Effect of biochar amended with beneficial microbes on establishment and growth of cranberry (*Vaccinium macrocarpon* L.) cuttings under controlled conditions

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List of abbreviations

BMP	Best management practices
CTAB	Cetyltrimethylammonium bromide
CFU	Colony forming unit
DNA	Deoxynucleic acid
ErM	Ericoid mycorrhizal
ISQ	Institue de la statistique du Québec
K	Potassium
LBA	Lauria Broth Agar
N	Nitrogen
NA	Nutrient Agar
PBS	Phosphate buffered saline
PGPR	Plant growth promoting rhizobacteria
P	Phosphorous
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
p/p	Poids / Poids
qPCR	Quantitative real-time polymerase chain reaction
WA	Water Agar
AMF	Arbruscular mycorrhizal fungi
PVP	Polyvinylpyrrolidone

Abstract

Sustainable production of cranberry (*Vaccinium macrocarpon*) in Quebec fields requires the adoption of best management practices. A promising approach is the application of biochar combined with beneficial microbes as soil amendment, leading to a faster establishment of cranberry fields and reduced use of agrichemicals. This study was undertaken in order to determine whether the application of maple bark biochar amended with selected plant growth promoting rhizobacteria (PGPR) would stimulate vegetative growth of "Stevens" cranberry cuttings under controlled growth bed conditions and also to evaluate biochar effect on microbial populations present in the potting mix. Biochar was added at 1% (w/w) and used directly or mixed with three selected bacterial strains known to stimulate plant growth compared to treatments in which biochar was not added. Shoot and root dry weights increased upon the addition of 1% biochar and beneficial microbes at both harvesting dates compared to those in potting mix fortified with full dose of Actisol®. Under lower dose of Actisol®, biochar and bacteria amendment significantly increased root dry weight at 120 days after transplanting. Depending on the date of harvesting, the addition of 1% biochar and beneficial microbes significantly *($P < 0.05$)* increased the total abundance of microbes present in the rhizosphere and bulk soil of cranberry cuttings. In particular, there was an increase in the abundance numbers of fungal and Actinomycets phyla in bulk soil. Quantitative-PCR assays using species-specific primers showed that DNA copy numbers of PGPR and ericoid mycorrhiza in soil and in roots of cranberry cuttings varied with date of harvesting and with the type of biological sample tested. Under the above conditions, our results indicate that the application of maple bark biochar yielded variable results and may not be the best-suited type of biochar for cranberry production in conjunction with selected beneficial microbes

Résumé

La culture durable de la canneberge (*Vaccinium macrocarpon*) au Québec requiert l'adoption de meilleures pratiques de gestion. Une approche prometteuse est l'application de biocharbon et de rhizobactéries comme amendement de sol, menant à un rapide établissement en champs et à une diminution des intrants chimiques. Cette étude a été réalisée afin de déterminer si l'application de biocharbon d'écorce d'érable et de rhizobactéries bénéfiques pouvait stimuler la croissance de boutures de canneberges du cultivar « Stevens » en serre et d'évaluer ses effets sur la flore microbienne. 1% de biocharbon (p/p) a été utilisé seul et en combinaison avec trois rhizobactéries reconnues pour stimuler la croissance, puis comparé à un contrôle. La masse des tiges et des racines a augmenté suite à l'addition de 1% de biocharbon et de rhizobact.éries aux deux dates de récoltes comparé au traitement contenant 100% de la dose d'Actisol®. Comparée à une plus faible dose d'Actisol®, la masse des racines a augmenté après 120 jours avec l'addition de biocharbon et rhizobactéries. Selon la date de récolte, l'addition de 1% de biocharbon et rhizobactéries a augmenté de façon significative ($P<0.05$) le nombre total de microorganisme présent dans le sol. Plus particulièrement, il y a eu une augmentation du nombre de populations fongiques et d'actinomycetes dans le sol non rhizosphérique des boutures. L'utilisation d'amorces spécifiques à l'espèce en PCR-quantitative, dans le but de déterminer le nombre de copies des rhizobactéries et de mycorhize éricoïdienne dans le sol et les racines des boutures, a démontré des résultats variant avec les temps d'échantillonnage et les types d'échantillons testés. Sous les conditions testées, nos résultats indiquent que l'application de biocharbon et de rhizobacteries bénéfiques en production de canneberge biologique produit des résultats variables et n'est peut-être pas la façon la plus adéquate d'améliorer l'établissement des boutures en champs.

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PREFACE

The following thesis was prepared according to the "Guidelines Concerning Thesis Preparation" of McGill University. David Bernard Perron was the primary researcher for each chapter. He designed the experiments with the guidance of Dr. Suha Jabaji, Department of Plant Science, Macdonald Campus of McGill University, Dr. Franz Lang, Département de Biochimie Université de Montréal helped in the overall design of the experiments and provided the bacterial inocula. Tanya Copley, Department of Plant Science, Macdonald Campus of McGill University, helped in the experimental procedures and provided advice on statistical analyses. Dr. S. Jabaji provided supervision, guidance and funding for all chapters.

