdR	1-aminocyclopropane-1-carboxylase deaminase regulatory protein
AcdS	1-aminocyclopropane-1-carboxylase deaminase structural protein
acdS	1-aminocyclopropane-1-carboxylase deaminase structural gene
CEC	Cation exchange capacity
E. cloacae UW5	*Enterobacter cloacae* UW5
E. coli DH5 α	*Escherichia coli* DH5α
ESEM	Environmental Scanning Electron Microscopy
GFP	Green fluorescent protein
HTT	Highest treatment temperature
IAA	Indole-3-acetic acid
IDP	Indole-3-pyruvate
IpdC	Indole-3-pyruvate decarboxylase
ipdC	Indole-3-pyruvate decarboxylase structural gene
MPS	Mineral phosphate solubilizing
P. putida UW4	*Pseudomonas putida* UW4
PGPR	Plant growth promoting rhizobacteria
P_i	Inorganic phosphorus
PLFA	Phospholipid fatty acid
qPCR	Quantitative polymerase chain reaction
RFP	Red fluorescent protein
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
SAM	S-adenosyl-L-methionine
TyrR	Tyrosine transcriptional repressor
WHC	Water holding capacity

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 PLANT GROWTH PROMOTING RHIZOBACTERIA

1.1.1 Brief history in agriculture

The current projection for the world population to reach 9 billion by 2050 strikes an alarm when considering global food security. This drives a need, even greater than already present, to ensure sustainable and resilient agricultural systems with maximal crop production. For decades, countless studies have exemplified the benefits of using plant growth promoting rhizobacteria (PGPR) as soil inocula to improve agronomic productivity (Compant *et al.*, 2010; Kloepper *et al.*, 1989; Vessey, 2003). Various strains of PGPR are able to fix nitrogen, solubilize phosphorus, sequester iron, suppress stress ethylene production by roots, produce plant growth hormones, antibiotics and anti-fungal compounds, and to enhance competitive exclusion of plant pathogens. The first recorded commercialization of PGPR was the use of rhizobia in 1895 (Herrmann and Lesueur, 2013). Since then, over a dozen different genera have been introduced to soils or seeds and many are sold commercially (Bakker *et al.*, 2010). In fact, Popular Science named Bio-Soil™, a cocktail of over 300 species of PGPR developed at Michigan State University, as one of the top 10 solutions for the future of farming (Rosner, 2009).

PGPR can be categorized based on their beneficial traits. For example; a nitrogen fixer would be termed a biofertilizer, an organism emitting plant growth hormones is a phytostimulator, and ones secreting antibiotics and antifungal compounds are bio-control agents. While PGPR offer a multitude of benefits for healthy plant growth and development, this body of work focuses on mineral phosphate solubilizing (MPS)

bacteria and phytostimulators. Root colonization by phytostimulators has been shown to enhance root development, resulting in greater total root surface area and enhanced nutrient and water absorption. In turn, phytostimulators play the greatest role in increasing crop yields in stressed agriculture, as plant-hormone interference can induce plant systemic tolerance to drought, flooding, salinity, and heavy metals (Vessey, 2003; Yang *et al.*, 2009).

1.1.2 Mineral phosphorus solubilization

Phosphorus (P) is a macronutrient essential for plant growth and development and current agricultural practices have become reliant on the application of P fertilizers, especially phosphate rock. Consequently, current reservoirs of phosphate rock are expected to become depleted in 50– 100 years (Cordell *et al.*, 2009). Inorganic P (P_i) accounts for approximately 35– 70% of total soil P (Jones and Oburger, 2011). Plants are able to uptake P_i in its soluble forms (*i.e.* HPO_4^{2-}, $H_2PO_4^-$). In soils with neutral to slightly acidic pH's an abundant amount of P_i is available in these soluble compounds, but unfortunately this can result in the loss of P from plant root zones via leaching. Also, P_i forms highly insoluble mineral complexes with Ca in alkaline soils and with Al and Fe in strongly acidic soils, such that it has maximum availability to plants only at near neutral pH (Jones and Oburger, 2011). These issues present a challenge as approximately 75% of P added to soil via fertilizers becomes non-plant available (Rodríguez and Fraga, 1999).

To release mineral-bound P_i some bacteria have MPS capabilities, typically achieved by the release of organic anions and correspondingly free protons (Jones and Oburger, 2011). This lowers the pH of the soil solution and can release P from Ca, Al, and Fe complexes (Rodríguez and Fraga, 1999). Hence, MPS bacteria can function as biological fertilizers, releasing mineral-bound P_i currently present in soils and reducing the need for additional P fertilization.

1.1.3 ACC deaminase

Plant growth promoting microorganisms can affect root development by generating growth hormones or depleting hormones that would otherwise cause stunted root development. Ethylene, a gaseous phytohormone, is critical for many plant developmental stages. While an initial peak in ethylene concentration in plant tissues is essential for root and stem growth and flower and fruit development, its continued accumulation triggers a cascade of responses including inhibition of root elongation, induction of hypertrophies, acceleration of aging, promotion of senescence and abscission, and inhibition of auxin transport (Glick *et al.,* 2007; Govindasamy *et al.*, 2011). Ethylene can also inhibit stimulation of cell proliferation and elongation by repressing auxin response factor synthesis (Dugardeyn and Van Der Straeten, 2008). The ethylene-mediated stress response can be activated by many environmental factors such as heavy metal contamination, high salinity, flooding, drought, and phytopathogens.

The pathway to ethylene biosynthesis in plants involves the conversion of methionine into S-adenosyl-L-methionine (SAM) by an enzyme SAM synthetase followed by the hydrolysis of SAM to form 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase. In the next step, plant produced ACC oxidases bind to ACC with high affinity, with K_m values range from about 50– 120 µM, and catalyze the conversion of ACC to ethylene, carbon dioxide and hydrogen cyanide (Glick *et al.*, 1998). Conversely, if ACC is exuded from root tissues, root colonizing bacteria can uptake the ACC and use it as a nitrogen source for growth. Microorganisms with ACC deaminase cleave the propane ring of ACC to produce ammonia and α-ketobutyrate, both of which are then metabolized by bacteria (Figure 1.1; Glick *et al.*, 2007). However, ACC deaminase enzymes have low binding affinities for ACC, with K_m values ranging from 1.5– 17.5 mM (Glick *et al.*, 2007). Thus, it has been suggested that ACC deaminase must be available at 100– 1000 fold greater amounts than ACC oxidase to reduce the accumulation of deleterious ethylene levels in plant tissues (Glick *et al.*, 1998).

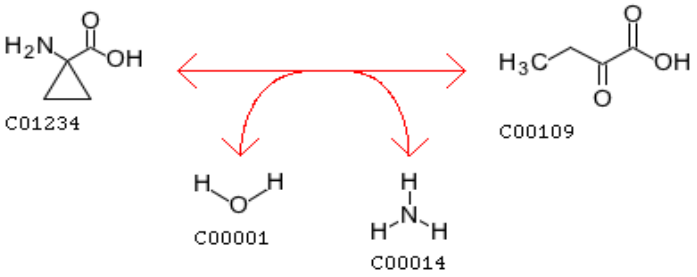


Figure 1.1 Depiction of ACC deaminase activity, NH_3 is cleaved from ACC yielding α-ketobutyrate and ammonia (adapted from Glick *et al.*, 2007).

The genetic regulation of the ACC deaminase structural gene, *acdS*, has been well described for *Pseudomonas putida* UW4, detailing an intricate regulation promoted by an ACC-bound complex and inhibited by a protein transcribed upstream of *acdS*, termed the ACC deaminase regulatory protein, AcdR. The intergenic space between these genes has been identified as the *P. putida* UW4 promoter region for AcdS, where binding of both regulatory complexes occurs (Cheng *et al.*, 2008).

1.1.4 Plant growth hormone production

Phytostimulating bacteria have been shown to generate exogenous plant growth hormones in the rhizosphere, namely; auxins, cytokinins, and gibberellins (Vessey, 2003). In particular, bacterial strains that generate an auxin compound, indole-3-acetic acid (IAA), stimulate total root length, root hair formation, and root branching when colonizing plant rhizospheres (Barbieri and Galli, 1993; Spaepen *et al.*, 2008). When applied as a seed coat, *Enterobacter cloacae* UW5 promoted increased root branching and total root length in both mung bean and canola in growth pouch assays (Patten and Glick, 2002). This activity was explicitly linked to IAA production by UW5 (Patten and Glick, 2002). Many soil bacteria have the ability to synthesize IAA through a diverse set of biosynthesis pathways (Figure 1.2).

The indole-3-pyruvate (IDP) pathway is considered to be the major pathway for IAA synthesis in plants and is also utilized by many bacteria (Spaepen *et al.*, 2007). The genetic regulation of a key enzyme, indole-3-pyruvate decarboxylase (IpdC), involved in this pathway has been well described. Strain UW5 has been shown to have a pathway to IAA production in which the genetic regulation of *ipdC* is induced by tryptophan (Ryu and Patten, 2008). The promoter region of the IpdC gene has a tyrosine transcriptional repressor (TyrR) recognition box (Ryu and Patten, 2008). The transcription of *ipdC* is promoted by the binding of the TyrR box by tryptophan, phenylalanine, and tyrosine, all of which are plant root exudates (Ryu, 2012).

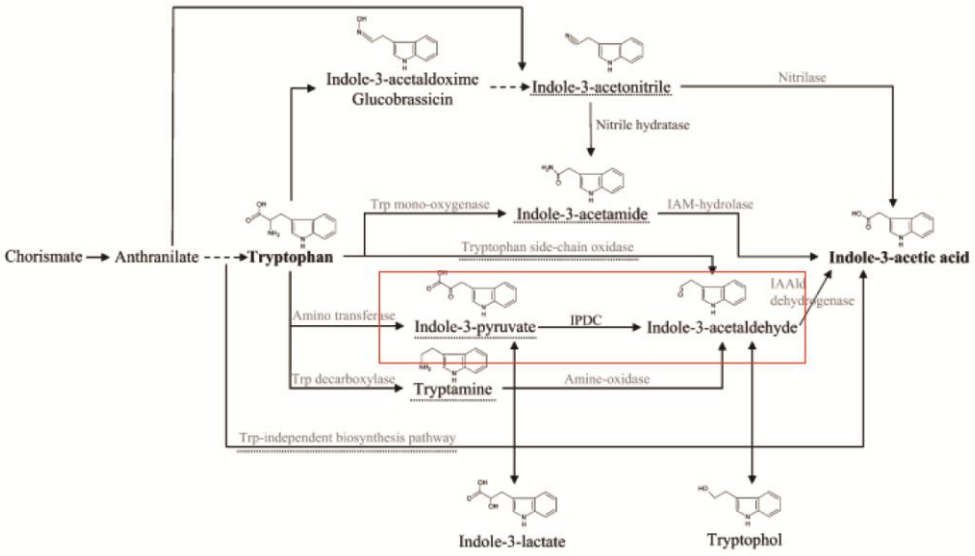


Figure 1.2 : Overview of the various bacterial pathways for IAA production. The names of the pathways are underlined by a dashed line. The indole-3-puruvate (IDP) pathway, involving indole-3-pyruvate decarboxylase (IpdC) enzymatic activity, is shown in red box (adapted from Spaepen *et al.*, 2007).

1.1.5 Limitations

The development of commercial biofertilizers is difficult as a result of poor or inconsistent survival rates of soil inocula, making quality assessment and verification of any given product very difficult (Acea *et al.*, 1988; Okon and Labandera-Gonzalez, 1994). For example, Acea *et al.* (1988) effectively demonstrated that declines in population densities of soil inocula, such as *Pseudomonas, Rhizobium*, and *Bacillus* spps. corresponded to increases in populations of bacterial predators and bacterial competitors (Figure 1.3). Additionally, direct incorporation of liquid inoculum into soils is complicated by bacterial adhesion to soil particles, which greatly reduces their vertical transport and the ability to colonize roots located in the subsurface soil profile (Huysman and Verstraete, 1993). Often, PGPR must be repeatedly added to a soil to maintain high population densities. One of the greatest research gaps limiting this field is inconsistent results when cultures are taken from lab to field. To better manage the ecosystem function of PGPR it is critical to study the survival and activity of both inoculated and native soil microorganisms in non-sterile soil.

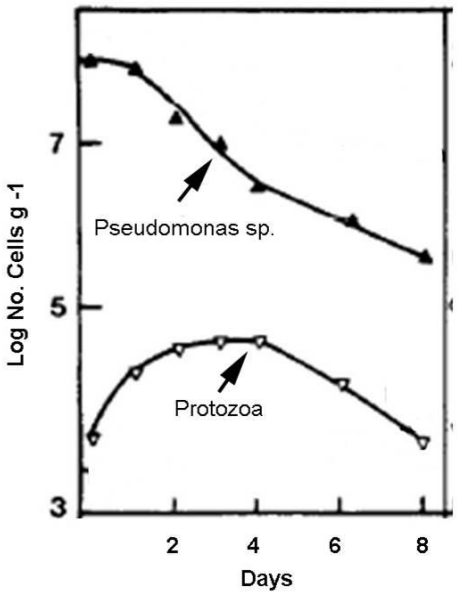


Figure 1.3 Inoculum population density decline in days following soil inoculation is coupled with rising population of protozoa (Modified from Acea, *et al.* (1998)).

1.2 CARRIERS

1.2.1 Commonly used carriers

Carrier materials can influence inoculum success by providing protective habitats and also by affecting soil aggregate formation, which provide protection from predation (Van Elsas *et al.*, 1992). Soil inoculants are commonly prepared in formulations of powders, granules, and liquids (Herrmann and Lesueur, 2013; Xavier *et al.*, 2004). Previous research has evaluated the use of carrier materials to improve survival and distribution of inocula, much of which was conducted for soil inoculation with rhizobia (Albareda *et al.*, 2008). Peat moss is commonly used a microbial carrier and its benefits increase markedly if sterilized prior to inoculation (Catroux *et al.*, 2001; Hume and Blair, 1992). Vermiculite, lignite, and sodium alginate encapsulation have all been studied as alternative carriers to peat. Also, charcoal maintains high ($>10^9$ cells g^{-1} soil) populations of inoculum suitable for use after 280 days of storage (Beck, 1991). While this information has existed for years, very little work has been continued to examine the suitability of charcoal as a carrier for microorganisms.

1.2.2 Economics and sustainability of obtaining carrier materials

There is a variety of well-studied carrier materials, all of which have limitations that restrict their widespread use. For example, peat has had success in the rhizobia industry, but it is a highly variable material and the extraction of peat from bogs is

unsustainable (Herrmann and Lesueur, 2013; Schoebitz *et al.*, 2013). The process of obtaining vermiculite also requires mining, which is associated with negative environmental impacts. As a result of limited sources, both peat and vermiculite are sometimes unavailable in regions where they are not naturally present (Bashan *et al.*, 2002; Herrmann and Lesueur, 2013). Alginate encapsulation of inoculum preparations offers a promising alternative to liquid inoculum, clay, and peat formulations. However, production of alginate beads is more expensive than the alternatives (Herrmann and Lesueur, 2013). Although the technology behind bioencapsulation has been reported since the 1980's, the current devices that produce the beads are still predominantly limited to lab-scale (Bashan, 1986; Schoebitz *et al.*, 2013)

1.2.3 Employment status of current carriers

Although countless studies have been performed on plant responses to soil inoculants, far less research has focused on inoculum preparation. A literature survey by Xavier *et al.* (2004) found that less than 0.5% of publications that cover rhizobia research discuss inoculum formulation. Many factors will differentially influence the survival of inoculum when applied to soils with diverse physical and chemical characteristics. For example, several abiotic soil parameters affected the survival of *Azospirillum brasilense* in bulk soil, including soil texture, water holding capacity, soil nitrogen, and organic matter (Bashan *et al.*, 1995). Accordingly, for PGPR to be considered a successful means to address major agricultural challenges, several things must be considered. Carrier

materials must be affordable, sustainable, and widely available. Also, the formulation of the inoculum must ensure that high PGPR populations can be stored and distributed into soils, and that once applied to soil, the PGPR can thrive, colonize roots, and commence plant-beneficial activities.

1.3 BIOCHAR

1.3.1 History

Although biochar is a relatively new term to the scientific community, it is rooted in an ancient tradition of native Amazonians. Their method for disposing organic wastes involved heating it in deep earth pits, under low levels of oxygen. This left behind carbon rich "Terra Preta", or black earth, which is still stable hundreds of years later and has proven to be an excellent soil amendment (Lehmann, 2006). Terra Preta soils contain high concentrations of nitrogen, phosphorus, potassium, calcium, and stable organic matter (Glaser *et al.*, 2001). These highly fertile pockets of soil are a stark contrast to the acidic indigenous soils, which are low in nutrients and organic matter and considered to be incapable of supporting agriculture (Glaser *et al.*, 2001). Interestingly, similar soil patches can be found throughout the world, and their high fertility is consistently associated with an abundance of black carbon (Lehmann and Joseph, 2009). The use of charcoal also has a long history in agriculture and has been used to promote agronomic productivity for centuries (Ogawa and Okimori, 2010). This tradition can be modernized by way of pyrolysis machinery specially engineered to heat solid or liquid biomass at designated temperatures in zero to low levels of oxygen. The resulting product has been termed biochar and biochar materials are receiving a lot of attention from scientists, engineers, farmers, and entrepreneurs (Maddox, 2013)

1.3.2 Carbon sequestration

Given the current annual increase in atmospheric carbon dioxide of 4.1×10^9 tons year^{-1}, reduction of anthropogenic greenhouse gas emissions is critical (Kuzyakov *et al.*, 2009; Steinbeiss *et al.*, 2009). Products of pyrolysis are carbonaceous and recalcitrant and can be incorporated into soils thereby serving as a stable carbon sink and climate change mitigation strategy (Woolf *et al.*, 2010). Biochars have been estimated to have mean residence times in soils of temperate climates of about 2000 years whereas fresh organic matter may be degraded in less than a decade (Kuzyakov *et al.*, 2009; Lehmann *et al.*, 2006). Pyrolyzing waste materials typically sequesters 50% of the source carbon as compared to traditional slash-and-burn techniques, which sequester only 3%, and natural decomposition, which retains 10– 15% (Lehmann *et al.*, 2006). Biochar production has been credited as a tool that could offset 12% of anthropogenic carbon dioxide carbon equivalents annually if implemented on a global scale (Woolf *et al.*, 2010). Therefore, biochar production from all bio-wastes offers a sustainable mechanism for land and waste management while providing a carbon negative system.

