Biochar as a potential inoculant carrier for plant-beneficial bacteria

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Abstract

In recent years, biochar has gained importance as a way to deal with global climate change, by sequestering C into soils, but also as a soil amendment and bioremediation tool. Many studies have demonstrated the positive influence of biochar on soil quality and subsequently, plant growth, although the results are not consistent and climate seems to be the main reason for this inconsistency. Biochar, with its highly porous structure, is believed to provide a suitable habitat for many microorganisms by protecting them from predation and desiccation; additionally it might also provide reduced carbon as an energy source and mineral nutrients. However, not much attention has been focused on biochar and microbial interactions. In this study, we propose the use of biochar as an inoculant carrier for plant-beneficial bacteria. Presently the most commonly used carrier for bacterial inoculants is peat moss. However, peat is a natural and effectively non-renewable resource; its overuse is of great environmental concern. Moreover, rapidly decreasing reserves of peat have led to price increases, which ultimately limit its use. A good bacterial carrier must possess a number of key features. It should be relatively unlimited, locally available and inexpensive; furthermore, it should have good water holding capacity and aeration properties and most important of all, it should sustain growth and survival of bacteria over time. Additionally, the right inoculant carrier should be non-toxic, environmentally friendly, and easily produced, sterilized and handled in the field. Finally, it should easily release bacteria into the soil, be simply converted to powder, mixable, packageable and adhere to the seed. I hypothesised that biochar can fulfill the conditions of a suitable carrier; its chemical and physical properties can create appropriate conditions for bacteria habitat and provide some nutrients that sustain survival and growth of bacteria over time. Moreover, it was expected that biochar can be successfully used as seed-coating material. Therefore the objectives of this research were to: 1) determine whether biochar can serve as a carrier for beneficial bacteria, 2) identify biochar(s) which sustains bacteria viability at the highest population density, 3) create a seed-coating system which could potentially be used in agriculture, 4) evaluate the effect of biochar inoculant and seed-coating on plant growth. Two bacteria, P- solubilizing *Pseudomonas libanensis* and N_2-fixing *Bradyrhizobium japonicum*, were selected as model bacteria. *P. libanensis* biochar inoculant and seed-coating were tested on corn plants, while *B. japonicum* was tested on soybean. The results of the storage time experiment demonstrated that

not every biochar can be a suitable carrier; however, two out of the four were able to sustain *B. japonicum* survival for as long as 8 months at a population density high enough to secure efficient soybean nodulation. Moreover, I demonstrated that biochar is a suitable material for seed-coating. The storage time experiment showed that biochar seed-coating can support the survival of *P.libanensis* and *B. japonicum* for up to four months at a relatively high population density; however, the abundance of the population strongly depends on storage temperature. I also demonstrated that the main reason behind a quick decline in the bacteria population is related to the pH of the carrier. A germination assay revealed that seed-coating does not affect or have a positive influence on seedling growth and germination characteristics. Finally, a greenhouse experiment was carried out to evaluate biochar inoculant and seed-coating effect on plant growth. This research clearly demonstrated that biochar can be a suitable bacterial carrier and can successfully replace commercially used peat moss. Biochar rhizobial inoculant can significantly improve soybean growth and reduce N fertilizer demand. Finally, biochar seed-coating might be considered an efficient end easy way to provide beneficial bacteria to the crop.

Résumé

Dans les dernières années, le biocharbon a gagné en importance, autant pour régler les changements climatiques mondiaux, que comme amendement pour le sol et outil de bioremédiation. De nombreuses études ont démontré l'influence positive du biocharbon sur la qualité du sol et sur la croissance des plantes qui y poussent. Par contre, les résultats de ces études manquent de cohérence, le climat semblant en être la cause principale. Il y a lieu de croire que la structure hautement poreuse du biocharbon pourrait offrir un habitat convenable aux microorganismes, en les protégeant de la prédation et de la dessiccation. De plus, le biocharbon pourrait offrir une source énergétique et des éléments minéraux sous forme de carbone. Jusqu'à maintenant, l'attention des chercheurs n'était toutefois pas rivée sur l'interaction entre le biocharbon et les microbes. Dans cette étude, nous proposons l'utilisation du biocharbon comme vecteur d'inoculation pour les bactéries. En ce moment, le vecteur le plus utilisé comme inoculant bactérien est la tourbe-mousse. Par contre, la tourbe est une ressource naturelle et non renouvelable, dont l'usage excessif devient problématique pour l'environnement. Qui plus est, la baisse des réserves de tourbe a provoqué une hausse des prix qui limite son usage. Un bon porteur doit posséder un certain nombre de caractéristiques pour accomplir sa fonction : être illimité, disponible localement et peu coûteux; avoir une bonne capacité de rétention d'eau et des propriétés d'aération; et, surtout, soutenir la croissance et la survie des bactéries pour une certaine période de temps. Il doit aussi être non toxique, respectueux de l'environnement, facile à produire et manipulé dans les champs. Finalement, il doit facilement libérer les bactéries dans le sol, se convertir aisément en poudre, se mélanger, s'emballer et coller à la graine. Nous avons formulé l'hypothèse que le biocharbon remplit les conditions de vecteur convenable. De plus, nous nous attendions à ce que le biocharbon soit utilisé avec succès comme matériel de pelliculage des semences. Les objectifs de cette recherche étaient donc de : 1) déterminer si le biocharbon peut servir de porteur pour des bactéries bénéfiques; 2) choisir le biocharbon qui soutient la viabilité bactérienne à la densité de population la plus élevée; 3) créer un système de pelliculage des semences qui pourrait être utilisé en agriculture; et 4) évaluer l'effet de l'inoculant de biocharbon et du pelliculage des semences sur la croissance des plantes. Deux bactéries, la *Pseudomonas libanensis* qui solubilise le phosphore et la *Bradyrhizobium japonicum* qui fixe l'azote atmosphérique, ont été choisies comme bactéries modèles. L'inoculant et le pelliculage des semences développés à partir du biocharbon *P. libanensis* ont

été testés sur des plants de maïs et ceux développés à partir de *B. japonicum* sur du soja. Les résultats de l'expérience de temps de conservation démontrent que ce ne sont pas tous les biocharbons qui peuvent être des porteurs adéquats, bien que deux porteurs sur quatre aient permis la survie de la bactérie *B. japonicum*, pour huit mois, à une densité de population suffisamment élevée pour assurer la nodulation efficace du soja. De plus, nous avons démontré que le biocharbon est un matériel adéquat au pelliculage des semences. Notre expérience de temps de conservation a démontré que le pelliculage de semences au biocharbon peut soutenir la survie des bactéries *P.libanensis* et *B. japonicum*, pour quatre mois, à une densité de population relativement élevée, bien que cette densité dépende fortement de la température de conservation. Nous avons aussi démontré que la raison principale du déclin rapide de la population de bactéries est liée au pH du vecteur. L'évaluation du taux de germination a révélé que le pelliculage des semences n'influe pas sur la croissance du plant et sur ses caractéristiques de germination. Finalement, des expériences en serre ont été menées pour évaluer les effets de l'inoculant et du pelliculage des semences développé à partir du biocharbon sur la croissance des plantes. Cette recherche a clairement démontré que le biocharbon peut être un porteur bactérien adéquat et peut remplacer la tourbe-mousse commerciale. L'inoculant rhizobien de biocharbon peut grandement améliorer la croissance du soja et réduire la demande en engrais d'azote. Finalement, le pelliculage des semences avec du biocharbon peut être considéré comme une façon simple et efficace de fournir des bactéries bénéfiques aux cultures.

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1. Introduction

Biochar is defined as a product of thermal degradation of organic based feedstock, through the process of pyrolysis, which requires elevated temperature and the absence of oxygen (Lehmann and Joseph, 2009). Growing interest in biochar is associated with its carbon sequestration ability. Biochar is a promising means of reducing the atmospheric CO_2 concentration because it slows the return of photosynthetically fixed carbon to the atmosphere (Xu et al., 2012). Furthermore, biochar has gained much attention as a soil amendment (Lehmann et al. 2006). Research has demonstrated that biochar application can significantly improve crop productivity (Chan et al. 2007); however, it strongly depends on climate and soil type. Many studies have shown positive effects of biochar on plant growth in tropical climates (Major et al. 2010); however, these have not been found in temperate regions. Therefore, an alternative, but not exclusive, use for biochar in temperate climates is using it as a carrier for beneficial microorganisms and applying it as a biofertilizer. In order for an ideal carrier to fulfill its function, it must possess appropriate characteristics. First it should be renewable, locally available and inexpensive; it should have good water holding capacity, good aeration properties, and sustain growth and survival of bacteria over time. Additionally, the right inoculant carrier should be non-toxic, environmentally friendly, easily manufactured, sterilized and manageable in the field (Khavazi et al. 2007). Moreover, it is important that the carrier be simply converted to powder, mixable and packageable and able to adhere to the seed. Finally, it should easily release bacteria into the soil (Smith 1992). One of the most commonly used carrier materials for commercial inoculants for the past half-century has been peat moss, particularly in legume inoculants. It is also used on a large scale in vegetable transplants production. However, we must not forget that peat moss is effectively a non-renewable resource and does not occur everywhere; in some areas its use has been limited by legislation, and if it is available its price is very high. In some countries that have programs to preserve wetlands, peat extraction is deemed inadvisable, if not forbidden (Temprano et al. 2002). Therefore it is necessary to find an alternative carrier that has the capacity to retain the viability of bacteria for long periods without loss of effectiveness. Many chemical and physical characteristics of biochar, such as high porosity, sorption capacity and high water holding capacity (WHC), might create suitable habitats for microorganisms, promoting their activities (Thies and Rillig, 2009).

World human population is likely to reach 9 to 11 billion during this century (Reid 2014). Modern agriculture faces a huge challenge to feed this number of people. Agricultural producers have increased yields mainly by increasing their applications of fertilizers, water, and pesticides, thereby doubling global cereal production over the past 40 years (Tilman et al. 2002). However, demands for food, particularly cereals, meat and oilseeds, as well as fiber, will grow as the human population increases during the coming decades. Doubling food production – again – and sustaining food production at the current level, in a way that would not compromise both short- and long-term environmental integrity and public health, will be a great challenge, which will be difficult to achieve without increasing fertilizers consumption.

In present days plant production is almost impossible without the application of mineral fertilizers. Fertilizers can increase productivity and result in better quality of products in agricultural systems (Savci 2012). However, the abuse of fertilizers, which is presently observed in modern, intensive agriculture, is the source of many undesirable effects on the environment, as well as undesirable effects on human health. Nitrogen and phosphorus are two essential mineral fertilizers for plant growth and development (Hameedaa et al. 2008). Learning how to reduce these inputs but, at the same time, sustain the sufficiency of nutrients for plant production should be prioritized.

Nitrogen is applied largely in the form of ammoniac fertilizers produced via the Haber-Bosch process (Smil, 2001). However it has been shown that only a small portion of applied nitrogen fertilizer is used directly by the plant (Savci et al. 2012). Furthermore, nitrate can cause the depletion of soil minerals, acidification of soil, and it can alter freshwater and coastal marine ecosystems (Peter et al. 1997). Excessive rates of N fertilizer can cause air pollution by emission of nitrous oxide, which is a very potent greenhouse gas. Water and food contaminated with nitrate can endanger human health by leading to methemoglobinemia, which is particularly dangerous for infants (Savci, 2012).

Phosphorus is the second most-used fertilizer in plant production. The global consumption of all phosphorus fertilizers surpassed 1 MMT (million metric tons) P yr^{-1} during the late 1930s and reached 14 MMT P yr^{-1} in the 1980s (Liu et al. 2008); moreover world phosphate fertilizer demand is expected to increase in the coming years. The biggest phosphorus-related problem for the agriculture industry is the immobilization of phosphorous in the soil. Applied phosphate is transformed into insoluble calcium, iron, or aluminium phosphates, which are unavailable for plants. On average, only about 15-20% of the total amount of phosphorus in a plant comes

directly from the fertilizers currently applied to the crop (Liu et al. 2008). Phosphorus is a non-renewable natural resource. The formation of phosphate rock is mainly due to diagenesis of phosphorus-containing sediments in freshwater and marine ecosystems, an extremely time consuming process of 10^8-10^9 years (Pierrou, 1976). Phosphorous is currently being mined at a rate of about 19.5 MMT P yr^{-1}. At this rate, the world's phosphate reserves could be exhausted in 120 years, with peak extraction occurring much sooner. Moreover, it has been projected that the utilization trend will probably increase at the rate of 0.7% to 1.3% annually (FAO 2000). This strongly suggests that phosphate rock, as a limited, non-renewable resource, may be effectively exhausted in the near future. Isherwood (2000) estimated that global reserves can sustain the current mining intensity for only another 80 years.

The overuse of nitrogen and phosphorus fertilizers is commonly observed in intensive agriculture systems, and this is not only environmentally problematic. It is also a problem which affects human health directly. These natural resources are being extracted at such a dramatically fast pace that our future generations are endangered by a potential food crisis. We urgently need to find a way to improve nitrogen and phosphorus fertilizer efficiency, and we need to minimize the loss of nutrient sources at the same time. To do so, intensive agriculture needs to apply more sustainable and pro-environmental practices in plant production. Biochar bacterial inoculants might be the way to sustain plant growth, increase soil fertility and finally decrease mineral fertilizers consumption without exhausting limited peat moss resources and compromising environmental integrity. Biochar bacterial seed-coating might facilitate the inoculants application and stimulate plant growth through nitrogen fixation and phosphorus mobilisation. Ultimately biochar inoculants might reduce demand for mineral fertilizers and thereby protect our environment from irreversible damages.

Therefore the main goal of this research is to determine whether biochar can serve as a suitable carrier for P-solubilizing bacteria, *Pseudomonas libanensis* and nitrogen fixing *Bradyrhizobium japonicum*, two beneficial bacteria which have the potential to reduce mineral fertilizer consumption. I will investigate for how long biochar can sustain the viability of cells. I will also attempt to create an efficient and optimal system of biochar and seed-coating inoculation, which could potentially be used in agriculture. Finally, I will evaluate the effect of inoculant on plant germination, growth and development.

2. Literature Review

1.1 Biochar

Biochar is not a modern invention. The incorporation of charcoal into the soil to enhance soil quality has been an agricultural practice for thousands of years (Xu et al. 2012). Pre-Columbian people left behind fertile soil that is rich in organic matter and nutrients today; it is known as *Terra Preta* - dark earth - soil of the Amazon, soil combined with charred residues of organic and inorganic wastes, such as excrements and household wastes (Van Zwieten et al. 2010). The oldest description of charcoal use in agriculture may be in the 17th century Encyclopedia of Agriculture by Yasusada Miyazaki, where he cited an even older textbook published in China. Rice husk charcoal has been used as a soil amendment, likely since the beginning of rice cultivation in Asia (Ogava and Okimori, 2010). Presently we use the term "biochar" to refer to a product of biomass pyrolysis, wherein plant-based materials are heated under anaerobic conditions to capture combustible gases. Originally, biochar production was associated with slow pyrolysis, characterized by a long period of time (more than 10 h) under relatively low temperature conditions. More recently, there has been growing interest in biochar production through fast pyrolysis, which is conducted quickly at a high temperature (Xu et al. 2012).

In the climate change era, biochar is a tool for agricultural resilience. The half-life of biochar in the soil is estimated to range from hundreds to thousands of years (Zimmerman 2010). Soil amendment with biochar is a strategy for long-term carbon sequestration.

1.1.1 Effect of biochar on soil proprieties

Many studies have demonstrated the positive influence of biochar on soil quality. Biochar application can enhance organic matter content in soil, and consequently improve soil fertility (Xu et al., 2012). Also, biochar has a positive effect on nutrient retention. Biochar reduces soil acidity and increases soil electrical conductivity and cation exchange capacity (CEC), which results in higher nutrient availability (Laird et al. 2010). The increase of soil pH by biochar application is a well-known mechanism for improving availability of such important nutrients as

phosphorus and potassium (Atkinson et al., 2010). Biochar has high total porosity. It can retain water in small pores and thus increase soil WHC or, after heavy rains, it can let the water flow from the topsoil, through the larger pores, to deeper layers (Asai et al., 2009). These effects may enhance water availability to crops and decrease erosion; thus it can be an important tool to manage water in agricultural production, particularly under water stress conditions. Artiola et al. (2012) report that biochar treatment significantly influenced Bermuda grass growth in a 1-month water-stress test where 100% of the control plants were killed. The plant survival rates of pots amended with 2 and 4% biochar were 50 and 100%, respectively. Biochar can reduce the availability of trace elements to plants. Namgay et al. (2010) found that concentrations of As, Cd and Cu in maize shoots significantly decreased owing to the application of biochar. However, the uptake of trace elements and their availability to the plant can vary, depending on the trace element and the rate of biochar application. Glaser et al. (2002) reported that biochar application reduced aluminium toxicity to plant roots and soil microbiota. Considering the problem of contamination of arable soils with heavy metals, mostly from mineral fertilizers, biochar shows the potential to remediate cultivated soils. Chan et al. (2012) provided some evidence showing that pine needle based biochar can be a very efficient tool in bioremediation of polycyclic aromatic hydrocarbons (PAHs) in contaminated soils. Sewage-sludge biochar decreased the plant-available Cu, Ni, Zn, Pb, the mobile forms of Cu, Ni, Zn, Cd and Pb, and also the risk of leaching of Cu, Zn, Ni and Cd. Freddo et al. (2012) reported that the concentrations of metals, metalloids and PAH in four plant-based biochars (bamboo, redwood, rice straw and maize) were lower than those reported as acceptable for sewage sludge and either lower, or in keeping with, those accepted for compost.

1.1.2 Effect of biochar on plant growth

There are a number of studies that report the positive influence of biochar on plant growth and development. Biochar can improve plant productivity in two ways: directly, as a result of its nutrient content and release characteristics; and indirectly, via improved retention of nutrients, increase in soil pH, CEC, soil water retention and alteration of soil microbial populations and functions (Graber et al. 2010). Biochar significantly improves soil fertility; it influences soil structure, texture, particle size distribution, porosity, and density (Xu et al., 2012). Uzoma (2011) reported that biochar application at a rate of 15 and 20 t ha $^{-1}$ increased maize

grain yield by 150 and 98%, respectively, under sandy soil conditions. Graber (2010) found that number of nodes and canopy dry weight of tomatoes treated with biochar amendment (0, 1, and 3 wt %) are significantly higher than the control. In the same experiment, he showed that tomatoes treated with biochar had more buds and, in the end, more fruit. What is more, he estimated that fruit weight, whole plant yield, and single fruit weight were all significantly higher. However, he did not report a difference between the two rates of biochar. Major et al. (2010) conducted a long-term (4 year) experiment in a Colombian savanna oxisol. Their study showed that biochar application effect is most visible in the third and fourth years. Maize yield did not significantly increase in the first year, but in the second, third and fourth years after application of biochar at a rate of 20 t ha $^{-1}$, maize grain yield increased over the control (28, 30 and 140%, respectively). Similarly, in other long term study, Jones et al. (2012) observed no crop improvement in the first year of application. However, in the second and third years after application of biochar, at rates of 25 and 50 t ha $^{-1}$, crop height and total dry biomass of grass, as well as foliar N, were significantly higher than the control. Plant growth can be directly affected by improved macro- and micronutrient uptake. Chan et al. (2007) showed that the concentrations of P, K and Ca in radish tissue increased significantly after applying biochar at a rate of 50 and 100 t ha^{-1}. They reported that the increase in P and K contents in the radishes that grew in biochar treated soil was related to high concentrations of available P and exchangeable K present in the biochar. Studies were conducted to investigate the effect of biochar on the germination process. Free at al. (2010) reported no significant effect of 5 different plant-based biochars on germination of maize in a paper towel assay. However, Solaiman et al. (2012) showed that biochar generally increased wheat seed germination at the lower application rates (10–50 t ha^{-1}) and decreased or had no effect at higher rates of application.

1.1.3 Biochar and fertilizer interactions

The effect of biochar and mineral fertilizer combinations has been studied. For example, Yamato et al. (2006) showed that an NPK-plus-charcoal amendment was more efficient than the control under field conditions. The fresh weights of harvested maize, cowpea and peanut crops were significantly higher following NPK-char amendment. In other research projects, biochar application at a rate of 10 and 40 t ha^{-1} increased rice yield by 12 and 14% in the Tai Lake plain, China. The same rates of application, in combination with N fertilizer, increased yield by 8.8%

and 12.1%, respectively (Zhang et al. 2010). Asai (2009) reported that grain yield of rice in northern Laos was significantly higher after the application of biochar at a rate of 4 and 8 t ha^{-1} in the presence of N fertilizer. Pot trials with romaine lettuce showed that fresh weight was significantly higher after biochar application (at a rate of 2 and 4% w/w) compared to the control; similar effects were seen as a result of pre-treatment with concentrated fertilizer solution. The authors speculated that this increase might be due to the storage and release of fertilizer chemicals by biochar particles. Similarly, Hossain (2010) observed 20% higher yields of cherry tomatoes owing to treatment with biochar with fertilizer, compared to the fertilizer treatment alone. In the same experiment, he showed 64% greater yield of cherry tomatoes under biochar treatment. A pot trial was carried out to study the effect of biochar that had been produced from green waste on the yield of radish (Chan et al. 2007). Three rates of biochar (10, 50 and 100 t ha^{-1}) with and without additional nitrogen application (100 kg N ha^{-1}) were studied. The soil used in the pot trial was a hard setting alfisol. In the absence of N fertilizer, application of biochar to the soil did not increase radish yield, even at the highest rate of 100 t ha^{-1}. However, a significant biochar × N fertilizer interaction was observed. For example, an additional increase in dry matter of radish, in the presence of N fertilizer, varied from 95% in the control to 266% in the 100 t ha^{-1} biochar amended soils (Chan et al. 2007). Schulz and Glaser (2012) investigated the effect of biochar on soil quality and plant growth under greenhouse conditions. They showed that the addition of biochar to sand can increase plant growth slightly. However, they observed a significant synergistic effect on plant growth when the biochar was combined with a mineral fertilizer or compost. These combinations have increased plant growth more than any increase due to pure biochar, compost or mineral fertilizer. Igarashi (1996) conducted a cultivation experiment for crops amended with rice husk charcoal. He applied charcoal with magnesium, phosphate, and lime, and rotated the crops soybean and maize. He reported that a charcoal application significantly increased plant growth, root nodulation and yield. The effect was sustained in the second crop of maize, and was observed continuously up to the tenth crop rotation. He also reported that the growth and yield of maize treated with charcoal were greater than those of the control treatment, which had been cultivated only with mineral fertilizers. All of the examples presented above highlight the capacity of biochar to improve the fertilizer use efficiency of crop plants. Some authors (Asai et al. 2009) speculate that the fertilizer efficiency improvement might be associated with the capacity of biochar particles to retain and slowly release fertilizer chemicals. Others (Chan et al. 2007; Lehmann et al. 2003)

attribute the fertilizer use efficiency to improved physical soil conditions or reduced fertilizer run-off as a result of biochar application. Although there is some speculation regarding the mechanism of this process, it is not completely understood, and there is still a need for more research.

1.1.4 Biochar and plant resistance

Several studies provide evidence that biochar-amended soils have an impact on plant resistance to pathogens. Matsubara et al. (2002) demonstrated that charcoal amendments had a suppressive effect on the soil borne pathogen *Fusarium* sp. They found that charcoal produced from coconut fiber suppressed fusarium crown and root rot, and increased arbuscular mycorrhizal (AM) colonization of asparagus seedlings. The possible ability of biochar to affect plant systemic resistance responses against disease microorganisms has been studied in several systems involving foliar pathogens. The intensity of diseases caused by necrotrophic (*Botrytis cinerea*) and biotrophic (*Oidiopsis sicula*) foliar pathogens in pepper and tomato (Elad et al. 2010) was significantly lower in wood-derived biochar application treatments. Reduced damage by broad mite (*Polyphagotarsonemus latus*) in biochar-amended pepper plants was also observed (Elad et al., 2010). Meller Harel et al. (2012), in an experiment on strawberry, showed that biochar application results in suppression of *Podosphaera aphanis, B. cinerea* (powdery mildew pathogen) and *Colletotrichum acutatum* on the leaves of plants.