Chapter I: General introduction

1.1. Introduction

Native to North America, cranberry (*Vaccinium macrocarpon* L.) is a high-value crop that over the years has gained popularity due to its outstanding content in vitamins, antioxidants and other health beneficial compounds (Bodet et al., 2008; Leahy, 2002; Neto, 2007). According to l'Institut de la statistique du Québec (ISQ, 2014), cranberries were commercially produced on 3,212 ha in Québec in 2012. This represented an increase of 11.8% over the 2011 production area. The value of the yearly production was $53 million for 2012 (ISQ, 2014). The Canadian situation is also prosperous; the cranberry area more than doubled to 7,100 acres, up from 3,348 acres from 2006 to 2011. The 2011 Census of Agriculture (Statistics Canada, 2014) showed that Quebec surpassed British Columbia to become the largest province for cultivation of cranberry in the country.

Even though cranberry production in Quebec has been thriving over the last 10 years, the production of fruit requires important costly cultural practices, in particular for the installation of productive planting beds: a period of 2-3 years is required, with full productivity often only starting by year 4, as well as long-term maintenance for high productivity. Regular cultural practices include pruning, sanding, fertilization, irrigation and water management, in addition to control of weeds, and fungal and insect diseases (UMass, 2010). The extensive use of water in modern cranberry production necessitates significant water holdings on operations. Coupled with the application of fertilizers for optimum growth, pesticides and herbicides against a large variety of cranberry pests, cranberry bed establishment represents a substantial financial and time investment that mandates that the growers to maximize vine colonization and minimize effects of weed competition (Sandler, 2004). Due to the augmentation of production, public concern over potential chemical runoff and

contamination of rivers, lakes and water supplies has heightened. In addition, there is concern over potential contamination of the fruit itself and over the health of farm workers applying pesticides. Together, this has resulted in the cranberry industry being increasingly scrutinized and regulated in recent decades (Caron, 2009). The growers' response has been towards the progressive adoption of integrated pest management strategies (IPM) and a shift towards organic production. IPM has the potential for a better economic return due to the high price of the organic product, however it requires substantial production know-how.

The cranberry market is also distinctive as it is essentially a North American market. The year 2013 was marked by the highest yields ever recorded in cranberry production history (APCQ, 2014). In Quebec, yields were 182 million pounds compared to 118 million in 2012 (APCQ, 2014), a 54% increase. Increases in yields occurred across all of the North American east coast in 2013, which led to a flooded cranberry market resulting in drastic dropping of the price of the berries. Price return for famers that were once on an average of 23 ¢/pound dropped under the production costs (APCQ, 2014). For the 2014 growing season, most of the Quebec growers were obliged to cut down production by 15% through a reduction on fertilizer applications and on the use of pollinators in order to reduce costs (J. Painchaud, MAPAQ, personal communication). Taken together, there is more than ever an urgent need to reduce production cost while keeping the cultural practices as environmentally friendly as possible. Quebec cranberry production needs to keep pushing research to develop new tools to help in this regard.

Cranberry organic crop production is the fastest growing portion of U.S. and Canadian agriculture, increasing a minimum of 20% annually during the last 15 years. The establishment of federal guidelines (OMAFRA, 2011) for organic certification in 2009 provided a structure for producers and processors to market certified organic foods. The guidelines provide the general provisions and processes for obtaining and maintaining organic certification, but do not specifically

determine the best management practices for crop production within the organically approved methods. The province of Québec is currently the world leader in organic cranberry production. This is due to the expertise and climatic conditions which are unique to Québec. Although in light of the current situation, best management practices and approaches are the corner stone of the industry and should be continually implemented. One such approach that is being promoted in agriculture is the application of biochar (Lehmann et al., 2011). Biochar, the solid co-product of pyrolysis has shown several beneficial effects once incorporated into the soil. Among them are enrichment of soil microbial content (Steiner et al, 2004), increase in microbe species diversity (Pietikäinen, 2000), and increase soil fertility and retention preventing nutrient leaching (Major, 2009). Adapted to organic cranberry production, biochar can play a key tool in the best management practices implementation and development.

As cranberries prosper in a high carbon soil, (Poirier, 2010) it is thus ideal to test the effect of biochar on cranberry growth parameters. In this context, this study describes the response of growth parameters of cranberry cuttings grown in pots to the application of maple bark biochar in combination with growth promoting bacteria.