1.3.3 Physico-chemical characteristics

Physico-chemical properties of biochar account for some of its agricultural benefits. The process of pyrolysis generates porous, charred particles that structurally resemble the parent material but have carboxylated aromatic cores with slight negative charge (Glaser *et al.*, 2001). During pyrolysis a lack of oxygen available to the system

results in a residual material rich in carbon. As pyrolysis temperatures are increased the resulting biochars become increasingly aromatic as oxygen-bound functional groups escape. NMR- based diagrams of slow and fast pyrolysis chars prepared with pyrolysis temperatures of 500°C, and gasification produced char generated at 750°C, display typical aromatic clusters that comprise biochar materials (Figure 1.4; Brewer *et al.*, 2009).

Chen *et al.* (2008) used elemental analysis and FTIR to examine the sorption behavior of eight pine needle biochars produced at pyrolysis temperatures ranging from 100°C to 700°C. Pyrolysis temperatures up to 300°C mark the initial removal of OH, aliphatic C-O, and ester C=O groups from outer surfaces of such structures. At 400 ° C there is complete destruction of aliphatic alkyl and ester C=O groups that shield the aromatic core. At temperatures above 500 °C, there is further removal of aromatic CO- and phenolic –OH groups. The removal of these outer groups and exposure of the aromatic core is a key determinant of the sorption behavior and cation exchange capacity of the biochar (Chen *et al.*, 2008). In general, for lignocellulosic materials, carbonization increases as combustion temperatures rise to 500°C and the materials approach full carbonization as temperatures reach 1000°C (Allen-King *et al.*, 2002).

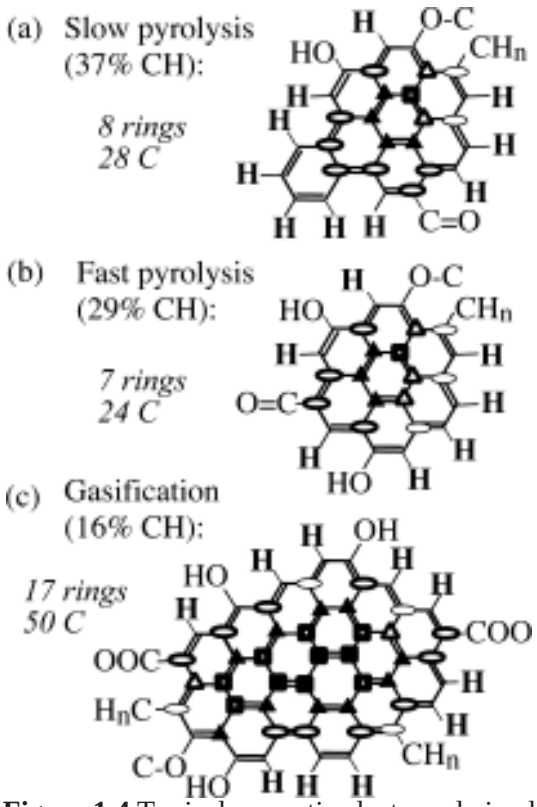


Figure 1.4 Typical aromatic clusters, derived from NMR, in (a) slow pyrolysis char, (b) fast pyrolysis char, and (c) gasification char from switchgrass. Symbols label the distance of carbons resonating between 107 and 142 ppm from the nearest proton(s). Thin-line ellipse: Two bonds from multiple H. Thick-line ellipse: Two bonds from one H. Open triangle: Three bonds from multiple H. Filled triangle: Three bonds from one H. Open square: Four bonds from multiple H. Filled square: Four bonds from one H, or more than four bonds from any H. (Brewer, 2010)

The exposure of aromatic structures lends these biochars the property of having many fine pores. The abundance of nano to meso-sized pores contributes to the large surface areas associated with higher-temperature chars as well as to their enhanced ability to adsorb non-polar compounds (Chen *et al.*, 2008; Chun *et al.*, 2004; Yu *et al.*, 2006). Biochar materials often retain the cellular structure of the feedstock, which can provide and intricate network of pores on the order of tens of micrometers in diameter (Brewer *et al.*, 2014; Wildman and Derbyshire, 1991). Hence, factors such as feedstock and pyrolysis conditions, the highest treatment temperature (HTT) achieved during pyrolysis in particular, will affect the porosity, specific surface area, cation exchange capacity (CEC), and adsorptivity of the resulting biochar. This allows the opportunity to derive biochar materials with optimized properties for specific uses, such as agronomy, soil remediation, water filtration, or soil inoculum delivery.

1.3.4 Biochar in soils

The use of biochar has been recommended to improve soil fertility and has been shown to condition soils when used in agriculture (Chan *et al.*, 2008; Lehmann, 2006; Major *et al.*, 2012; Novak *et al.*, 2009; Singh *et al.*, 2010; Yuan and Xu, 2011). Direct effects of biochar on soil properties include decreased soil bulk density, general increases in soil water holding capacity (WHC), CEC, porosity, and alteration of soil pH (Chan *et al.*, 2008; Karhu *et al.*, 2011; Lehmann *et al.*, 2003; Major *et al.*, 2012). For example, after 20 t biochar ha^{-1} were incorporated into an oxisol nutrient leaching of Ca, Mg, NO_3-

N, and K were all reduced significantly as compared to unamended controls (Major, *et al.*, 2012). Also, attributable to its macroporous nature, biochar can modify soil WHC, enhancing that of sandy soils and lowering matric potential in clay soils (Sohi, *et al.*, 2010). Indirect effects of biochar amendments include improved soil aggregate stability, root growth, drainage and aeration. Additionally, biochar application results in greater soil nutrient retention and therefore improved fertilizer efficiency and reductions in and runoff and nitrous oxide emissions, thereby lowering some of the detrimental environmental impacts associated with fertilizer use.

1.3.5 Biochar influence on crop productivity

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) and a recent meta-analysis, which incorporated publications up to April, 2013 demonstrated an overall mean increase in crop productivity of approximately 11% with biochar application (Liu *et al.*, 2013). From each of these meta-analyses it becomes apparent that biochar will have different effects on plant biomass and yield that is influenced by soil and biochar characteristics, application rate, crop variety, and time post amendment. These reviews concur that biochar had the greatest positive influence on crop productivity when incorporated into acidic, clay, or sandy soils with low water

holding capacity or low organic matter (Biederman and Harpole, 2013; Jeffery *et al.*, 2011; Liu *et al.*, 2013).

1.3.6 Biochar economics

The economic cost associated with biochar production and application has become a major limiting factor to its wide-spread use (Brown *et al.*, 2011; Mukherjee and Lal, 2014). Estimates by Brown *et al.* (2011) projected that current biochar production will only be profitable if bio-oil is simultaneously generated and carbon offsets have values in the range of 20 USD or more per metric ton. Shackley *et al.* (2011) provided a total assessment of the costs and benefits associated with biochar deployment and determined that biochar production costs are best reduced when feedstocks are waste feedstocks that would otherwise have a gate fee or landfill charge associated with their disposal. The greatest economic benefits associated with biochar use were related to energy production, as agronomic cost benefits can be highly inconsistent (Shackley *et al.*, 2011). Hence, adjustment to biochar products to ensure greater agronomic benefits could transition biochar use into a profitable sector.

1.3.7 Effect on soil biota

It is important to gain a fundamental understanding of how biochar application will affect soil biota before its use can be recommended on a broad scale. When applied to soils, biochar has many effects on soil physical and chemical properties that, in turn,

affect the properties of the soil as a habitat for microbial growth. To date several exploratory studies have assessed the response of bacteria, fungi, and enzymes to biochar incorporation in a soil. In Figure 1.5, scanning electron micrographs depict clear images of fungal hyphae extending into biochar pores and bacterial cells located on char surfaces (Jin, 2010; Ogawa and Okimori, 2010). Furthermore, microscopic, chromatographic, and spectroscopic studies have shown root hairs entering water-filled macropores or bonding to biochar surfaces (Joseph *et al.*, 2010). At this interface the biochar particles can adsorb organic compounds released from growing roots. Thus, biochar pores may serve as an ideal microenvironment for biological activity. However, contrary to these findings Quilliam *et al.* (2013) report minimal colonization of biochar by native soil microorganisms 3 years post 2– 4% amendment with a wood-derived biochar.

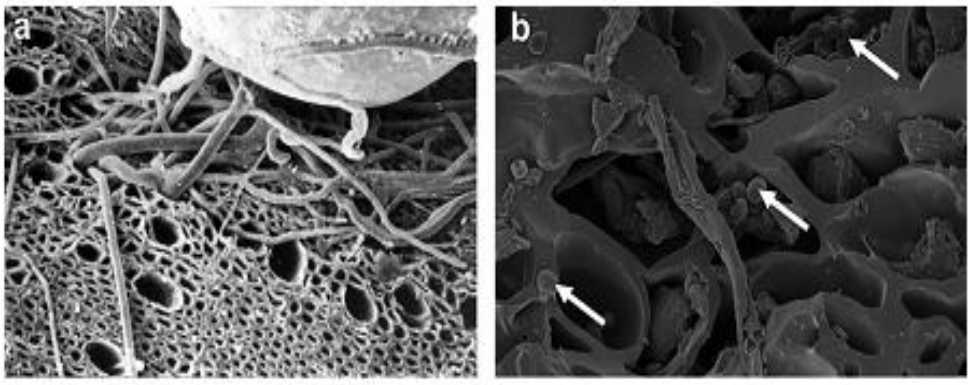


Figure 1.5: a. Scanning electron micrograph of fungal hyphae extending into biochar pores (Ogawa and Okimori, 2010) **b**. Scanning electron micrograph depicting bacteria (above arrows) on char surface (Jin, 2010). Images obtained from Lehmann, *et al.* (2011)

In examining the influence of biochar on native soil bacteria many investigations have focused on diversity profiles. In a pioneering study, Kim *et al.* (2007) compared Terra Preta soils to adjacent pristine soils and found the Terra Preta to contain 25% greater bacterial species richness and hundreds of novel bacteria taxa (Figure 1.6). Also, Kolton *et al.* (2011) found that changes in bacterial community structures were observed after soils were amended with a fresh, citrus wood-derived biochar. Pyrosequencing of 16S rRNA genetic markers revealed a decrease in the total numbers of proteobacteria when biochar was added to a soil and an increase in bacteroidetes, and particularly flavobacteria (Kolton *et al.*, 2011). These are noted chitin degraders that secrete anti-fungal compounds. Nielsen *et al.* (2014) utilized an ultra high-throughput sequencing platform to obtain 16S rRNA gene sequences of bacteria with low abundance, as low as 0.01% of the total population. Their results agreed with previous findings, that biochar applications resulted in shifts in abundances of various taxonomic groups and also indicated that taxa correlation patterns are altered with biochar application (Nielsen *et al.*, 2014).

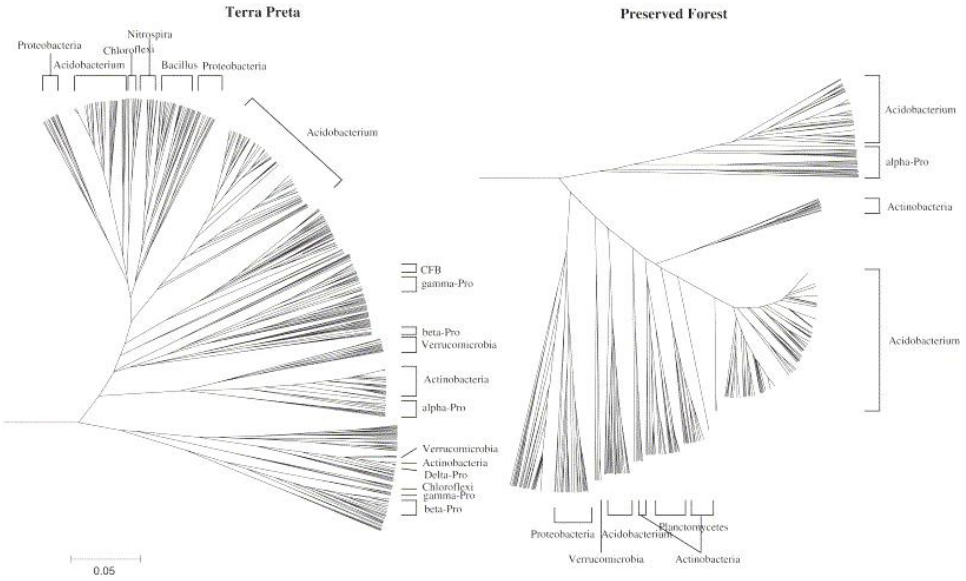


Figure 1.6: Representing overall bacterial diversity: taxonomic cluster analysis of 16S rRNA sequences from terra preta soils (left) and neighboring pristine soils (right) (Kim, et al, 2007)

A phospholipid fatty acid (PLFA) analysis by Steinbeiss *et al.* (2009) revealed that once incorporated into soils, biochar prepared from a protein rich feedstock (low C:N ratio) selected for fungi while biochar derived from a cellulose-based feedstock (high C:N ratio) selected for bacteria. In agreement with this finding, another recent study demonstrated a positive correlation 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. Additionally, 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 and plant responses to biochar (Spokas *et al.*, 2011).

Soil enzyme activities are differentially affected by biochar application (Bailey *et al.*, 2011; Jin, 2010; Wu *et al.*, 2013). Most notably enzymes with enhanced activities in alkaline conditions showed higher activity post biochar amendment (Bailey *et al.*, 2011; Jin, 2010). This is reasonable, as pH's of many biochars tend to be alkaline, a property that is dependent on pyrolysis temperature. Furthermore, Harter *et al.* (2013) used molecular techniques to assay abundance and expression of bacterial genes involved in nitrogen cycling as affected by biochar application. They found that N_2O reductase transcript numbers were increased when soils were amended with 10% (w/w) biochar (Harter *et al.*, 2013). This altered microbial activity is especially important when considering greenhouse gas emissions and reduced N_2O emissions from soil, a common phenomenon reported with biochar application (Van Zwieten *et al.*, 2010; Wang *et al.*,

2011). All together, it is clear that with biochar addition to a soil there will likely be changes in soil biological community structures and functions and that pyrolysis temperature, feedstock, and application rate will all have significant influence on these induced changes.

1.3.8 Biochar as a carrier for soil inoculants

Pre-inoculation of biochar with PGPR could add value for biochar marketing (Lehmann *et al.*, 2011) and provides a means to evenly distribute and potentially improve the survival of inoculants following their introduction into soils. Many biochars have characteristics that also are conducive for use as inoculum carriers including high internal porosity, large specific surface area, and the ability to adsorb organic compounds and bacteria (Abit *et al.*, 2012; Chen *et al.*, 2008; Chun *et al.*, 2004; Rivera-Utrilla *et al.*, 2001). As apparent in Figure 1.7, biochar materials, specifically those prepared from lignocellulosic feedstocks, are covered in pores averaging about 20 µm in diameter. Pores of these sizes are considered to be accessible to bacteria, but will likely exclude entry of bacterial predators such as nematodes and protozoa. Biochar is sterilized during pyrolysis, which can better ensure high quality preparations of carrier materials (Herrmann and Lesueur, 2013). Establishment of a plant-beneficial bacterial population on biochar also provides preemptive colonization of the biochar substrate and may help to competitively exclude colonization by plant pathogens (Cunniffe and Gilligan, 2011).

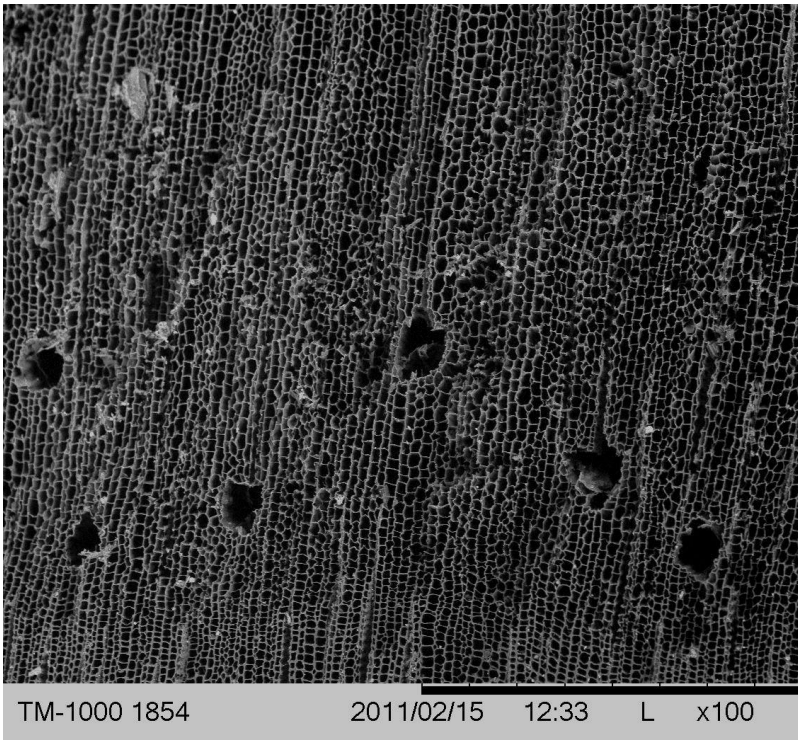


Figure 1.7. Preserved vascular structure of pinewood biochar visualized using a Hitachi Environmental Scanning Electron Microscope, 100×

The PGPR activity of a strain in the introduced soil environment is a requirement for an effective biological fertilizer. There is concern that bacterial enzymes or substrates could adsorb to char surfaces or be regulated by small molecules incorporated with char. Biochar had an effect on plant gene regulation (Viger *et al.*, 2014) and was shown to interfere with microbial signaling (Masiello *et al.*, 2013). Thus, it is essential to ensure that biochar does not interfere with plant-microbe signaling essential for root colonization and that the PGPR activity of a strain of interest is not impeded in the presence of biochar.