1.1.5 Effect of biochar on microbial communities

Soil microbial ecosystems and their function are maximized under good soil conditions, including good soil fertility. The most important services provided by soil microbiota are decomposing organic matter, cycling and immobilizing inorganic nutrients, and bioremediation of contaminated soils. Some bacteria have the capacity to suppress plant pathogens and disease; they influence soil porosity, aggregation and water infiltration (Paul 2007). Another very important feature of soil microorganisms is their interaction with the rhizosphere. Some bacteria strongly influence the ability of plants to take up macro- and micro-nutrients. This might be a direct effect of mutualistic relations between bacteria and plant roots. Undeniably, soil microbiota influence soil quality and, as a consequence, crop growth and yield.

The environmental factors which most significantly influence bacterial abundance, diversity and activity are moisture, temperature and hydrogen ion concentration (pH) (Wardle 1998). The pH of biochar depends on feedstock as well as type of pyrolysis conducted during biochar production. However, as was shown before, in most cases biochar might be considered to be a good tool to increase the soil pH. Most bacteria prefer neutral pH, therefore it might be expected that adding biochar to the soil significantly influences bacterial populations. This may also apply to moisture; because of the high WHC of biochar, it retains water and, at the same time, creates suitable and more stable habitats for bacteria. The biochar high WHC can be considered as suitable protection for microorganisms against desiccation. Moisture is the main factor crucial to the higher survival rate of microbial inoculants (Lehmann et al. 2011). Biochar may retain moist pore spaces that allow continued hydration of microorganisms in a drying soil.

A number of research efforts in Japan, as well as recent investigations from the US, have shown that biochar supports the activity of many soil microorganisms important to agriculture. A recent review of biochar and its effect on soil biota (Lehmann et al. 2011) provides considerable evidence that application of biochar to the soil has significant effects on micro-organisms. Moreover, most published studies show positive influences of biochar-amended soil on microbial biomass.

Biochar and its highly porous structure can provide a suitable habitat for many microorganisms by protecting them from predation and desiccation, providing carbon (C), energy and mineral nutrient needs (Saito and Muramoto 2002). Presently there is a lack of quantitative evidence regarding the hypothesis that bacteria are better protected against grazers and competitors by colonization of biochar pores. However, pore size distribution of microorganisms and biochar, as well as visual observations, weaken this hypothesis (Thies and Rillig, 2009). The increase in bacterial abundance might be associated with sorption to the biochar surface, which prevents microbial leaching and thus stabilizes the population (Pietikäinen et al., 2000). Bacteria might be attached to biochar particles in several ways: flocculation, adsorption on the surface, covalent bonding to carriers, cross-linking of cells, encapsulation in a polymer gel or entrapment in a matrix (Lehmann et al., 2011) (Fig. 1).

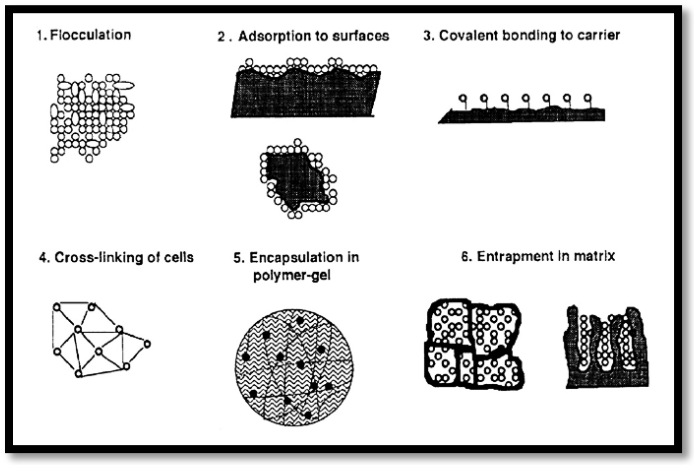

Figure 1.1 Possible attachments of viable microbial cells to biochar surfaces (Cassidy et al, 1996).

Adhesion of bacteria to a biochar surface might depend on hydrophobic attraction, electrostatic forces and biochar properties such as ash content, pore size, and volatility (Lehmann et al. 2011). However, to understand this process better, more studies are required.

Biochar is also considered to be suitable protection for microorganisms against desiccation. Seasonal drying of soil leads to stress and, in effect, dormancy or mortality of bacteria, with significant differences between gram-negative and gram-positive bacteria (Schimel et al., 2007). Because of the large surface area of biochar (Downie et al., 2009) and greater WHC after addition to light-textured soils (Asai et al., 2009), biochar may retain water in pore spaces that allow for the continued hydration of microorganisms in a drying soil.

A number of investigations provide evidence that biochar influences the composition of soil bacterial communities. It is commonly accepted that introduction of biochar changes the physicochemical properties of soil and provides metabolically available sources of carbon (C), which may result in shifts in soil microbial community structure. Terminal restriction fragment length polymorphism (TRFLP) studies conducted by Anderson and colleagues (2011) show that biochar treatment enhanced the abundance of *Bradyhrizobiaceae* (~8%), *Hyphomicrobiacea* (~14%), *Streptosporangiaceae* (~6%) and *Thermomonosporaceae* (~8%). There were negative effects of biochar on the *Streptomycataceae* (~-11%) and *Micromonosporaceae* (~-8%) families. Increases in N_2-fixing rhizobia were recorded in the rhizosphere whereas N_2-fixing *Frankiaceae* increased in both bulk and rhizosphere soil amended with biochar. The authors conclude that biochar treatment potentially enhances the growth of bacteria involved in N cycling in the soil, particularly of those which may decrease the flux of N_2O. In addition, they speculate that

biochar might play an integral role in supporting the proliferation of and interaction between these bacteria, possibly because of easily available C-compounds. A similar study conducted by Kolton et al. (2011) shows that the genus most significantly affected by biochar treatment (3% wt/wt) was *Flavobacterium*. The total relative abundance of this group was 4.2% of total genus-defined root-associated operational taxonomic units (OTUs) in the control treatment and 19.6% in biochar-amended soil. Simultaneously, there was a decrease in relative abundance from 71 to 47% for the genus *Proteobacteria*. The authors conclude that biochar-amended soil led to important changes in the root-associated microbial community, characterized by induction of several chitin- and aromatic compound-degrading genera. Ameloot et al. (2013) suggest that the temperature of pyrolysis is an important influence on the microbial community. In their research, they observed a correlation between Gram-negative bacteria and low temperature pyrolysis biochar. They report a high abundance of Gram-negative bacteria in a low 350°C biochar treatment and increases in Gram-positive bacteria in all types of biochar except that produced at 700°C and made out of swine manure. Abit et al. (2012) showed that biochar pyrolyzed at high temperature (700°C), and made out of pine chips, significantly reduced transport of *E. coli* through a fine sandy soil under water-saturated and partially saturated conditions when compared to the control, in column experiments. The authors explain that the difference between biochar types is due to differences in pore size distribution for biochars produced from different feedstocks. They suggest that incorporation of high temperature biochar made out of plant-based feedstocks to the soil might be a potential method for reducing microbial movement through soils, and it might be considered as a management practice for protecting shallow groundwater from pathogenic microorganisms. Luo et al. (2013) provide the evidence that plant-based biochar pyrolyzed at low temperature (350°C) increases microbial colonization at high soil pH compared to biochar pyrolyzed at 700°C. They speculate that it might be because the availabilities of C, N and other nutrients were low in 700°C biochar, as most were lost during pyrolysis. They also speculate that lower colonization of the 700°C biochar might be because biochar produced at higher temperatures is characterized by fewer structured fragments and finer pores, which results in fewer physical niches for bacteria.

Probably one of the first research efforts showing that biochar might serve as a medium for bacteria was conducted by Gorelick et al (1951). Their investigation showed that an unusually high concentration of *Brucella suis* bacteria can be obtained by cultivation in a charcoal-cellophane system. However, the stimulatory effect of the charcoal in cellophane cultures exists

only during the active growth of the microorganisms. Over 20 years ago, Beck (1991) proved that charcoal mixed with soil in a ratio 1:3 supported the growth of rhizobia to the point that it could easily replace commercially used peat moss. Saranya et al. (2011) showed that acacia wood and coconut shell based biochar can be used as a superior alternative carrier material to lignin for the preparation a microbial inoculant. The results of the experiment demonstrate that *Azospirillum lipoferum* is able to survive in a biochar carrier as long as in commercially used lignin (180 days). Moreover, in the case of coconut shell based biochar, the percent reduction in the original population of bacteria is lower than for lignin, 14.22 and 17.37% respectively. Viveganandan and Jauhri (2000) investigated charcoal-soil (3:1), calcium alginate, and charcoal-soil plus calcium alginate as a carrier for the phosphate-solubilizing bacteria *Pseudomonas striata* and *Bacillus polymyxa.* Storage time experiments show that all three carriers support the survival of bacteria at relatively high levels. However, the bacterial population was slightly higher in calcium alginate than in the other two carriers.

The literature review presented above suggests that biochar is a material that significantly affects soil quality, plant growth, fertilizer efficiency and finally, microorganism dynamics. In general, most of the studies reveal positive or neutral influence of biochar on these bio components. Many of the chemical and physical properties, as well as some previous studies, suggest that biochar can be used as an alternative for peat moss and lignin as an inoculant carrier.

1.2 Seed-coating

The first trials with inoculation of seeds with rhizobia come from the end of the nineteenth century (Voelecker, 1896). Seed-coating technology has developed rapidly over the past two decades, and has resulted in new economical approaches to seed improvement. Interest in seed-coating technique has been observed, particularly, for the most important agronomic and horticultural crops (TeKrony, 2006). Seed coating is a technique that might combine several components, such as nutrient elements, fertilizers, plant growth regulators and pesticides at much lower rates than the traditional application, by applying them to seed with adhesive agents to increase seed performance (Zeng and Zhang, 2010). Very few studies have been conducted on seed coating with bacteria. Microbes are living organisms, which is why seed-coating with

inoculated carriers is a very complex process, requiring suitable moisture, temperature and nutrient content to keep the bacteria alive for a sufficiently long period. However, treated seeds might be a simpler and more efficient technique than most currently available commercial inoculants, which need to be applied and mixed with the seed by the farmer, immediately prior to planting. Vidhyasekaran and Muthamilan (1995) treated chickpea seeds with a talc-based formulation of three *P. fluorescens* strains (control of chickpea wilt). The results show that field emergence, chickpea yield and suppression of disease were improved by seed treatment. Temprano et al. (2002) investigated the survival of a *B. japonicum* strain in peat inoculant on *Lupinus albus*. The studies revealed that the bacterial dynamic was comparable in both the seed and the powdered carrier. A decline of one order of magnitude was recorded, with respect to the initial population; one month after inoculation, viable-cell count was still high enough to ensure good nodulation (10^6 bacteria $seed^{-1}$). The same authors studied the influence of storage temperature on survival of bacteria on the seed-coat. They report that, in the case of peat and vermiculite carriers, survival of rhizobia is higher when stored at 4 °C than at 28 °C. However, the perlite carrier supports the survival of bacteria at a higher level at 28 °C. Gaind and Gaur (1990) explain that lower temperatures (4°-10°C) retard division and metabolic activities of bacterial cells, which results in a reduced consumption of nutrients and reduced loss of moisture in the carrier, and thus favours the storage of inoculants. Environmental temperature is one of the important factors that affect the population of phosphate-solubilizing bacteria in inoculants during storage. Higher temperatures result in greater losses of moisture from inoculants, thereby reducing the phosphorus solubilizing bacteria (PSB) population.

There are very few studies regarding the influence of seed-coating on the germination process. O'Callaghan et al. (2006) showed that the germination rate of onion seeds coated with biopolymer gel and *Pseudomonas fluorescents* F113 tended to be lower than that of untreated seeds. Similarly, Slinginger et al. (1996) reported that seeds inoculated with *P. fluorescents* 2-79 formulated in methylcellulose suffered significant germination reductions. The authors attributed this decrease in germination to phytotoxic metabolites produced by the bacteria. Also, the decrease in germination of coated seeds could be explained by the physical barrier to the radical and hypocotyl of the seed. However, Zeng and Zhang (2010) found that seed-coating (without bacteria) does not inhibit the germination process; on the contrary, single plant fresh weight, germination energy, germination percentage, germination index and vigor index of coated seeds was significantly higher than in uncoated seeds. Likewise, Gholami et al. (2009)

found that maize seeds coated with gum arabic as an adhesive and rolled in a suspension of bacteria (PGPR) with perlite significantly increase the seed germination and seedling vigour of maize. Therefore, the hypothesis that seed-coating might be a mechanical barrier cannot be confirmed.

Testing a seed-coating system with bacteria *in situ* can probably provide the most reliable data and a full image of the functionality of this technique. Very few studies have been conducted so far; however, some of them report that coated seeds perform better than control plants in field experiments. For example, Gholami et al. (2009) showed that seeds treated with a talc-based formulation with PGPRs resulted in better yield than untreated seeds. They reported that plant height, leaf area, seed dry weight, 100-seed weight and shoot dry weight were significantly higher for coated seeds than for controls. Similarly, Alagawadi and Gaur (1988) showed that seeds inoculated (soaked in liquid culture) with rhizobia or *Pseudomonas straiata* produced more dry matter, grain and straw yield of chickpea than untreated seeds. Moreover, the combined inoculation of these two bacteria gave even better results.

1.3 *Pseudomonas libanensis* – characteristics and influence on plant

Pseudomonas libanensis is a gram-negative, non-spore-forming rod, strict aerobe, considered a plant growth promoting rhizobacteria (PGPR). PGPR are the group of bacteria that actively colonize plant roots and by a number of different actions, have the capacity to increase plant growth and yield (Gholami et al. 2009). Some of the mechanisms by which PGPR's promote plant growth include: asymbiotic N_2 fixation, the ability to produce phytohormones, the synthesis of antibiotics, enzymes and/or fungicidal compounds, and also solubilisation of mineral phosphates and other nutrients. *Rhizobium*, *Pseudomonas* and *Bacillus* are considered the most powerful solubilizers of bound soil phosphorus (Rodrigiuez et al. 1999). A number of studies have shown that *Pseudomonas* bacteria can significantly improve growth and yield of agronomically important crops in response to inoculation. For example, Glick et al. (1997) reported that *Pseudomonas putida* GR12-2 can increase root and shoot elongation in canola during the first month of growth. Weller and Cook (1986) inoculated winter wheat with

fluorescent pseudomonads and obtained yield increases of 27% in field trials. Also, Freitas and Germid (1992) investigated the colonization capacity and plant growth promoting actions of several *Pseudomonas* strains on winter wheat. The results showed that *P. cepaciu* MR85 and *P. putida* MR111 increased grain yield by 6 and 11%, respectively. Inoculant populations in the wheat rhizosphere were controlled throughout the growing season and reached levels of 10^4 - 10^8 cfu g^{-1} of roots. Moreover, the results showed that these inoculants colonized winter wheat roots and survived in the rhizosphere even over the winter. Gholami et al. (2009) investigated the effect of six bacteria, including *Pseudomonas* strains *P. putida* R-168 and DSM298, and *P. fluorescens* DSM 50090 and R-93 on germination, seedling growth and yield of maize. The experiments proved that seed inoculation enhanced seed germination and seedling vigour. The rate of increase varied with bacterial strains; however, all bacteria increased seed germination up to 18.5% over the untreated control. Also, shoot and leaf dry weight, leaf area, and 100-seed weight were significantly enhanced by bacterial treatment. The experiment also showed that bacterial inoculation had a more stimulating effect on growth and development of plants in non-sterile soil than under sterile conditions. Several studies have been conducted to examine survival of various strains of *Pseudomonas* in a range of carriers. For example, O'Callaghan et al. (2006) studied the biocontrol strain *Pseudomonas fluorescens* F113. Onion seeds were coated with bacteria using various formulations of biopolymer gels. The experiment was conducted up to 70 days after inoculation. A result showed that shelf-life of environmentally-sensitive (particularly to temperature and desiccation) bacteria is relatively short and strongly depends on storage temperature as well as type of packaging seeds were kept in. However, the authors reported stable bacterial survival (with an average1.4 x 10^8 CFU/g seed) after 70 days under 4°C conditions. Vidhyasekaran and Muthamilan (1995) examined 6 different carriers for *Pseudomonas fluorescens* including talc, vermiculite, kaolinite, lignite, peat and farmyard manure. In all of them, bacteria were able to survive more than 120 days with an average 11-2.8 x10^7 CFU g^{-1}. Moreover, the authors reported that the most suitable carrier supporting the survival of *P. fluorescens* is talc, where bacteria survived at 1.3 x 10^7 CFU g^{-1} for up to 240 days.

Biochar is a rich source of carbon and it can retain other nutrients, and possibly provide suitable protection and habitat; therefore it is expected that it will support *P. libanensis* viability and growth. Lehmann et al. (2002) reported that phosphate is easily adsorbed by the biochar surface. This could suggest that bacteria might have easier access to inorganic phosphorus, and potentially solubilize it more effectively when biochar is present.

1.4 *Bradyrhizobium japonicum* – characteristics and influence on plants

Bradyrhizobium japonicum is a facultatively anaerobic soil bacterium which has the capacity to form nodules and fix nitrogen in the rhizosphere of the agronomically important legume soybean. Inside the nodules, *B. japonicum* fixes atmospheric N_2 in symbiosis with the plant. In this environmentally sustainable process, the bacterium is providing N to soybean crops in a way similar to applying mineral fertilizer (Quelas et al. 2006). Symbiotic nitrogen fixation is a complex process involving both plant and bacterial physiological and biochemical aspects. The soybean plant (host) secretes flavonoids which lead to the expression of *nod* genes in *B. japonicum*. Subsequently, bacteria produce Nod factors (lipo-chitooligosacharides), which serve as a signal for the host plant and incite the process of root hair curling. Bacteria form infection threads which move towards the nodule primordium in the root cortex. Afterwards, bacteria are released into the cytoplasm where they differentiate into bacteroids, and nitrogen fixation in the nodule begins (Sorensen and Sessitsch 2007). Under short season conditions, soybean plants in symbiosis with *B. japonicum* can fix 100 to 200 kg ha^{-1} yr^{-1} of nitrogen from the atmosphere. Soybean is a subtropical legume which requires a 25 to 30 °C root zone temperature (RZT) for optimal symbiotic nitrogen fixation. However, during the first month of the growing season in Canadian soybean culture areas, temperature is often below 15°C and until July, it might be lower than 20°C (Zhang and Smith, 1996). Low RZTs negatively influence all stages of nodulation as well as nodule functions (Lynch and Smith, 1993). Another problem can be low levels of highly effective rhizobia in many soils. That is why seed inoculation has gained more attention in recent decades. In the American Midwest, attempts to improve the yield of soybean by inoculation with highly efficient strains of *B. japonicum* have usually failed. In soils that contain resident populations of bradyrhizobia, inoculant strains usually represent only 5 to 20% of the bacteria in the nodules formed (McDermott and Graham, 1989). A number of strategies have been applied to improve nodule occupancy, but these have met with limited success. Several factors influence the ability of inoculated rhizobia to nodulate. Depending on the geographic region and climate, as mentioned above, it might be the root zone temperature that limits nodulation and nitrogen fixation. Many tropical soils have low pH and high soil temperature, which limits the survival of applied rhizobial cells (Owiredu and Dansot, 1988).

Some studies (Kamicker and Brill, 1987) indicate that the movement of inoculant rhizobia in the soil is limited. Salema et al. (1982) showed that relatively few rhizobia from the seed coat come in contact with and are dislocated to the emerging radical. The authors explain that it might be because of rapid extension of the root system and possible further dilution of inoculant cells in the infectible root zone. The results of a study by McDermott and Graham (1989) support this concept. Their investigation shows that inoculant strain number in the rhizoplane fell dramatically with increasing distance from the point of inoculation. However, there is some research demonstrating that movement of bacteria in the soil depends on many factors. Breitenbeck et al. (1988) performed a study to assess the influence of percolating water and an advancing wetting front on the transport of *Bradyrhizobium japonicum* in sand and silt loam soils. The data obtained showed that percolation with the equivalent of 10 cm of rainfall dispersed *B. japonicum* throughout 40-cm columns containing sand and silt loam soils. Percolation with 5 cm of water was sufficient to disperse bacteria throughout 20-cm columns of the same soils. This investigation shows that cells of *B. japonicum* are readily transported by an advancing wetting front and indicates that non-saturated flow of soil water contributes to dispersal of inoculum in soils.

Although there are a number of factors which reduce the effectiveness of *B. japonicum* inoculants, there are a number of research investigations providing information about positive impacts of inoculants on soybean productivity. For example, Hume and Shelp (1990) investigated the response of soybean seed yield to a variety of *B. japonicum* strains as inoculants in several years of field trails. The results showed that strain 532C resulted in average yields of 3.08 t ha^{-1} compared to 2.70 t $ha^{-1}$for strain 61A89, 2.84 t ha^{-1} for 61A133, 2.83 t ha^{-1} for a commercial inoculant, and 1.96 t ha^{-1} for the uninoculated control. The highest number of nodules per plant was observed in the treatment with strains 61A133 and 61A148. Owiredu and Dansot (1988) observed significantly higher nodulation, N content in plant tissue and dry matter yield of shoots after inoculation with *B. japonicum* strains SRJ-S, SX-S or Sl4-S at the rate of 10^5 cells g^{-1} soil compared to an uninoculated control. Daramola et al. (1994) conducted a number of experiments on *B. japonicum* effective strain 61A124a and ineffective strain THA I in various applications (on seed, into the soil). The results demonstrated higher nodule numbers as well as nodule fresh weight when an effective strain was inoculated directly into the soil and an effective strain was applied to the soil and ineffective strain on the seeds. However, the highest dry matter yield was noted in treatments with seeds inoculated with an effective strain. Another

field investigation (Zhang et al. 2003) showed the positive effect of *B. japonicum* inoculant on soybean culture in a short season area. Three inoculants were syringe-applied directly onto the seed along the furrow: 532C, USDA 30 and USDA 31. All of these showed superior nodule number, nodule weight and shoot nitrogen yield compared to a control. Biochar is expected to provide suitable habitat, protection from predation and desiccation and provide nutrients needed for survival of *B. japonicum*, and thereby establish high enough bacterial populations in the rhizosphere to be able to compete with other indigenous soil bacteria.

3. Research Question and Objectives

The main research question of this Master's project is: *Can biochar serve as a suitable and efficient bacterial inoculant carrier?* I hypothesise that biochar can provide suitable habitat for bacteria, serve as an efficient inoculant carrier for phosphorus solubilizing and nitrogen fixing bacteria, and finally that biochar inoculant and seed-coating can improve plant growth. I also hypothesise that biochar chemical and physical proprieties can provide an appropriate habitat for bacteria, and sustain survival and growth of bacteria over time. It was expected that biochar can be successfully used as seed-coating material. The whole project is divided into two main parts. The first part focuses on the suitability of biochar for phosphorus solubilizing bacteria, development of *Pseudomonas libanensis* inoculant and evaluation of its influence on corn growth. The second part investigates whether biochar can be appropriate for *Bradyrhizobium japonicum* inoculant and how it affects soybean growth. In order to answer the principal question, the following objectives were formulated for both parts:

1. To determine chemical and physical properties of specific biochars.
    - In particular, I was interested in characterising properties which might affect bacterial viability, such as porosity, elemental composition, water holding capacity and pH.

2. To evaluate how long biochar will sustain viable bacterial cells in powdered biochar as well as in seed-coating and what will be the abundance of the population after various incubation periods.
3. To test seed-coating treatment effects on germination.
    - The specific objective was to determine whether seed-coating negatively affects the germination process or inhibits seedling growth and development.
4. Finally, I wanted to evaluate how seed treatment, as well as applying biochar inoculants, will affect plant growth.
    - I was interested in determining how *Pseudomonas libanensis* inoculant and seed-coating affect the growth and nutrient uptake of corn.
    - I also wanted to see the influence of *Bradyrhizobium japonicum* inoculant and seed-coating on soybean growth and nodulation.