1.2. Research hypotheses

1. Cranberry cuttings in amended biochar potting mix will establish faster leading to increased plant growth
2. Biochar amendment increases total foliar N, P, K levels
3. Biochar amendment increases the type and abundance of microbial populations in bulk and rhizosphere soils and also in and on cranberry roots.

1.3. Objectives

The global objective of this research was to develop a sustainable approach for the establishment of cranberry beds under organic cranberry practices. More specifically, the short-term objectives were the following:

1. To study the effect of biochar amended with specific rhizospheric bacteria on the establishment and growth of cranberry runners under controlled conditions.
2. To conduct comparative studies of biochar amended with microbes and/or with organic fertilizer on the total concentrations of N, P, K in cranberry shoot tissues
3. To conduct comparative studies on the taxonomic groups and total number of microbial populations isolated from bulk and rhizosphere soils of cranberry cuttings treated or not with amended biochar.

Chapter II: Literature review

2.1. Cranberry production and cultural practices

Native to North America, cranberry (*Vaccinium macrocarpon* L.) is a perennial plant that belongs to the ericaceous family and grows in bogs (Poirier, 2010; Thomas, 2003). Cranberry has become a high-value crop and has gained popularity over the years because of its outstanding nutritional and medicinal proprieties including vitamin and antioxidant properties (Thomas, 2003). The majority of the world`s cranberry production is concentrated in the USA and Canada, with Wisconsin, Massachusetts, and Quebec as the world`s top three cranberry producing regions covering production areas of 17,700, 13,000, and 6,242 acres, respectively (Poirier, 2010). Canada commercially cultivates cranberries on 6,093 ha, with an annual production value (farm-gate value) of $88 million (Statistics Canada 2012). Approximately half of all Canadian production occurs in Quebec (2,872 ha) with a market value of $41.8 million (MAPAQ, 2012).

Quebec is known as the world leader in organic cranberry production, with 350 ha cultivated. This represents 8% of their total cranberry production, which is exceptionally high when compared to other agricultural industries that have an average of 1-2% dedicated to organic production (APCQ, 2012; Poirier, 2010). The average growth for organic production has increased nearly 29% between 2005 and 2009, and is projected to reach 425 ha by 2013 (Poirier, 2010). The reasons for such an expansion are due to the geographical advantages that are inherent to the landscape and climate. The northern geographical situation of Quebec grants the organic cranberry industry with natural protection against pests and diseases such as fruit rot, as diseases and pests die-off during the winter and therefore diminish the need for fungicide and pesticide use (Poirier, 2010).

2.2. Establishment of cranberry beds and cultural requirements

The transition from chemical to organic production takes several years and requires substantial know-how and is costly. For example, establishing a new cranberry field will cost an average of $100,000/ha (Poirier, 2010). This is compounded by additional costs required for cultural practices such as pruning, sanding, fertilization, irrigation and water management, control of weeds, fungal disease and insects, as well as complete mechanization for fruit production in the third year of growth. However, organic production has the potential for better economic return due to the high price of the organic product. This emphasizes the importance of achieving the industry's highest priority: increased average yield at a low cost. To do so, adoption of best management practices and tools is urgently required for sustainable, long-term viability of cranberry production in Québec.

2.3. Best management practices

Best management practices (BMPs) are designed to maximize productivity while preserving the environment. There are guidelines that provide the farmer with expert knowledge on several aspects of crop production. In the case of cranberry production, BMPs facilitate the transition period from conventional to organic production, thus providing easier access to organic certification. The most effective BMPs in organic cranberry production are sanding and flooding. Sanding provides control over insect and fungal pathogens while promoting the growth of upright shoots which bear fruit and increase the overall vigor of the cranberry plants (UMass, 2010). Weed management in organic production is costly, and sanding has been shown to improve weed control (Sandler, 1997; Stonehouse, 1996). Also, sanding is reported to stimulate plant growth as less fertilizer is required.

Flooding is required to protect cranberries from cold damage in winter, but it requires proper management of oxygen as anaerobic conditions may develop during the flooded period and lead to vine damage (UMass, 2010). Flooding can also be used to control fungal and insect pests (UMass, 2010). Not only cultural practices can be implemented as best management practice, but the choice of native cultivars and integrated pest management as well as early disease scouting is also key to organic cultivation (UMass, 2010).

Irrigation practices and fertilizer application coupled with pesticide and herbicide applications are required to protect against a large variety of pests (e.g., fruit worm, cutworms, southern red mite and fruit rot) and increase optimal growth. Also, extensive use of water in modern cranberry production requires a good quality supply of surface water adjacent to the cranberry farm coupled with significant water-holding capacity at the operation site (Poirier, 2010). These factors have heightened public concern over potential chemical runoff and contamination of rivers, lakes and water supplies. In response to this concern, a study in 2006 commissioned by the Ministère de l'agriculture, des pêcheries et de l'alimentation du Québec (MAPAQ) to look into the effect of water runoff on cranberry production. The study conducted by Marchand (2006) concluded that the establishment of closed circuit water systems could drastically reduce farm runoff. This recommendation is currently enforced for organic and conventional farming of cranberry (Poirier, 2010) by the Ministère du development durable et des parcs (MDDEP), Quebec.