1.3.9 Methodology to study biochar-amended soils

Current methodology employed to study microbial presence and activity in soil is dependent upon the extraction of whole cells, enzymes, or nucleic acids from soil. For instance, many methods for analyzing soil microbial diversity and microbial activity are reliant upon on extraction of highly-pure DNA directly from soils (Dobrovol'skaya *et al.*, 2001). However, DNA is negatively charged due to the phosphate ions in its structure and could adsorb to newly-made biochar particles. As biochars weather, they develop a dominant negative charge and thus may interact with DNA in other ways such as a cation bridging type of bonding (Joseph *et al.*, 2010). Furthermore, macropores may shelter bacterial colonies during extractions and whole cells or DNA from these microorganisms will be omitted from further analysis. In previous work biochar reduced DNA extraction efficiencies from soil samples (Jin, 2010). Furthermore, components of soil organic

matter can contaminate extracted DNA with inhibitors of polymerase chain reaction (PCR)-based downstream applications. Humic acids, which are very common in soils, are renowned for interfering with reagents used in DNA extraction kits. Hindrance of extraction efficiency has also previously been demonstrated in soils with heavy clays (Kaestli *et al.*, 2007).

1.4 GOALS AND OBJECTIVES

In this work methods were optimized to study biochar-amended soils to ensure that bias was not generated from samples containing biochar, due to low-quality DNA preparations or biochar sorption of analyzed materials. The survival and activity of PGPR strains were assayed when biochar was used as a carrier. Specific biochar properties that relate to microbial colonization were sought out to better understand these complex interactions. Because biochar can be made widely-available, offers intrinsic benefits to agricultural soils, and has properties that can be fine tuned, we hypothesize that this will serve as an ideal carrier material.

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CHAPTER TWO

DEVELOPMENT OF TECHNIQUES TO ASSESS CARRIER SUTIABILITY OF BIOCHAR: A CASE STUDY USING LOW-TEMPERATURE PINEWOOD BIOCHAR

ABSTRACT

Several assays were designed to evaluate the suitability of a low-temperature, pinewood biochar as a carrier for well-studied PGPR strain, *Enterobacter cloacae* UW5. This strain was genetically modified to carry a rhizosphere-stable plasmid that contained a green fluorescent protein (GFP). The GFP functioned as an identifiable marker for tracking of the inoculum via culture-based selective plate count assays and quantitative PCR (qPCR). The GFP marker was also used to determine the efficiency of soil DNA extractions and for optimization of a soil DNA extraction protocol from the biochar-amended soils. Total 16S rRNA genes were quantified using qPCR to distinguish the impact of biochar on total bacterial abundance from its influence on inoculum survival. Plant assays provided insight into root colonization by UW5 and cucumber root growth and architecture as influenced by the PGPR and biochar.

Biochar pre-inoculated with UW5 was incorporated into soils and compared to biochar-amended and non-amended soil inoculated directly with a liquid UW5 suspension. Results of both the molecular and culture-based inoculum survival assays confirmed that cell survival was slightly improved by addition of bacteria to soil using biochar as a carrier. Total bacterial abundance was not influenced by biochar. Cucumber plants grown in the biochar-amended soils had significantly greater biomass and root development than those planted in un-amended soil, regardless of the presence of inoculum. Strain UW5 colonized cucumber roots at population densities of approximately 10^5 CFU g^{-1} root mass in all treatments. However, UW5 inoculum did not promote root development in cucumber in any of the soils tested here. Overall, these

experiments suggested that the inoculation protocol allowed for even distribution of the inoculum into soil, that the culture-based and molecular protocols both serve well to track inoculum survival, and that the biochar had a positive influence on plant development without impacting root colonization by strain UW5.

2.1 INTRODUCTION

The large-scale production of biochar for carbon sequestration provides an opportunity for using these materials as inoculum carriers to deliver plant growth-promoting rhizobacteria (PGPR) into agricultural soils. Biochar could serve as a cost effective, widely-available carrier. Previously, an *Azospirillum* biofertilizer was demonstrated to have a shelf life of at least 6 months at room temperature when carried on biochar (Saranya *et al.*, 2011). However, little is known about the outcome of the inoculum if introduced to soils. Whether biochar actually can promote survival of bacterial inoculants in soil is a topic that has not been rigorously investigated.

The gene encoding a green fluorescent protein (GFP) was originally isolated from the jellyfish, *Aequorea victoria*, and is unlikely to be present naturally in agricultural soils (Shimomura *et al.*, 1962). Hence, it serves as an excellent molecular and observable marker and has successfully been used to monitor bacteria added to non-sterile environments (Bloemberg *et al.*, 1997; Ibekwe *et al.*, 2004; Park and Crowley, 2005; Rochat *et al.*, 2010). Both molecular and culture-based methods have shortcomings when used to assay soil bacteria (Errampalli *et al.*, 1999). The combination of these approaches, using quantitative PCR (qPCR) and colony forming unit (CFU) counts to simultaneously track GFP-tagged UW5 offers a reliable picture of inoculum population densities over time. If biochar stimulates microbial growth in soils, quantitative data on the UW5 population size may reflect this consequence and not necessarily improved inoculum survival rates. The response of soil bacterial population size to biochar

amendment was also monitored using universal 16S DNA primers. An ideal carrier will not only prolong inoculum survival but also will not interfere with processes essential for microbial induced plant growth promotion. These experiments also examined differences in root colonization and assayed cucumber root and shoot development following different methods of soil inoculation. The objectives of this study were to optimize several methods that can be applied to samples of biochar-amended soils.

Here we examine the effects of a pinewood biochar that was produced with a highest treatment temperature (HTT) achieved during pyrolysis of 300 °C. Cell densities of *E. cloacae* UW5 were enumerated in soil after incorporation into soil already containing biochar or when added to soil using biochar as an inoculum carrier. Seed treatment with strain UW5 has been shown to increase total root length and lateral root branching in mung bean and canola which was correlated to exogenous production of indole-3-acetic acid (IAA) (C. L. Patten and Glick, 2002). The phytohormone, IAA, result in stimulation of plant root growth and disease resistance when produced by PGPR (Kazan and Manners, 2009; Spaepen *et al.*, 2007). Strain UW5 serves as a well-studied bacterium that has an understood, tryptophan-dependent pathway for IAA production. We hypothesized that a pinewood biochar, with large internal porosities, could provide protected habitats for inoculum, thereby reducing predation by nematodes and protozoa and prolonging survival.

2.2 MATERIALS AND METHODS

2.2.1 Soil, biochar, and planting conditions

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 experiments. Pine-wood biochar produced at a maximum pyrolysis temperature of 300 °C was provided by Alterna Biocarbon (Prince George, BC, Canada) and was applied to soil at a rate of 1% (w/w) two years after its production. This biochar can be classified as a low-temperature and was characterized according to protocols outline by the International Biochar Initiative ("IBI Certification Program Manual: Requirements and Procedures for Biochar Certification," 2013). Triplicate measurements were taken using an Accumet® basic AB15 pH meter for pH and for electrical conductivity (EC), using an Accumet® model 20 pH/conductivity meter (Fisher Scientific). The biochar was 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). To sterilize, soil was autoclaved in polyethylene bags (121°C, 30 min) and autoclaved again after 48 h to ensure sterility.

Cucumber seeds, *Cucumis sativus* cv. Spacesaver, (Ferry-Morse, Fulton, KY, USA) were uniformly germinated by heating in 45°C deionized (DI) water for 5– 10 min. The heat-treated seeds were maintained on DI water soaked filter papers in glass Petri dishes in the dark for 2– 3 d before planting. Germinated seeds were planted in 50 cm^3

plastic cones (Conetainers, Bend Oregon) for samples collected at 3 week or less, or in 1700 cm^3 pots for plants grown for more than 3 weeks. Seeds were covered with 1– 2 cm soil, and the plants were maintained in growth chambers at 25 °C, with 50% humidity, under 12 h photoperiods provided by fluorescent and incandescent light. One week after plant emergence, pots were mulched to decrease soil drying. Plants were fertilized bi-weekly with half strength complete nutrient solution (Hershey, 1994) and watered with 50 ml (pots) or 5 ml (cones) every other day in the first 3 weeks and then daily thereafter.

2.2.2 Culture conditions

Strain *Enterobacter cloacae* UW5 was kindly provided by Dr. Cheryl Patten (University of New Brunswick, Canada). This strain was previously identified as *Pseudomonas putida* and was later classified as *E. cloacae* by 16S rRNA gene sequencing (Cheryl L Patten and Glick, 2002; Ryu and Patten, 2008). Unless otherwise specified, cultures were grown at 28°C, with shaking at 170 r min^{-1}, on Luria-Bertani (LB) medium (Difco). Strain UW5 is naturally resistant to 100 µg ml^{-1} ampicillin. When used for selective purposes antibiotics were incorporated into medium at the following concentrations: ampicillin, 100 µg ml^{-1}; kanamycin, 25 µg ml^{-1}; and cycloheximide, 100 µg ml^{-1}.

2.2.3 Bacterial transformation by electroporation

UW5 cells were tagged with a bright mutant of green fluorescent protein carried on rhizosphere stable plasmid, pSMC21, a derivative of pSMC2 developed by (Bloemberg *et al.*, 1997). Electrocompetent UW5 cells were prepared using methods described by (Peloquin *et al.*, 2000). Transformation was carried out using 500 ng pSMC21 combined with 200 µl of competent UW5 cells, electroporated at 2.5 kV, 25µF, 250Ω, using a 2 mm gap cuvette in a Biorad GenePulser (Hercules, CA). SOC medium was added immediately after electroporation and cultures were incubated at 28 ℃ for 1.5 h, after which they were spread plated onto LB agar containing kanamycin. Integration of pSMC21 was verified by sequencing of PCR products amplified from the GFP gene (protocol listed in section 2.6) and by microscopic observation of GFP expressing cells. Fluorescent microscopy was performed on an Olympus IX71fluorescent microscope scope using a light excitation range 533– 583 nm, with an emission range of 607– 684 nm. UW5-pSMC21 transformants were screened for altered growth response relative to the wild type strain by growth curve analysis on nutrient rich LB medium and a carbon and nitrogen starvation response medium prepared according to (Voigt *et al.*, 2007). IAA production levels by the transformed cells were compared to those of wild type UW5 using Salkowski reagent and the S2/1 method described by (Glickmann and Dessaux, 1995). The stability of plasmid pSMC21 in strain UW5 was assayed on Voigt carbon and nitrogen starvation media. Cells were transferred daily over a 2-week period, to fresh Voigt medium without kanamycin and at three day intervals cultures were serially diluted

and spread onto plates with and without kanamycin. The percent of cells retaining the plasmids was calculated based on differences in CFU counts on these plates.

2.2.4 Inoculum preparation

To prepare the liquid inoculum used for all treatments, 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. Dilutions of this suspension were spread onto LB + kanamycin plates and CFU's were counted to determine starting inoculum concentrations for all experiments. The washed inoculum was left shaking at 25 °C for 24 h without biochar, for liquid inoculum, or in a 5:1 inoculum: biochar (v/w) mixture, to be used as inoculated biochar. Direct inoculation treatments were prepared by thoroughly mixing 50 ml liquid inoculum into 1 kg soil by hand, ensuring even wetting. Inoculated biochar treatments were prepared in a similar fashion, using a 50 ml liquid, 10 g biochar mixture for each kg soil prepared. All inocula were added to soils with or without biochar carriers at a rate between 7×10^6 and 7×10^7 CFU g^{-1}soil. Soils receiving no inoculum were treated with 0.85% NaCl.

The presence of UW5 on biochar surfaces was verified using ESEM and fluorescence microscopy. Biochar particles suspended in sterile 0.85% NaCl served as negative controls. ESEM images were obtained using a Hitachi TM 1000 tabletop microscope. To preserve microbial cells prior to imaging, biochar samples were flash-

frozen in an isopentane bath chilled with liquid nitrogen. For each treatment, 10– 15 electron micrographs were collected. GFP expressing UW5-pSMC21 cells were imaged on fine biochar pieces using fluorescence microscopy and conditions previously described.

2.2.5 Determination of inoculum survival

Five treatments were used to monitor the effect of biochar on the survival of strain UW5 (listed in Table 2.1). Each of the 5 soil treatments were prepared in triplicate microcosms consisting of 135 cm^3 open-ended, steel cylinders capped with cheesecloth and foam plugs to allow drainage and airflow. Microcosms were maintained at room temperature and were routinely watered to maintain moisture levels at 60% of field capacity. Soil samples were collected at the initial start point and again after 3 d, 7 d, and 3 week.

Table 2.1. Treatments used in plant development (PD), inoculum survival (IS), and root colonization (RC) assays

Assay	Treatment	Biochar	Inoculum	Soil	Inoculation method
PD	Non-inoculated soil	0%	No inoculum	Non-sterile	N/A
PD	Biochar-amended soil	1% (w/w)	No inoculum	Non-sterile	N/A
PD, IS, and RC	UW5-pSMC21- inoculated biochar	1% (w/w)	50 ml kg^{-1}soil	Non-sterile	Pre-inoculated biochar
PD, IS, and RC	Biochar-amended soil with UW5-pSMC21	1% (w/w)	50 ml kg^{-1}soil	Non-sterile	Direct soil inoculation
PD, IS, and RC	Soil with UW5-pSMC21	0%	50 ml kg^{-1}soil	Non-sterile	Direct soil inoculation
IS	Sterilized biochar-amended soil with UW5-pSMC21	1% (w/w)	50 ml kg^{-1}soil	Sterile	Direct soil inoculation
IS	Sterilized soil with UW5-pSMC21	0%	50 ml kg^{-1}soil	Sterile	Direct soil inoculation

2.2.6 CFU-based enumeration

For determination of UW5 population density by CFU counts, bacterial cells were extracted from soils (Heijnen and van Veen, 1991). Briefly, 10 g of soil were added to a 250 ml flask with 95 ml of sterile 0.1% sodium pyrophosphate and small marbles (to aid in dispersal). Flasks were shaken at 200 r min^{-1} for 10 min and allowed to settle 1 h. Using standard techniques, suspensions were serially diluted and spread onto LB agar plates supplemented with cycloheximide, ampicillin, and kanamycin. Plates were incubated at 28 °C for 20 h, then moved to 4 °C for 24 h to allow GFP development. Only colonies expressing green fluorescence under UV excitation were counted. At each collection point, 3 cell extracts were collected per treatment, 1 from each replicate microcosm. All extracts were spread in triplicate.

2.2.7 Molecular quantification

At the same time points DNA was extracted from 0.25 g of soil using the PowerSoil® DNA isolation kit from MO BIO Laboratories (Carlsbad, CA, USA). The provided protocol was modified to increase DNA yield and purity as suggested by MO BIO Laboratories. The modified protocol (CPI protocol) involved replacement of 200 µl of bead solution with 25:24:1 phenol:chloroform:isoamyl alcohol , pH 7– 8 (AMRESCO, Solon, OH, USA) prior to initial cell lysis. Instead of the vortex stp, initial cell lysis was performed using a FastPrep® FP120 cell disrupter, speed setting 5 m s^{-1}, for 45 s

(Qbiogene, Carlsbad, CA, USA). Upon addition of solution C4, a mixture of equal parts lysate, solution C4, and 100% ethanol are vortexed and washed through the spin column 650 µl at a time. Then, a mixture of 300 µl C4 and 370 µl 100% ethanol is used to wash each column, followed by a wash step with 500 µl 100% ethanol and a final wash step with 500 µl solution C5. The 60 µl DNA elutions were treated with 1 µl of 10 µg ml^{-1} RNase to remove RNA that is concurrently extracted using this protocol. All extractions were tested for purity and concentration using a NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, DE, USA). At each time interval, 3 DNA extractions were obtained from each soil treatment, 1 from each replicate microcosm.

2.2.8 Quantitative PCR

Quantitative PCR was performed on a MyiQ® Thermal Cycler (BioRad Laboratories, Hercules, CA) using a QuantiTect SYBR green PCR kit (Qiagen, Valencia, CA). Primer concentrations were 0.3 µM per reaction volume and 1 µl DNA extractions served as templates. DNA extractions from non-UW5-pSMC21-inoculated soils served as negative controls for GFP-based qPCR and ultra pure DI water served as negative control for 16S-targeted reactions. Primers used to target an 89 bp region of mutGFP were designed using Beacon Designer 7 and their sequences were; GFP1f (5'-GAAGATGGAAGCGTTCAA) and GFP1r (5'- AGGTAATGGTTGTCTGGTA). The cycle conditions for qPCR detection of GFP included a 15 min initial denaturing step, 40 cycles of 94 ℃ 15 s, 55℃ for 30 s, 72℃ for 15 s followed by a melt curve analysis,

55°C to 95°C with 0.5°C temperature increases over 30 s dwell times. Universal 16S rRNA gene primers and cycle conditions were taken from literature (Park and Crowley, 2005) and primer sequences were; 338f (5'-ACTCCTACGGGAGGCAGCAG) and 518r (5'-ATTACCGCGGCTGCTGG). For both primer sets, assessment of primer efficiencies, melt curve analysis, and product sequences were verified in accordance with MIQE guidelines (Bustin *et al.*, 2009). Target PCR products derived from UW5 chromosomal DNA (16S) or pSMC21 plasmid (GFP) were ligated into pGEM T vectors (Promega Coorporation, Madison, WI) which were used for DNA standards. DNA standard curves were prepared using 4, 10-fold dilutions, ranging from 2.72×10^{5} to 2.72×10^{8} (pGEM-T-UW516S) or 4.64×10^{4} to 4.64×10^{7}(pGEM-T-GFP1). All qPCR reactions were performed in triplicate.