Preface to Chapter 4

Chapter 4 describes the ability of *Pseudomonas libanensis* to solubilize inorganic phosphorus. It provides procedures for biochar characterisation, inoculation and incubation, as well as seed coating techniques, which are also used in Chapter 5. The main focus of this chapter is on biochar and how it sustains bacterial viability. A germination assay and greenhouse experiment demonstrated how *P. libanensis* inoculant and seed-coating affect corn growth, development and nutrient uptake. This was accomplished using a combination of classical microbiological approaches and standard plant bioassays, under controlled growth conditions. Chapter 4 is co-authored by the candidate's supervisor Dr. Donald L. Smith and lab member Timothy Schwinghamer. Chapter 4 will be submitted to *Applied and Environmental Microbiology* for publication.

4. Biochar as an inoculant carrier for the novel phosphate solubilizing bacterial strain *Pseudomonas libanensis* and its potential to improve corn growth

4.1 Abstract

Considering that peat moss, which is presently used as a standard inoculant carrier, is non-renewable resource, and its price is expected to increase in coming years, there is an urgent need to find alternatives to it. In this study I suggest biochar as a potential inoculant carrier. Due to its chemical and physical characteristics and availability, we believe that biochar can take the place of peat moss. In this investigation we evaluate 4 different biochars, in comparison with peat moss, as inoculant carriers for novel phosphorus solubilizing bacteria strain, *Pseudomonas libanensis*, and the influence of the developed inoculant on the growth of the agriculturally important crop corn. The results showed that Dynamotive (DM) and Pyrovac (PR) biochars sustained the viability of the bacteria at a higher level than peat moss (PM) and other biochars. Also, in a seed-coating system with DM and PR biochars, which we developed, bacteria tend to survive much longer than in other carriers. Moreover, results showed that seed coating positively affects germination of corn; however, no significant differences were found in subsequent plant growth.

Key words: biochar, inoculant carrier, *Pseudomonas libanensis*, phosphorus solubilization, *Zea mays* L,

4.2 Introduction

Peat moss is the most common and widely used substrate for potted plant production. Most growing substrates contain significant amounts of peat. Because of its properties, such as a good WHC, appropriate consistency and high porosity, peat is also recognized worldwide as a carrier for most of the commercially available bacterial inoculants (Eudoxie and Alexander 2011). However, peat is a natural and effectively non-renewable resource, and its overuse is of great environmental concern. Moreover, rapidly decreasing reserves of peat have led to price increases which will ultimately limit its use (Tariq et al. 2012). Over the past few years, researchers have been looking for worthwhile and efficient alternatives to peat as an inoculant carrier and component of growth media for potted plants.

Due to its chemical and physical characteristics such as high porosity and WHC, as well as elevated concentrations of some nutrients, including organic carbon, and its general availability, biochar can be considered as a potential inoculant carrier. Biochar refers to a product of biomass pyrolysis. Many studies have demonstrated positive effects of biochar on soil quality and plant growth (Xu et al., 2012; Graber et al. 2010; Major et al. 2010); however, there have not been as many investigations looking at biochar–microbe interactions, and almost none evaluating biochar as an inoculant carrier.

Pseudomonas libanensis, a phosphorus solubilizing bacteria isolated from the soybean rhizosphere, was used in this study. It is considered to be a plant growth promoting rhizobacteria (PGPR), because of its ability to solubilize inorganic phosphorus, make it available and thereby stimulate plant growth. Phosphorus (P) is one of the essential macronutrients for plant growth and development (Hameedaa et al. 2008). In the soil, phosphorus occurs at levels of 400-1200 ppm. Microorganisms play a substantial role in the natural phosphorus cycle. Most agricultural soils contain large reserves of phosphorus. However, the concentration of soluble P in soil is very low, normally at levels of 1ppm or less. A considerable part of this macronutrient has accumulated as a consequence of regular application of P fertilizers (Goldstein, 1994). A large part of soluble inorganic phosphate applied to soil as a chemical fertilizer is immobilized shortly after application and becomes unavailable to plants. It is mostly because many P compounds are high-molecular weight materials, which must first be bio-converted to either soluble ionic phosphate (Pi, HPO_4^{2-}, $H_2PO_4^-$) or low molecular weight organic phosphate in order to be

assimilated by the plant (Goldstein, 1994). It is commonly accepted that the major mechanism of mineral phosphate solubilisation is the action of organic acids synthesized by soil microorganisms (Banik and Dey, 1982). Moreover, some PGPR can convert insoluble phosphate into soluble forms through the processes of acidification, chelation and exchange reactions (Chung et al. 2005). Several phosphate solubilizing bacteria occur in soils; however their numbers are usually not high enough to compete with other bacteria commonly established in the rhizosphere. Also, the amount of P liberated by these bacteria is generally not sufficient for a significant increase in plant growth. In consequence, inoculation of plants with target microorganisms at significantly higher concentrations than normally found in soil is needed to take advantage of the phosphate solubilizing properties of bacteria to improve plant growth and enhance the yield.

Corn (*Z. mays* L.) was selected for this study as an experimental plant because of its importance as a crop and its high phosphorus demand. *Zea* is a genus of the family *Graminae* (*Poaceae*), commonly known as the grass family. Maize is a tall, monoecious annual C_4 grass. It is used mainly for human consumption and animal feed. Although corn comes originally from central Mexico, the majority of production is located in temperate climates. Maize has been cultivated by the indigenous peoples of North America, including Canada, for thousands of years. Corn is presently the most important of all crops in terms of total mass and grain production. Global maize production increased from 265 million tonnes (Mt) in 1970 to 844 Mt in 2010, which places it in first position in global grain production next to rice (696 Mt) and wheat (654 Mt) (Long et al. 2013, FAO 2012). Corn is one of the major crops cultivated in Canada. The production of grain increased from 663 gigatonnes (GT) in 1960 to 1420 GT in 2013. In 2013 in Canada, 1,475,079 acres were under corn production. In Québec alone, farmers planted 4,046.85 acres of corn for grain (Statistics Canada, 2013).

The present investigation was undertaken with the aims to: 1) determine whether biochar can serve as a suitable carrier for *Pseudomonas libanensis*; 2) create a seed-coating system with biochar and *P. libanensis*, which could potentially be used in the agriculture industry to improve P availability; 3) evaluate the potential of *Pseudomonas libanensis* to solubilize inorganic phosphorus; and 4) evaluate the effect of the inoculum and seed treatment on corn growth.

4.3 Materials and Methods

4.3.1 Biochar source, characterization, and carrier preparation

Four types of biochar, two produced from hardwood feedstocks by Dynamotive, West Lorne, Canada (DM), Basque, Rimouski, Canada (BQ), and two produced from softwood feedstocks by BlueLeaf, Drummondville, Canada (BL), and Pyrovac, Saguenay, Canada (PR), were selected for examination as potential carriers for bacteria inoculants. Peat moss, PRO-MOSS TBK, Rivière-du-Loup, Canada (PM) was used as a standard inoculant carrier. WHC, as well as the pH of each material was determined (Table 4.1). All biochars were subjected to complete elemental analysis, as well as particle size and composition analysis, which were performed by the Soil Control Laboratory in Watsonville, California (Table 4.2). Additionally, porosity analyses were performed by Micromeritics Analytical Services Lab, Norcross, Georgia (Table 4.1).

Two hundred grams of each material were oven-dried at 75°C for 3 consecutive days, finely powdered and passed through a 500 µm sieve. Afterwards, material was placed in autoclavable polypropylene bags and sterilized for 3 consecutive days at 121°C for 20 minutes each time.

4.3.2 *Pseudomonas libanensis*: culture and phosphate solubilisation

Source of bacteria culture

Pseudomonas libanensis, a phosphate solubilizing strain, was isolated from the soybean rhizosphere through work conducted in Professor Donald Smith's laboratory, Department of Plant Science, Macdonald Campus, McGill University, Ste Anne-de-Bellevue, Québec, Canada.

Preparation of broth culture and incubation of Pseudomonas libanensis

The *Pseudomonas libanensis* strain was grown on King's B (KB) medium (containing: 20 g peptone, 1.5 g $MgSO_4$, 1.5 g K_2HPO, 10 mL glycerol per 1 L of distilled water) with the pH

adjusted to 7.0. KB medium was sterilized for 20 minutes at 121°C, cooled and inoculated with bacteria. The culture was incubated in a gyratory shaker for 7 days at 25°C in darkness. After 7 days of incubation, a log-phase culture of *Pseudomonas libanensis* was plated onto Petri plates to evaluate the condition and viability of the population. The cell count of the broth culture was performed and CFUs were determined.

Phosphate solubilisation in solid media

The capacity of the *Pseudomonas libanensis* strain to solubilize inorganic phosphate in a solid medium was determined in a Petri plate assay according to the method of Nautiyal (1999). National Botanical Research Institute's phosphate growth (NBRIP) medium was prepared: per L dH_2O and the pH was adjusted to 7.0: glucose, 10g; $Ca_3(PO4)_2$, 5g; $(NH_4)_2SO_4$, 0.5g; $MgCl_2 \cdot 6H_2O$, 5g; $MgSO_4 \cdot 7H_2O$, 0.25g; KCl, 0.2g; $(NH_4)_2SO_4$, 0.1g; 1.5% Bacto-agar. Five µL of bacteria culture in the late-log phase (4.9×10^9 CFU/mL) were stabbed into the medium in triplicate using sterile pipette tips. The Petri plates were incubated for 14 days at 25°C in darkness. The halo and colony diameter (mm) was measured using Vernier calipers. Halo size was calculated by subtracting colony diameter from the total diameter.

Phosphate solubilisation in liquid media

Quantitative estimation of phosphate solubilisation in broth was carried out according to Nautiyal (1999) in Erlenmeyer flasks (150 mL) containing 100 mL of NBRIP medium with the pH adjusted to 7.0 with tricalcium phosphate ($Ca_3 (PO_4)_2$). One mL of the *Pseudomonas libanensis* strain (in the range of 10^8 CFU mL^{-1}) was added to the flask; the control flasks remained uninoculated. The experiment was performed in triplicate. The flasks were incubated for 5 weeks at 25°C on an orbital shaker (Forma Scientific, 4530 S/N) at 100 RPM. After 1, 7, 14, 21, 28 and 35 days of incubation, 10 mL of aliquots of cultures were aseptically taken from each flask. The cultures were harvested by centrifugation at 10,000 RPM for 10 min. Phosphate in culture supernatant was estimated using the Murphy and Riley (1962) method with ascorbic acid and ammonium molybdate. Samples were analysed by spectrophotometer (UV/Visible Spectrophotometer, Ultraspec 4300pro) at 870 nm. Values obtained with the uninoculated controls were subtracted from the respective treatments.

4.3.3 Response of *P. libanensis* to biochar carrier

Biochar inoculation

To evaluate which biochar carrier best supports the growth, survival and abundance of a *P. libanensis* population over time, a storage time experiment was conducted. Four biochar carriers (BL, PR, DM, and BQ) and peat moss (PM) as a standard inoculant carrier were used. Bacteria culture (1.2 mL) in late log-phase (4.9×10^9 CFU/mL) was mixed with 11 mL of sterile water to obtained the desired 40% moisture of biochar/peat moss and aseptically injected into sterilized bags containing 30 g of carrier. The bags with inoculated carriers were thoroughly kneaded and mixed manually to ensure absorption and distribution of the liquid culture into the carrier and closed with two clips. Every treatment was replicated 3 times and kept at room temperature (21°C) in a completely randomized design.

Seed-coating

Preliminary experiments were conducted to determine the most suitable adhesive for the seed-coating. The following were evaluated: gum arabic, guar gum, molasses, corn syrup and carboxy methyl cellulose (CMC). The results indicated guar gum and gum arabic as the best performing sticking agents. With regard to the price of both, guar gum was chosen as the less expensive adhesive. Furthermore, different proportions of components were tested to determine the most appropriate texture and thickness of seed-coating. The results are presented in Table 4.3.

For the seed-coating preparation, previously prepared biochars and peat moss were mixed with suitable amounts of sterile water, liquid culture of *P. libanensis* and guar gum as an adhesive under a laminar hood, to avoid contamination. A number of trials were performed until a mixture of appropriate texture and thickness was obtained. Formulations were determined for further experimentation. Sets of corn seeds were sterilized in 70% alcohol, washed twice in sterile water and then dried in a laminar hood for 2 h. Each seed was dipped separately in a prepared formulation, placed in a sterile Petri plate (20 seeds/Petri plate) and left to dry under the laminar hood for 2 h. Two sets of seeds were prepared. Each treatment was replicated 5 times. When the seeds were completely dry, Petri plates were sealed with Parafilm and one set of seeds

was subjected to 7°C, while the other set was kept at 25°C to determine the influence of temperature on storage time.

4.3.4 Biochar and seed-coating storage time experiment

Storage time and enumeration of bacteria population in the biochar and seed-coating

The survival of bacteria (CFU mL^{-1}) as a function of time was determined for each biochar type (BL, PR, DM, and BQ) and peat moss (PM) at day 0, 1-week and later at 2-weeks intervals until the colony count dropped to 0 in the carrier. Serial dilutions of the inoculated biochar and seed-coating were carried out by aseptically transferring 1mL (≈ 0.26 g) of biochar or one seed (≈ 0.03 g of seed-coating) into sterile Eppendorf tubes and filling each to 2 mL with 0.85% NaCl, vortex mixed for 1 min (Analog MiniVortexer, Fisher Scientific), and then 2 mL of the biochar or coat in NaCl suspension were aseptically transferred into a sterile Eppendorf tube (performed in duplicate). The tubes with the suspension (inoculated biochar and seed-coating) were allowed to sit at 4 °C for 4 hours to let the particles fall to the bottom of the tube. Fifty microliter aliquots were spread-plated in appropriate dilutions on KB Petri plates. Afterwards, Petri plates were parafilmed to prevent desiccation, inverted and incubated at 25 °C in darkness for 4 days for colony development. Number of colony forming units per milliliter (CFU mL^{-1}) was determined with the following formula:

CFU mL^{-1} = (∑ No. of colonies per plate / No. of plates counted) x (1 / mL of aliquot) x (1 / decimal dilution)

4.3.5 Evaluation of seed-coating and inoculated biochar on plant growth

Germination experiment

A germination experiment on the two biochars showing the highest colony count over time was conducted utilizing corn seeds (*Zea mays* Blue River, 19k19) to determine the influence of seed-coating on the germination process, and to investigate how seed-coating affects basic germination variables. The following corn seed treatments were used: control-uncoated and

uninoculated seeds (UC), Pyrovac coated seeds (PR), Pyrovac coated seeds and inoculated with *P. libanensis* (PR+I), Dynamotive coated seeds (DM), Dynamotive coated seeds and inoculated with *P. libanensis* (DM+I). For each treatment, 100 seeds were examined. Seeds were arranged on two layers of Fisherbrand P8 filter papers (Fisherbrand, Mississauga, Canada) and soaked with 5 mL of sterile distilled water in Petri plates (150 mm diameter). Each Petri plate contained 10 seeds and each treatment was repeated 10 times. All Petri plates were kept in a growth chamber at a constant temperature of 25 °C and relative humidity of 60-80% in darkness for 7 days. A daily (intervals of 24 h) germination count was performed until no further germination occurred, generally for seven consecutive days. Root and shoot length and seedling dry weight was recorded. Additionally, germination indicators were determined with the following formulas: Germination percentage GP= (Ga/Gn) x100% (Gn - total number of experimental seeds, Ga - the number of normal germinating seeds on the 7th day); Germination Index GI = ΣGt/Dt, (Gt - number of normal germinating seeds and Dt - a number of germination days); Seedling vigor index VI = GP x seedling root length (mm), Speed of germination (SG), SG = [n1/d1 + n2-n1/d2 +……nn-nn-1/dn] (n = no of seeds germinated on day (d), d = serial number of days).

Greenhouse experiment

A greenhouse experiment was conducted to determine the efficiency of *Pseudomonas libanensis* and the influence of inoculated biochar and seed-coating on development and growth variables of corn, as well as the capacity of *P. libanensis* to colonize the rhizosphere. For this experiment, only the best-performing biochar from the previous experiments was selected. *P. libanensis* strain was tested in sand and turface (2:1), nutrient-less medium in plastic pots (50 cm in diameter). The treatments tested included: uncoated and uninoculated seeds (UC), uncoated seeds inoculated with *P.libanensis* (UC+I), DM biochar coated seeds (DM), DM biochar coated seeds and inoculated with *P. libanensis* (DM+I), biochar inoculated with *P. libanensis* at a ratio of 5t ha^{-1} (UC + IN). To be able to determine the influence of bacteria on phosphorus availability, two sets of each treatment were prepared. One set was watered with Hoagland's solution containing tricalcium phosphate (Ca_3 $(PO_4)_{2)}$ (TCP) at the same concentration as KH_2PO_4 - in normal Hoagland's solution, to provide all necessary nutrients except soluble P. Another set of pots was watered with complete Hoagland's solution containing soluble P

(Hoagland's No. 2 Basal Salt Mixture, Sigma-Aldrich). Pots were supplied with the nutrients twice a week and also watered, with distilled water, twice a week. All treatments were replicated 5 times, resulting in a total of 45 pots. Each pot contained 5 seeds. Plants were grown in a greenhouse at 25 °C day temperature and 20 °C at night, with 16 h of light and 8 h of darkness and a relative humidity of 60-80%. After 1 week, the number of seedlings was reduced to 3 per pot, which gave a total of 15 plants per treatment. Pots were arranged in a completely randomized design. The following growth variables were determined: germination percentage, above ground fresh weight, dry weight, plant height, main root length, basal steam diameter, leaf area and chlorophyll content using a chlorophyll meter (SPAD-502, Konica Minolta).

P concentration in plant tissue was determined using a colorimetric method, as described by Murphy and Riley (1962). Standards were prepared to establish the slope of the calibration line. The concentration was read at 660 nm with a spectrophotometer (UV/Visible Spectrophotometer, Ultraspec 4300pro).

4.3.6 Statistical analysis

SAS System software (Version 9.3, SAS Institute, Inc, 1999, Cary, NC, USA) was used for statistical analyses. Regarding the bacterial growth data, non-linear regression was performed for repeated measurements using SAS PROC NLIN. The most appropriate function to model the data was selected based on its low mean square error (MSE) (Sit and Poulin-Costello 1994). For final and repeated measurements from plant growth, generalized linear mixed models were fit to the data using SAS PROC GLIMMIX. For the repeated measurements, the best covariance structure was selected based on the Bayesian Information Criterion (BIC) (Lindsey and Jones 1997). Percentages were expressed as continuous values between 0 and 1, and therefore the Beta Distribution, DIST=BETA, was specified, except in the case of carbon %, for which the traditional arcsine square root transformation was required and PROC MIXED was used for inference, after the Shapiro-Wilk statistic confirmed that the transformed data was normally distributed. To make inferences, the LSMEANS statement and Bonferroni adjustment were used when fewer than 6 treatments were compared with each other. Scheffe's adjustment was used when more than 6 treatment groups were compared. SAS PROC SGPLOT was used with the

LOESS statement to produce the scatter-diagram smoothing. A single polynomial function was fit to each treatment group for each plot (smooth=1).

4.4 Results and Discussion

4.4.1 Quality of carrier

pH and WHC

The biochars utilized were manufactured from various feedstocks and under a range of pyrolysis conditions, thus physical and chemical analysis revealed important differences between carriers (Table 4.1). It was expected that different properties of biochars would have a significant effect on viability and survival of bacteria over time. DM, BQ and PR showed similar pH values of 7.40, 7.25 and 6.38, respectively. PM revealed a slightly acidic pH of 5.03; on the other hand, an alkaline pH was recorded for BL biochar (8.59). Out of the 5 carriers studied, the lowest WHC was recorded for BQ (33.9 %) and PM (42.1 %). All other carriers had similar WHC, varying between 70.7 % and 80.1 %.

Biochar pH might vary from below 4 to above 12, depending on the feedstock, pyrolysis temperature and degree of oxidation (Lehmann 2007), which creates very different living conditions for bacteria in the biochar pore spaces. Considering the often observed proximity of microorganisms to the biochar surface, biochar pH might be considered to have an important effect on total microbial abundance (Lehmann et al. 2011). A neutral pH of 7.0 – 7.5 has been reported to be optimal for the growth medium of *Pseudomonas* strains (Thomas et al. 1994). Therefore it was expected that biochars close to this pH (DM, PR and BQ) would sustain the number and viability of *P.libanensis* at higher levels than other carriers. Moisture is another factor affecting the survival rate of microbial inoculants (Lehmann et al., 2011). It is considered that water contents between 40 and 80% of soil capacity are generally optimal for microbial activity (Morgan and Watkinson 1989). At lower water contents, osmotic and matric forces limit the availability of water and, consequently, microbial growth (Aislabie et al. 2006). Although in

the storage time experiment of inoculated biochar the moisture of carriers was adjusted to 40%, which was optimal for handling carriers and suitable for bacteria, it could be expected that under field conditions carriers with higher WHC will support bacterial growth better and create a more suitable environment.

Chemical composition and porosity

Chemical composition of dissimilar biochars varied considerably depending upon element (Table 4.2). Generally BQ biochar contained higher concentrations of macronutrients such as Al, Fe, Mg, K, P, ammonium (NH_4-N) and nitrate (NO_3-N), but also elevated concentrations of heavy metals such as As, Cd, Zn and Cu. DM biochar revealed elevated concentrations of Cr (26 mg kg^{-1}) and Sn (7 mg kg^{-1}) and PR biochar had the greatest concentrations of As (2.1 mg kg^{-1}), Ni (9.4 mg kg^{-1}), B (35 mg kg^{-1}), Na (330 mg kg^{-1}) and available P (45.5 mg kg^{-1}). The highest percentage of organic carbon was recorded for BL biochar (68 %). DM and PR revealed the same amount of 44%. Mercury porosimetry analysis showed that PR biochar has the highest total pore area (17.3 m^2 g^{-1}). DM and BQ biochars also revealed relatively high total pore area of 13.369 m^2 g^{-1} and 13.2 m^2 g^{-1}, respectively. The highest average pore diameter and porosity was recorded for BL and PM, 5.15 and 6.03 µm, respectively, which was about 90 % higher compared to other carriers. The lowest porosity of 69.91 % was for BQ biochar. DM biochar showed far higher median pore diameter by volume (42.5 µm) compared to other biochars, which were in a range of 12.2 and 14.7 µm.

Concentration and availability of essential elements in the carriers might have a significant effect on viability and abundance of bacterial populations. For example, it is well known that the availability of organic carbon is the key factor regulating microbial regrowth. Miettinen et al. (1997) showed that addition of phosphorus (50 mg of PO_4-P $liter^{-1}$) increased microbial growth in fresh drinking water. However, other inorganic nutrients (N, K, Mg, and Ca) did not significantly affect the microbial growth. The difference between biochar types is due to differences in pore size distribution of biochars produced from different feedstocks. Abit et al. (2012) report that wood-derived biochar retains the configuration of plant cells and its pores resemble interconnected chambers 5-10 µm in diameter, which is an optimum pore size for retention and inhabitation of bacteria. In contrast, biochar based on poultry manure is relatively amorphous with few larger pores (up to 300 µm). On average, bacteria have a diameter range of

0.3-3 μm (Swift et al. 1979), thus average pore diameter for BQ, DM and PR seems to be just about this size.