2.4 Biochar production and physical properties

Biochar is the solid co-product of biomass pyrolysis, a technique used for carbon-negative production of second-generation bio-fuels. Pyrolysis consists of heating biomass in a very low, near zero oxygen environment. The absence of oxygen prevents combustion and yields combustible

liquid and gas as well as charcoal, which was dubbed biochar (Lehmann et al., 2012). The biochar produced by pyrolysis is composed of organic carbon and its residence time in soil is estimated to be from hundreds to thousands of years (Lehmann et al., 2006).

Due to its porous structure, an immense active surface, and high cation exchange capacity (Hibbett et al., 2007), biochar adsorbs and holds large quantities of soil nutrients (for example, 1 g of activated biochar has an active surface of several 1000 m^2) and water. This particular characteristic is very attractive to farmers since it leads to the reduction in fertilizer requirements and water use. This is especially interesting in the particularly permeable, sandy soils that are commonly used for cranberry production.

2.4.1. Beneficial properties

Biochar has been shown to increase dry matter content and reduce fertilizer requirements for some crops (Chan et al., 2008; Major et al., 2010). As a result, fertilization costs are minimized and fertilizers (organic or chemical) are retained in the soil, reducing runoff into rivers and lakes (Laird et al., 2010; Major et al., 2009) that contributes to algal and cyanobacterial pollution. In addition, since chemical fertilizers are typically fossil-fuel based, biochar provides indirect climate change benefits by reducing fertilizer needs, in addition to immobilizing carbon in the soil for long periodsof time (Woolf et al., 2010). More importantly in an agricultural context, biochar additions to soil have been shown to significantly improve soil structure and the development and maintenance of beneficial soil microbe communities, including rhizospheric bacteria, and free-living and mycorrhizal fungi (Kolbet al., 2009; Solaiman et al., 2010)

The health and diversity of soil microbial populations are essential to soil functions and have a great impact on soil structure, stability, nutrient cycling, aeration, water use efficiency,

disease resistance and carbon storage (Brussard, 1997). Biochar holds promise as an amendment for soil quality improvement and sequestration of atmospheric carbon dioxide. However, knowledge on how biochar influences soil properties, especially soil microorganisms is limited. Multiple studies have reported on plant growth promotion when soils were amended with biochar. Plant growth promotion was related to the increases in rhizospheric microbial populations (Graber et al., 2010; Steineret al., 2008) and in mycorrhizal colonization (Makoto et al., 2010). On the other hand, there are contradictory reports in which biochar amendment decreased the abundance of arbuscular mycorrhizal fungi (Birk et al., 2009; Warnock et al., 2010) in the soil and in roots of plants. The reasons for this decrease are not fully understood and need further investigation.

Biochar also has a direct beneficial effect on plant growth. Many crops were affected by biochar application. For example, application of biochar caused an increase in: root biomass and root tip numbers of larch seedlings (Makato et al. 2010); number of storage roots in asparagus (Matsubara et al., 2002) and rice root length (Noguera et al., 2010). The rational behind the beneficial impact of biochar on plant growth is rarely well defined, and most reports allude to improvement of soil fertility, water availability, pH and aeration as factors that may play a role (Lehmann et al., 2011).

It is well known that optimal combinations of soil microbes and mycorrhizae lead to a better nutrient availability to plants, crop productivity, and more vigorous plants that have a better resistance to pests (Lugtenberg et al., 2009). In addition, more vigorous plant growth reduces both the time required for establishing a productive cranberry field and the time needed for weed control that is most costly during the period in which cranberry plants have not completely covered the ground. All these properties make biochar when combined with selected

beneficial microbes an attractive soil amendment in cranberry production (and agricultural crops in general), and this was the basis of the study of this thesis.