2.2.9 Root colonization assay

Germinated cucumber seeds were planted treatments soils listed in Table 2.1. After 7 d, roots were harvested and CFU's g^{-1} root were enumerated (Xin *et al.*, 2005). Briefly, roots were gently washed with sterile DI then weighed and placed in 150 ml flasks with 1:100 (w/v) sterile 0.85% NaCl, shaken at 100 r min^{-1} for 5 min, and left to stand for 10 min. Serial dilutions of this suspension were plated onto triplicate LB plates supplemented with cycloheximide, ampicillin, and kanamycin and only CFU's which fluoresced under UV excitation were counted. There were 5 replicate plants per treatment and the experiment was performed twice.

2.2.10 Plant development assay

To assess the effects of biochar and UW5 on plant development five differing treatments were prepared (Table 2.1). Germinated cucumber seeds were planted according to conditions previously described with 5 replicates per treatment for each sampling time point. Plants were destructively harvested after 1, 3, and 5 weeks. To determine total root length and lateral root formation, roots were washed then stained with 0.1% Toludine O Blue solution for 5 min. Residual stain was removed by washing twice with DI water and shaking in DI water for 5 minutes. After staining, the roots were imaged using an Epson Expression 1680 scanner and images were processed with WinRHIZO Pro 2005a software (Régent Instruments, Quebec City, QC, CA). This program provided measurements on total root length (determined by dark pixels on scanned images) as well as total number of forks (defined as points where 3 lines intersect) for scanned root clusters. After imaging roots were oven dried at 80 °C for 24 h and weighed to determine dry weight biomass. At week 5, plant fresh and dry weights, total leaf counts, leaf widths, and plant heights were also measured.

2.2.10 Statistical analyses

Statistical analyses were performed using JMP 11 (SAS Institute, Cary, NC, USA). Quantitative PCR and CFU count data were analyzed with repeated measures ANOVAs then Tukey-Kramer HSD tests were used to compare means of qPCR experimental replicates and replicate plate CFU counts. Other data were analyzed using

one-way ANOVAs and Tukey tests or where appropriate, ANOVAs based on ranks coupled with Dunn's method. Plots and graphs and were generated with SigmaPlot 11.0 (Systat Software, San Jose, CA, US).

2.3. RESULTS

2.3.1 UW5 transformation, biochar inoculation, and DNA extraction

Plasmid pSMC21 was retained in greater than 95% of the UW5 cells after 42 generations of growth on carbon and nitrogen limited medium. No difference in the growth rate or IAA production was observed between UW5-pSCM21 and the wild type. The biochar and had a pH of 10.07 ± 0.03 and an EC of 2.16 ± 0.07 mS cm^{-1}. Biochar pores were consistent in size, cylindrical in shape, and the majority of pores that were visible at 1000 X magnification had diameters of 20 µm. Following biochar application, sieved soil bulk density decreased from 1.33 ± 0.01 g cm^{-3} to 1.10 ± 0.002 g cm^{-3}, water holding capacity increased from 0.21 ± .001 g H_2O g^{-1} dry soil to 0.25 ± .002 g H_2O g^{-1} dry soil, and pH increased from 7.46 ± 0.01 to 7.81 ± 0.05. Inoculation of biochar with UW5 was visually confirmed (Figure 2.1). ESEM micrographs of UW5-inoculated biochar depict many regions comprised of globular matrices with refraction indices that differed from biochar surfaces (Figure 2.1.a). These regions were typical of all inoculated biochar particles and were not on non-inoculated biochar surfaces (Figure 2.1.b). Additionally, igures 2.1 c-d show images indicating the presence of viable GFP-expressing cells adhering to the biochar. This provides visual confirmation of inoculation specific to live UW5-pSMC21 cells. It should be noted that the Heijnen and van Veen (1991) method, described for soil cell extractions for enumeration of UW5 CFUs, was employed using inoculated biochar only. This was an attempt to enumerate UW5 cells

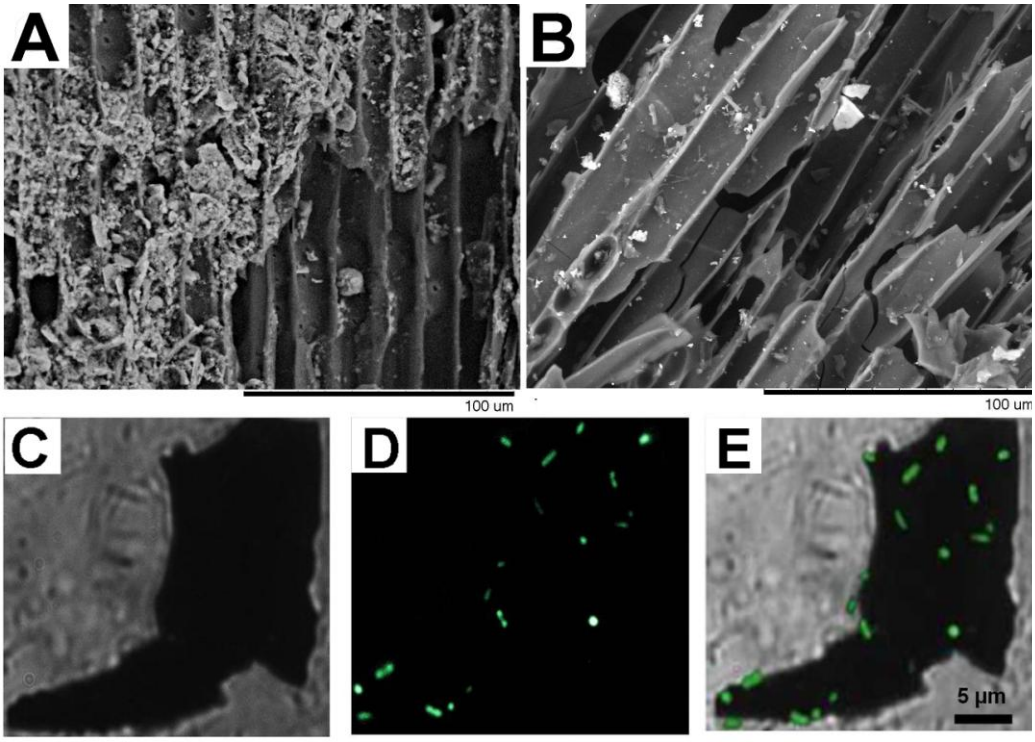


Figure 2.1. ESEM images of inoculated biochar (A) and biochar treated with 0.85% NaCl (B) and fluorescent microscopy images of UW5-psmc21 on biochar particle; bright field (C), GFP excitation (D), and an overlay of bright field and

initially adhered to the boichar. However, when applied to the biochar alone, this method never yielded reproducable results and therefore was not useful for this purpose.

The soil DNA extracts obtained using the CPI protocol were all considered to be of high purity (260/280 ratios between 1.7 and 2.0 and 260/230 ratios above 1.3) and concentrations ranged between 10 and 30 ng μl^{-1}. DNA purity and yield did not correlate with the presence or absence of biochar in the soil. This was an improvement over the PowerSoil®-provided protocol, which yielded 2– 7 ng μl^{-1} DNA with 260/280 ratios between 1.40 and 2.70 and 260/230 ratos between 0.53 and 1.85.

2.3.3 Inoculum survival

Both the culture-based and molecular methods used to monitor UW5 cell densities in non-sterile and sterile soils yielded similar results for the proportion of initial inoculum that survived after the cells were introduced into soil. Quantitative PCR efficiencies were 96-97% for the GFP primer sets and 105% for the 16S rRNA gene primer set. The R^2 values for calibration curves were all above 0.98. The GFP copy numbers determined from microcosms prepared using non-sterilized soils were not significantly different at any time point (repeated measures ANOVA, $P<0.05$, Figure 2.2a). However, at week 3 the UW5 population densities were significantly different in non-sterilized soils with and without biochar with respect to $\log_{10}$ CFU counts (repeated measures ANOVA, $P<0.05$, Figure 2.2b). Specifically, microcosms prepared with previously-inoculated biochar had significantly greater CFU counts than those in which

cells were directly added to soil (Tukey-Kramer HSD, *P<0.05*, Figure 2.2b). Regardless of the presence of biochar, UW5 inoculated into previously sterilized soils persisted at greater population densities as compared to cells added to non-sterile soils at 1 and 3 weeks after inoculation (Figure 2.2). Quantification of total bacteria present in the non-sterile soils, as measured by16S rRNA gene copy numbers, revealed that the population sizes stayed relatively constant and were not significantly affected by the presence of biochar (Figure 2.3)

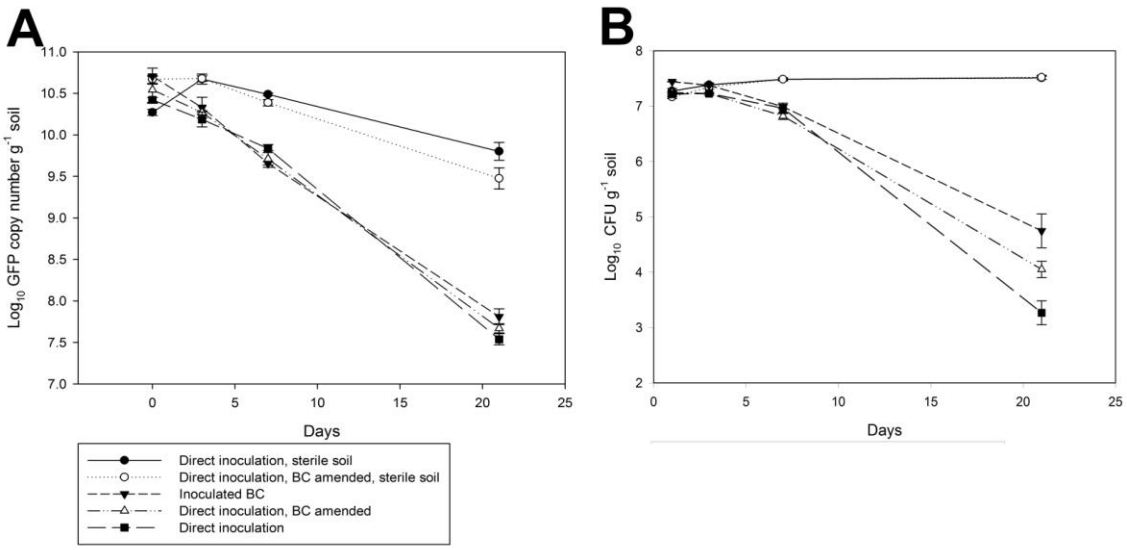


Figure 2.2. Inoculum Survival. Per gram soil GFP copy numbers of UW5-pSMC21 inoculated treatments (A) or Log_{10} CFU counts for UW5-PSMC21, GFP-expressing colonies (B). Data points are means of 3 biological x 3 experimental replicates. Error bars reflect SEM's based on biological replicates

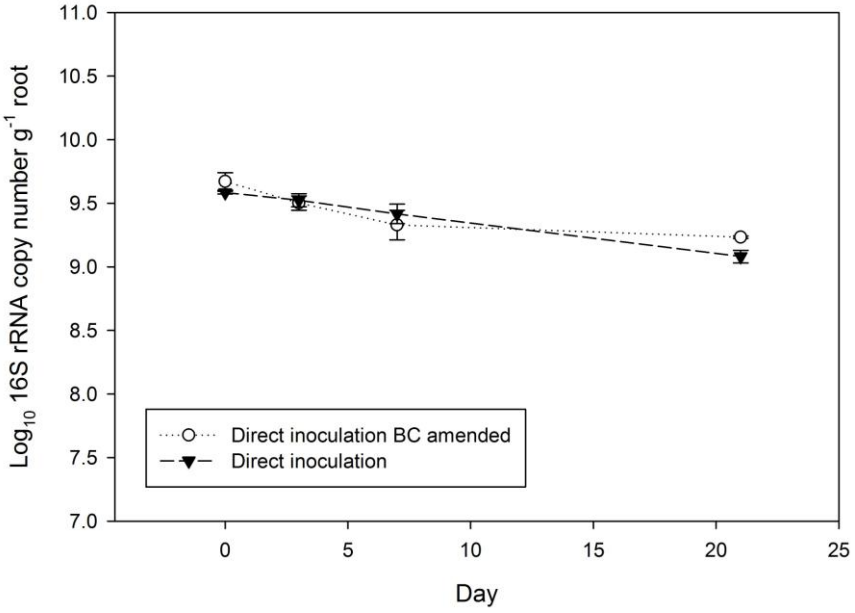


Figure 2.3. Total Bacterial Populations. Per g soil copy numbers of total 16S rRNA gene sequences in soil treatments. Data points are means of 3 biological x 3 experimental replicates. Error bars reflect SEM's based on biological replicates.

2.3.4 Root colonization and plant development

In all of the treatments, UW5 was present on the surface of 1-week-old cucumber roots at high population densities that ranged between $2.37x10^5$ –$1.44x10^6$ cfu g^{-1} root fresh wt. (Figure 2.4). Root colonization by UW5 was not significantly different in soils amended with biochar prior to inoculation or when inoculated into the soil using biochar as a carrier (ANOVA, $P<0.05$). The 1% biochar application lead to significant increases in root dry weights, total root lengths, plant heights, leaf counts, and plant dry weights (Figure 2.5) and also decreased soil bulk density by 17% and increased soil water holding capacity by 4%. The addition of UW5 as a cell suspension or on the biochar carrier did

not result in any significant increases in plant growth parameters. It should be noted that root branching was determined by dividing the number of forks output from WinRhizo by the total root length. As previously reported, these estimations can yield high values because overlapping roots can be counted as root forks (Okubara *et al.*, 2004). Hence, these values serve for internal comparison between scans of roots with similar densities. Plant root growth was not significantly different in the non-biochar-amended treatments, irrespective of inoculation with strain UW5 (Figure 2.5 a-f).

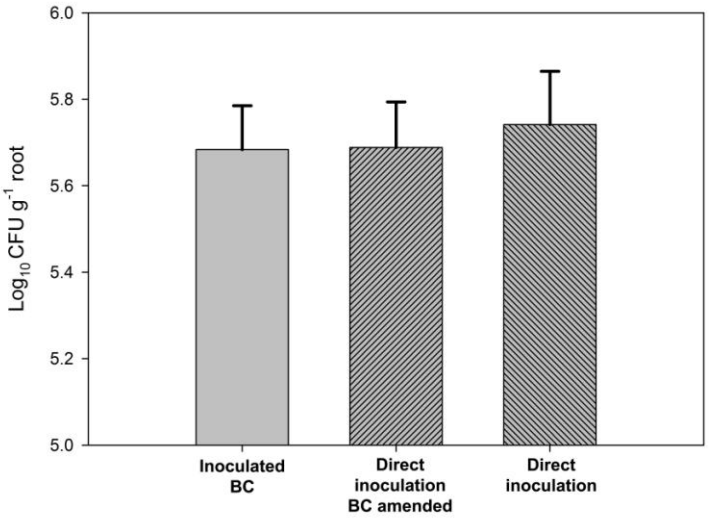


Figure 2.4. Root colonization of 1 week old cucumber roots. Log_{10} CFU of UW5 removed from fresh roots grown in soil treatments 1– 3. Bar plots represent means and SEM's of values obtained from duplicate experiments. Each experiment consisted of 5 biological and 3 experimental replicates per treatment. There was no significant difference among treatments (repeated measures *ANOVA, P<0.05*).