4.4.2 Phosphate solubilisation by *Pseudomonas libanensis*

The efficiency of the *P. libanensis* strain to solubilize TCP on agar medium was revealed by the halo zone formation around the colony (Figure 4.1). On average, the halo diameter of 10 replicates was 25.6 mm and the Halo/Colony diameter ratio was 10.45. The quantitative estimation of inorganic phosphate solubilisation (TCP) by tested strain was also revealed. P solubilisation in a liquid medium was calculated based on the calibration curve. The spectrophotometric analysis showed that a significant amount of 465.11 mg/L of phosphorus was solubilized in liquid medium containing TCP, two weeks after inoculation (Table 4.4). After 24h of incubation 17.4% of TCP had been solubilized by the bacteria; however, the maximum P solubilizing activity was recorded the first week after inoculation (98.2%) compared to the control. After the first week, the amount of TCP solubilized remained stable until the end of the experiment (5 weeks). This standstill in further solubilisation of TCP was probably caused by decreased activities of bacteria after one week from inoculation.

High solubilisation of TCP suggests that *Pseudomonas* are considered as some of the most powerful phosphorus solubilizers. Abbas-Zadeh et al. (2009) studied plant growth promoting activities of 40 different strains of *Pseudomonas fluorescens* and *Pseudomonas putida*. Halo/Colony ratio recorded for the most efficient fluorescent strain Wf93 was 3.58 and the amount of solubilized P after 5 days reached 335.26 μg mL^{-1}. Chen et al. (2006) investigated the ability of some soil bacteria to convert insoluble forms of phosphorus to accessible P. The maximum P solubilisation was recorded by *Arthrobacter* sp. (CC-BC03) followed by *S. marcescens* (CC-BC14), which solubilized 519.7 and 421.8 mg L^{-1}, respectively, classifying these bacteria as the most efficient solubilizesr and potential biofertilizers.
Hariprasad and Niranjana (2008) isolated 33 phosphate solubilizing rhizobacteria (PSRB) which could potentially improve tomato health and growth. The most efficient was strain PSRB7, which solubilized 143 mg L^{-1} in liquid culture and produced a halo size of 20 mm in a Petri plate assay. Our research and its consistency with previous work on the *Pseudomonas* strains, suggesting that *Pseudomonas libanensis* might be considered as an efficient P solubilizer. It can

be expected that bacteria will be capable of mobilizing insoluble forms of phosphorus, making it available for plants and thus improving plant growth and development.

4.4.3 Storage time of *P. libanensis* inoculated biochar and seed-coating

Biochar storage time experiment

Viable cell count was performed on each treatment at day 0, 1-week and later at 2-week intervals until 22 weeks. There was variability in ability of the 5 carrier materials to sustain the survival and viability of *Pseudomonas libanensis* over time. After 22 weeks viable cells were present in 3 carriers, PR, DM and BQ; population abundance was 1 x 10^6, 3.3 x 10^5 and 5.3 x 10^5, respectively (Table 4.5). In general, these 3 biochars sustain bacteria populations at similar levels (Figure 4.2). Moreover, an increase in bacteria population was recorded until the 5^{th} week of incubation; 10 weeks after inoculation the bacteria population started to decrease slightly. In the last week of the experiment the highest Log CFU of 6.0 and 5.7 was recorded for PR and BQ biochar, respectively (Figure 4.3). Surprisingly, PM as well as BL biochar sustains bacteria viability for a very short period of time. After two weeks of incubation no viable cells were found in either carrier. Not many studies using biochar as a carrier have been performed so far.

Saranya et al. (2011) showed that acacia wood and coconut shell based biochars can be used as good alternative carrier materials for lignin for the preparation of microbial inoculant. The results of the experiment demonstrate that *Azospirillum lipoferum* is able to survive in a biochar carrier as long as in commercially used lignin (180 days). Vidhyasekaran and Muthamilan (1995) showed that various carrier materials support the survival of *P. fluorescens* strains (control of chickpea wilt) at different rates and over different time periods. The results showed that although the bacterial population declined over time, in the most efficient carrier (talc) the bacteria population was 1.3 x 10^7 after 240 days of incubation. Results from the current study suggest that biochars of appropriate pH and porosity can be used successfully as a carrier for bacterial inoculants and efficiently replace commercially used peat moss.

Seed-coating storage time experiment

Two sets of biochar coated corn seeds inoculated with *P. libanensis* were prepared and stored at two different temperatures, 4°C and 21°C to determine the survival of bacteria in seed-coating and the influence of temperature on the abundance of the bacteria population over time. Viable cell counts were performed for each treatment at day 0, 1-week and later at 2-week intervals until 16 weeks. As with previous experiment, DM, PR and BQ biochar seed-coating supported the survival of bacteria for the longest time period (Figure 4.4). After 16 weeks of storage at 21°C, the highest Log CFUs of 3.42, 2.69 and 2.45 were recorded for PR, BQ and DM biochars, respectively (Table 4.6). Viability of bacterial cells was higher when biochar-coated seeds were stored at a low temperature (Figure 4.5). Again PR biochar sustained the survival of bacteria at the highest population level. After 16 weeks of storage at 4°C, Log CFU was 4.24, 4.07, 3.26 and 2.93 for PR, DM, BL and BQ biochar, respectively. PM was the least suitable carrier for *P. libanensis.* When stored at 21°C, no viable cells were found in the seed-coating 1 week after inoculation. However, when seeds were stored at 4°C, bacteria were present until the 8^{th} week; nonetheless, the population abundance was quite low (1.34 Log CFU). Also, when seeds were stored at a low temperature, the decrease in bacteria population was much slower than at room temperature. For example, DM coated seeds stored at 21°C led to a decrease in bacterial abundance of up to 63%, but when stored at 4°C, the same treatment resulted in a decline of 36% compared to the initial bacteria population. A Kruskal-Wallis test indicated that the powdered biochar treatments produced higher intercepts than the seed coating treatments ($\alpha = 0.05$). Also, statistical analysis for the logarithmic functions showed that 4°C is a more suitable storage temperature than 21°C ($\alpha = 0.05$). Gaind and Gaur (1990) explain that lower temperatures (4°-10°C) retard division and metabolic activities of bacterial cells, which results in a reduced consumption of nutrients and reduced loss of moisture in the carrier, and thus favours the storage of inoculants. Environmental temperature is one of the important factors that affect the population of phosphate-solubilizing bacteria (PSB) in inoculants during storage. Higher temperatures result in greater loss of moisture from inoculants, thereby reducing the PSB population.

In general, the abundance of *P. libanensis* was higher in powdered biochar than in seed-coating. For example, CFU in PR coated seeds after 16 weeks of incubation at 21°C was 49.7 % lower, and when seeds were stored at 4°C, the population was 37.6 % lower than in powdered biochar stored at room temperature (21°C). Burton (1967), reported that the survival of bacteria

on the seed surface is usually lower than on the solid carriers. This is probably because of lack of protection against desiccation, high temperature, lack of a source of nutrients and sometimes because of toxic compounds on the seed coat. In our studies, seed-coating before storing was dried to prevent precocious germination of seeds, while biochar was kept at 40 % moisture; thus desiccation was the most probable reason for a decrease in *P. libanensis* numbers in the seed-coating.

4.4.4 Evaluation of seed-coating and biochar inoculant on corn growth

Germination experiment

To evaluate corn seedling growth and the influence of seed-coating on corn seedling development, a germination test was carried out by Petri plate assay. Following from the results of previous experiments, PR and DM biochars were selected as the most suitable carriers for *P. libanensis.* Generally, DM coated seeds and DM coated seeds inoculated with *P. libanensis* performed better than the control and other treatments (Table 4.7). Results indicated that these treatments significantly ($p < 0.05$) enhance fresh weight of corn seedlings. The same treatments produced significantly ($p < 0.05$) longer roots of 8.5 cm and 9.7 cm, respectively. Similarly, shoot lengths of seedlings of DM coated seeds and DM coated seeds inoculated with *P. libanensis* were improved. Both uninoculated and inoculated DM coated seeds produced significantly ($p < 0.05$) longer shoots than the control by 41% and 44%, respectively. Also, uninoculated as well as inoculated PR coated seeds resulted in significantly ($p < 0.05$) longer shoots than the control. Although not statistically different, PR inoculated with *P. libanensis* seeds resulted in 13 % longer shoots than PR coated but uninoculated seeds. The results of a germination experiment showed that neither seed-coating nor inoculation with bacteria affected seedling dry weight. Germination percentages (Table 4.8) revealed that only DM coated and inoculated seeds germinated as well as the control (99 %). Except for uninoculated PR seeds which showed a decrease in germination percentage (93 %), all other treatments had similar results. Speed of germination was increased in both PR inoculated and uninoculated seeds compared to the control; however, only uninoculated seeds significantly ($\alpha = 0.05$) increased speed of germination, although they had the lowest final germination percentage. There was no

statistical difference among treatments for the germination index. A significantly higher (α = 0.05) Seedling Vigor Index resulted for DM uninoculated seeds.

There are very few studies regarding the influence of seed-coating on the germination process. O'Callaghan et al. (2006) showed that the germination rate of onion seeds coated with biopolymer gel and *Pseudomonas fluorescens* F113 tended to be lower than for untreated seeds. Similarly Slinginger et al. (1996) reported that seeds inoculated with *P. fluorescens* 2-79 formulated in methylcellulose suffered significant germination reductions. The authors attributed this decrease in germination to phytotoxic metabolites produced by the bacteria. Also, the decrease in germination of coated seeds could be explained by the physical barrier to emergence of the radical and hypocotyl of the seed. Nevertheless, Zeng and Zhang (2010) found that seed-coating (without bacteria) does not inhibit the germination process; on the contrary, single plant fresh weight, germination energy, germination percentage, germination index and vigour index of coated seeds were significantly higher than in uncoated seeds. Likewise Gholami et al. (2009) found that maize seeds coated with gum arabic, as an adhesive, and rolled in a suspension of bacteria (PGPR) with perlite significantly increased the seed germination and seedling vigour of maize. Therefore, the hypothesis that seed-coating might be a mechanical barrier cannot be confirmed.

Greenhouse experiment

A greenhouse experiment was conducted to evaluate the influence of bacterial seed-coating as well as inoculated biochar on corn growth characteristics. DM, the best performing biochar in the germination assay, was selected for the greenhouse experiment. Corn plant growth indicators are presented in Table 4.9. Between all the plants supplied with complete Hoagland's solution, only DM coated seeds showed statistically significant higher ($p < 0.05$) fresh weight, plant height, stem diameter, total leaf area and dry weight compared to the control and other treatments. Germination percentage was higher for both DM coated uninoculated (100 %) and inoculated (92 %) seeds than uncoated seeds (88 %). It was expected that corn seeds inoculated with *P. libanensis* would result in better growth of plants than uninoculated seeds when plants were supplied with inorganic phosphorus ($Ca_3 (PO_4)_2$); however, no significant differences in growth were recorded. Application of inoculated biochar at a rate of 5 t ha^{-1} seemed to perform slightly better than other treatments; however, this was not statistically

significant. Also, biochar seed-coating did not significantly affect plant growth when TCP was applied (Table 4.9).

Root characteristics for the plants supplied with soluble P reflect the above ground plant characteristics (Table 4.10). DM coated seeds revealed the most developed root system, and fresh and dry weights of roots were also higher for this treatment than for the others.

Although there were no direct effects on plant physiology of corn supplied with insoluble P, roots showed that all seed treatments significantly improved fresh and also dry weights of roots compared to untreated seeds. Uncoated seeds inoculated with *P. libanensis* (UC+I), as well as application of inoculated biochar at a rate of 5 t h^{-1} (UN+DMI) resulted in plants with significantly heavier roots ($\alpha = 0.05$) compared to the control. DM coated seeds (DM) as well as DM coated and inoculated seeds (DM+I) also resulted in a significant ($\alpha = 0.05$) increase in root weight, compared to uncoated seeds, by 30 % and 32 %, respectively. Similarly root dry weight statistically increased ($\alpha = 0.05$) for all treatments, compared to the control.

Hameedaa et al. (2008) screened five bacterial strains with phosphate-solubilizing ability for their influence on plant growth. Glasshouse and field experiments were conducted using the two most effective strains, *Serratia marcescens* EB 67 and *Pseudomonas* sp. CDB 35. Results showed that when rock phosphate was applied as the only source of phosphorus, a significant increase in plant biomass was recorded. On the other hand, Hariprasad and Niranjana (2008) showed that strains showing strong P-solubilizing abilities improved tomato seedling growth under laboratory conditions; however, plant growth was not affected under greenhouse conditions. The authors suggest that this might be due to the ability to stimulate plant growth directly by production of phytohormones along with phosphate solubilisation. Gamalero et al. (2003) demonstrated that inoculation of tomato plants with *Pseudomonas fluorescens* 92rk significantly improved total root length, total root area, total root volume, number of root tips and root branching degree. The authors hypothesized that changes in root architecture, most probably induced by the bacteria, could be related to increased P acquisition. It is well known that root systems with increased root surface area and volume are indeed characterized by a higher absorptive surface. Thus it could be possible that improved architecture of the corn root system was an effect of bacterial activity, although not necessarily phosphorus solubilizing activity.

Chemical analysis of corn tissue revealed some differences between treatments (Table 4.11). Plants grown from uncoated seeds inoculated with *P. libanensis* and supplied with

complete Hoagland's solution showed higher concentrations of P (24.2 mg/kg) compared to other treatments. This is most probably the direct result of seed inoculation. This treatment also results in elevated percentages of carbon and nitrogen; however the differences are not statistically significant. The N/C ratio did not reveal much difference between treatments, only the DM coated and uninoculated treatment resulted in a significantly lower ($\alpha = 0.05$) N/C ratios than the control. Chlorophyll measurement demonstrated that the biggest decrease in chlorophyll content was between the 3rd and 4th weeks, and then remained stable until the 6th week (Figure 4.5). Statistical analysis revealed that DM coated seeds resulted in plants with significantly ($\alpha = 0.05$) higher chlorophyll content.

When plants were supplied with Hoagland's solution containing insoluble P, the elevated concentration of P in the plant tissues was recorded for DM coated seeds (32.7 mg kg^{-1}) and uncoated seeds with 5 t ha^{-1} of biochar inoculant (32.6 mg kg^{-1}), which were marginally higher ($\alpha = 0.1$) than the rest of the treatments. Elevated carbon and nitrogen percentage resulted from DM+I coating of seeds (coated with biochar and inoculated with *P.libanensis*); however, only nitrogen percentage was statistically significant ($\alpha = 0.05$). DM coated and inoculated seeds (DM+I) revealed a nitrogen to carbon ratio (N/C) of 0.0218, which was statistically higher ($\alpha = 0.05$) than other treatments. No statistically significant differences were found in chlorophyll content of the corn plants supplied with Hoagland's solution containing TCP. All treatments resulted in similar levels of chlorophyll in plant tissue.

It was observed that all treatments with DM biochar (DM, DM+I, UN+DMI) resulted in higher concentrations of P in corn tissue. This could suggest that inoculation with *P. libanensis* affects P uptake less than biochar treatment. Furthermore, it might be possible that an elevated concentration of P is the result of direct uptake of P from the biochar, which contains a considerable amount of available P (17.5 mg kg^{-1}). It was previously demonstrated that inoculation with P-solubilizing bacteria improves plant P and N uptake. Hameedaa et al. (2009) showed that inoculation of corn plants with *Pseudomonas* sp. CDB improved uptake of P not only in shoots, but also in grain tissue. Moreover, chemical analysis revealed improved N uptake in corn shoots. On the other hand, Freitas and Germid (1997) showed that none of the P-solubilizing rhizobacteria significantly enhanced the growth and yield of canola; furthermore, the results of P analysis showed that the seed-P concentrations in the controls were not significantly different from those of the seeds of inoculated plants.

4.5 Conclusions

Bacteria belonging to the genus *Pseudomonas* are known to solubilize insoluble forms of phosphorus and thereby aid in plant growth (Rodriguez and Fraga, 1999). In the present investigation, *P. libanensis* (isolated from the soybean rhizosphere) showed elevated phosphate solubilizing abilities in solid as well as liquid medium. It was also shown that not all carriers sustain bacteria viability and survival in the same way. Clearly, peat moss (PM) and Blue Leaf (BL) biochar did not create suitable environments for bacteria. One of the reasons might be pH, too low in the case of PM (4.93) or too high in the case of BL (8.59). Another reason might be relatively low total pore area compared to other carriers. Pore size and distribution might have an important effect on the colonization of the carrier, thereby influencing the bacterial populations. Furthermore, these two carriers have a much higher average pore diameter than the other three biochars, which might result in a less suitable habitat for the bacteria. In the storage time experiments, PR and DM biochars turned out to be the most suitable carriers, capable of supporting a bacterial population for the longest period of time and at a relatively higher abundance. Both DM and PR had very similar porosities of 78.27 % and 78.56 %, respectively. Also, both carriers were manufactured in a fast pyrolysis process, which is known to have an impact on the final chemical and physical properties (Lehmann et al 2007). Chemical compositions of biochars have shown many differences in the concentration of some elements. It was expected that BQ biochar, which exhibited the highest concentration of most macro- and micronutrients, would sustain survival of *P. libanensis* better than other carriers. In spite of relatively good results in the biochar storage experiment, this biochar performed worse than PR and DM biochars in a coated seed storage experiment. This may be related to the relatively high content of some heavy metals, which might be toxic to the bacterium. Furthermore, a storage experiment of coated seeds clearly showed that low temperature (4°C) is more suitable for the survival of bacteria when compared to room temperature (21°C). As was explained by Gaind and Gaur (1990), lower temperatures decrease metabolic activities, reduce nutrient exhaustion and extend the viability of bacteria.

A germination experiment revealed the positive influence of seed-coating on corn seedling growth under laboratory conditions. Clearly DM biochar contributed to improved germination characteristics of corn; however, the impact of bacteria and their P-solubilizing

activity is questionable. It is hypothesized that the main reason for improved seedling growth is the biochar coat, which retains water around the seed and thus creates favourable conditions for seedling development. Also, due to the high content of macro- and microelements, biochar might be a direct source of some nutrients. P-solubilisation is one of many mechanisms by which bacteria might directly or indirectly affect plant growth. The production of gibberellins, cytokinin, ACC deaminase and volatile compounds can be considered as other types of these mechanisms (Podile and Kishor 2006); however, they were not characterized in the present study. Therefore, it is also possible that *P. libanensis* produces plant-like compounds which may stimulate plant growth. In fact, Freitas and Germid (1997) showed that many of the P-solubilizing bacteria synthetize IAA-like hormones which are well known to influence plant growth development.

The results of plant growth promotion studies under laboratory and greenhouse conditions did not correlate and it may be due to the varied ability of *P. libanensis* to perform under different environmental conditions. There was also no relationship between TCP solubilisation in liquid cultures and plant growth. Although seedling growth was improved in the germination experiment (under sterile conditions), no increase in plant growth was recorded in the greenhouse experiment. The only seed treatment which distinctly improved corn growth when plants were supplied with complete Hoagland's solution was DM biochar coated seeds. It is difficult to explain why the same treatment but with bacterial inoculation (DM+I) did not perform similarly or better. The medium used in the greenhouse experiment was not sterile, hence it is highly possible that *P. libanensis* was not able to compete with, or was completely suppressed by microbiota present in the medium. It was also surprising that inoculation did not improve growth of plants supplied with inorganic P Hoagland's solution. It was expected that P-solubilizing abilities of *P.libanensis* would provide available P and thereby increase corn growth. Although there was no significant difference in above ground growth indicators, seeds treatments clearly improved root architecture and development. Chemical analysis revealed that all plants provided with complete Hoagland's solution only (UN+I) had slightly higher P contents than other treatments, which I explain as the result of bacterial activity. Simultaneously, this treatment showed the highest nitrogen concentration. When plants are supplied with Hoagland's solution with TCP, biochar treatment seems to improve P-uptake; however, inoculation with bacteria did not seem to affect P content in plant tissue. One explanation could be the relatively high concentration of available P in biochar, which could be used directly by the plant; however,

the amount of biochar in the seed-coating was probably too small to affect P concentration in the plant. Also, if biochar were a considerable source of P, an elevated concentration of this element would also be observed in treatment UC+DMI (inoculated biochar applied at a rate of 5t ha^{-1}), since the amount of biochar was much higher.

This study demonstrated that biochar might be an efficient bacterial carrier and it can successfully replace peat moss, which is considered to be an effectively non-renewable resource. However, not all biochars perform in the same way, making it important to classify them according to their properties and characteristics. Further studies are required to gain a better understanding of *P. libanensis* root colonization and bacteria distribution in the rhizosphere. It would also be a good idea to investigate the P-solubilisation mechanism of *P. libanensis*.

4.6 Tables and figures

Table 4.1 Characteristics of selected carriers

Characteristics	Peat moss (PM)	Dynamotive (DM)	Pyrovac (PR)	Basque (BQ)	BlueLeaf (BL)
Source of biomass	peat	hardwood	softwood	hardwood	softwood
Pyrolysis					
Temperature (°C)	---	> 700	> 700	N/A [a]	+/- 450
Method	---	fast	fast	N/A	slow
Time	---	Few minutes	Few minutes	N/A	2.5 h
pH	4.93	7.40	6.38	7.25	8.59
WHC [b] (%)	42.1	70.7	73.3	33.9	80.1
Porosity					
Total pore area (m^2 g^{-1})	3.06	13.36	17.32	13.21	2.54
Median pore diameter: area (µm)	0.7026	0.0098	0.0061	0.0075	2.9538
Median pore diameter: volume (µm)	26.0	42.5	14.7	12.7	12.2
Average pore diameter (4V/A) (µm)	5.15	0.68	0.49	0.38	6.03
Porosity (%)	83.3	78.2	78.5	69.9	89.9

[a] Not Available N/A
[b] Water Holding Capacity (WHC)

Table 4.2 Biochar elemental analysis

Element concentrations [b] (mg kg^{-1})

	Al	Fe	Mg	Mn	Sn	As	Cd	Cr	Organic C %
DM [a]	58	1000	370	112	7	0.2	<0.01	26	44
PR	1000	1580	1580	1310	6	2.1	<0.1	4.4	44
BQ	5530	7660	4600	2320	<5	1.2	1.6	3.1	22
BL	N/A	N/A	N/A	N/A	N/A	0.7	1.5	7.4	68
	Hg	**Ni**	**Se**	**Zn**	**B**	**Cl**	**Na**	**K**	**Inorg. C %**
DM	<0.1	2.1	<0.01	13	19	37	73	4010	0.07
PR	<0.1	9.4	<0.1	140	35	23	330	3060	0.37
BQ	<0.1	4.2	<0.1	157	30	62	146	11600	0.08
BL	<0.1	2.7	<0.1	95	25	18	115	4496	0.46
	Cu	**P**	**Pb**	**Mo**	**Co**	**NH_4-N**	**NO_3-N**	**P (Av.)**	**Total N %**
DM	<10	163	3.4	0.3	0.4	38	4.2	17.5	0.25
PR	10	572	9.6	<.01	2.4	24	2.4	45.5	0.54
BQ	16	1580	4.1	<.01	0.5	165	7.4	34.9	0.07
BL	11	820	3.4	< 0.1	0.3	13	5.2	13	0.51

[a] Dynamotive (DM), Pyrovac (PR), Basque, (BQ), BlueLeaf (BL)
[b] Analysis was performed on dry biochar samples

Table 4.3 Seed-coating formulations for each carrier

	H_2O (mL)	**Carrier (g)**	**Guar gum (g)**	**Bacteria culture 4.9 x10^9 CFU/mL (mL)**
PM	25	2.1	0.5	3
DM	25	2.6	0.5	3
PR	25	2.1	0.5	3
BQ	25	3.2	2	3
BL	25	2.1	2	3

Dynamotive (DM), Pyrovac (PR), Basque, (BQ), BlueLeaf (BL)

Table 4.4 P solubilization of *P. libanensis* in solid and liquid medium 2 weeks after inoculation

	P solubilization			
	Halo size* (mm)	Halo/Colony* Diameter ratio	Broth ** (mg/L P solubilized)	% of solubilization compared to control
P. libanensis	25.6	10.45	465.11	98.2

Reported statistics are means of *10 or **3 replicates

Table 4.5 Viable count in Log_{10} CFU mL^{-1} of *Pseudomonas libanensis* in different carrier materials

Carrier	Weeks after inoculation						
	1	**2**	**5**	**10**	**16**	**20**	**22**
DM	6.1	6.2	6.3	6.3	6.7	6.5	5.5
PR	6.9	7.0	7.5	7.0	6.8	6.8	6.0
BQ	7.0	7.0	7.7	6.4	6.7	6.7	5.7
BL	5.4	5.8	0.0	0.0	0.0	0.0	0.0
PM	3.8	0.0	0.0	0.0	0.0	0.0	0.0

Dynamotive (DM), Pyrovac (PR), Basque, (BQ), BlueLeaf (BL)

Table 4.6 Viable count in Log_{10} CFU mL^{-1} of *P. libanensis* in coated seeds stored at 21°C and 4°C

Carrier	Weeks							
	0	**1**	**3**	**6**	**8**	**10**	**13**	**16**
21°C								
DM	6.65	5.29	5.28	4.11	3.60	4.06	4.13	2.45
PR	6.77	5.07	4.91	4.24	3.78	3.94	3.88	3.42
BQ	5.39	5.65	3.95	2.74	2.65	3.30	2.67	2.69
BL	4.82	4.62	0.00	0.00	0.00	0.00	0.00	0.00
PM	4.94	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4°C								
DM	6.44	6.23	5.43	4.84	4.97	4.49	3.62	4.07
PR	6.00	5.83	5.29	4.57	4.89	4.26	4.25	4.24
BQ	4.12	3.24	3.10	2.19	3.35	2.49	2.38	2.93
BL	3.94	3.89	3.95	3.64	3.58	3.53	2.34	3.26
PM	3.64	3.30	2.30	2.12	1.34	0.00	0.00	0.00

Dynamotive (DM), Pyrovac (PR), Basque, (BQ), BlueLeaf (BL)

Table 4.7 Growth characteristics of corn seedlings

Seed coating	*P. libanensis* inoculation	Fresh weight (g) (lsm ± se)*	Root length (cm) (lsm ± se)*	Shoot length (cm) (μ ± se) †	Dry weight [g]
Uncoated	No	0.56 ± 0.009^{b}	7.0 ± 0.26^{c}	3.3 ± 0.13^{c}	0.193 ± 0.002
PR	No	0.49 ± 0.010^{c}	5.9 ± 0.28^{c}	4.0 ± 0.19^{b}	0.198 ± 0.002
PR+I	Yes	0.55 ± 0.009^{b}	8.5 ± 0.26^{b}	4.6 ± 0.15^{b}	0.192 ± 0.002
DM	No	0.62 ± 0.009^{a}	9.7 ± 0.27^{a}	5.6 ± 0.19^{a}	0.194 ± 0.002
DM+I	Yes	0.60 ± 0.009^{a}	8.6 ± 0.26^{b}	5.9 ± 0.25^{a}	0.203 ± 0.002

Pyrovac biochar coated seeds (PR), Dynamotive biochar coated seeds (DM), inoculation (+I)
* Bonferroni adjusted pdiff < 0.05
† $\alpha = 0.05$. LS-means with the same letter are not significantly different.