2.5. Plant growth promoting rhizobacteria (PGPR)

Plant growth promoting rhizobacteria (PGPR) are beneficial bacteria that directly promote plant growth. They help in the uptake of nutrients that would otherwise not be available to plants or mycorrhizal fungi. They also help via production of phytohormones, solubilization of inorganic phosphates, increased iron availability through iron-chelating siderophores, and volatile compounds that affect the plant signalling pathways (Bhattacharyya et al., 2012; Podile et al, 2006). In the case of nitrogen-fixing bacteria, the uptake/conversion of nitrogen is not from the soil but from the air, providing a constant supply of free nitrogen, which is advantageous because it corresponds to a slow-release fertilizer. There are two types of beneficial bacteria, those that invade the plant root system but only of specific types of plants (e.g., *Rhizobium* relatives that interact with leguminous plants; unknown for cranberry), and others that are either free-living and/or in external contact with either plant roots or the colonizing hyphae of mycorrhizal fungi (Vessey, 2003). Known free-living beneficial bacteria include members of the *Azospirillum, Bacillus, and Variovorax* genera. Although the modes of action of PGPR are often not well understood (that is, nitrogen fixation, plant hormone secretion, solubilization of inaccessible nutrients in the soil, synergy with plant symbionts) (Vessey, 2003), PGPR appear to significantly enhance plant growth of a large variety of plants, and whatever the precise mechanism(s) is, this merits to be examined in cranberry plantations.

2.5.1. *Azospirillum* species

Inoculation of crop plants with PGPR is a contemporary agricultural practice used to improve crop yields. The *Azospirillum* genus consists of gram-negative free-living nitrogen-fixing rhizosphere bacteria (Steenhoudt et al., 2000). It is one of most well-known and well studied PGPR and has been isolated from the rhizosphere of many crops world-wide (Bashan et al., 2004). Strains of *Azospirillum* are reported to increase yield and biomass of cereal crops both in greenhouse and field trials and have been used extensively in cereal crops as a biofertilizer for many years (Okon, 1994). Cells of *Azospirillum* are flagellated and show chemotaxis toward organic acids, sugars, amino acids and root exudates (Heinrich et al., 1985). *Azospirillum* species under stress conditions form internal cyst-like structures for storage of poly-β-hydroxybutyrate granules (Sadasivan et al., 1987; Tal et al., 1990). These structures help the bacteria to resist abiotic stresses such as drought and environmental starvation. *Azospirillum* species can fix nitrogen and also produce phytohormones such as indole acetic acid (IAA) (Dobbelaere et al., 1999; Okon et al., 1995). A*zospirillum brasilense* has been shown to have beneficial impacts on non-cereals crops by improving seed germination and overall plant performance in different crops (Felici et al., 2008; Puente et al, 1993).

2.5.2. *Variovorax* species

Another group of bacteria that is a known PGPR is the *Variovorax* genus whose members exert beneficial effects on plant growth. These strains are aerobic soil bacteria that belong to the subclass of Proteobacteria and are primarily associated with bio-degradation processes in nature. Species of *Variovorax* can enhance the host plant's stress tolerance, disease resistance and aid in nutrient availability and uptake (Belimov et al., 2009). The effectiveness of *Variovorax* as a PGPR is

likely to be more potent because it also appears to be a good endophytic symbiont (Sessitsch et al. 2004) and thus interacts more closely with the host plant.

Variovorax paradoxus possesses all of the genetic material to behave both as a heterotrophic and autotrophic bacterium. This allows *V. paradoxus* to develop metabolic features that enable it to survive independently or as a symbiont (Han et al., 2011). Many reports have shown the importance of the growth promoting effect of *V. paradoxus* on plants*:* it has been shown to increase root elongation in contaminated soil (Belimov et al., 2001) and has been shown to have beneficial effects on the growth of *Arabidopsis thaliana* and *Glycine max* due to its ability to oxidize H_2 in soil (Maimaiti et al., 2007). *V. paradoxus* can also promote growth by detoxifying the plant environment thus preventing stress and promoting growth. It also increases stress and disease tolerance of the plants (Belimov et al., 2005; Belimov et al., 2008). Other research demonstrated its beneficial endophytic ability in many crops (Reiter et al., 2006; Ryan et al., 2007; Sessitsch et al., 2004). *V. paradoxus* can also be found in close association with the mycelium of the symbiotic fungal group of arbuscular mycorrhizal fungi (AMF) (Lecomte, 2011).

2.5.3. *Microbacterium* species

Species belonging to the genus *Microbacterium* are gram-positive and heterotrophic bacteria (Park et al., 2008). They are one of the predominant species isolated as endophytes from agronomic crops (Zinniel et al., 2002). These groups of bacteria are effective as bio-remediators, having the potential of solubilizing heavy metals as well as degrading hydrocarbon compounds and thus promoting plant growth in contaminated soils (Harwati et al., 2007; Sheng et al., , 2008). *Microbacterium ginsengisoli* has been found to be associated with the mycelium of the AMF *Glomus irregulare*. The role of the bacterium is, however, not clear. It is believed that the presence of the

bacterium may improve the nutrient availability to the AMF (Lecomte et al., 2011).