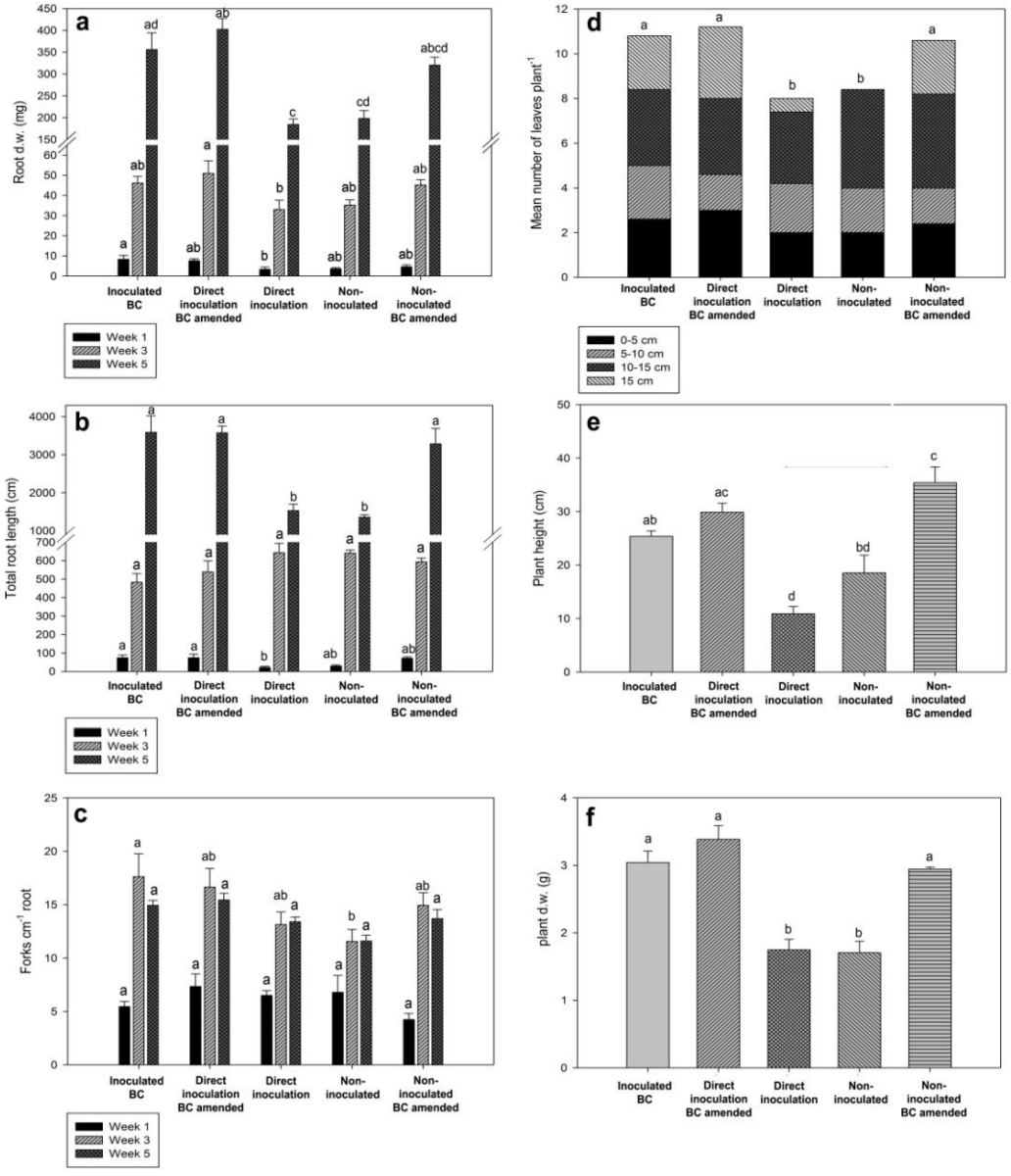


Figure 2.5. **Plant development.** Root (a-c) and plant (d-f) measurements for cucumbers planted in soil treatments. Data shown are the means and SEM's of 5 biological replicates. Different letters indicate significant differences between treatments within the same week (Tukeys, $P<0.05$).

2.4 DISCUSSION

2.4.1 Methodology development for assessing biochar as a carrier

GFP served as an excellent molecular and observable marker. Population dynamics were studied using both molecular methods and culture-based methods and the results concurred. The population size estimates determined from each method were approximately 3 orders of magnitude apart, but this was expected. The molecular method quantified total cells, including those that have diminished in vigor to become nonviable, whereas the culture-based method quantified only cells that are metabolically active and cell aggregates can be counted as a single colony. Also, there are multiple copies of GFP per UW5 cell. In fact, the pSMC21 parent plasmid, pUC18, is maintained in *Escherichia coli* at approximately 50 copies per genome (Lin-Chao *et al.*, 1992). Although biochar materials have been demonstrated to reduce soil DNA extraction efficiencies in previous research (Jin, 2010), that was not the case with our modified PowerSoil® protocol. Initial GFP copy numbers, obtained for DNA extracted immediately after UW5-pSMC21 inoculum were spiked into soils, did not differ when biochar was present (Figure 2.2). Hence, we recommend the modified DNA extraction method for use with biochar-amended soils.

2.4.2 Evaluation of inoculation protocol

The main advantages of the inoculation method described here were the non-abrasive preparation of the microorganisms and the simplicity of the bacterial loading step. Some of the starting population densities were slightly higher in inoculated biochar treatments (Figure 2.2). Possibly, bacteria applied to the char may have used residual nutrients from the biochar to increase in population. However, these increases were not significant and do not affect the ability to contrast population densities at later time points. Biochar particles observed after UW5 inoculation had regions that looked similar to biofilms observed at similar magnifications using ESEM (Figure 2.1) (Schwartz *et al.*, 2009; Zammit *et al.*, 2011). However, the dispersal of these films over the biochar was not continuous, leaving regions of char surfaces exposed. This could be beneficial if a goal of biochar amendment is to affect soil properties, such as cation exchange capacity, or to be used as a sorbent for contaminants. Additionally, the degree to which bacteria adsorb to biochar surfaces will be influenced by biochar pyrolysis temperature, feedstock, and microbial surface properties (Abit *et al.*, 2012; Rivera-Utrilla *et al.*, 2001). Many studies have demonstrated that biochar produced at temperatures near 600 °C have much higher specific surface areas and are more adsorptive than biochars made from the same feedstocks at lower pyrolysis temperatures (Chen *et al.*, 2008; Chun *et al.*, 2004; Yu *et al.*, 2006). The shelf life of the inoculum on the biochar was not tested here and would be an important parameter to assess the efficacy of this inoculation method.

2.4.5 Inoculum survival

While our original hypothesis speculated that biochar would improve the long-term survival of the soil inoculant, the results of this research do not indicate a profound impact of biochar on the fate of the inoculant, whether added to the soil prior to inoculation, or when used as a carrier to deliver the inoculum into the soil. Both culture and molecular-based methods depicted much greater survival rates of UW5 when incorporated into sterile soils. The sterile soils were presumed to have no bacterial, fungal, protozoan, or nematode populations, the presence of which would potentially decrease UW5 cell densities over time. Hence, these serve as controls for survival when competition and predation are non-issues. When introduced into non-sterile soils, UW5 cell numbers decreased 100 to 1000 fold after 3 wks, regardless of the presence or absence of biochar. Low temperature biochar products commonly contain a large number of adsorbed volatile organic compounds (VOCs) that may hinder microbial growth (Spokas *et al.*, 2011). These findings indicate that the addition of 2-yr old 300°C pinewood biochar to soil microcosms was not detrimental to inoculum population densities and provided a means to assure even distribution of the inoculum throughout the soils. The population density of viable cells was more than 10-fold greater, when the cells were introduced on the biochar carrier as compared the treatment in which the cells were directly added to unamended soil (Figure 2.2). In contrast, cell survival based on GFP copy numbers showed only a slight improvement in survival when the inoculum was introduced into the soil on the biochar carrier (Figure 2.2). In this regard, earlier research

by (Van Elsas *et al.*, 1992) suggest that alginate encapsulation may be more effective for preventing this population decline in nonsterile soils.

Biochar additions had no significant effects on the total bacterial density in non-sterile soils, as determined by quantification of total 16S rRNA genes. Hence, we ruled out the possibility that the greater UW5 population densities associated with biochar amendment were simply a factor of biochar's stimulation of total bacterial abundance (Figure 2.3). In contrast, (Chen *et al.*, 2013) used similar soil DNA extraction techniques and 16S rRNA qPCR methods to show that addition of wheat-straw biochar to soil increased the abundance of soil bacteria. These opposing findings suggest that different types of biochar may have varying amounts of residual labile carbon and will support microbial growth differently. Overall, the 300 °C pinewood biochar served as a delivery mechanism to evenly mix viable UW5 cells into soils but the degree to which its use resulted in increased inoculum survival should be greater if this material is to be recommended as an inoculum carrier over other common carrier materials. Likewise, other biochar preparations may prove to be superior to the low temperature pine pyrolysis material tested here based on internal porosity and surface area and charge properties. Other variations not evaluated here include methods for mixing and infiltrating the biochar with bacteria, and use of supplemental nutrients to cultivate the bacteria in the char following inoculation of the char particles.

2.4.6 Root colonization and plant development

Recent work demonstrated that biochar could interfere with microbial signaling and that this hindrance was different depending on biochar type (Masiello *et al.*, 2013). Here we observed that after one week UW5 root colonization was not hindered even when bacteria pre-colonized biochar surfaces (Figure 2.4). This time point indicates that UW5 can efficiently colonize plant roots during early development regardless of biochar presence. *Enterobacter cloacae* strains 501R3 and GS1 were shown to colonize cucumber and rice roots in un-amended soils at similar population densities to those observed here (Lohrke *et al.*, 2002; Shankar *et al.*, 2011).

Biochar provided a significant influence on both shoot and root development, which was likely a result of increased soil porosity and water holding capacity post biochar amendment (Figure 2.5). However, cucumber plant development was not significantly impacted by UW5 inoculation (Figure 2.5). Previously, (C. L. Patten and Glick, 2002) demonstrated significant increases in lateral root formation and total root length when UW5 colonized canola roots at a rate of 10^6 following seed treatment and development in growth pouches. Here we only observed slight increases in these parameters when seeds were germinated in the absence of UW5and root population densities were closer to 10^5. Plant response to UW5 may be more significant during germination or may require higher population densities. Although this density may be too low for UW5 induced plant growth promotion, *Rhizobium* in soils at a rates of 10^3– 10^5 CFU g^{-1} soil were shown to result in significant increases in nodulation (Nazih and Weaver, 1994; Weaver and Frederick, 1972). Hence, while the pinewood biochar–UW5

combination is not ideal, the combination of biochar with other strains of PGPR may result in more direct benefits to plant development.

In future work it will be important to evaluate different types of biochar in an effort to strike a balance between adsorptive properties and porosity to best optimize inoculum survival, without hindering plant-microbe interactions caused by sorption of hormones or signal compounds on the biochar surfaces.

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CHAPTER THREE

BIOCHAR CHARACTERISTICS RELATE TO CARRIER SUITABILITY

ABSTRACT

Biochar materials have greatly variable physical and chemical properties that 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) achieved during slow pyrolysis. Peat and vermiculite, which are traditional inoculum carriers, were included for comparison. All of the carriers were incorporated into non-sterile soil after being inoculated with a plant growth promoting rhizobacterial (PGPR) strain, *Enterobacter cloacae* UW5, carrying a green fluorescent protein (GFP) marker. Inoculum survival after 4 weeks was determined using quantitative PCR to enumerate the GFP markers. 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 in an effort to identify specific attributes that have the greatest influence on the survival of the inoculant. 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.

3.1 INTRODUCTION

Plant growth promoting rhizobacteria (PGPR) are currently being develop for use as biofertilizers to improve agronomic productivity (Compant *et al.*, 2010; Kloepper *et al.*, 1989; Vessey, 2003). One of the major challenges in the development of commercial biofertilizers is assuring consistent, high population densities 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 traditionally served as a carrier for Rhizobia and often alternative carriers are assayed in comparison with peat (Albareda *et al.*, 2008; Smith, 1992). Vermiculite is another well-studied carrier and has been assessed for several decades (Sangeetha, 2012; Thompson, 1984). 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, biochar has been shown to condition soils, effectively decreasing the bulk density and improving aggregate formation, soil water holding capacity, and nutrient retention (Chan *et al.*, 2008; Karhu *et al.*, 2011; Lehmann *et al.*, 2003; Major *et al.*, 2012). To date, the economic costs associated with biochar production, transportation, and application are

major factors that limit its wide-spread 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 biotechnology products for agriculture including PGPR, plant-disease suppressive bacteria, and microorganisms that are useful for bioremediation of contaminated soils.

While biochar can be produced from a variety of 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 (Enders *et al.*, 2012; Kloss *et al.*, 2012; Novak *et al.*, 2009). 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, various biochars show divergent effects on soil microbial activity, transport, and diversity, likely caused by indirect changes to the soil's chemical properties (Abit *et al.*, 2012; Muhammad *et al.*, 2014; Steinbeiss *et al.*, 2009). While not yet well investigated, both the feedstock and HTT will likely affect the suitability of biochars as carrier materials.

The objectives of this study were to compare biochar materials to standard carriers with respect to promoted inoculum survival. Improved survival was related to physico-chemical properties of the biochar materials. Ideal physico-chemical properties were recognized and related to either feedstock or pyrolysis temperature. Overall, specific biochar feedstocks and production methods are identified for optimizing biochar-inocula formulations.

3.2 MATERIALS AND METHODS

3.2.1 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 2,128 cm^3 steel cylinder within a $42\times19\times14$ 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 hrs (300 °C) or 1– 1.5 hrs (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).

3.2.2 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) measurements were determined using previously described methods (Rajkovich *et al.*, 2012; Thompson *et al.*, 2002). Briefly, 1 g of biochar was suspended in 20 mL deionized water and left shaking 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 of an ethanol drop (MED) test (Doerr, 1998; Kinney *et al.*, 2012). The MED values from 1– 2 indicate hydrophilic samples, 3– 4 are slightly to moderately hydrophobic, and 5-7 are strongly to extremely hydrophobic.

As recommended by the International Biochar Initiative (IBI, 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 percent water holding capacity (WHC) for the carriers were determined after the materials were saturated in water for 24 hours, then allowed to air dry for 1 hr. Values for %WHC were calculated using the mass of water retained in the material per g dry material x 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).

3.2.3 Bacterial strains, culture conditions, and transformation

Enterobacter cloacae UW5 was generously provided by Dr. Cheryl Patten (University of New Brunswick, Canada). Microbial cultures were grown at 30°C, shaking

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). UW5 cells were transformed with a rhizosphere stable plasmid, pSMC21, carrying a bright mutant of green fluorescent protein (GFP), provided here by Dr. Yanbin Guo. Transformation was carried out using 500 ng plasmid combined with100- 200 µL of competent cells, electroporated at 2.5 kV, 25µF, 250Ω, using a 2 mm gap cuvette in a Biorad GenePulser (Hercules, CA). Integration of plasmids was verified by selection on kanamycin (25 µg ml^{-1}) medium and by microscopic observation of GFP expressing cells. Fluorescent microscopy visualization was performed on an Olympus IX71fluorescent microscope scope using a light excitation range 533– 583 nm, with an emission range of 607– 684 nm. The quantity of indole compounds produced by transformed cells was compared to that of wild type UW5 using Salkowski reagent and the S2/1 method described by (Glickmann and Dessaux, 1995). The UW5-pSMC21 transformants were screened for growth inhibition using growth curve analysis on a nutrient rich LB media and a carbon and nitrogen starvation response media prepared according to (Voigt *et al.*, 2007). The stability of plasmid pSMC21 in strain UW5 was assayed over a 2 week period. Cells were transferred daily to fresh Voigt media without kanamycin and at 3 d intervals cultures were serially diluted and spread onto plates with and without kanamycin. The percent of cells retaining plasmids was calculated based on differences in CFU counts on these plates.

3.2.4 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, 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 left shaking 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 for 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 deionized water to maintain microcosms at 60% field capacity. After 4 weeks a second round of DNA extractions were performed on all replicate microcosms. The soil DNA extractions served as templates for qPCR used to quantify GFP gene copy numbers.

3.2.5 DNA extractions and quantitative PCR

DNA was extracted from 0.25 g of soil using the PowerSoil® DNA isolation kit from MoBio Laboratories (Carlsbad, CA, USA) with modifications (described in chapter 2). All extractions were tested for purity and concentration using a NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, DE, USA). All qPCR reagents, protocols, and data analysis were performed within the standards outline 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). For the survival study, GFP primers and qPCR cycle conditions and melt curve analyses were identical to those described in Chapter 2. All qPCR reactions involving sample DNA or control DNA templates were prepared in duplicate.

3.2.6 Statistical analyses

SigmaPlot 11.0 was used to generate plots and to perform all ANOVA and pair-wise 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.

3.3 RESULTS

3.3.1 Survival study

Figure 3.1 displays the 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. The Pine300 biochar consistently demonstrated detrimental effects on population 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 UW5 (Student-Newman-Keuls Method, $P<0.05$). With respect to week 4 population density 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 served as a carrier as compared to all other biochar carriers and vermiculite (Tukey Test, $P<0.05$). All other biochars had similar sustained populations to the vermiculite and none showed reduced population densities as compared to the soil-only control (Tukey Test and Student-Newman-Keuls Method, $P<0.05$)(Figure 3.1).

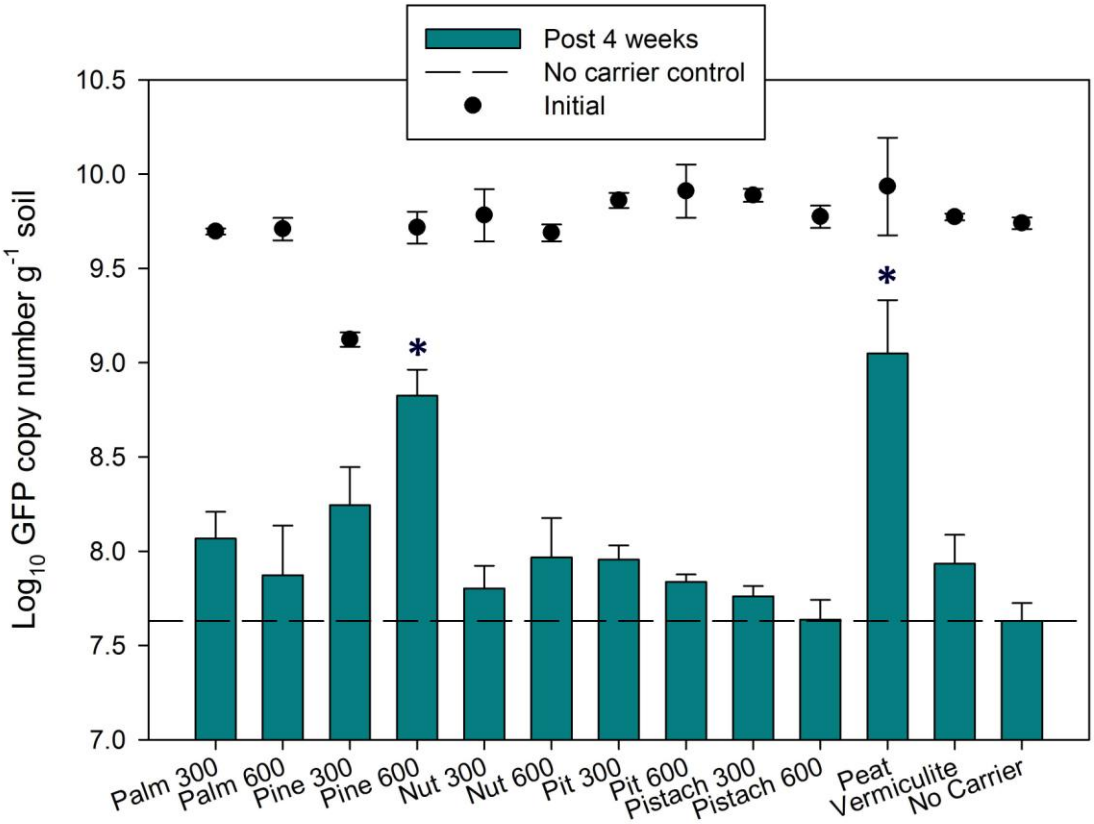


Figure 3.1. Log_{10} copy number GFP after inoculation and soil incubation using 10 types of biochar, peat, or vermiculite carriers or liquid inoculum with no carrier. Values obtained from means and SEMS of DNA extractions from 4 replicate microcosms performed initially after inoculation and after a 4-week incubation. Asterisks reflect treatments in which week 4 GFP copy numbers are significantly different than that of the no-carrier control (Student-Newman-Keuls Method, $P<0.05$).