Table 4.8 Germination characteristics of corn seeds

Seed coating	*P.libanensis* inoculation	Germination percentage	Speed of Germination	Germination Index	Seedling Vigour index
Uncoated	No	99 ± 1.0a	5.17 ± 0.19b	14.1 ± 0.14a	6831bc
PR	No	93 ± 3.3a	6.14 ± 0.20a	13.3 ± 0.47a	5487c
PR+I	Yes	98 ± 1.3a	5.70 ± 0.14ab	14.0 ± 0.19a	8330ab
DM	No	96 ± 1.6a	5.17 ± 0.21b	13.7 ± 0.23a	9312a
DM+I	Yes	99 ± 1a	5.33 ± 0.20b	14.1 ± 0.14a	8514ab

Pyrovac biochar coated seeds (PR), Dynamotive biochar coated seeds (DM), inoculation (+I)
Bonferroni Grouping for Least Squares Means ($\alpha = 0.05$). LS-means with the same letter are not significantly different.

Table 4.9 Corn growth characteristics

Seed coating	Germ %	Fresh weight [g]	Plant high [cm]	Stem diameter [mm]	Total leaves area [cm^2]	Dry weight [g]
+ Complete Hoagland's solution						
UC	88	11.6 ± 0.93a	85.5 ± 2.5a	5.5 ± 0.22a	173 ±18.8a	1.77 ± 0.20a
DM	100	17.4 ± 0.90b	101.7 ±1.5b	6.7 ± 0.16b	297±16.1b	2.93 ± 0.25b
UCI	88	11.8 ± 0.63a	86.6 ± 2.4a	5.5 ± 0.15a	234 ± 14.3a	1.76 ± 0.11a
DM+I	92	11.6 ± 1.12a	86.9 ± 3.0a	5.4 ± 0.24a	202 ± 17.3a	1.81 ± 0.19a
+ Hoagland solution with TCP						
UC	100	24.4 ± 1.28	102.4 ± 1.2	8.2 ± 0.20	361 ± 18.1	4.0 ± 0.32
DM	100	21.8 ± 0.93	103.6 ± 1.1	7.9 ± 0.17	337 ± 19.3	2.9 ± 0.20
UC+I	96	22.5 ± 1.02	104.4 ± 1.3	8.4 ± 0.22	358 ± 17.9	3.9 ± 0.26
DM+I	96	21.5 ± 1.12	103.6 ± 1.3	7.8 ± 0.25	319 ± 20.8	4.2 ± 0.33
UC+DMI	100	23.2 ± 0.79	102.5 ± 1.0	8.6 ± 0.13	391 ± 18.8	4.0 ± 0.22

Dynamotive coated seeds (DM), Uncoated seeds (UC), Uncoated seeds + biochar inoculant applied at rate of 5 t ha^{-1} (UC + IN), inoculation (+I),
Values with the same letters within each column are not significantly different from each other at $P < 0.05$

Table 4.10 Corn root characteristics

Seed coating	*P.libanensis* inoculation	Root Fresh Weight	Root Length	Root Dry Weight
+ Complete Hoagland's solution				
UC	No	10.4 ± 2.04	48.4 ± 3.2	0.6 ± 0.10
DM	No	14.4 ± 2.90	45.4 ± 3.2	1.1± 0.32
UC+I	Yes	6.6 ± 1.04	46.6 ± 2.3	0.6 ± 0.09
DM+I	Yes	8.4 ± 2.51	47.5 ± 5.8	0.6 ± 0.19
+ Hoagland solution with TCP				
UC	No	8.8 ± 0.81b	50.6 ± 1.2	0.7 ± 0.03c
DM	No	12.6 ± 0.95ab	55.4 ± 3.2	1.0 ± 0.03b
UC+I	Yes	16.6 ± 2.68a	51.0 ± 4.3	1.4 ± 0.18ab
DM+I	Yes	12.9 ± 0.73ab	58.6 ± 2.5	0.9 ± 0.07bc
UC+DMI	Yes	18.6 ± 1.95a	50.2 ± 7.6	1.3 ± 0.06a

Dynamotive coated seeds (DM), Uncoated seeds (UC), Uncoated seeds + biochar inoculant applied at rate of 5 t ha^{-1} (UC + IN), inoculation (+I)
Bonferroni Grouping for Least Squares Means ($\alpha = 0.05$). LS-means with the same letter are not significantly different.

Table 4.11 Chemical composition of corn tissue

Seed coating	*P.libanensis* inoculation	Carbon (%) **	Nitrogen (%) **	N/C **	Phosphorus (mg/kg)*
+ Complete Hoagland's solution					
UC	No	42.1 ± 0.29a	3.17 ± 0.059a	0.029 ± 0.0004a	22.0 ± 1.36b
DM	No	47.0 ± 3.80a	2.75 ± 0.234a	0.022 ± 0.0003b	22.6 ± 1.56b
UC+I	Yes	46.8 ± 5.18a	3.23 ± 0.429a	0.027 ± 0.0013ab	24.3 ± 1.44ba
DM+I	Yes	41.7 ± 0.21a	2.86 ± 0.152a	0.027 ± 0.0017ab	22.3 ± 1.53b
+ Hoagland solution with TCP					
UC	No	41.8 ± 0.17a	2.39 ± 0.076ab	0.0218 ± 0.00074a	27.1 ± 0.77ba
DM	No	41.1 ± 0.64a	2.56 ± 0.139ab	0.0246 ± 0.00140ab	32.7 ± 2.96a
UC+I	Yes	37.3 ± 3.30a	2.15 ± 0.196b	0.0223 ± 0.00055a	28.8 ± 0.46ba
DM+I	Yes	42.9 ± 5.40a	3.33 ± 0.247a	0.0294 ± 0.00047b	31.3 ± 2.79ba
UC+DMI	Yes	40.8 ± 1.82a	2.49 ± 0.162ab	0.0243 ± 0.00093ab	32.6 ± 0.74a

Dynamotive coated seeds (DM), Uncoated seeds (UC), Uncoated seeds + biochar inoculant applied at rate of 5 t ha^{-1} (UC + IN), inoculation (+I)
Scheffe Grouping for Least Squares Means* (α =0.1), ** (α =0.05). LS-means with the same letter are not significantly different.

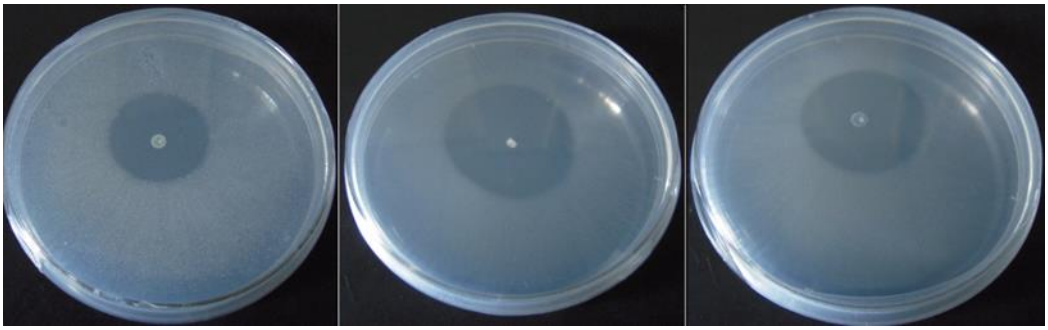

Figure 4.1 TCP solubilization by *Pseudomonas libanensis* on NBRIP medium. Halo around the colonies shows P-solubilization of $Ca_3(PO_4)_2$ by the bacteria. Experiment was replicated 10 times.

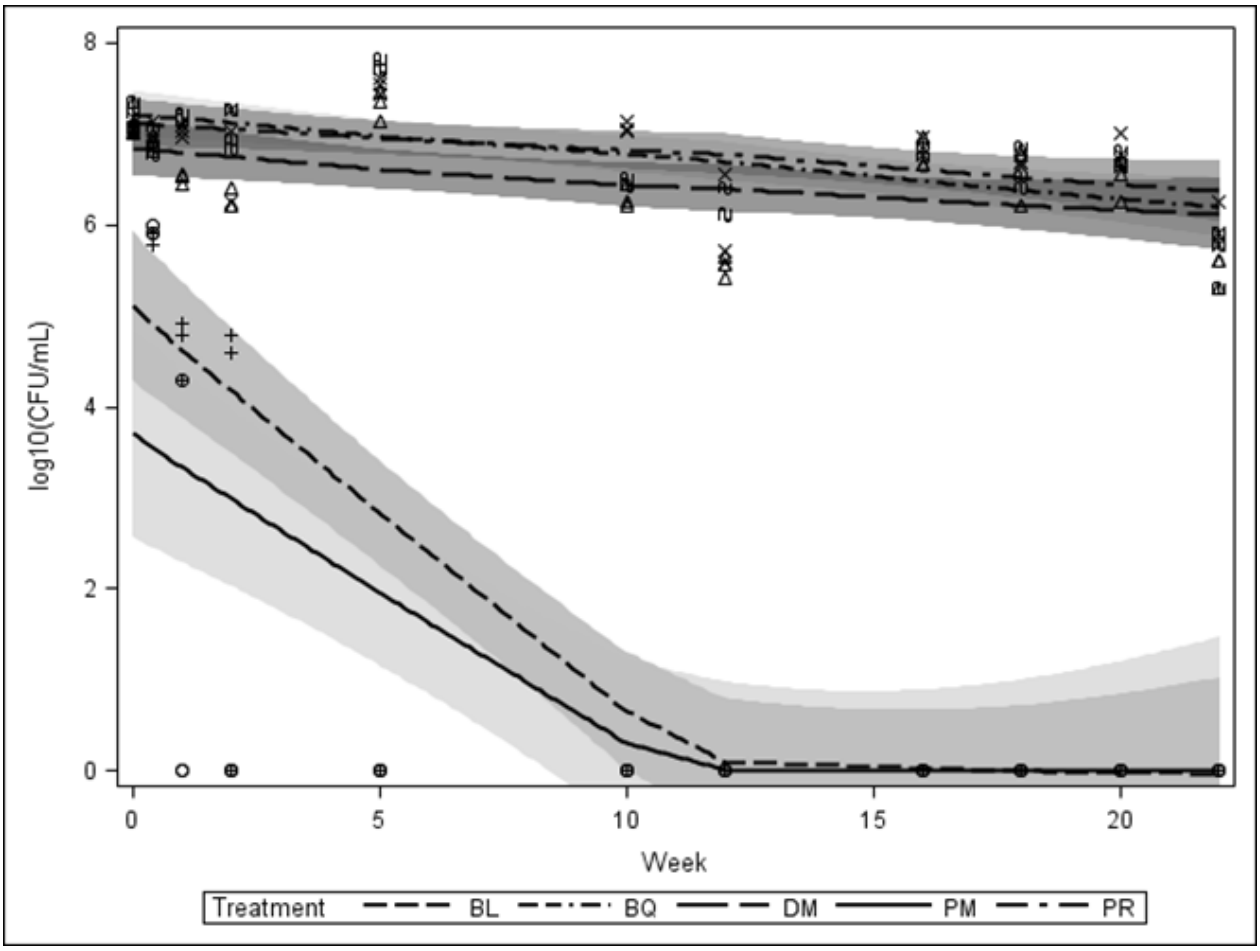

Figure 4.2 Scatter-diagram smoothing/local regressions for Log_{10} CFU mL^{-1} of *P. libanensis* in powdered biochar stored at 21°C as a function of time. Shaded areas represent the confidence limits ($\alpha = 0.05$)
Peat moss (PM), BlueLeaf (BL), Pyrovac (PR), Dynamotive (DM), Basque (BQ)

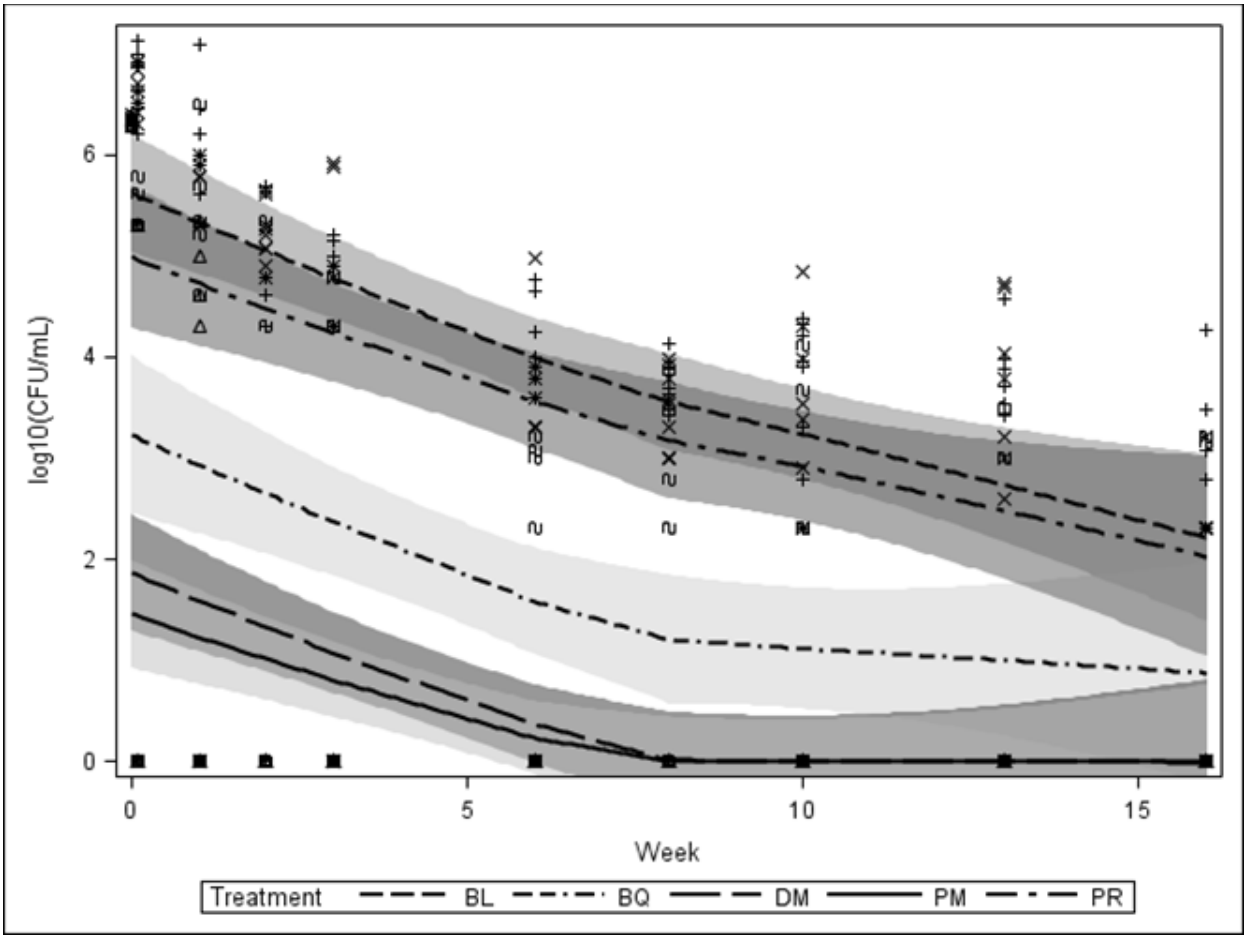

Figure 4.3 Scatter-diagram smoothing/local regressions for Log_{10} CFU mL^{-1} of *P. libanensis* in seed stored at 21°C as a function of time. Shaded areas represent the confidence limits (α = 0.05). Seed-coating carriers: Peat moss (PM), BlueLeaf (BL), Pyrovac (PR), Dynamotive (DM), Basque (BQ)

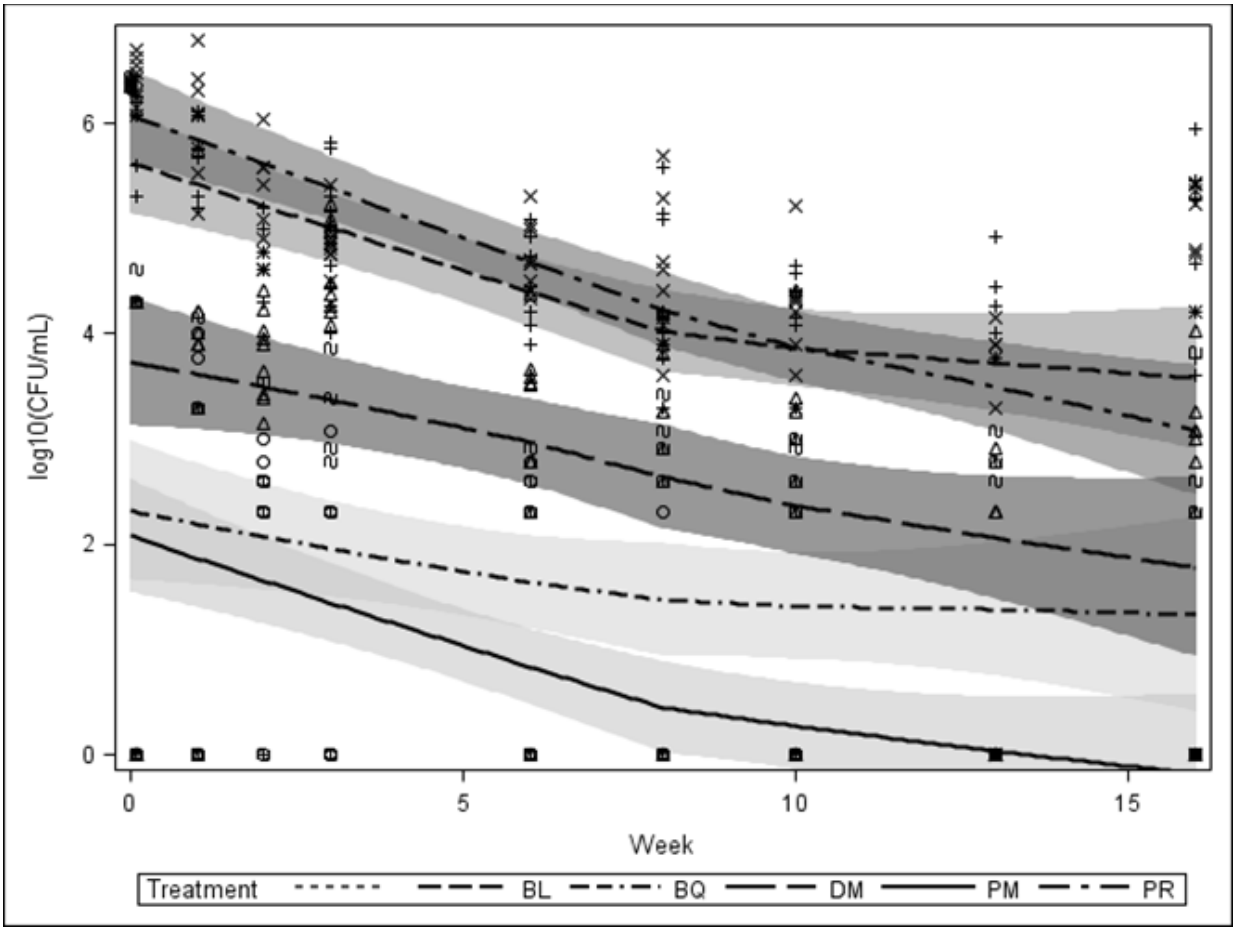

Figure 4.4 Scatter-diagram smoothing/local regressions for Log_{10} CFU mL^{-1} of *P. libanensis* in seed stored at 4°C as a function of time. Shaded areas represent the confidence limits ($\alpha = 0.05$). Seed-coating carriers: Peat moss (PM), BlueLeaf (BL), Pyrovac (PR), Dynamotive (DM), Basque (BQ)

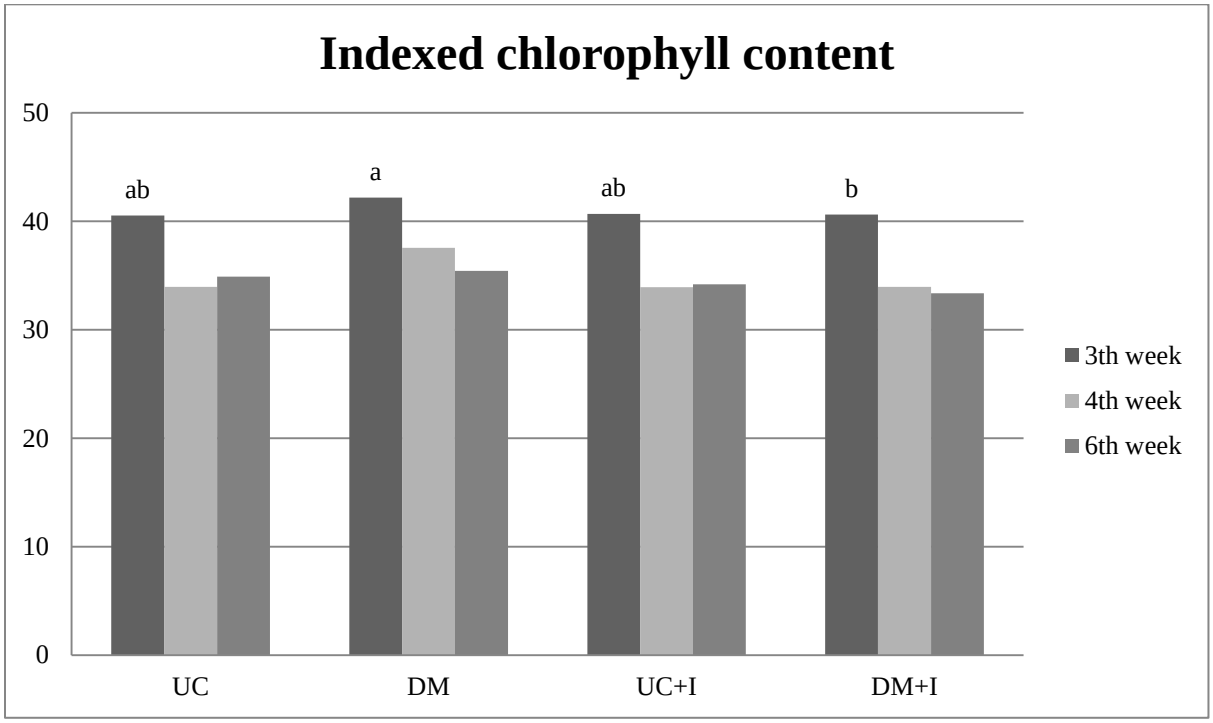

Figure 4.5 Indexed chlorophyll content for the corn plants supplied with complete Hoagland's solution. Measurements were taken 3 times during growth period (3, 4, 6 weeks after seeding). Bonferroni Grouping for Least Squares Means ($\alpha = 0.05$). LS-means with the same letter are not significantly different.