2.6. Symbiotic and ericaceous fungi

The hair roots of ericaceous shrubs from pine forest and open heathland contain a large diversity of ericoid mycorrhizal (ErM) fungi, which are regarded as important mutualistic associates. The fungal genus *Oidiodendron* inhabits the roots of several ericaceous plants forming symbiotic relations. *O. maius* is the most commonly encountered species and is commonly isolated from roots of ericaceous plants (Dalpé, 1986). ErM fungi positively influence growth, survival and competitiveness of their host species by enhancing nutrient uptake (Read et al., 2003) and alleviating heavy metal toxicity (Perotto et al., 2002). In addition to ErM fungi, root endophytes belonging to the group of fungi known as dark septate endophytes (DSE) are reported to colonize roots of ericaceous plants (Jumpponen, 2001). The DSE comprise ascomycetous fungi with a wide range distribution of host plants. Inoculation with DSE often causes apparent effects on biomass production or nutrient uptake by host plants in pot cultures (Vohník et al., 2003). The most commonly isolated fungal group belongs to the genera *Phialocephala* and *Sebacinaceae*.

In natural sites, it is expected with ericaceous plant species that both ErM and DSE co-exist together. Direct observations have confirmed the simultaneous occurrence of ErM and DSE with multiple colonizations in roots of ericaceous species (Urcelay, 2002). Also, molecular methods proved the simultaneous presence of ErM and DSE fungi within the same root system (Midgley et al, 2004).

2.6.1. *Phialocephala* species

The fungal genus *Phialocephala* belongs to the so-called dark septate endophyte (SDE), which is characterized by melanized and septate hyphae (Currah, 1993; Jumpponen, 2001). The effect of *Phialocephala* species on plants seems to be host and strain-dependent. Root colonization by DSE fungi including *P. fortinii* has been reported to cause a variety of host growth responses (see review by Jumpponen and Trappe 1998). Host growth responses ranged from negative through neutral to positive (Vohník, 2005*;* Vohník, 2003; Newsham 2011). These observations were related to variations between strains, and experimental conditions which might have influenced the outcome of the symbiotic associations. Ericaceous plants including blueberries, black huckleberry, and cranberries are hosts for *Phialocephala* species (Gorzelak et al., 2012; Sadowsky et al., 2012)

2.6.2. *Sebacinaceae* species

Sebacinales are basal Hymenomycetes with diverse mycorrhizal abilities, ranging from ectomycorrhizae to ericoid and orchid mycorrhizae (Selosse et al., 2007; Weiss, 2004). Some Sebacinales strains are commonly considered as endophytes because they significantly enhance plant growth and seed yield, and induce systemic resistance of their host plants against abiotic stress and fungal pathogens. Experimental studies suggest that the fungus improves the nutritional status of its host plants and promotes plant growth and performance in barely and tobacco (Barazani et al., 2005; Deshmukh et al., 2006; Waller et al., 2005).

Chapter III: Materials and methods

3.1. Biochar

3.1.1. Production

Biochar was supplied by Award Rubber & Plastic Industries, Ltd. (Plessisville, Quebec). It was produced from the pyrolysis of maple tree bark at 700ºC for 4 hours and was used as a soil amendment in all experiments. For biochar to be most effective as a soil amendment it was crushed to obtain particles of a maximum diameter of 2 mm, and for the planned experiments, it was sieved to obtain a uniform 1-2 mm particle size (Fig. 1).

3.1.2. Biochar acidification and analysis

Biochar used for the greenhouse experiment was acidified (Doydora et al., 2011) to an optimal pH of 4.5 in order to support cranberry growth. Batches of 600 g of biochar were treated with 4.5 moles of hydrochloric acid (Fisher scientific, Hampton, New Hampshire, USA) over a period of one week with daily agitation. Each 600 g batch was placed in a 4 L Erlenmeyer flask with 363 mL of 38% HCl solution. Once the pH of the solution became stable for 24 h, the biochar was filtered on Whatmann paper filter paper of 125 mm diameter (Cat No 1001 125) using a vacuum pump and dried overnight at 90°C. The pH of the acidified biochar was measured to ensure that it was in the range of 4 to 5. For pH readings, a sample of biochar was mixed with 0.01 M of $CaCl_2$ at

a ratio of 1:2 biochar:$CaCl_2$ and agitated for 30 minutes (Van Zwieten et al., 2010). Multiple pH readings were taken over a 24 h period to ensure that the biochar buffering capacity had been neutralized and the pH of the biochar was stable in the optimal pH range for cranberry growth. Total chemical analysis of acidified biochar (Table 1) was done in Dr. William H. Hendershot's Environmental Soil Analysis laboratory, at the Department of Natural Resource Sciences, McGill University, Macdonald campus, Ste. Anne de Bellevue, Quebec by acid digestion according to the method of Parkinson (1975).