3.3.2 Biochar physico-chemical characteristics relating to cell survival

The 10 biochars assessed here demonstrated high variability in every physical and chemical parameter tested (Table 3.1 and 3.2). Figure 3.2 shows representative ESEM images, which display surface features and pore-opening orientations of biochars generated from each of the 5 feedstocks. The biochar pH's and SSA's 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, $R^2< 0.3$).

Table 3.1. Chemical characteristics of biochars and traditional carriers**.**

Material	% C	% N	C:N ratio	Oxidizable C[a] (mg kg^{-1})	pH[a]	Ec[a] (mS cm^{-1})	MED index
Palm300	59.22	1.33	45	740 ± 12	6.47 ± 0.08	1.33 ± .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 ± .01	1
Pine600	72.35	0.21	350	83 ± 23	6.75 ± 0.10	0.06 ± .01	4
Nut300	60.60	0.35	174	150 ± 10	6.88 ± 0.14	0.72 ± .12	1
Nut600	71.27	0.66	108	142 ± 12	9.85 ± 0.02	1.60 ± .0 9	1
Pit300	78.94	3.02	26	69 ± 27	7.39 ± 0.01	0.17 ± .01	6
Pit600	72.41	1.96	37	194 ± 3	8.26 ± 0.13	0.78 ± .12	1
Shell300	69.63	1.79	39	200 ± 9	6.11 ± 0.04	0.31 ± .07	1
Shell600	79.77	0.62	128	250 ± 5	9.06 ± 0.44	1.01 ± .15	1
Peat	41.21	1.24	33	1019 ± 16	4.27 ± 0.02	0.54 ± .01	7
Vermiculite	N/A[b]	N/A	N/A	N/A	8.19 ± 0.32	0.04 ± .01	1

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

Table 3.2. 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)

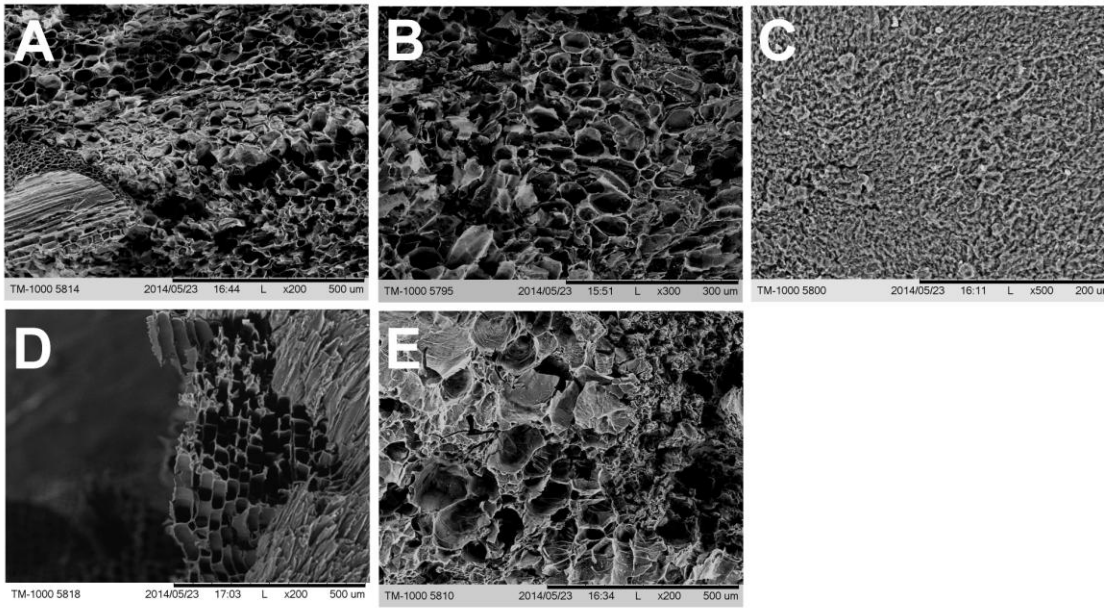


Figure 3.2. ESEM images of biochars prepared at 300°C from palm fronds (A), coconut shells (B), pistachio nut shells (C), pinewood (D), and stone fruit pits (E) at magnifications 200– 500x.

There was no significant difference in GFP copy numbers among the initial DNA extractions (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. Biochar pH has the greatest influence on the initial inoculum density. A Gaussian peak model fit the pH and initial microbial population density data well (R^2=0.837) and indicated that the optimal biochar pH was between 7.9 and 8.3. To a lesser extent, the C:N ratios were negatively correlated and the % N's were positively correlated with initial inoculum population density (linear regression analysis, R^2=0.37 and 0.29). All other biochar characteristics tested here showed no linear or Gaussian relationships to initial inoculum density (R^2 <0.3).

Inoculum survival after 4 weeks in soil was related to biochar feedstock, but not the pyrolysis temperature (2-way ANOVA, *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*). Of the biochar physico-chemical characteristics tested here C:N ratios, SSA, % WHC, and pore-opening diameters were significantly correlated to GFP copy number at the end of the 4-week soil incubation. 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) (Figure 3.3).

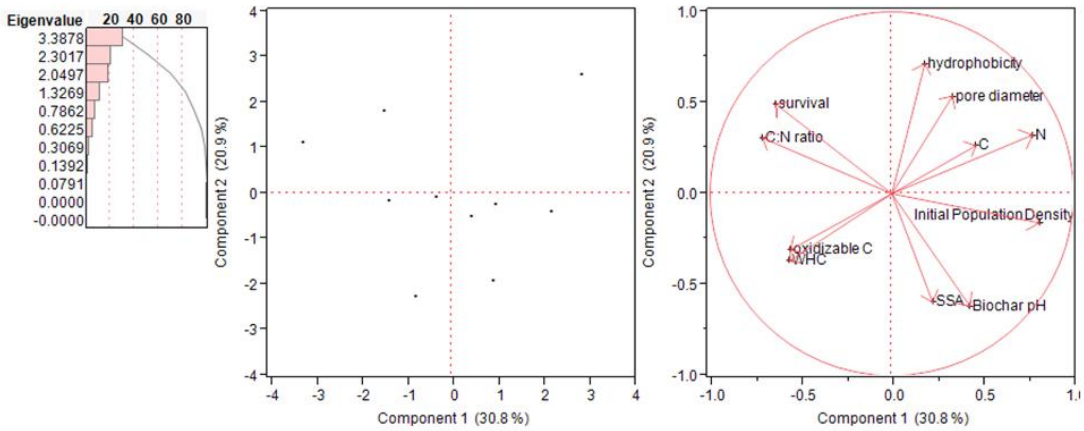


Figure 3.3. Principal components analysis of biochar physio-chemical characteristics and survival.

The results reflected that week 4 survival and C:N ratio had similar effects on variance within the dataset. The C:N relationship to survival was a positive, linear correlation (linear regression analysis, R^2=0.59). Also, SSA, pore-opening diameters, and %WHC were fit to week 4 survival data with Gaussian models (R^2= 0.61, 0.46, and

0.35). The Gaussian peak for SSA and survival centered around a SSA of 112 m^2 g^{-1} and had 95 percentile range between 94 and 129 m^2 g^{-1}. The %WHC critical point is at 184% with 138- 230% range. The mean pore-opening diameter Gaussian model showed a critical point at 36 µm with a 95 percentile range between 26 and 46 µm.

3.4 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 materials, Pine600 was identified as the best biochar for use as an inoculum carrier. It performed as well as the industry standard carrier, peat moss, and its use resulted in higher sustained population 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 in samples analyzed after the 24 h inoculation procedure and also promoted the greatest survival after the 4-week soil incubation, which was slightly higher than that of the Pine600. This was associated with the high availability of labile carbon and high nitrogen content of the peat (Table 3.1). To identify specific characteristics that related to the survival outcomes, the biochars were assessed based on several chemical and physical parameters. All characteristics analyzed were highly variable among the various biochar materials, which is consistent with previously reported findings (Mukome *et al.*, 2013; Uchimiya *et al.*, 2011). The pyrolysis temperature had the greatest effects on pH and SSA, whereas feedstock type largely determined the % WHC of the biochars.

Biochar pH and C:N ratio had the greatest effect on initial GFP copy numbers, which reflect 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 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 (Figure 3.1). However, after 4 weeks in the soil, this material supported cell densities that were similar to those supported by the other biochars and vermiculite. Also, when cell densities were compared after the 4-week incubation, there was no correlation between the biochar pH and survival. Hence, while the pH may have been initially influential, after application to the soil, this effect was no longer detected. Other variables associated with higher initial population densities were related to nitrogen in the char, lower C:N ratios and higher N contents. Saranya *et al.* (2011) also observed a positive influence of nitrogen when testing the shelf life of *Azospriliium lipoferum* soil inoculants on various biochars.

However, in the present study, there was no relationship between biochar nitrogen contents and cell densities after 4 weeks of incubation. 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 % WHC's. The hydrophobicity was assayed on dry materials, but % WHC values were obtained after 24 h of saturation. Hence, the hydrophobicity of the dry biochar 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.

Survival of the introduced PGPR strain UW5 after 4 weeks in non-sterile soil was strongly correlated with the C:N ratios of the 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, a 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 variable soil types.

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 final HTT's (Downie *et al.*, 2009; Sun *et al.*, 2012; Yu *et al.*, 2010). These materials may have a large volume of nano–micropores, which are not accessible to bacteria and thus do not reflect the functional carrier capacity of the material. In fact, macroporosity often makes up only a small portion of the total surface area on biochar particles (Brewer *et al.*, 2014; 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 to nanoporosity which was measured by SSA, not the macropores we visualized using ESEM. The materials closely resemble 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, pre-treatment 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.

3.5 CONCLUSIONS

Altogether, chemical properties of biochar, such as nitrogen 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 have 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 population densities, which is a common association observed between soil properties and microbial populations.

Results of this study showed that various types of biochar 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 with moderate pH's, and the use of compost-biochar mixtures for development of new carriers for soil inoculation.

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CHAPTER FOUR

THE INFLUENCE OF BIOCHAR ON PLANT GROWTH PROMOTING ACTIVITY OF RHIZOBACTERIA

ABSTRACT

If biochar interferes with plant-growth-promoting rhizobacterial (PGPR) activities, be it those of native soil bacteria or inoculum introduced on biochar carriers, then it is rendered ineffective as a carrier material and should be added to agricultural soils with caution. To better understand the impact of biochar on native soil PGPR, soil samples from biochar-amended soils were surveyed for bacteria harboring 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase enzymes. Strains were also assayed for the ability to solubilize mineral-complexed phosphate and produce indole compounds, like indole-3-acetic acid (IAA). Spectrophotometric assays and selective and differential media were used to indentify portions of microbial populations harboring these traits and quantify PGPR activity in mixed consortia. Results indicated that biochar derived from biosolids or willow wood did not negatively impact the assayed PGPR communities.

Activities of PGPR were assayed as influenced by the presence of biochar. Two strains served as models for the production of a plant growth hormone, IAA, and ACC deaminase activity. The strains, *Enterobacter cloacae* UW5 (IAA-producer) and *Pseudomonas putida* UW4 (ACC deaminase activity) have been well-studied in previous literature with respect to PGPR activity. Spectrophotometric assays and red fluorescent protein (RFP) reporter systems, which incorporated promoters for genes involved in these traits, did not indicate reduced PGPR activity in cell cultures grown with 2% biochar. To provide higher sensitivity, quantitative PCR assays were performed on cDNA generated from RNA extractions from cultures grown with and without biochar. Again, biochar

showed no influence on the expression of the structural gene for ACC deaminase or the indole-3-pyruvate decarboxylase enzyme involved in the IAA biosynthesis pathway. Hence, the results of this research indicate that biochar is not likely to interfere with PGPR function by sorption of substances involved in bacterial plant growth promotion.

4.1 INTRODUCTION

The effect of PGPR on native microbial communities will significantly impact its utility as an agricultural soil amendment. To assess the bioavailability of residual phosphorus (P) in biosolid-derived biochars it is important to consider the mineral phosphate solubilizing (MPS) microbial community. In soils phosphate is frequently complexed by calcium (Ca), iron (Fe), or aluminum (Al), making it insoluble and unavailable to plants (Jones and Oburger, 2011). This is also the case with the residual P in biosolid-derived biochar studied here, where P is predominantly complexed as Al and possibly Ca phosphates (Wang *et al.*, 2012). Mineral phosphate solubilization (MPS) by bacteria is often correlated with the release of organic anions and corresponding free protons, which lowers the pH in the surrounding soil water solution, and thereby induces chelation of the metal complexes (Rodríguez and Fraga, 1999). If MPS bacterial populations are affected by biochar, this could have a significant impact on P availability from biosolid-derived biochars and plant P uptake. The abilities of the native soil bacteria to solubilize mineral phosphate in treatments prepared with biosoild-derrived biochars generated at 4 HTT's were quantified using spectrophotometric assays and indicator media.

Also, biochar could impact phytoremediation processes utilized in efforts to reduce concentrations of heavy metals and organochlorines pesticides in contaminated soils. Biochar has been shown to enhance root development in arsenic contaminated soils and influence sorption of heavy metals in soils (Brennan *et al.*, 2014; Paz-Ferreiro *et al.*, 2014). Some ferns have the ability to remove heavy metals from soils by translocating them into their biomass. Root development in contaminated soils can also stimulate the

growth of soil microorganisms by release of root exudates that serve as substrates. Many soil microorganisms degrade organic pollutants, such as organochlorine pesticides. Also, survival of the accumulator plants is essential for successful soil remediation. In this study root-colonizing strains were isolated from soils contaminated with dichlorodiphenyltrichloroethane (DDT) and arsenic and assayed for ACC deaminase activity and auxin production, microbial traits that play a major role in assisting survival of plants stressed by heavy metals (Arshad *et al.*, 2007; Rajkumar and Freitas, 2008). These PGPR activities were quantified across isolated cultures and mixed consortia taken from the rhizospheres of 2 fern species in contaminated soils amended with biochars prepared at 2 HTT's. The ferns included a New Zealand native species, *Blechnum novae-zelandiae,* and a species originally from China, *Pteris cretica*, which has been demonstrated to be an arsenic-hyperaccumulating species (Chen *et al.*, 2002). The main objective of these studies were to determine if PGPR communities were affected by different biochar materials and if this effect could be related to plant development in the given treatment soils.

Changes in the expression of enzymes brought about by biochar will shed light on the physiochemical processes in the soil environment that shape the structure and function of microbial communities in the rhizosphere. Current strategies for sustainable soil management entail the use of methods that selectively enrich indigenous plant growth promoting bacteria (PGPR), or carrier materials, such as biochar, that deliver beneficial soil inoculants to plant root zones. Many PGPR have the capacity to produce exogenous plant growth hormones, an activity that has been correlated with increased

total root length, branched root architecture, and root hair formation (Patten and Glick, 2002; Spaepen *et al.*, 2008). *Enterobacter cloacae* UW5 serves as a well-studied strain for production of plant growth hormone, indole-3-acetic acid (IAA) by the indole pyruvate pathway. 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 (Ryu and Patten, 2008; Spaepen *et al.*, 2007). Another significant PGPR trait is the ability of some microorganisms to produce 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase. Under conditions of abiotic stress plants generate ethylene, which can accumulate in the rhizosphere and, in turn, elicit a stunting response in the plant, drastically reducing crop yields (Yang *et al.*, 2009). Diverse soil bacteria have the enzyme ACC deaminase, which allows them to utilize the precursor to ethylene, ACC, as a nitrogen source, degrading the ACC into ammonia and α-ketobutyrate (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). ACC deaminase has been best studied in *Pseudomonas putida* UW4, and the expression of the gene encoding this enzyme is induced in the presence of ACC (Cheng *et al.*, 2008). The activities of each of these enzymes were determined to be essential to plant-growth-promotion by the given strains (Li *et al.*, 2000; Patten and Glick, 2002). Hence, any interference of biochar with the expression of these genes could result in loss of benefit associated with inoculum harboring these traits. In previous work soil-microbial enzymatic activity was shown to be increased or decreased in the presence of biochar (Bailey *et al.*, 2011; Jin, 2010). Thus, it is important to better understand the

influence of biochar on beneficial PGPR enzyme activity.