Preface to Chapter 5

In Chapter 4 we characterized the chemical and physical properties of all selected carriers, which revealed essential differences between them. Inoculation and storage time assays demonstrated that only two out of the four biochars were suitable as a carrier for *Pseudomonas libanensis.* To further evaluate biochar as an inoculant carrier in Chapter 5, we used *Bradyrhizobium japonicum* strain 532, which is commonly used in rhizobial inoculants, as model bacteria. Chapter 5 uses the same experimental procedures and protocols that were used in Chapter 4 for biochar inoculation and seed-coating. Storage time experiments were designed in the similar way; only data collection points were different. Germination assay and greenhouse experiment were also conducted following the procedures described in Chapter 4.

Chapter 5 was co-authored by the candidate's supervisor Dr. Donald L. Smith, and lab member Timothy Schwinghamer. Chapter 5 will be submitted to *Applied and Environmental Microbiology* for publication.

5. Biochar as an inoculant carrier for *Bradyrhizobium japonicum* and its potential to improve soybean growth

5.1 Abstract

Experiments were designed to evaluate the suitability of four different types of biochar as a bradyrhizobia carrier for inoculant and seed-coat production. Biochars, as well as peat moss as a standard inoculant carrier, were inoculated with *Bradyrhizobium japonicum* strain 532 and storage time of inoculants was assessed. A seed coating system was developed using biochar, bacteria liquid culture, water and guar gum as an adhesive, and viability of bacteria in seed-coating was evaluated as a function of time at two temperature conditions of 4°C and 21°C. The best performing biochars were selected for a germination assay, which demonstrated that seed-coating does not negatively affect soybean germination. Based on the results, one biochar was chosen for the greenhouse experiment. Soybean growth characteristics, nutrient uptake and chlorophyll content were differently affected depending on the applied fertilizer. When plants were supplied with nitrogen, no differences were found between treatments, while plants supplied with N-free nutrient solution showed significant differences in growth, root characteristics, chemical composition and chlorophyll content

5.2 Introduction

Biochar is the product of biomass combustion under low-oxygen or anaerobic conditions. In recent years, biochar has become a product of interest as a way to prevent climate change by carbon sequestration, and as a soil amendment and as a tool for bioremediation. (Xu et al., 2012; Yamato et al. 2006; Graber 2010; Chan et al. 2012; Freddo et al. 2012). However, marginal

attention was focused on biochar as a potential inoculant carrier. In order to fulfill its function, a rhizobial carrier, similar to any other inoculant carrier, must possess the appropriate characteristics. It should be unlimited, locally available and inexpensive; it should have good water holding capacity, good aeration properties and sustain growth and survival of bacteria over time. The right inoculant carrier should also be non-toxic, environmentally friendly, easily produced, sterilized and handled in the field. Finally, the perfect carrier should easily release rhizobia into the soil, be simply converted to powder, mixable, packageable and adhere to the seed (Smith 1992; Khavazi et al. 2007).

Crop plants use only 50% of the nitrogenous fertilizers applied to soil: 2-20% is lost by volatilization, 15-25% reacts with organic compounds or clay soils, and the remaining 2-10% reaches surface and ground water (Savci 2012). When N is applied to the soil at an inappropriate time, or at excessive rates, it can leach through the soil, into surface and ground water, leading to eutrophication and pollution of drinking water. Environmental and economic issues are the main reasons for the growing interest in rhizobia inoculants. To inoculate 20 mha of agricultural lands, 2000 tonnes of rhizobium inoculant with a high number (10^9 g^{-1}) of bacteria, storable for 3- 6 months, with a suitable carbon source, are required for biological nitrogen fixation (Ben Rebah et al. 2007).

Regarding the requirements for a carrier to be efficient, biochar might be considered as a suitable material. Depending on feedstock and the pyrolysis process, biochar can have very different characteristics; however, most biochars have very high water holding capacities (WHC), high porosity and surface area, and often elevated concentrations of available carbon and other nutrients. It can also be produced locally using organic wastes such as agricultural remains. Although there are not many studies evaluating biochar as a potential carrier, previous experiments suggest that biochar can be a suitable carrier for *Pseudomonas libanensis*. One of the very first investigations regarding the use of combusted organic matter such as charcoal as a carrier was conducted by Gorelick et al. (1951). The study showed that a very high concentration of *Brucella suis* bacteria can be sustained by cultivation in a charcoal-cellophane system. However, the stimulatory effect of the charcoal in cellophane cultures exists only during active growth of the microorganisms. Furthermore, almost a quarter of a century ago, Beck (1991) demonstrated that charcoal mixed with soil in a ratio 1:3 supported the growth of rhizobia to the point that it could successfully replace commercially used peat moss. Viveganandan and Jauhri (2000) also studied charcoal-soil (3:1), as well as calcium alginate and charcoal-soil plus

calcium alginate as a carrier for the phosphate-solubilizing bacteria *Pseudomonas striata and Bacillus polymyxa*. Storage time experiments show that all three carriers support the survival of bacteria at relatively high levels. However, the bacterial population was slightly higher in calcium alginate than in the other two carriers.

Bradyrhizobium japonicum is a well-studied symbiotic bacterium, nodulating soybean plants. Symbiotic nitrogen fixation is a complex process involving both plant and bacterial physiological and biochemical aspects. Inside the nodules, *B. japonicum* fixes atmospheric N_2 in symbiosis with the plant. In this environmentally sustainable process, the bacterium is providing N to soybean crops in a way similar to applying mineral fertilizer (Quelas et al. 2006). Soybean in symbiosis with *Bradyrhizobium japonicum*, depending on the conditions, can fix up to 300 kg N ha^{-1} (Keyser and Li, 1992). However, low root zone temperature (RZT) negatively influences nodulation as well as nodule functions, thereby significantly decreasing N_2 fixation. Soybean is a subtropical legume which requires a 25 to 30 °C RZT for optimal symbiotic nitrogen fixation. Therefore, under short season conditions such as in Canada, soybean plants in symbiosis with *B. japonicum* can fix only 100 to 200 kg ha^{-1} yr^{-1} of nitrogen from the atmosphere (Zhang and Smith, 1996). Some other factors affecting biological N fixation include available soil N, genetic determinants of compatibility in symbiotic partners, and other yield-limiting factors. Another problem can be low levels of highly effective rhizobia in many soils (Keyser and Li, 1992). This is why seed inoculation has become an increasingly common practice. It results in early nodulation and causes the formation of prominent nodules clustered mostly around the crown of the root, considered as crucial for nitrogen fixation in the early stages of crop development (Daramola et al. 1994).

Soybean (*Glycine max*) was selected as an experimental plant for this study. The reasons behind this choice are first, the symbiotic relationship with *Bradyrhizobium japonicum*; second, the importance of soybean as a crop. Soybean is a tropical legume, originally from East Asia; however nowadays it is cultivated worldwide, even in cool climate areas such as Canada, which is one of the major soybean exporters. Soybean gained global importance as a crop mostly because it is a great source of oil and protein; therefore it is cultivated for both human and animal nutrition. Production in Canada increased significantly in recent years. In 2013, 1 829 thousands hectares of Canadian fields were under soybean cultivation, which produced 5.2 million tonnes of soybean grains. In Quebec alone, 288 506 ha were covered with soybean in 2013 (Statistics Canada).

The ideal soybean inoculant would be one that could be applied to seeds in the early winter, when the seed companies clean and bag seed for the coming growing season, and still contain high numbers of living cells at planting time, in the spring. Such an inoculant would add value to the seeds and would remove the need for crop producers to manually add and mix inoculants into seed immediately prior to seeding; this requires time at a very busy point in the crop production cycle. Past attempts at this have generally resulted in insufficient cell counts by seeding time.

The main objectives of this study were to: 1) determine whether biochar can serve as a suitable carrier for *B. japonicum*; 2) assess how long bacteria can survive in biochar and what is the abundance of the population; 3) create a seed-coating system with biochar and *B. japonicum* which could potentially be used in the agriculture industry; and 4) evaluate the effect of seed-coating and biochar inoculant on soybean growth.

5.3 Materials and methods

The same four biochars (Dynamotive, Pyrovac, Basque and BlueLeaf) and peat moss were used in this study. For biochar source, characterisation and inoculation please refer to Chapter 4.3.1.

5.3.1 *Bradyrhizobium japonicum* source and culture

Source of bacteria culture

Bradyrhizobium japonicum strain 532 was obtained from the stock culture in Professor Donald Smith's laboratory, Department of Plant Science, McGill University, Ste Anne-de-Bellevue, Québec, Canada.

Preparation of broth culture and incubation of Bradyrhizobium japonicum

YEM (yeast extract mannitol) broth (containing 0.5 g K_2HPO_4; 0.2 g $MgSO_4 \cdot 7\ H_2O$; 0.4 g yeast extract; 0.1 NaCl; 10 g mannitol per 1 liter of distilled water) was prepared. The pH of the broth was verified and adjusted to 7.0 with HCl. YEM broth was sterilized for 20 minutes at 121°C. 250 mL of broth were aseptically inoculated with 2.5 mL of bacterial culture. The culture was incubated in a gyratory shaker for 14 days at 25°C in darkness. After 7 days of incubation the cell count of the broth culture was estimated. After 14 days of incubation, a log-phase culture of *B. japonicum* strain 532 was plated onto Petri plates to evaluate the condition and viability of the population.

Response of *Bradyrhizobium japonicum* to the biochar carrier was performed using the same protocol as for *Pseudomonas libanensis.* Refer to Chapter 4.3.3

A biochar and seed-coating storage experiment was conducted following the same procedure as for *Pseudomonas libanensis*. Refer to Chapter 4.3.4 with the following changes; peat moss pH for the seed-coating storage time experiment was adjusted to 7 with NaOH.

5.3.2 Evaluation of *B. japonicum* seed-coating and biochar inoculant on soybean growth

Germination experiment

A germination experiment was performed to determine the influence of seed-coating on the germination process and to investigate how seed-coating affects basic germination variables. For this experiment only the two best performing biochars from the previous experiment were selected. The following soybean seed (Absolut RR) treatments were used; Pyrovac coated seeds (PR), Pyrovac coated seeds and inoculated with *B. japonicum* (PR+I), Dynamotive coated seeds (DM), DM coated seeds and inoculated with *B. japonicum* (DM+I), Control-uncoated and uninoculated seeds (UC). For each treatment 100 seeds were examined. Seeds were arranged on two layers of Fisherbrand P8 filter papers (Fisherbrand, Mississauga, Canada) and soaked with 5

mL of sterile distilled water in a Petri plate (150 mm diameter). Each Petri plate contained 10 seeds and each treatment was repeated 10 times. All Petri plates were sealed with Parafilm and kept in a growth chamber at a constant temperature of 25 °C and relative humidity 60-80% in the dark for 7 days. A daily (intervals of 24 h) germination count was performed until no further germination occurred, generally for seven consecutive days. Root length and seedling fresh and dry weight were recorded. Additionally, germination indicators were determined using the following formulas: Germination percentage GP= (Ga/Gn) x100% (Gn- total number of experimental seeds, Ga- the number of normal germinating seeds on the 7th day); Germination Index GI= ΣGt/Dt, (Gt- number of normal germinating seeds and Dt- number of germination days); Seedling vigour index VI= GP x seedling root length (mm), Speed of germination (SG), SG = [n1/d1 + n2-n1/d2 +……nn-nn-1/dn] (n= no of seeds germinated on day (d), d= serial number of days).

Greenhouse experiment

To evaluate the efficiency of *Bradyrhizobium japonicum* seed-coating and biochar inoculant on development and growth variables of soybean, a greenhouse experiment was conducted. For the experiment only the best performing biochar from the previous experiments was selected. Coated seeds inoculated with *B. japonicum*, as well as inoculated biochar were tested in sand and turface (2:1), nutrient-less medium. Plastic pots of 50 cm in diameter were used. Plants were grown in a greenhouse at 25 °C day temperature and 20 °C at night, with 16 h of light and 8 h of darkness, and a relative humidity of 60-80%. The following soybean seed treatments were tested: control-uncoated and uninoculated seeds (UC), uncoated seeds inoculated with *B. japonicum* (UC+I), Pyrovac coated seeds (PR), Pyrovac coated seeds and inoculated with *B. japonicum* (PR+I), uncoated seeds + Pyrovac inoculated with *B. japonicum* at a rate of 5 t ha^{-1} (UC+PRI). To determine the influence of inoculated seed-coating and inoculated biochar on nitrogen fixation and soybean nodulation, two sets of each treatment were prepared. One set was watered with complete Hoagland's solution (+N) to provide all necessary nutrients including nitrogen. Another set of pots was watered with N-free Hoagland's solution (-N). Pots were supplied with the nutrients twice a week and watered with distilled water also twice a week. All treatments were replicated 5 times, with every pot containing 5 seeds. After 1 week, the number of seedlings was reduced to 3 per pot, which gave a total of 15 plants per treatment. Pots

were arranged in a completely randomized design. The following growth variables were determined: germination percentage, above ground fresh weight and dry weight, plant height, leaf area, and number and weight of pods. Chlorophyll content was measured three times during the growth period using a SPAD-meter (SPAD-502, Konica Minolta). Furthermore, the following root characteristics were recorded: main root length, root fresh and dry weight, and number and weight of nodules.

Leaf and stem samples were collected and oven-dried at 75°C for 48 h. Dried biomass was ground in a laboratory grinder (IKA M 20 Universal mill) and about 2-3 mg of plant material were analyzed for total nitrogen, carbon and nitrogen/carbon ratio (N/C) using an elemental analyzer (Thermo Quest NC2500).

P concentration in plant tissue was determined using a colorimetric method, as described by Murphy and Riley (1962). Standards were prepared to establish the slope of the calibration line. The concentration was read at 410 nm with a spectrophotometer (UV/Visible Spectrophotometer, Ultraspec 4300pro).

Statistical analysis was performed following the same methods as described in Chapter 4.3.6

5.4 Results and Discussion

5.4.1 Storage time of *B. japonicum* inoculated biochar and seed-coating

Storage time of inoculated biochar

Viable cell count was performed on each treatment at days 0 and 1 and at 2-weeks intervals until 37 weeks. There was clear variability in the ability of the 5 carrier materials to sustain the survival and viability of *Bradyrhizobium japonicum* over time. After 37 weeks, viable cell were present in only 2 carriers, PR and DM. CFU count revealed differences of two orders of magnitude between PR and DM biochars; population abundance was 2×10^6 and 7.9×10^4, respectively (Table 5.1). Surprisingly, PM sustained survival of bacteria for a very short time.

Four weeks post- inoculation, no viable cells were found in the PM carrier. This confirms the results from *P. libanensis* inoculation storage time, where PM also sustained bacterial survival for a very limited time period. A sudden decline in the bacteria population was observed in the BQ carrier, where after 21 weeks of incubation, the *B. japonicum* population dropped from 1.5 x 10^5 to 0 (Figure 5.1). Similar to the storage time experiments with *P. libanensis*, BL biochar was not a suitable carrier for *B. japonicum*. After 9 weeks of storage, no viable cells were found in this carrier.

In summary, PR and DM biochar turned out to be the most appropriate carriers to host *B. japonicum.* This is consistent with the results of *P. libanensis*, where PR and DM, together with BQ biochar, were the best performing carriers. Again, we hypothesize that pH was one of the main factors affecting bacterial population. It was reported that the optimal pH condition for *Bradyrhizobium japonicum* growth is between 6 and 7, although a lower optimum may be exhibited by strains from acid soils (Mubarik et al. 2012). Owiredu and Dansot (1988) suggested that because of low pH and high soil temperature, the survival of applied rhizobial cells in many tropical soils is limited. Therefore, this might confirm the theory that the low pH of PM (4.93) was the factor limiting bacteria viability over time. On the other hand, BL biochar had a higher pH of 8.59, which could be too high for *B. japonicum*, thus this carrier supported cell viability for only 9 weeks at a relatively low population density (1.3 x 10^3). The two best performing biochars, PR and DM, are characterized by elevated total pore areas, 17.32 m^2 g^{-1} and 13.36 m^2 g^{-1}, respectively, which is significantly higher than for PM and BL. Also, the porosity of these carriers is nearly the same (78.2 % and 78.5 %, respectively), while PM and BL revealed higher porosities of 83.3 % and 89.9 %, respectively. Thus similar porosity characteristics of the best and worst carriers might suggest that these are the factors that affect the population density. Quilliam et al. (2013) hypothesized that most bacteria, as well as fungal hyphae, cannot penetrate pores <1 µm in size, thus 17.5 $\pm$ 0.1% of the total pore volume of the biochar which was used in their study is uninhabitable. While the porosity of biochar varies significantly, depending on the feedstock and pyrolysis process, the average pore diameter of DM and PR biochars is 0.68 µm and 0.49 µm, respectively, so we can assume that these biochars provide a suitable habitat for bacteria. It could be expected that PM and BL, which have much higher average pore diameters of 5.15 µm and 6.03 µm, respectively, would be less suitable carriers. However, this cannot be confirmed by our results. Another hypothesis is that bacteria do not necessarily occupy the pore,

but actually colonize the surface of the biochar. However, this cannot be confirmed without scanning electron micrograph work.

Okereke and Okeho (2007) conducted a short-duration experiment on three *Bradyrhizobium* strains. They investigated the suitability of rice husk, charcoal and coal as carriers. The results showed that the most suitable carrier was rice husk, which sustained *Bradyrhizobium* strains at Log CFU between 5.8 and 7.5, after 6 weeks of incubation at 26 °C. It can be assumed that PR biochar investigated in this study is much more efficient, since Log CFU after 37 weeks from inoculation was 6.3. Khavazi et al. (2007) studied the effects of carrier, sterilization method and incubation on survival of *B. japonicum* in soybean inoculants. The results showed that bacterial populations were greatest after 180 days of incubation (Log No. bacteria g^{-1} inoculant about 9) in perlite mixtures. Our results showed that the population of *B. japonicum* in PR biochar was slightly lower; however, the experiment lasted 80 days longer.

Storage time of inoculated seeds

Two sets of biochar coated and inoculated with *B. japonicum* soybean seeds were prepared and stored at two different temperatures of 4°C and 21°C to determine the survival of bacteria in seed-coating and the influence of temperature on the population density of bacteria over time. Viable cell count was performed on each treatment at 1-week and later at 2-week intervals until 19 weeks. Results of this study are consistent with the biochar storage time experiment (Table 5.2). Again, biochars with the highest bacterial population after 19 weeks post-inoculation were PR and DM, which sustained the bacterial populations at 1.1×10^5 and 8.1×10^4, respectively, with seeds stored at 4°C (Figure 5.3). Clearly, the temperature significantly affected the survival of bacteria with regard to time. Low temperature (4°C) sustains the viability of *B .japonicum* for longer periods of time. Likewise, the population density of bacteria was significantly higher when seeds were stored at a low temperature. The decline in the number of colonies was also affected. For example, DM coated seeds stored at 21°C revealed a 62 % decline in population, but when seeds were stored at 4°C, the same treatment resulted in a decline of 22 % compared to the initial bacteria population. The carrier least suitable for *B. japonicum* seed-coating again turned out to be BQ biochar. After 4 weeks of incubation at 21°C, no viable cells were found (Figure 5.2). The survival of bacteria in this carrier was significantly higher at 4°C; however, the abundance of bacteria was much lower (2×10^2) compared to other

treatments. In addition, it was observed that peat moss (PM) sustained the bacterial abundance at a much higher level than in the biochar inoculation experiment. This distinctly shows that the main cause of a decrease in the bacteria population was pH. Once pH had been adjusted to neutral (pH 7.0), PM sustained the survival of *B. japonicum* for up to 19 weeks (both at 21°C and 4°C) at a level similar to DM biochar, 2.5 and 4.9 Log CFU at 21°C and 4°C, respectively. As with the powdered biochar inoculation, PR and DM biochar were identified as the most efficient carriers for *B. japonicum* seed-coating. It can be expected that the seed-coating system developed will ensure effective nodulation of soybean plants even after 4 months of storage.

Temprano et al. (2002) investigated the survival of a *B. japonicum* strain in a peat seed-coating formulation of *Lupinus albus*. The studies showed that bacteria had abundance on the seed similar to the powdered carrier. A slow decline in population of one order of magnitude with respect to the initial population was recorded; however, one month after inoculation viable-cell count was still sufficiently high to ensure good nodulation (10^6 bacteria seed^{-1}). The influence of storage temperature on survival of bacteria on the seed-coat was also determined. Temprano et al. (2002) reported that, in the case of peat and vermiculite carriers, survival of rhizobia is higher when stored at a 4 °C temperature than at 28 °C. These results correspond to the results of our study. As was previously explained by Gaind and Gaur (1990), lower temperatures (4°-10°C) reduce cell division and metabolic processes, thus retarding nutrient depletion. It was expected that the *B. japonicum* population density would be significantly lower in seed-coating compared to powdered biochar. This is probably due to the reduced moisture of the seed-coating, which was dried for two hours under a laminar hood before storage to prevent premature germination of soybean seeds. In the end, the density of *B. japonicum* in the PR seed-coating was sufficient to inoculate soybean seeds and meet the recommended doses of 10^5-10^6 rhizobia seed^{-1} (Lupyawi et al., 2000).

5.4.2 Evaluation of seed-coating and biochar inoculant on soybean growth

Germination experiment

To evaluate soybean seedling growth and the influence of seed-coating on corn seedling development, a germination test was carried out by Petri plate assay. Based on results from

previous experiments, PR and DM biochars were selected as the most appropriate carriers for *B. japonicum.* Seedling growth characteristics are presented in Table 5.4. The Shapiro-Wilk statistic indicated that the fresh weight data was not distributed normally. Nonparametric Kruskal-Wallis tests with adjustments for multiple comparisons (Dunn's test) indicated that there were differences between the treatments at $\alpha = 0.05$ level. Results showed that treatment PR+I produced significantly heavier soybean seedlings than all other seed treatments except the control (UC). PR coated seeds also resulted in heavier seedlings; however, the difference was not statistically significant. The root length data was normalized by power transformation. Likewise, seed treatments had no significant effect on dry weight of soybean seedlings. Regarding germination characteristics (Table 5.3), statistical analysis did not detect any significant differences between seed treatments. Although control seeds (UC) revealed a marginally better germination percentage, seedling vigour index as well as germination index differences were not significant. Even though there was no difference between PR and CU, most of the germination characteristics showed improved germination of PR coated-seeds over DM. It can also be observed that for biochar treated seeds, germination indicators such as germination percentage, seedling vigour index, speed of germination and root length were better, although not significantly, when seeds were inoculated with *B. japonicum*.