3.2. Potting substrate

The potting substrate consisted of 5 parts of sand and 1 part of peat (v/v). The sand was obtained from the farm Les Atocas St-Louis, situated in St-Louis de Blandford, Quebec, Canada in the spring of 2012. Peat moss was purchased from Fafard (Saint-Bonaventure, Québec, Canada). The sand and peat moss were autoclaved separately for two cycles of 6 hours each (121°C, 15 psi). Both potting constituents were left at room temperature in sealed bags for 3 days between autoclaving cycles. Autoclaved sand and peat were mixed together as previously described (5:1 ratio) and 1 kg of potting mixture was added to each pot.

Chicken manure in the form of Actisol®, (St-Wenceslas, Quebec, Canada), an organic fertilizer, was supplied by Mr. Yvan Montreil, an organic cranberry grower and owner of the farm Les Atocas St-Louis associated with the project. The manure was ground into a fine powder using a coffee grinder and then placed in an Erlenmeyer flask and pasteurized at 72°C overnight (8 h). Colony forming units (CFU) were estimated in pasteurized and unpasteurized manure to ensure the efficiency of the pasteurization. The pasteurized manure was then thoroughly mixed into the potting mix. Treatments received either 100% of the normal organic fertilizer dose (1500 lb/ha amounting

to 15.6 g of manure per pot) or 25% of the organic fertilizer dose (3.9 g/pot) or no manure (control).

The other amendment that has been used was acidified biochar (see section 3.1.2). Treatments received either a 1% concentration of biochar by weight (10 g/pot) or no biochar (control).

3.3. Biological material

3.3.1. Cranberry cuttings

Cranberry cuttings of the cultivar Stevens were provided by Les Atocas de l'érables Inc, (Notre-Dames-de-Lourde, Québec, Canada). The cuttings (5 cm in length) were prepared from runners of field-grown plants in the spring of 2012, and planted in 281 cell trays in a mixture of 5:1 peat:sand. The cuttings were placed outside in a cold frame structure and watered as required (always moist) for 2-3 months until fully rooted. Mycorrhizal colonized (see section 3.2) and fully rooted cranberry cuttings with vertical shoot lengths of 8.5 to 11.0 cm were selected for the experiment (Fig. 2).

3.3.2. Visualization of mycorrhizae in cranberry roots

Cranberry plants are typically colonized by ericoid mycorrhizal (ErM) fungi in their native environment, and are capable of utilizing organic nitrogen sources that are unavailable to non-mycorrhizal plants. Therefore, it is critical that ErM uniformly colonizes the starting cranberry cuttings for greenhouse trials and re-establishment of new cranberry beds. Additionally, rooted-

ErM cuttings offer greater resistance to transplantation shock and offer flexibility in planting dates (Jansa et al., 2000).

To confirm that the roots colonization by ErM prior to planting, root samples were randomly taken from several cuttings, pooled and assessed for the presence of mycorrhizae using chlorozal staining methods (Brundrett, 1996). Briefly, the roots were bleached in 10% KOH at 121°C 15 psi for 15 minutes, and rinsed under running water to neutralize the KOH followed by staining with chlorozal black at 121°C 15 psi for 15 minutes. Stained root segments were stored in lactoglycerol (1:1:1 v/v/v water, lactic acid and glycerol). Visualization of ErM as hyphal coils inside the roots was observed using a dissecting microscope and pictures were recorded using a Moticam 2300 camera (Motic, Hong Kong).

3.3.3. Bacterial inoculum, maintenance and inoculum preparation

The bacteria *Microbacterium gensengnii, Azospirillum brasilense* and *Variovorax paradoxus* were provided by Dr. Franz Lang (Dept. of Biochemistry, Université de Montréal, Montréal, Québec). Two rounds of single-cell colony isolations were performed to ensure the purity of the cultures. A single colony of each bacterium was cultured in Lauria Broth (LB) under agitation (250 rpm) for 24 h at 30°C. A 500 µL aliquot of each bacterial broth was mixed with 500µl of 50% glycerol solution and placed a 1.5mL Ependorff tube, flash frozen in liquid nitrogen and stored at -80°C until further use.

3.3.3. Bacterial growth curve and inoculum preparation

A 24 hour LB culture of each bacterium was grown under agitation in large LB batches (500 mL) for 24 h at 30°C. For each microorganism, three absorbance readings at λ=600nm (A_{600}) were taken at three different time points during the exponential growth phase of the bacteria. For each absorbance reading a serial dilution of the LB culture was made and 100 µL of a specific dilution was plated on Lauria Broth Agar (LBA) plates for colony forming unit (CFU) counts after 24 h. Bacterial growth curves were extrapolated to correlate the absorbance A_{600} and the number of cells per mL of LB. The bacteria were then diluted in phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) pH of 7.4 in order to obtain a concentration of 10^5 CFU/mL for plant inoculation experiments. Three days after planting, each cranberry cutting received either 30 mL of sterile PBS (control) or 10 mL of each of the three bacteria at a concentration of 10^5 CFU/mL for a total of 10^6 CFU per microorganism per cutting. Additionally, DNA was purified from each bacterium using the DNeasy Blood & Tissue Kit (QIAGEN, Limburg, Netherlands) for the development of standard curves for qPCR analyses.