Several methods were employed to analyze the expression of genes involved in ACC deaminase activity and IAA biosynthesis. Spectrophotometric assays provided initial insight into the PGPR activity of these strains as influenced by biochar in culturing media. Positive gene expression was also verified using a red fluorescent protein (RFP) reporter system similar to that described by Rochat *et al.* (2010). Promoters for the *acdS* or *ipdC* genes were previously determined (Cheng *et al.*, 2008; Ryu and Patten, 2008) and inserted into a plasmid in front of a promoter-less RFP. The RFP-reporter cells were grown in the presence of biochar and RFP expression was verified using fluorescent microscopy. To obtain quantitative data on gene expression qPCR primers were designed to target either the *ipdC* or *acdS* gene sequence. Then, RNA extractions and subsequent RT-qPCR analysis were used to enumerate gene transcripts in cultures grown in the presence or absence of biochar.

4.2 MATERIALS AND METHODS

4.2.1 Spectrophotometric assays

The quantity of IAA produced by UW5 cultures and ACC deaminase activity of UW4 cultures grown with 0% or 2% (w/v) biochar was determined. IAA production was quantified for cell-free supernatants using the method described by Khalid *et al.* (2004). To induce the expression of *ipdC*, a 0.5-mM filter-sterilized tryptophan solution was added to each UW5 culture. After 48 h of growth at a 30°C with shaking at 170 rpm, cultures were centrifuged for 5 min at 13000×g at room temperature. Cell-free supernatant (0.8 ml) was mixed well with 0.8 ml of Salkowski coloring reagent (4.5 g $FeCl_3$ per liter in 10.8 M H_2SO_4, Glickmann and Dessaux, 1995) and left in the dark for 30 min at room temperature. Following color development the absorbances of the solutions were read at 540 nm using a spectrophotometer. Supernatant solutions were compared to a set of standard solutions, ranging from 0 to 100 ppm IAA in water, also mixed 1:1 with Salkowski reagent. Cultures of UW4 were amended with 3 mM filter sterilized ACC compound to induce *acdS*. The ACC deaminase activities of UW4 cultures were quantified using a protocol described by (Penrose and Glick, 2003). All assays were performed on 4 replicate cell culture supernatants per treatment.

4.2.2 Strains and DNA manipulations

Strain *E. cloacae* UW5 was generously provided Dr. Cheryl Patten (University of New Brunswick, Canada) and a 520 bp promoter region for *ipdC* was isolated using primers previously verified by Ryu and Patten (2008) (Table 4.1). *Pseudomonas putida* UW4 was kindly donated by Dr. Bernard Glick (University of Waterloo, Canada). The ACC deaminase promoter is in the intergenic space between the ACC deaminase structural gene and regulatory protein (accession number AF047710) (Grichko and Glick, 2000; Shah *et al.*, 1998). A 371 bp product containing this region was amplified using primers designed for this study which target sequences inside the AcdR and AcdS flanking regions (Table 4.1). Restriction endonucleases were purchased from New England Biolabs (Ipswich, MA, USA) and digested products were purified with either the MinElute PCR Purification Kit (Qiagen;Valencia, CA, USA) or the E.Z.N.A. Plasmid Mini Kit I (Omega Biotek; Norcross, GA, USA). Digested PCR products were then ligated into pME9010 vectors digested with the same enzymes, using standard techniques. Plasmid pME9010 was provided here by Dr. Christoph Keel (University of Lausanne, Switzerland).

4.2.3 Bacterial transformations

Electrocompetent UW5 cells were prepared using methods described by Peloquin *et al.* (2000) and UW4 cells were prepared with respect to a protocol described by Choi *et al.* (2006). Transformations were carried out using 500 ng plasmid combined with

Table 4.1 Strains, primers, and plasmids used for gene expression studies

Material	Relevant properties	Source
Strains		
Enterobacter cloacae UW5	IAA production	Patten and Glick, 2002
Pseudomonas putida UW4	ACC deaminase activity	Glick *et al.*, 1995
Escherichia coli DH5α	Competent cells	C.H. Yang
Primers		
IPIF ATAT**GAATTC**GCCATGGCAGGAAATCTTC	Upstream of *ipdC* promoter, EcoR1 in bold	Ryu and Patten, 2008
IPIR ATAT**GGATCC**GACGGTCCAGCAGGTAATG	Downstream of *ipdC* promoter, BamH1in bold	Ryu and Patten, 2008
AcdiF ATAT**GAATTC**CAGAATGGCACGGTCGGTT	Upstream *acdS* promoter, EcoR1 in bold	This study
AcdiR ATAT**GGATCC**CAGTCTTCACGCTTGGCATA	Downstream of *acdS* promoter, BamH1 in bold	This study
AcdS.F CTACGAGGGCAAATCCAT	Within *acdS* structural gene	This study
AcdS. R AGGTGGGCATAAAGAACT	Within *acdS* structural gene	This study
IpdcF AATGAACCAGGCGATTGA	Within *ipdC* structural gene	This study
IpdcR GGATGATGACGACATAAGG	Within *ipdC* structural gene	This study
Plasmids		
pME9010	Promoterless mCherry- based reporter vector derived of pPROBE'*gfp*[AAV];Km^r	Rochat *et al.*, 2010
pME9010-acdi	pME9010 with acdi insert into MCS	This study
PME9010-ipi	pME9010 with ipi insert in MCS	This study

100–200 µL of competent cells, electroporated at 2.5 kV, 25µF, 250Ω, using a 2 mm gap cuvette in a Biorad GenePulser (Hercules, CA). SOC medium was added immediately after electroporation and cultures were incubated at 28 °C for 1.5 h before being spread onto selective LB plates. Integration of plasmids was verified by selection on kanamycin.medium (25 µg ml^{-1}) and by microscopic observation of RFP expressing cells. Fluorescent microscopy visualization was performed on an Olympus IX71fluorescent microscope using a light excitation range of 530-558, and emission range of 594-691, appropriate for mCherry. Transformants were screened for growth inhibition using growth curve analysis on a nutrient rich LB media and a carbon and nitrogen starvation response media prepared according to Voigt *et al.* (2007). IAA production levels of transformed cells were compared to those of wild-type UW5 using Salkowski reagent and the S2/1 method described by Glickmann and Dessaux (1995). Using the method described by Penrose and Glick (2003) ACC deaminase activity was assayed for wild-type and transformed UW4 cells. Proper induction of the RFP reporter was checked using inducing and non-inducing culturing conditions. Induction of RFP was dependent upon tryptophan in pME9010-IPI carrying UW5 and on ACC in pME9010-acdi carrying UW4.

4.2.4 Qualitative expression analysis with fluorescent microscopy

Transformed cells were grown in Dworkin and Foster (DF) minimal salts medium, prepared with $(NH_4)_2SO_4$ as a nitrogen source, described by Penrose and Glick (2003). To induce the expression of *ipdC,* a 0.5 mM filter sterilized tryptophan solution

was added to each UW5 culture. Cultures of UW4 were amended with 3 mM filter sterilized ACC compound to induce *acdS*. Cell cultures (50 ml) were shaken at 170 rpm at 30°C in 125 ml flasks in the presence of 0%, 2%, or 5% (w/v) Pine600 biochar. After 24 h, wet mounts of cell cultures were prepared for fluorescence microscopic observation of RFP expressing cells using parameters described in section 4.2.3.

4.2. 5 Culture-based RNA extractions and **RT-qPCR** 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. For this, 25 ml DF broth cultures were maintained in125 ml flasks to which 0%, 2%, or 5% (w/v) Pine600 biochar was added. Non-induced cultures were used to inoculate inducing cultures. Cultures were induced using tryptophan or ACC (described previously) and grown at 30°C, on a shaker at 170 rpm. RNA was extracted from cell pellets after 6 h of growth. Negative controls were prepared using RNA extracted from non-induced cultures, lacking tryptophan or ACC.

Total RNA extractions were obtained from bacterial cultures using the Aurum™ For each treatment 4 RNA extractions were obtained from 4 individual flask cultures**.** Total RNA Mini Kit and were subsequently treated with the iScript™ cDNA synthesis kit, following the 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).

4.2.6 Quantitative PCR

All qPCR reactions, protocols, and data analyses were performed according to the standards outlined by the MIQE guidelines (Bustin *et al.*, 2009). Reactions were prepared using the SsoAdvanced universal SYBR® Green supermix and were run on a MyiQ® Thermal Cycler (BioRad Laboratories, Hercules, CA). Primers were designed using Beacon Designer 7.8 (PREMIER Biosoft International, Palo Alto, CA). For expression analysis of ACC deaminase primers AcdSF/R were designed to target an 87 bp region of the *acdS* gene in UW4, which was previously sequenced and functionally verified (Accession number: AY823987, Table 4.1). The *ipdC* gene has been previously sequenced and verified (Accession number: AF285632.2, Patten and Glick, 2002). Primers IpdcF/R were used to target a 76 bp region of the *ipdC* gene sequence (Table 4.1). Both the *acdS* and *ipdC* target oglios were amplified using a protocol that used a 15 min initial denaturing step followed by 30 cycles at 94 °C 15 sec, 57°C for 30 sec, 72°C for 15 sec. After each qPCR run, a melt curve analysis was performed using the following conditions; 55°C to 95°C with 0.5°C temperature increases over 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 determined for RNA samples without reverse transcriptase treatment. Amplicons obtained from PCR products derived from UW5 (*ipdC*) or UW4 (*acdS*) chromosomal DNA were ligated into pGEM-T vectors using the kit protocol and competent *Escherichia coli* DH5α cells (Promega Coorporation, Madison, WI). These plasmids were isolated and used to develop DNA standard curves, which included 4 plasmid-based standards, diluted 10

fold, ranging from 10^4 to 10^7 gene copies ul^{-1}, respectively, prepared in triplicate. All qPCR reactions involving sample or control DNA or RNA templates were prepared in duplicate.

4.2.7 Isolation of strains and preparation of mixed consortia

Root samples were collected after 3 weeks of growth in soil treatments described in section 4.2.8 and 4.2.11. Roots were gently shaken to remove sand particles and 5 g of roots and closely adhering sand were stored at -20°C until ready for use. Roots and soil were added to 25 ml of sterile water and left shaking at 170 rpm for 40 min. Then 200 µl of each solution, 10^{-2}, and 10^{-1} diluted solutions were spread onto Tryptic Soy Agar (Becton, Dickinson) + 75 mg l^{-1} cycloheximide to ensure rapid growth and select against fungi. All plates were made in triplicate. Ten strains with varying morphologies were selected from each treatment and streaked 3– 4 times for isolation. Mixed consortia were obtained using 200 µl the soil solution to inoculate 10 ml TSB broth + 75 mg/L cycloheximide, incubated shaking for 24 hours at 30°C. Then 200 µl of these cultures were used to inoculate 10 ml of TSB and cultured in the same conditions. Optical densities were adjusted to 0.5 (A600) and used to inoculate appropriate media for the varying assays.

4.2.8 Biochar, soil, and planting conditions for MPS Study

Biosolid-derived biochars were pyrolyzed at 4 HTT's, 250°C (BS250), 350°C (BS350), 450°C (BS450), and 550°C (BS550), using conditions previously described (Wang *et al.*, 2012) and were incorporated into a Waitarere sandy soil at a rate of 5 t h^{-1}. Moata' tetraploid Italian ryegrass *(Lolium multiflorum Lam*.) was grown in biochar-amended soils, in soils amended with fresh biosoilds, Sechura phosphate rocks (SPR), or calcium dihydrogen phosphate (CaP), and unamended soil. More details on plant growth conditions are described by Wang *et al.* (2012).

4.2.9 Strain MPS analysis

Isolated colonies were used to inoculate 5 ml of a minimal medium based on AT salts containing the following (per liter): glucose, 10.0 g; KH_2PO_4, 10.9 g; $(NH_4)_2SO_4$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.16g; $FeSO_4 \cdot 7H_2O$, 0.005g; $CaCl_2 \cdot 2H_2O$, 0.011 g; and $MnCl_2 \cdot 4H_2O$, 0.002 g, adjusted to pH 7 (Mehta and Nautiyal, 2001). Cultures were adjusted to an optical density of 0.6 at an absorbance of 600 nm and 10 µl of these cultures were used to inoculate 5 ml of National Botanical Research Institute's phosphate growth medium with bromophenol blue (NBRIP-BPB). The medium was prepared as described by Mehta and Nautiyal (2001) to contain the following (per liter); glucose, 10 g; $MgCl_2 \cdot 6H_2O$, 5 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; KCl, 0.2 g; $(NH_4)_2SO_4$, 0.1 g; BPB, 0.025 g; and either $Ca_3(PO_4)_2$, 5 g or 8% Florida rock phosphate powder suspension, 45 ml and adjusted to a pH of 7.0 prior to BPB addition and autoclaving. All cultures were prepared

in duplicate. The ability of individual isolates to produce organic anions, corresponding to a decrease in pH of the surrounding media, was visually verified based on decolorization of BPB. Cultures were assessed for color change on day five and recorded as showing 0, medium, or high levels of anion production.

4.2.10 Mixed consortia MPS

The mixed consortium cultures (40 ml) were used to inoculate NBRIP-BPB in 250 ml flasks. All flasks were incubated with shaking at 30°C. On days 3, 5, 7, and 10, 8 ml of liquid culture were removed from each flask and centrifuged at 18,000 rpm for 8 min. Supernatants were removed for soluble phosphorus analysis using a vanadomolybdate colorimetric method for phosphorus determination, described by Wang *et al*. (2012). Vanadomolybdate solution was prepared using the following per 5 liters; ammonium metavanadate, 5 g; ammonium molybdate, 100g; concentrated HNO_3, 700 ml. The reagent was added to diluted sample solutions (1:3 v/v), mixed well, allowed 15 minutes for color development, and absorbencies at 420 nm are read on a spectrophotometer. A set of standards is compared to the samples using oven dried KH_2PO_4 at 100 pm and below.

4.2.11 Phytoremediation experiment soil, biochar, and planting conditions

A potted greenhouse experiment was prepared using an Orthic Pumice soil contaminated with arsenic and organochlorines, included isomers of hexachlorocyclohexane (HCH), aldrin, dieldrin, and dichlorodiphenyltrichloroethane (DDT), collected from a former sheep dip site in New Zealand. Biochar made from willow feedstock (*Salix sp*) was prepared at HTT's of 350 (W350) or 550°C (W550) and added to the soil at a rate of 2% (w/w). *Blechnum novae-zelandiae* and *Pteris cretica* were seeded in the contaminated soils. After 3 weeks of growth roots were harvested for isolation of rhizobacteria. Additional details on the soil analysis and biochar preparation are described by Gregory *et al.* (2014).

4.2.12 ACC deaminase and IAA production in strains and mixed consortia

Populations with ACC deaminase activity were selected using a modified Dworkin Foster media as described by Penrose and Glick, 2002. Strains growing on agar cultures with ACC as sole nitrogen source were qualitatively determined to be ACC deaminase harboring strains. The ACC deaminase activities of mixed consortia were quantified using a protocol described by (Penrose and Glick, 2003).

Individual strains and mixed consortia were grown in glucose peptone agar medium (GPAM) to an optical density of 0.6 at 550 nm. Then, 0.5 ml of this culture was used to inoculate 10 ml of GPAM broth with or without L-tryptophan (0.5 mM). After 48 h, cultures from strains and consortia were assayed for the ability to produce IAA with

and without tryptophan supplemented media with respect to methods described in section 4.2.1 (Khalid *et al.*, 2004).

4.2.13 Statistical Analyses

SigmaPlot 11.0 was used to generate plots and to perform all ANOVA and pairwise multiple comparison procedures (Systat Software, San Jose, CA, USA). Dunnett's Test was used to compare means of treatments to controls and was performed using R Statistical Software (Foundation of Statistical Computing, Vienna, Austria).

4.3 RESULTS

4.3.1 Spectrophotometric assays

There were no significant differences in IAA concentrations in the culture medium of induced UW5 cells with and without 2% biochar (T-test, $P<0.05$, Figure 4.1 A). Also, ACC deaminase activity of UW4 cells induced in the presence of 2% biochar was not lower than that of UW4 cultures grown in inducing media alone (T-test, $P<0.05$, Figure 4.1 B).

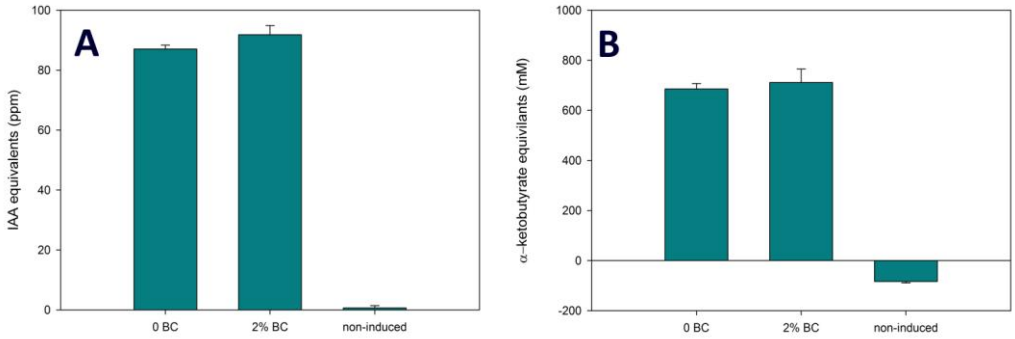


Figure 4.1 Results of spectrophotometric assays to determine; **A.** IAA production by UW5 cells and **B.** ACC deaminase activity of UW4 cells, growth in the presence of 0%, or 2% biochar with inducing compounds or non-induced. Values represent means and SEMs of 4 replicates

4.3.2 Red fluorescent protein reporter systems

The growth and PGPR activities of the transformed UW4 and UW5 strains were not significantly different from the growth and activities of the wild-type strains (ANOVA, $P<0.01$). Transformed cells only expressed RFP in the presence of the proper inducing compounds. When grown in the presence of 2% or 5% (w/v) Pine600 biochar, there was no observable difference in RFP expression by cell cultures. Figure 4.2 displays UW4-9010-*acdi* and UW5-9010-*ipi* cells grown in cultures amended with inducing compounds and 5% Pine600. These images are representative of those acquired for all induced cells.