Results of the germination experiment demonstrate that biochar seed-coating does not negatively affect soybean germination characteristics, and even improved some of them slightly. At this stage of growth, the effect on inoculation was not expected to be present, since nodule formation usually begins no earlier than 10 days after sowing when inoculated with compatible rhizobia (Ohyama et al. 2009). Retarded germination and slightly decreased germination of soybean seed might be due to biochar seed-coating. A hardened layer of biochar around the seed can create a mechanical barrier for the radical, slowing down emergence and germination. Not many studies regarding influence of seed-coating, and none of biochar seed-coating, on the germination process are currently available. Cassán et al. (2009) investigated the response of soybean seed germination to *B. japonicum* strain E109 inoculation. The results showed that inoculation positively affected seed germination and improved shoot fresh and dry weight, as well as shoot and root length. The authors hypothesized that this capacity could be due at least partially to bacterial phytohormone biosynthesis. It was shown that strain E109 is able to excrete plant growth regulator compounds into the culture medium, at a concentration sufficient to produce morphological and physiological changes in young seed tissues. These results can be

confirmed with our study. Although the improvements were not statistically significant, better performance of biochar coated seeds was recorded for inoculated treatments over uninoculated ones. Phytohormone production by our strain was not determined, and the germination improvements are not as clearly evident; thus we cannot assign them to bacterial compounds. However, we cannot exclude the idea that they are synthetized by our strain; perhaps they are retained in the biochar coating and do not affect the seed directly. Summarizing our germination study, seed-coating neither improves nor decreases soybean germination. However, due to the better performance of PR over DM biochar both in the storage time experiment and in the germination experiment, this biochar has been selected for further studies.

Greenhouse experiment

The greenhouse experiment was conducted to evaluate the influence of bacterial seed-coating as well as inoculated biochar on soybean growth characteristics. DM biochar was selected for this study. The soybean growth characteristics for plants supplied with complete (+N) and N-free (-N) Hoagland's solution are presented in Table 5.5. Results demonstrated that when plants were watered with complete Hoagland's solution, no variation in soybean growth was recorded across all treatments. Fresh weight of plants was slightly better for the UC+I treatment; however, the difference was not statistically meaningful. The same treatment resulted in 17 % higher plants compared to the control (UC). Also, application of biochar inoculant at the rate of 5 t ha^{-1} (UC + PRI) improved plant height by 22 % compared to the control; nevertheless, in both cases the differences were statistically insignificant. There were no variations between seed treatments with regard to pod number, total leaf area and dry weight of soybean plants. Slightly better pod fresh and dry weights were recorded for PR and UC+I treatments.

Root analysis results (Table 5.6) of the plants supplied with complete Hoagland's (+N) solution are consistent with above ground plant characteristics. When plants were supplied with nitrogen (+N), no variability between treatments was observed. Neither inoculation nor biochar significantly affected the roots of soybean. Although there was no statistical difference in root fresh and dry weight, it was observed that inoculated seeds developed slightly smaller roots of decreased weight compared to those which were not inoculated. Furthermore, very low numbers of nodules were recorded for inoculated plants. Biochar coated and inoculated seeds (PR+I)

produced an average of 4.2 ± 0.49 nodules per plant, while uncoated and inoculated with *B. japonicum* seeds (UC+I) only produced an average of 3.1 ± 0.83.

Chemical analysis of soybean tissue was performed to investigate the influence of seed-coating and biochar inoculant on basic uptake of elements (Table 5.7). Although the control (UC) revealed a slightly higher nitrogen percentage (N %), results showed that there was no statistically significant variation across the treatments of soybean plants supplied with complete Hoagland's solution (+N). Similarly, no statistical difference in carbon percentage (C %) was found; however, UC once again revealed slightly elevated C%. Results indicate that the control (UC) presents a marginally higher nitrogen to carbon ratio (C/N) compared to other treatments; however, it is only significantly ($\alpha = 0.05$) higher compared to PR+I treatment. A statistically higher ($\alpha = 0.05$) concentration of phosphorus (P) was recorded for UC and PR treatments. On the other hand, both treatments with *B. japonicum* inoculation resulted in decreased P content compared to uninoculated treatments, but only PR+I was significantly lower ($\alpha = 0.05$).

A number of studies were conducted to evaluate the impact of N fertilizer on biological N-fixation. Antagonism between nitrate concentration in the soil solution and the N_2 fixation process in the nodules is considered to be one of the constraints in terms of improving overall N uptake (Salvagiotti et al. 2008). Most studies on the effect of fertilizer-N on soybean growth and N_2 fixation by rhizobium concluded that fertilization reduces N fixation through a reduction in the number, weight and activity of nodules (Starling et al., 1998; Chen et al., 1992). Our results clearly demonstrate that N_2-fixation was significantly affected by N-fertilizer application. Nodule number was significantly reduced compared to that of plants grown in the absence of nitrate. This is consistent with the study of Albareds et al. (2009). They showed that addition of N-fertilizer to inoculated soybean did not improve seed yields when compared with inoculated only treatment. Furthermore, the addition of 50 Kg N ha^{-1} at the beginning of flowering resulted in a reduction of nodulation in inoculated plots. Hungria et al. (2005) showed that early application of even small amounts of N results in temporary suspension of nodule establishment and decreases their activity. A negative relationship was observed between N-fertilizer dose and N_2 fixation when nitrate was applied in the top 0-20 cm of the soil surface (Salvagiotti et al. 2008). It appears that nitrate inhibition has a local effect and the inhibitory effect may be reduced when the nitrate concentration in the zone surrounding the nodules is not increased (Arrese-Igor et al. 1997). In our experiment, N fertilizer diminished the effect of *B. japonicum* inoculant.

Growth characteristics as well as chemical composition and chlorophyll content showed no difference between inoculated and uninoculated seeds. Furthermore, no biochar effect was recorded either. This strongly suggests that with the application of rhizobium inoculants when also supplying the plants mineral nitrogen is both unnecessary and economically undesirable, as the effect of bacteria-plant symbiosis is suppressed. However, use of inoculants, instead of mineral nitrogen is both economically and environmentally desirable.

Variability in soybean growth was recorded for plants supplied with N-free Hoagland solution. The main source of variation is the effect of inoculant. Fresh weight of plants ($\alpha = 0.05$) improved significantly for all inoculated seed treatments. However, neither biochar seed-coating (PR+I) nor application of inoculated biochar (UC+PRI) improved soybean fresh weight compared to uncoated inoculated seeds (UC+I). The same was observed for soybean dry weight, which was significantly ($\alpha = 0.05$) improved when seeds were inoculated. Significantly taller plants ($\alpha = 0.05$) resulted when seeds were inoculated. Again, treatment with UC+I resulted in better growth than biochar treated seeds. Statistically ($\alpha = 0.05$) more pods were produced by all treatments involving inoculation with *B. japonicum.* Once again, biochar treatment did not affect pod number compared to uncoated seeds and pod fresh and dry weight was higher for seeds coated with biochar (PR+I) than for uncoated seeds supplied with inoculated biochar (UC+PRI). The number of trifoliate leaves was much higher for inoculated plants; thus total leaf area was also significantly ($\alpha = 0.05$) larger for inoculated plants. UC+PRI treatment resulted in 17 % and 6.8 % larger total leaf area compared to UC+I and PR+I, respectively.

Variation in root characteristics were recorded when plants were watered with N-free Hoagland`s solution (Table 5.6). Although root fresh and dry weight was slightly better for inoculated seeds, no significant difference was found between treatments. Insignificant numbers of nodules were recorded for uncoated seeds (UC) which served as a control, as well as for Pyrovac coated seeds (PR), which was the result of contamination, most probably during seeding or watering. Inoculated seeds produced statistically ($\alpha = 0.05$) more nodules compared to uninoculated seeds. UC+PRI treatment resulted in the highest number of nodules per plant, which was 29.6 ± 5.08; although it was 34 % more than UN+I, the difference was statistically insignificant. UC+PRI treatment produced 48 % more nodules than UC+PRI, which was marginally more ($\alpha = 0.2$) than PR+I treatment. Fresh and dry weight of nodules was highest when uncoated seeds were supplied with inoculated biochar at a rate of 5 t ha^{-1} (UC+PRI);

however, there was no significant difference across other treatments involving *B. japonicum* inoculation.

Plants supplied with N-free Hoagland's solution and grown from inoculated seeds showed a significant ($\alpha = 0.05$) increase in N percentage compared to uninoculated seed treatments (Table 5.7). The highest N % was recorded for UC+PRI, which was 4.35 ± 0.087. UC+I and PR + I resulted in similar N % values of 3.65 ± 0.052 and 3.82 ± 0.111, respectively; however, the differences between these treatments were not statistically significant. C % was also increased in plants inoculated with *B. japonicum*. Likewise, the nitrogen to carbon ratio (N/C) was improved significantly ($\alpha = 0.05$) for the inoculated treatments. Additionally, application of inoculated biochar (UC+PRI) caused a statistical increase ($\alpha = 0.05$) N/C compared to other treatments involving *B. japonicum* inoculation. P analysis showed that inoculation significantly affects P concentration in soybean tissue. Uninoculated treatments (UC and PR) resulted in a statistically ($\alpha = 0.05$) higher content of P compared to inoculated plants (UC+I, PR+I, UC+PRI). Significantly higher ($\alpha = 0.05$) chlorophyll index contents were caused by seed treatment with *B. japonicum* (Figure 5.4). Furthermore, results demonstrated that across all inoculated treatments, ones with biochar (PR+I and UC+PRI) showed no severe decrease in chlorophyll content between the 3rd and 4th week of soybean growth, as was generally the case for the control plants.

Many studies have demonstrated a positive influence of rhizobium inoculants on soybean growth. Albareda et al. (2009) showed that soybean inoculated with *Bradyrhizobium japonicum* strain USDA101 showed an important increase in number and dry weight of nodules, seed yield and seed N content. Thuita and colleagues (2011) tested a number of commercially available rhizobial inoculants on two different soybean varieties. The results of this study showed that different soybean varieties respond differently to inoculation; however, nodulation, biological nitrogen fixation, and biomass yield are improved. The authors suggest that commercial products manufactured elsewhere can be an important source of effective strains for use in regions where soybean is being introduced, or where populations of indigenous rhizobia poorly nodulate soybean plants. Similarly, Kubota et al. (2008) demonstrated that peat moss seed-coating with commercially available *B. japonicum* inoculant improves shoot and nodule dry weight. On the other hand, Okereke and Okeh (2007) demonstrated the importance of the strain used as an inoculant. They studied 4 different *B. japonicum* strains (TAL 209, 173, 658 and 379)

and their influence on cowpea. Although all strains improved plant dry weight and total N content, some differences between strains were recorded. Additionally, the experiment was conducted in sandy and silty soils, which also affect inoculant efficiency. Therefore it is strongly suggested to select the inoculant which is appropriate for the plant, but also for the type of soil. In general, our results confirm the previous study and clearly demonstrate the positive effect of inoculation on soybean growth. Although seed-coating and biochar inoculant application does not seem to have an important effect on plant growth, the nodulation process was significantly improved for the plants supplied with inoculated biochar at a rate of 5 t ha^{-1}. Increased nodule number and weight suggest that during later growth stages, these plants will be capable of fixing more nitrogen and thus the final yield will be probably higher compared to plants with lower nodule numbers. For example, Albareda et al. (2008) showed that the soybean plants with higher numbers and dry weight of nodules result in significantly higher seed yield; moreover, seed N content was also significantly higher. Furthermore, by comparing our plant growth results of –N and +N soybean, we can assume that seed inoculation as well as direct application of inoculated biochar, will result in increased nodulation and adequate N_2 fixation, which will cover the soybean demand for nitrogen and produce the same biomass as the plant supplied with complete Hoagland's solution. In our experiment the pod fresh weight during the R1/R3 growth stage was higher for –N but inoculated plants (particularly for UC+I and PR+I) than for the +N plants, both uninoculated as well as inoculated (Figure 1). Although there was no significant difference between uncoated seeds and biochar coated seeds, we must not forget that seeds used in our experiment were inoculated 1 day prior to seeding; therefore the bacteria population was very similar for both coated and uncoated seeds. However, it can be expected that after 3 months of storage, the bacteria population on uncoated seeds will decline drastically and will not be able to ensure high enough soybean nodulation, while our previous experiment showed that even after 4 months of storage at 4°C, the bacteria density in PR coated seeds was high enough to meet the recommended doses of 10^5-10^6 for efficient nodulation.

5.5 Conclusions

Bradyrhizobium japonicum is the symbiotic bacteria nodulating soybean plants. Its capacity to fix atmospheric nitrogen and therefore supply the soybean nitrogen demand and significantly reduce N fertilizer application is one of the main reasons why, in recent years, a number of commercially available rhizobial inoculants have appeared on the market (Ben Rebah et al. 2007). In our study, we proposed biochar as an inoculant carrier for *B. japonicum* as an alternative to traditionally used peat moss. Moreover, we developed a seed-coating system which permits the maintenance of a high population of *B. japonicum* for up to 4 months, which can ensure efficient nodulation of soybean. Our investigation demonstrated that chemical and physical properties of biochars strongly affect survival of bacteria over time. Out of five inoculated carriers, Pyrovac (PR) and Dynamotive (DM) demonstrated the most suitable conditions to support viability of bacteria for up to nearly 9 months. We suspect that the key factor was chemical composition as well as porosity of biochar. Moreover, by adjusting the pH of peat moss, we demonstrated that this was the main factor limiting bacterial viability. Once the pH was raised to 7, peat moss performed as well to the two best biochars. However, it can be considered to be economically undesirable since it is a costly and labour intensive process. A seed-coating storage time experiment confirmed that PR and DM biochars are the most suitable carriers and they were selected for further studies.

A germination experiment revealed that seed-coating does not negatively affect soybean germination. Although there was no significant difference in germination characteristics and seedling growth, PR performed better than DM biochar and similarly or slightly better than the control (UC). Finally, a greenhouse experiment showed that application of nitrogen fertilizer significantly affects the efficiency of the inoculant. Our results support previous studies and show that nitrate application decreases the nodulation process and reduces the number of nodules. When soybean plants were provided with complete Hoagland's solution (+N), a difference in plant growth and nutrient uptake between treatments was not found. However, when plants were watered with N-free Hoagland's solution (-N), significant differences in plant growth, chemical composition and chlorophyll content were found. Clearly, *B. japonicum* inoculation was the main reason for variation; it improved plant growth and uptake of some nutrients. We observed that application of inoculated biochar at a rate of 5 t ha^{-1} increased

nodulation of soybean. This strongly suggests that in later development stages, these plants would probably grow better due to higher nitrogen availability.

Further investigations are necessary to assess seed-coating efficiency. It is essential to evaluate the performance of inoculated and coated with biochar seeds after longer periods of storage. Moreover, to better understand the bacterial colonization of biochar, scanning electron micrographs should be performed and bacterial residency and distribution should be evaluated. It is also recommended that the dispersal and the facility of bacteria to move from the biochar coat be investigated.

5.6 Tables and Figures

Table 5.1 Viable count in Log_{10} CFU mL^{-1} of *B. japonicum* in different carrier materials

Carriers	Weeks after inoculation											
	1	**2**	**4**	**6**	**9**	**13**	**17**	**21**	**26**	**31**	**34**	**37**
DM	5.3	6.1	6.7	6.5	6.8	6.9	6.4	5.6	5.3	4.5	4.6	4.8
PR	7.1	7.8	7.9	8.0	8.1	7.9	7.6	7.6	7.0	6.4	6.8	6.3
BQ	7.4	6.6	7.0	6.9	7.7	6.2	5.1	0.0	0.0	0.0	0.0	0.0
BL	3.7	3.8	3.0	3.1	3.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
PM	1.6	1.6	1.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Dynamotive biochar (DM), Pyrovac (PR), Basque (BQ), BlueLeaf (BL), peat moss (PM)

Table 5.2 Viable count in Log CFU_{10} mL^{-1} of *B. japonicum* biochar coated seed stored at 21°C and 4°C

Carrier	Weeks after inoculation									
	24h	**1**	**2**	**4**	**6**	**8**	**10**	**13**	**16**	**19**
21°C										
DM	6.2	5.5	5.2	4.6	4.3	3.8	3.6	3.6	3.2	2.3
PR	6.0	5.6	5.3	5.3	4.8	4.4	4.5	3.9	3.3	3.0
BQ	4.9	4.7	3.2	1.8	0.0	0.0	0.0	0.0	0.0	0.0
BL	5.7	4.9	4.6	3.6	3.1	2.5	0.0	0.0	0.0	0.0
PM	6.5	5.5	5.0	4.6	4.2	3.7	3.6	2.7	2.3	2.5
4°C										
DM	6.3	5.8	5.6	6.6	5.5	5.6	5.1	5.3	4.7	4.9
PR	6.3	6.1	5.8	5.9	6.0	5.9	5.7	5.7	5.1	5.0
BQ	5.3	4.1	4.6	4.1	3.5	3.6	3.5	3.9	2.5	2.3
BL	6.0	5.4	5.3	4.8	6.0	4.7	4.5	4.8	4.5	4.5
PM	6.4	5.8	6.0	5.9	5.6	5.5	5.5	5.4	4.8	4.9

Dynamotive biochar (DM), Pyrovac (PR), Basque (BQ), BlueLeaf (BL), peat moss (PM)

Table 5.3 Soybean seedling germination characteristics

Seed Treatment	Germination Percentage	Seedling Vigour Index	Speed of Germination	Germination Index
PR	97 ± 1.5	8656 ± 742	4.7 ± 0.15	13.8 ± 0.2
PR+I	95 ± 2.2	8601 ± 616	5.7 ± 0.30	13.6 ± 0.3
DM	90 ± 3.3	7595 ± 576	4.6 ± 0.29	12.9 ± 0.4
DM+I	93 ± 2.1	8000 ± 671	4.7 ± 0.12	13.2 ± 0.3
UC	99 ± 1.0	9389 ± 1039	5.0 ± 0.19	14.1 ± 0.1

Uncoated seeds (UC), Pyrovac coated seeds (PR), Uncoated inoculated with *B. japonicum* seeds (UC+I), Pyrovac coated inoculated with *B. japonicum* seeds (PR+I), and uncoated seeds + 5 t ha-1 *B. japonicum* inoculated Pyrovac (UC + PRI).
Bonferroni Grouping for Least Squares Means at α=0.05 showed no significant differences in germination characteristics

Table 5.4 Soybean seedling growth characteristics

Seed Treatment	Fresh weight * [g]	Root length [cm]	Dry weight [g]
PR	0.5153 ± 0.01564b	9.3 ± 0.60	0.1318 ± 0.0022
PR+I	0.5492 ± 0.01258a	9.0 ± 0.54	0.1330 ± 0.0022
DM	0.4786 ± 0.00996b	8.4 ± 0.66	0.1357 ± 0.0021
DM+I	0.4718 ± 0.00811b	8.6 ± 0.54	0.1293 ± 0.0021
UC	0.5541 ± 0.01485a	9.5 ± 0.63	0.1349 ± 0.0039

Uncoated seeds (UC), Pyrovac coated seeds (PR), Uncoated inoculated with *B. japonicum* seeds (UC+I), Pyrovac coated inoculated with *B. japonicum* seeds (PR+I), and uncoated seeds + 5 t ha^{-1} *B. japonicum* inoculated Pyrovac (UC + PRI).
Bonferroni Grouping for Least Squares Means (α=0.05). LS-means with the same letter are not significantly different.

Table 5.5 Soybean growth characteristics

Seed Coating	Fresh Weight (g)	Plant Height (cm)	Pod Number	Leaf Area (cm^2)	Dry Weight (g)	Pod Fresh Weight (g)	Pod Dry Weight (g)
+N							
UC	3.8 ± 0.25	60.5 ± 4.9	2.5 ± 0.3	156 ± 8.7	1.4 ± 0.08	0.47 ± 0.07	0.113 ± 0.02
PR	3.8 ± 0.17	62.2 ± 4.6	2.5 ± 0.2	157 ± 8.5	1.3 ± 0.06	0.63 ± 0.10	0.122 ± 0.02
UC+I	4.1 ± 0.19	72.6 ± 6.3	2.5 ±.0.3	158 ± 6.4	1.4 ± 0.06	0.62 ± 0.11	0.135 ± 0.02
PR+I	3.8 ± 0.21	77.3 ± 5.4	2.7 ± 0.2	150 ± 7.9	1.4 ± 0.11	0.35 ± 0.05	0.080 ± 0.01
-N							
UC	1.6 ± 0.15b	52.7 ± 4.5b	1.1 ± 0.3b	49 ± 6.5b	0.5 ± 0.04b	0.32 ± 0.06b	0.067 ± 0.012bc
PR	1.5 ± 0.11b	53.5 ± 4.2b	0.8 ± 0.3b	46 ± 6.5 b	0.5 ± 0.04b	0.13 ± 0.03c	0.032 ± 0.006c
UC+I	4.1 ± 0.30a	81.6 ± 6.8a	3.2 ± 0.4a	169 ± 13.2a	1.3 ± 0.11a	0.75 ± 0.11a	0.154 ± 0.021a
PR+I	3.6 ± 0.21a	61.0 ± 6.2ab	3.3 ± 0.3a	190 ± 10.8a	1.1 ± 0.10a	0.72 ± 0.10a	0.145 ± 0.019a
UC+PRI	4.0 ± 0.35a	60.5 ± 5.6ab	3.0 ± 0.4a	204 ± 13.6a	1.2 ± 0.08a	0.59 ± 0.09ab	0.132 ± 0.018ab

Uncoated seeds (UC), Pyrovac coated seeds (PR), Uncoated inoculated with *B. japonicum* seeds (UC+I), Pyrovac coated inoculated with *B. japonicum* seeds (PR+I), and uncoated seeds + 5 t ha^{-1} *B. japonicum* inoculated Pyrovac (UC + PRI). Plants supplied with complete Hoagland's solution (+N), and N-free Hoagland's solution (-N)
Bonferroni Grouping for Least Squares Means ($\alpha = 0.05$). LS-means with the same letter are not significantly different

Table 5.6 Soybean root system characteristics

Seed treatment	Root fresh weight (g)	Nodules number/plant	Nodule fresh weight (g)	Nodule dry weight (g)	Root dry weight (g)
+N					
UC	4.07 ± 0.34	0.0	0.00	0.0000	0.49 ± 0.05
PR	4.25 ± 0.29	0.0	0.00	0.0000	0.58 ± 0.05
UC+I	3.95 ± 0.33	3.1 ± 0.83	0.057 ± 0.031	0.0078 ± 0.0013	0.49 ± 0.03
PR+I	3.54 ± 0.27	4.2 ± 0.49	0.088 ± 0.048	0.0084 ± 0.0007	0.41 ± 0.03
-N					
UC	2.25 ± 0.22	0.83 ± 0.29b	0.041 ± 0.053b	0.0071 ± 0.0028b	0.29 ± 0.03
PR	2.33 ± 0.14	0.91 ± 0.37b	0.038 ± 0.015b	0.0040 ± 0.0017b	0.22 ± 0.01
UC+I	2.98 ± 0.19	19.4± 1.81a	0.509 ± 0.035a	0.1066 ± 0.0073a	0.32 ± 0.03
PR+I	2.96 ± 0.23	15.3 ± 3.04a	0.447 ± 0.036a	0.0904 ± 0.0072a	0.33 ± 0.03
UC+PRI	3.05 ± 0.22	29.6 ± 5.08a	0.534 ± 0.038a	0.1117 ± 0.0076a	0.38 ± 0.05

Uncoated seeds (UC), Pyrovac coated seeds (PR), Uncoated inoculated with *B. japonicum* seeds (UC+I), Pyrovac coated inoculated with *B. japonicum* seeds (PR+I), and uncoated seeds + 5 t ha^{-1} *B. japonicum* inoculated Pyrovac (UC + PRI). Plants supplied with complete Hoagland's solution (+N), and N-free Hoagland's solution (-N). Bonferroni Grouping for Least Squares Means at $\alpha = 0.05$ did not showed significant differences between treatments

Table 5.7 Soybean chemical composition

Seed Coating	N % **	C %	N/C *	P (ppm) *
+ N				
UC	2.91 ± 0.070bc	44.2 ± 0.068	0.026 ± 0.0018a	14.8 ± 0.4a
PR	2.52 ± 0.143dc	43.8 ± 0.188	0.023 ± 0.0013ab	14.7 ± 1.5a
UC + I	2.56 ± 0.085dc	44.1 ± 0.142	0.023 ± 0.0007ab	13.4 ± 0.6ab
PR + I	2.34 ± 0.073dc	43.9 ± 0.116	0.022 ± 0.0007b	10.6± 0.6b
- N				
UC	2.02 ±0.167de	41.4 ± 0.367b	0.019 ± 0.0017c	84.8 ± 1.9a
PR	1.78 ± 0.187e	40.5 ± 0.285b	0.017 ± 0.0017c	91.1 ± 3.7a
UC + I	3.65 ± 0.052ba	44.6 ± 0.132a	0.034 ± 0.0004b	53.3 ± 4.5ba
PR + I	3.82 ± 0.111a	44.5 ± 0.146a	0.035 ± 0.0009bc	62.9 ± 3.9ba
UC + PRI	4.35 ± 0.087a	44.4 ± 0.101a	0.041 ± 0.0009a	68.3 ± 2.6b

Uncoated seeds (UC), Pyrovac coated seeds (PR), Uncoated inoculated with *B. japonicum* seeds (UC+I), Pyrovac coated inoculated with *B. japonicum* seeds (PR+I), and uncoated seeds + 5 t ha^{-1} *B. japonicum* inoculated Pyrovac (UC + PRI). Plants supplied with complete Hoagland's solution (+N), and N-free Hoagland's solution (-N).

* Bonferroni Grouping for Least Squares Means (α=0.05). LS-means with the same letter are not significantly different.

** Scheffe Grouping for Least Squares Means (α=0.05).

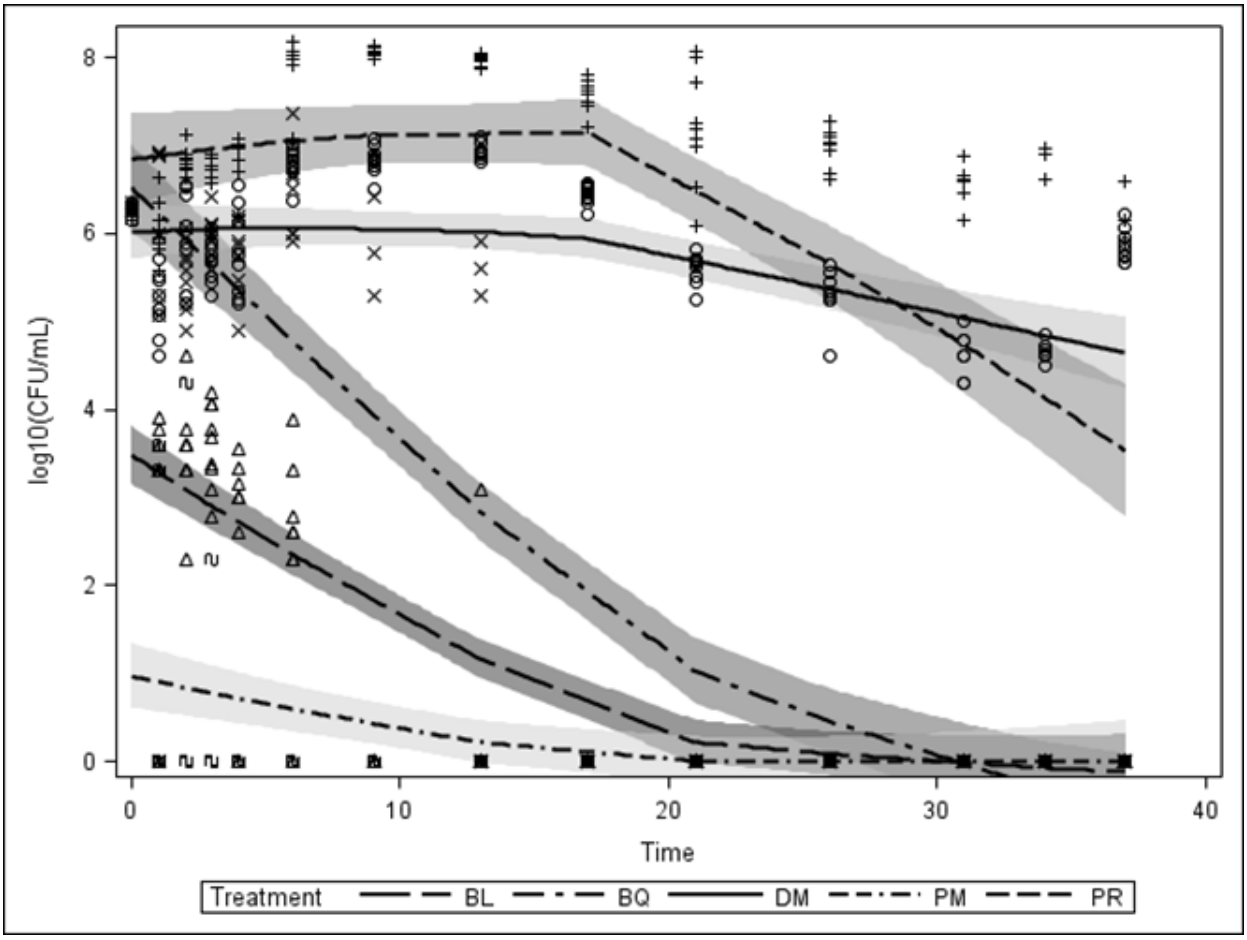

Figure 5.3 Scatter-diagram smoothing/local regression for Log_{10} CFU mL^{-1} of *P. libanensis* in powdered biochar stored at 21°C as a function of time. Shaded areas represent the confidence limits ($\alpha = 0.05$). Peat moss (PM), BlueLeaf (BL), Pyrovac (PR), Dynamotive (DM), Basque (BQ)

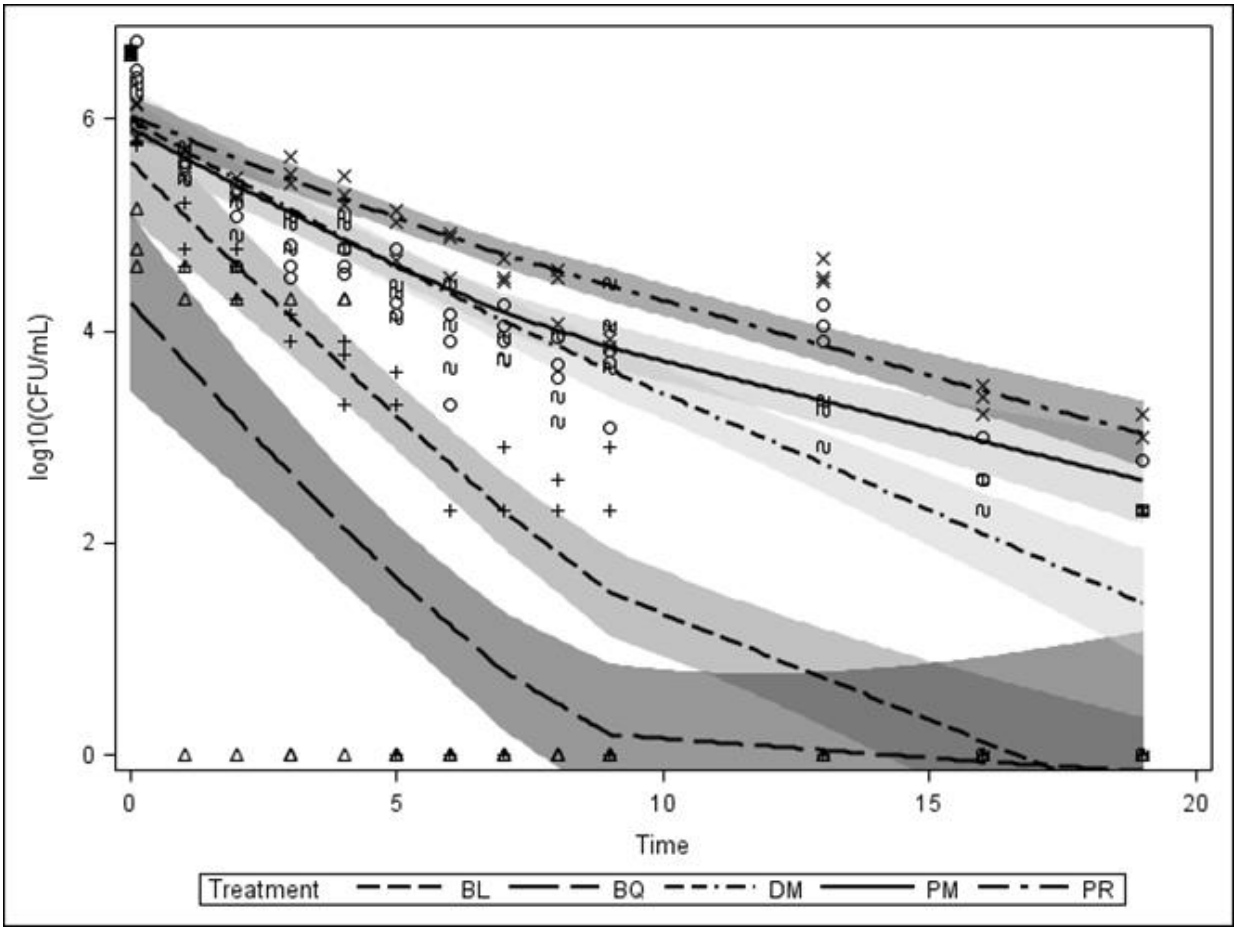

Figure 5.4 Scatter-diagram smoothing of local regression for Log_{10} CFU mL^{-1} of *P. libanensis* in coated seeds stored at 21°C as a function of time. Shaded areas represent the confidence limits (α = 0.05). Seed-coating carriers: Peat moss (PM), BlueLeaf (BL), Pyrovac (PR), Dynamotive (DM), Basque (BQ)

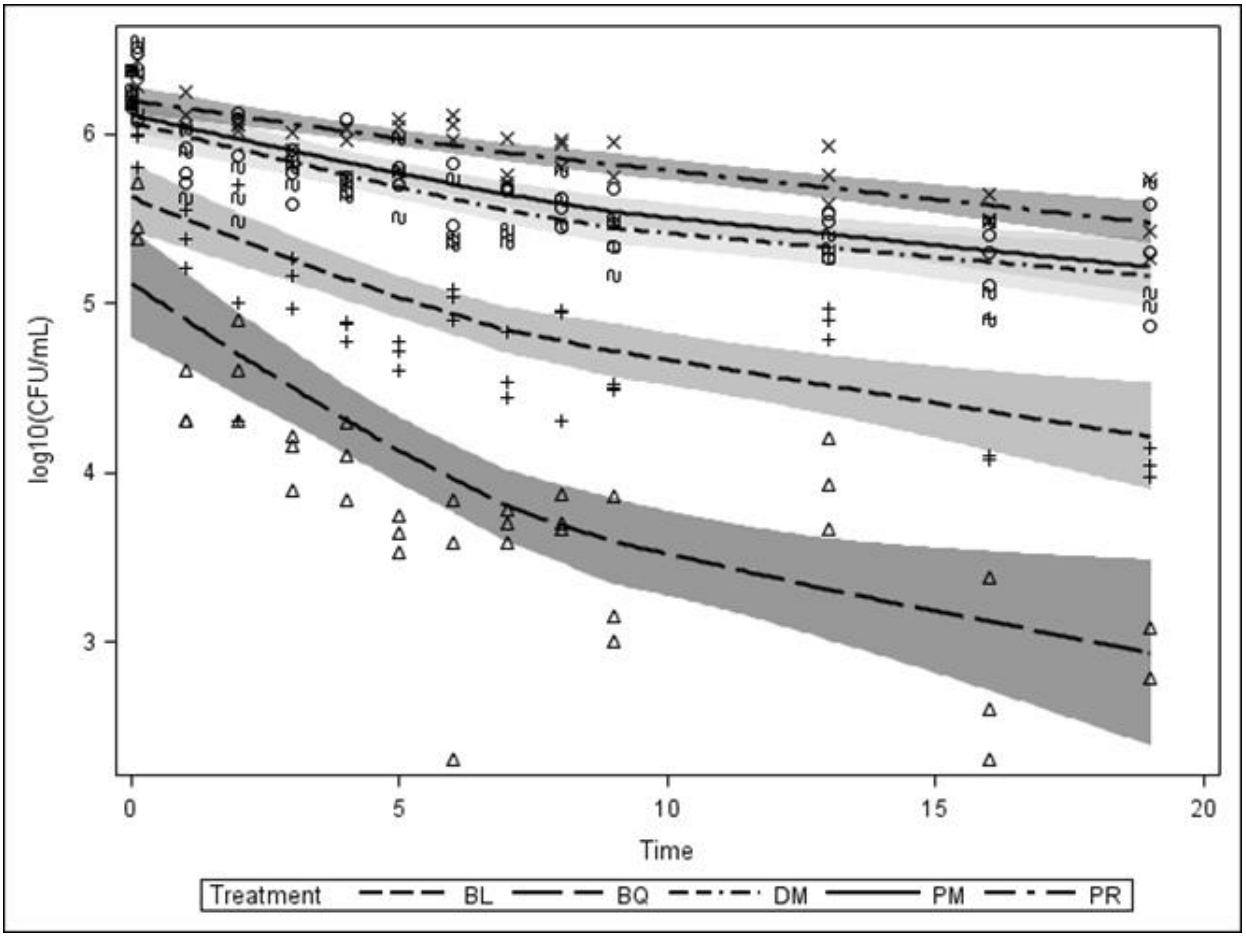

Figure 5.5 Scatter-diagram smoothing/local regression for Log_{10} CFU mL^{-1} of *P. libanensis* in coated seed stored at 4°C as a function of time. Shaded areas represent the confidence limits (α = 0.05). Seed-coating carriers: Peat moss (PM), BlueLeaf (BL), Pyrovac (PR), Dynamotive (DM), Basque (BQ)

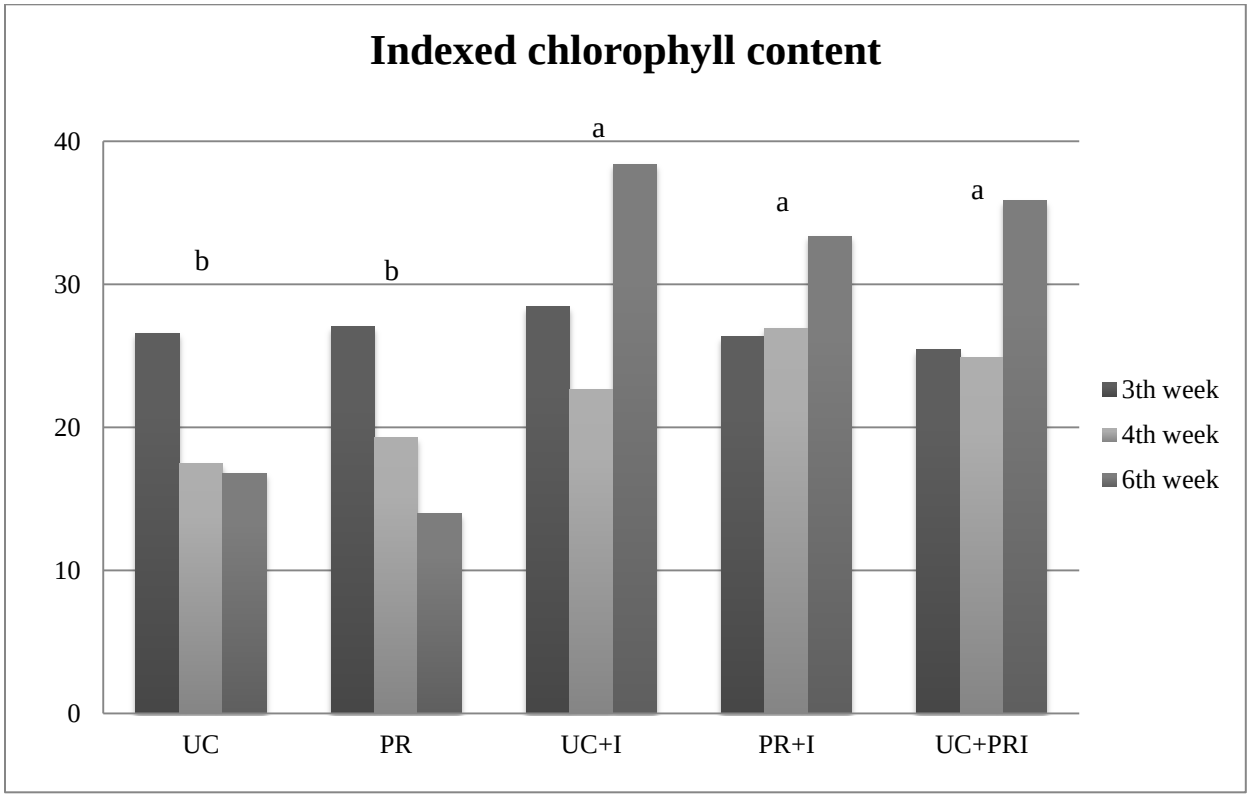

Figure 5.4 Indexed chlorophyll content for the soybean plants supplied with N-free Hoagland's solution (-N). Measurements were taken 3 times during growth period (3, 4, 6 weeks after seeding). Scheffe Grouping for Least Squares Means ($\alpha = 0.05$). LS-means with the same letter are not significantly different.

6. Summary, Conclusions and Future Research

In recent years, with both environmental and economic concerns, the urgent need to reduce fertilizer consumption has become the reason behind research to find alternatives. One successful solution has turned out to be bacterial inoculants, many of which are currently available on the market. However, the efficiency of these products is often questionable. Moreover, they are not always easy to handle. Most commercial inoculants are available in a liquid form which needs to be mixed or applied directly to the soil prior to seeding. Often it is a time and labour consuming process, which farmers would rather avoid. Furthermore, the majority of commonly accessible inoculants use peat moss as a carrier, which is a limited non-renewable natural resource (Temprano et al. 2002). The current situation and observations initiated this research which proposes biochar as a carrier for beneficial bacteria. Biochar is the product of biomass combustion under low-oxygen or anaerobic conditions. Many of its chemical and physical characteristics suggest that this material might be an appropriate carrier for microbial inoculants. In this thesis we investigated the potential of 4 selected biochar carriers for two beneficial bacteria strains: P-solubilizing *Pseudomonas libanensis* and N_2-fixing *Bradyrhizobium japonicum.* We developed a seed-coating system using biochar, which can facilitate inoculant application and save time and labour for potential users.

In Chapter 4 we demonstrated that chemical and physical characteristics of biochar strongly affect the viability of bacteria as a function of time. Clearly, not every biochar can sustain the survival of bacteria in the same way. We think that pH, chemical composition and porosity are the main factors which determine the suitability of biochar as a carrier. Furthermore, in Chapter 4 we showed that when biochar possesses the appropriate characteristics, it can sustain viability of *P. libanensis* for more than 5 months in powdered biochar, and for 4 months in seed-coating. I also demonstrated that storage temperature significantly affects viability. Evidently low temperature (4°C) is more suitable to maintaining high bacteria density in biochar seed-coating. To evaluate the efficiency of the inoculants, a greenhouse experiment was conducted. Because of its importance as a crop and its high phosphorus demand, corn (*Zea mays*) was selected as a model plant. A germination assay demonstrated that seed-coating positively

affects corn germination. However, a greenhouse experiment did not confirm these results. Depending on the nutrients provided, plants responded differently to seed treatments. Surprisingly, when plants were supplied with all necessary nutrients (+P), the only treatment which significantly improved corn growth was uninoculated biochar coated seeds (DM). However, the same treatment with *P. libanensis* inoculation (DM+I) did not affect plant growth. I concluded that biochar can serve as a suitable carrier for *P. libanensis*, although the influence of the inoculant and seed-coating on plant growth is not confirmed. I hypothesise that *P. libanensis* might be unable to compete, or even be completely suppressed, by indigenous bacteria occurring in an unsterile environment.

Chapter 5 confirmed the results of biochar inoculation and the storage time experiment in Chapter 4. Although different bacteria were used in this study, the same two biochars as in the previous chapter (PR and DM) sustained the viability of bacteria for a significantly longer period of time. Inoculation of biochar revealed that the best performing biochar (PR) can support the survival of *B. japonicum* for up to 9 months at a population density high enough to ensure efficient soybean nodulation. A seed-coating storage time experiment also showed that PR and DM biochars are the most efficient carriers. Additionally, I decided to adjust the pH of peat moss from 4.9 to 7.0 to further use it for seed-coating. The results clearly demonstrated that pH was the key factor limiting bacteria survival. Once the pH was increased to 7, peat moss seed-coating revealed results similar to the two best performing biochars (PR and DM). Results of the *B. japonicum* seed-coating storage experiment were consistent with results from Chapter 4. Again, the same biochars performed similarly in both experiments and viability of bacteria was improved when seeds were stored at a low temperature (4°C). A final count of viable cells revealed that PR biochar supports the survival of *B. japonicum* for almost 5 months at a population density which meets the recommended population density for efficient rhizobia inoculants. Furthermore, the biochar inoculant and seed-coating developed were assessed in a germination assay. Soybean (*Glycine max*), as a symbiont of *B. japonicum* and an important crop, was selected for the study. Results showed that seed-coating affects neither germination characteristics nor seedling growth. Finally, a greenhouse experiment was carried out. It clearly demonstrated that nodulation was significantly reduced when plants were supplied with N-fertilizers, such that inoculation resulted in no differences in plant growth and nutrient uptake. On the other hand, when plants were watered with N-free nutrient solution, seed inoculation as well as application of inoculated biochar significantly improved soybean growth and nutrient

content, as well as chlorophyll content. We were not able to find statistically significant differences between inoculated treatments, which means that uncoated seed performed similarly to biochar coated seed and to biochar itself. However, we observed that application of inoculated biochar at a rate of 5 t ha^{-1} resulted in significantly higher numbers of nodules than other inoculated treatments, which could suggest potentially better yield at the end of the growing period. We concluded that once again, biochar turned out to be a suitable carrier for bacteria. We explain the lack of clear differences between inoculated treatments by too short storage time used in the greenhouse experiment seeds. We suspect that after 3 months of storage, biochar-coated seeds would perform much better than just inoculated seeds. We think that a biochar coat provides suitable protection against desiccation is source of nutrients and provides a fitting habitat for bacteria.

The general objective of this research was to determine whether biochar can serve as a carrier for bacteria, as well as to evaluate how developed inoculants and seed-coating affect plant growth. While all of these objectives were met, several potential research questions arose from this investigation. There are a number of areas which still need to be explored and better understood. Thus, my recommendations for further research include:

1. Further characterization of biochar as a carrier. Classification by its physical and chemical characteristics could significantly facilitate the selection of biochar as a potential carrier. Moreover, it is important to determine the niche bacteria occupy in biochar, whether it is the surface or the pores, and if it is the pores, which pore dimensions are most appropriate.
2. Better understanding of bacterial dispersal. How bacteria are retained in biochar and whether they can be easily released from the biochar coat to colonise the root system.
3. To clearly asses the efficiency of seed-coating on plant growth, a greenhouse experiment should be conducted with seeds, inoculated and not, which have been subjected to different storage times.
4. Finally, the capacity of inoculated bacteria to compete with indigenous strains in a natural environment should be assessed. Field experiments should be carried out to determine whether seed-coating improves plant growth under field conditions.

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