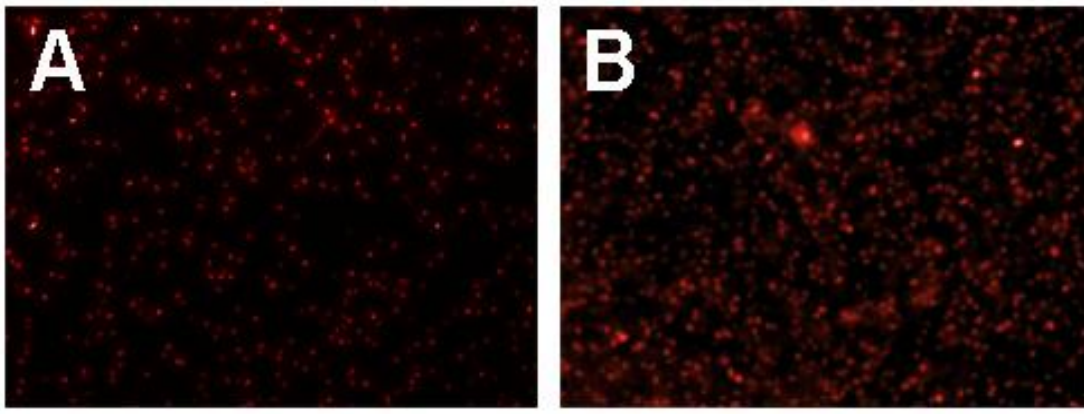


Figure 4.2 RFP expression in the presence of biochar by UW4-9010-*acdi* induced with ACC (A) and by UW5-9010-*ipi* induced with tryptophan (B)

4.3.3 Expression of *acdS* and *ipdC* as Monitored by qPCR

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 samples, RNA with no reverse transcriptase treatment served as negative controls and resulted in no qPCR amplification. Figure 4.3 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 were no significant differences in transcript numbers between cultures with 0%, 2%, or 5% biochar for either gene assayed (ANOVA, $P<0.05$).

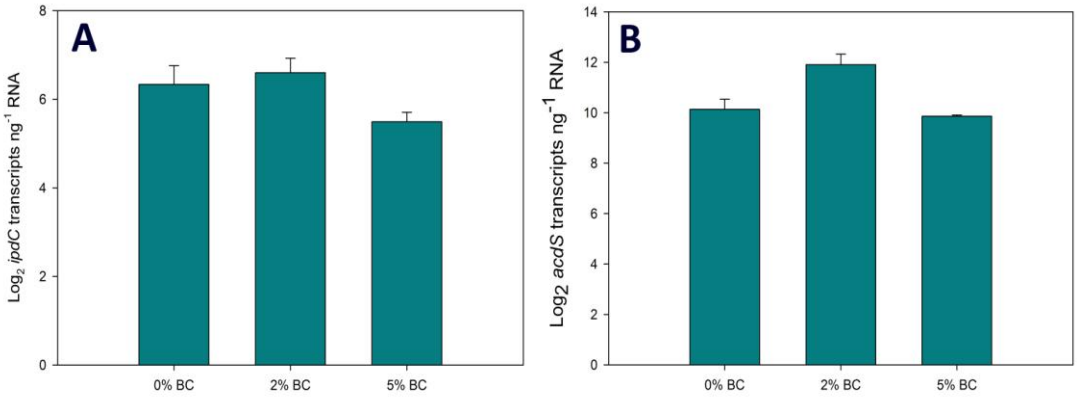


Figure 4.3. Log_2 transcript ng^{-1} RNA of gene *ipdC* or *acdS* obtained from RT-qPCR. Values reflect means and SEMS from cDNA templates obtained from 4 replicate cultures of strain UW5 (*ipdC*) or UW4 (*acdS*) induced in the presence of 0%, 2%, or 5% (w/v) Pine600. There

4.3.4 MPS of native bacteria from biochar-amended soils

Mineral phosphate solubilization was analyzed *in vitro* in media containing either insoluble calcium dihydrogen phosphate (CaP) or an Al and Fe-rich rock phosphate (RP) as phosphorus sources. The percentages of strains isolated from each treatment soil that had MPS activity are provided in Table 4.2. Of these, soils amended with the BS250 had significantly fewer MPS bacterial strains than did the unamended soil, when assayed using both CaP and RP-medium (Dunnett's test, $P<0.05$, Table 4.2). When the soils were used to directly inoculate medium, the subsequent mixed consortia from the BS250 soil also had lower levels of solubilized phosphate and generated a higher culture pH in RP-medium than that of the consortia grown from the un-amended soil (Dunnett's test, $P<0.05$, Table 4.2). The mixed consortium from the biosolid amended soil also solubilized less P in CaP and RP-media and had a significantly higher culture pH in RP medium (Dunnett's test, $P<0.05$, Table 4.2).

Table 4.2 Results of MPS study on bacteria isolated from biochar and P fertilized soils

Treatment	Strains with MPS activity (%)		Mixed Consortia Solubilized P (ppm)		Mixed Consortia Media pH	
	CaP	RP	CaP	RP	CaP	RP
BS250	10± 0*	20± 0*	502± 21	111± 27*	5.07 ± 0.23	3.65± 0.08*
BS350	35± 7	67± 0	530± 3	176± 3	4.68± 0.04	3.38± 0.03
BS450	60± 0*	60± 0	526± 9	188± 37	4.81± 0.23	3.33± 0.04
BS550	30± 0	35± 7*	135± 18*	125± 2	4.72± 0.01	3.56± 0.01*
CaP	40± 0	50± 0	529± 1	170± 18	4.9± 0.06	3.35± 0.04
RP	56± 0*	60± 0	461± 0	182± 5	5.18± 0.04	3.36± 0
Biosolids	55± 7*	65± 7	289± 20*	115± 15*	5.48± 0.39	3.52± 0.08*
Soil only	40± 0	60± 0	467± 11	159± 4	5.24± 0.03	3.26± 0.01

Results reflect means and SEMs of 4 replicates. *indicates a value significantly different from the soil only treatment (Dunnett Test, $P<0.05$)

4.3.5 ACC deaminase activity and production of IAA by native bacteria from biochar-amended soils

When assaying rhizosphere isolates from the contaminated soils amended with W350 and W550 biochars, the proportion of bacteria with ACC deaminase and the ACC deaminase activity of mixed consortia did not show significant differences (ANOVA, $P<0.05$, Table 4.3). However, when monitoring IAA equivalents produced by strains isolated from the *B. novae-zelandiae* rhizosphere, the soil amended with W550 had strains that produced significantly greater quantities of IAA equivalents than did strains isolated from the non-amended rhizospheres (Dunnett's test, $P<0.05$, Table 4.3). However, this effect was not seen for isolates from the *P. cretica* rhizosphere in the same W550-amendended soil (Table 4.3).

Table 4.3 Results of ACC deaminase and IAA assays for bacteria isolated from biochar-amended and non-amended rhizosphere soils

Fern Genus	Biochar Treatment	Strains with ACC Deaminase (%)	α-ketobutyrate in Mixed Consortia (mMol)	Mean IAA Equivalents Per Isolate (ppm)	
				+ tryptophan	-tryptophan
Blechnum	**No biochar**	60	109± 8	5.06± 1.19	1.94± 0.62
Blechnum	**W350**	50	85± 6	5.66± 1.23	0.63± 0.26
Blechnum	**W550**	40	96± 8	12± 1.84*	2.48± 0.65
Pteris	**No biochar**	70	114± 5	4.59± 1.74	1.89± 0.77
Pteris	**W350**	60	108± 3	10.82± 2.15	1.93± 0.41
Pteris	**W550**	50	74± 8	1.43± 1.39	1.43± 0.54

Results reflect means and SEMs of 4 replicates. *indicates a value significantly different from the non-biochar-amended treatment from the same fern species (Dunnett Test, $P<0.05$)

4.4 DISCUSSION

The assays for PGPR traits among the native soil microbial communities indicated whether biochar amendment is influencing the microbial communities, possibly shaping the communities to select for more or less plant beneficial strains. For the most part, microorganisms isolated and mixed consortia cultured from the amended soils showed similar MPS bacteria proportions and activities to that of the unamended soil. However, the soils amended with BS250 had significantly lower percentages of MPS bacteria or MPS activity in mixed consortia. Of the amendment chemical properties analysed by Wang *et al.* (2012) the BS250 had notably higher volatile matter/ (volatile matter + fixed C) ratio and lower pH than did the other amendments. These properties may have had an influence on the soil MPS bacteria.

In the case of the PGPR activities assayed in the phytoremediation study, bacteria with ACC deaminase were present in all rhizospheres and did not correlate significantly to the soil treatments. However, the W550 treatment had a positive influence on bacteria that produce IAA in the *B. novae-zelandiae* rhizosphere. This species of fern was found growing in the area from which the contaminated soil was extracted. The *P. cretica* fern species is not native to this area. Soil microbial communities may have been better adapted to the *B. novae-zelandiae* rhizosphere and this may exhibit a greater indication of response to biochar amendment. In other work using the same soil and biochars, microbial dehydrogense activity was significantly increased in the presence of W350 and

even more so by W550 (Gregory *et al.*, 2014). This could indicate the benefit of using an indigenous plant species for phytoremediation of contaminated soils involving biochar.

The results of this research indicated that the biochar amendments did not affect population sizes of bacteria harboring the plant-growth promoting traits tested. During the course of this research, several PGPR strains were isolated and characterized based on PGPR activity. The use of these strains offers New Zealand organic farmers biological fertilizers composed of native species.

Many beneficial microbial traits involve enzymatic activity. Enzymes and their substrates could adsorb to char surfaces or be regulated by signaling molecules that interact with biochar. Recent studies generated concern over this phenomenon and showed that biochar had an effect on plant gene regulation (Viger *et al.*, 2014) and also interfered with microbial signaling (Masiello *et al.*, 2013). Figure 4.1 demonstrates that levels of indole production and ACC deaminase activity assayed from cells grown in the presence of 2% Pine600 biochar were not significantly different than those for cells grown in biochar-free medium. However, the spectrophotometric assay measures only the accumulation of these compounds in culturing medium. Hence, more sophisticated assays were developed to better monitor the expression of genes essential for these beneficial traits in aims to better resolve responses to biochar. In the *in vitro* gene expression studies conducted here, we did not see a biochar-induced change in the promoter activity or expression of genes involved in IAA production or ACC deaminase. The RFP reporters provided a qualitative assessment that promoter activities were positively regulated in the presence of biochar. Furthermore, the RT-qPCR provided quantitative verification that

gene expression was not significantly impacted by the presence of 2% or 5% biochar. It appears that precursor compounds, such as tryptophan, were not irreversibly adsorbed to the biochars, which would have resulted in lower bacterial gene expression in the presence of biochar. This addresses the concern that biochar may interfere with PGPR activities and lower the efficacy of beneficial soil inoculants.

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CHAPTER 5

GENERAL CONCLUSIONS

This research directly addressed the hypothesis that biochar could serve as a carrier for PGPR soil inoculants. Previously, little work was done on this subject and most assessments of biochar and microorganisms related to soil diversity profiles as affected by biochar. This is the first body of work to quantify inoculant survival in soil when introduced via biochar carriers. It is somewhat misleading to continue biochar discussions as thought biochar is a single material when, in fact, the term biochar encompasses a wide array of materials with highly variable chemical and physical properties. Hence, these investigations focused on various biochar materials that were well-characterized for attributes that had the greatest potential of affecting carrier suitability. Experiments were carefully designed to best reflect the benefit of biochar to microbial survival with respect to physical protection, pre-colonization of surface, and sorption of soil nutrients or organic matter.

It was vital to develop methodologies that could be implemented to assay bacteria in close contact with biochar without hindrance by biochar on cell or DNA extractions. Using a commercial biochar product it was determined that a modified DNA extraction protocol, involving chloroform-phenol-isoamyl alcohol, and traditional protocols for extraction of cells from roots and were sufficient. Downstream applications of DNA and cell extractions indicated that biochar did not create a bias in extraction efficiencies using these techniques. Furthermore, the use of plasmid pSMC21 was thoroughly tested as it is foreseeable that without the selection of the antibiotic, the plasmid would be quickly lost from cells. From the results of the assays, in particular the *in vitro* plasmid stability assay and the sterilized soil assay, it is clear that there was not notable plasmid loss. The key

findings from this set of experiments indicated that the use of 300 °C pinewood biochar as a carrier material resulted in slight increases in survival of *Enterobacter cloacae* UW5 inoculum as compared to direct soil application. Furthermore, cucumber did not demonstrate significantly different growth in relation to UW5 inoculation but there were significant increases in root branching and total root length with 2% biochar application. Also, UW5 colonized cucumber roots at high densities in soils with and without biochar amendment.

This set of assays provides fundamental insight into the effect of biochar on bacterial survival. The UW5-pinewood biochar combination does not represent an optimized material. Strain UW5 is an opportunistic pathogen, and was used here for the sole purpose of examining the influence of biochar on the ability of an IAA-producing strain to promote plant growth. The fact that plant growth promotion was not shown could result from the lower root surface population density than shown previously when UW5 promoted root elongation. But, it could also reflect that the effect of a single strain is diminished when applied to a soil with an indigenous population of bacteria. In this case the non-inoculated control plants likely also have beneficial strains colonizing roots and interacting with the plant. Even still, amendment with inoculated biochar resulted in much improved plant biomass. Here the plants were all well-fertilized and watered. The improved growth is likely a factor of the improved soil structure associated with the biochar application. Also, the low-temperature pinewood biochar had characteristics which allowed for thorough inoculation and cell mobility for root colonization, all together representing several advantages.

These results provided motivation to further assay various biochars as inoculum carriers in order to identify a carrier that has a greater positive impact on inoculum survival, but also retains some of the beneficial properties that the low temperature pinewood biochar offered. Thus, in the next set of experiments 10 types of biochar were generated from 5 feedstocks at two HTT's and compared to industry standards, peat moss and vermiculite. The same GFP-qPCR based assay employed in the first study was used to quantify inoculum survival in all carriers. Then, the biochars were assessed based on several physical and chemical parameters to best identify characteristics which relate to carrier capacity of biochar. Of the 10 biochar materials tested, all performed as well as vermiculite and none demonstrated detrimental effects on the inoculum population. One biochar, made from pinewood at a HTT of 600 ° (Pine600), performed as well as peat moss and sustained higher population densities than did vermiculite. Chemical properties of biochar were among the most important characteristics affecting initial inoculum survival. However, once incorporated into soil, physical features were more closely associated with inoculum survival.

Overall, the results of these experiments are very promising. The survival of the inoculants delivered through the Pine600 biochar was similar to that of those distributed on peat. However, the peat offers nutritional benefits to the inoculum in the form of liable carbon and nitrogen. The biochar did not offer nutritional advantages to the inoculum. Thus, the improved survival can be related mainly to physical protection and interactions in the soil. If the biochar were supplemented with nutrients or aged with compost to adsorb nutrient compounds, then it could prove to be an improvement over peat moss.

The use of biochar as an inoculant carrier is more sustainable than the use of peat moss. Thus, optimized formulations of biochar and PGPR have great commercial potential.

Biochar carriers improved inoculum survival. But, it is also important to ensure that there is not hindrance of microbial beneficial traits caused by biochar. The last set of assays examined the effect of biochar on the expression of beneficial traits and the abundance of bacteria with beneficial traits in biochar-amended soils. The results of three *in vitro* assays concurred that activity of ACC deaminase and production of IAA or expression of the *ipdC* gene involved in IAA biosynthesis were not impeded by 2% or 5 % biochar (w/v). Furthermore, several surveys of native soil bacteria were conducted to assess if biochar application alters native soil community structures in ways that are detrimental to PGPR abundance. The results suggest that while VOC's on biochar may be a concern, in general MPS bacteria, bacteria with ACC deaminase, and auxin-producing bacteria are not present in lower abundances in biochar-amended soils. Also, the tested PGPR activities of mixed consortia isolated from biochar-amended soils were not lower than those from the unamended soils. Interestingly, IAA production was significantly greater in a mixed consortia isolated from biochar-amended rhizosphere soils of a native fern species. This could indicate the benefit of using an indigenous plant species for phytoremediation of contaminated soils and it is reassuring that IAA-producing bacteria were abundant when the soil was amended with biochar.

This body of work addressed several important questions regarding the use of biochar and PGPR as co-amendments for agricultural soils. It is clear that biochar can serve as an effective carrier material for soil inoculation. In particular, biochars from

woody feedstocks, with pore-openings around 30 µm in diameter and median water holding capacities and surface areas are especially well-suited for this purpose. Furthermore, the use of biochar did not impede activities which are essential for PGPR benefits to plants, such as root colonization and expression of beneficial traits.

Future work should address optimized formulations of biochar and PGPR. If supplemented with nutrients, biochar can better support initial microbial colonization. This will also be a key factor in improving the shelf life of any given product. Furthermore, isolated strains were used for these investigations to gain a clear picture of biochar's effect on inoculum survival. However, for practical purposes mixed consortia of PGPR species can offer a diverse array of benefits to plants and will have broader applicability for use with a variety of soil types and crops.