3.4. Experimental design

The greenhouse study examined the effect of amended biochar on various growth parameters of cranberry cuttings. Rooted-ErM cranberry cuttings were transplanted into 1 L pots (10 cm X 10 cm X 10 cm) bottom-lined with a Geotextile membrane and containing 1 kg of potting mix. Another Geotextile membrane was placed on the surface of the potting mix in order prevent cross contamination of the bacterial inocula among treatments.

The experiment consisted of six treatments (Table 2). They are: (1) potting mix not amended with biochar but fortified with 100% standard organic fertilization rate; (2) potting mix not amended with biochar but fortified with 25% of the standard organic fertilization rate; (3) potting mix not amended with biochar but fortified with the addition of the 3 PGPR; (4) potting mix amended with 1% biochar and 25% of standard organic fertilization rate*;* (5) potting mix amended with 1% biochar, 25% of standard organic fertilization rate and the addition of the 3 PGPR. There were nine (9) replicates per treatment.

Plants were placed on a growth bench following a completely randomized design (CRD) in the research greenhouse at the Macdonald Campus under light of 200 μmol photons•m^2•s^{-1} and day-light length of 18 hours followed 6 hours of dark cycle. The light quality was a mix of incandescent and fluorescent light bulbs. Day temperature was 22°C and night temperature was 18°C. A drip irrigation system was installed to ensure even watering of all the replicates (Fig. 3). The pH of the water was adjusted to be between 4 and 5 pH units to ensure optimal cranberry growth. Citric acid was used to lower the water pH as it is the only acid certified for organic production. At harvesting, soil pH was measured after 30 minutes of agitation in a solution of 0.01 M of $CaCl_2$ at ratio of 1:2 soil:$CaCl_2$ (Van Zwieten et al., 2010). Plants were harvested 120 and 160 days after cutting transplantation. Plants of both time points were grown on different growth benches.

3.5. Harvesting, growth parameters and microbial counts

Extension growth of upright and runner shoots was measured as well as the number of newly emerged branches every 30 days up to 160 days. Only shoots longer than 0.3 cm were measured and counted. Total plant growth and average shoot length were calculated using those measurements. Two harvests of plant tissue were done: one at 120 days and one at 160 days. At each harvesting

time, shoots and roots were separated, in order to determine: (i) dry mass of each tissue, (ii) nutrient composition of leaves and (iii) DNA copies of bacteria and ErM in roots. Bulk and rhizospheric potting mix were also collected to determine total microbial abundance (CFU number) and to estimate DNA copy numbers of the introduced rhizospheric PGPR bacteria and ErM.

3.5.1. Dry mass of tissue and nutrient analysis

Shoots were dried by placing them in paper bags for three days at 71°C. Roots were carefully washed under running tap water until they were clean, blotted dry with paper towel to remove extra water, placed into 15 mL Falcon™ tubes, flash frozen in liquid nitrogen, and lyophilized at -70°C for 48h. Dry mass of shoots and roots were determined and values were expressed in grams.

Leaves from each replicate in each treatment were removed from the stems, pooled, reduced to powder in liquid nitrogen using a mortar and pestle. Three subsamples of each pooled replicate from each treatment were used for the digestion. Nutrient analysis of leaves was performed by digestion with sulfuric acid and hydrogen peroxide according to the method Parkinson (1975). Briefly, 0.160 g of leaf tissue were digested at 340°C for 1 hr with an acid mixture containing 5.05 g/L of lithium sulfate, 0.15 g/L of selenium powder, 0.038% hydrogen peroxide and 2.66 mol/L of sulfuric acid and analyzed colorimetrically for the different elements using a flow injection analyzer QuickChem 8000 (Lachat instrument, Milwaukee, WI, USA). Values were expressed in mg per gram of dry plant tissue.

3.5.2. Abundance of culturable microbes

Rhizopsheric and bulk potting mix from each treatment were pooled together and three subsamples from each type of soil for each treatment were subjected to enumeration and DNA extraction to determine copy number. The samples were split in two portions: one portion was used for microbial CFU counts and the other portion was flash frozen with liquid nitrogen, stored at -80°C and used for DNA extraction.

In order to determine whether organic fertilizer, biochar and biochar amended with bacteria affect microbial population abundances in bulk and rhizospheric potting mix, CFU counts were performed on different microbiological culture media. Serial dilutions were plated on 3 different selective microbial media (Graber et al., 2010): (i) potato dextrose agar (PDA) amended with antibiotics (100 mg/L penicillin, 100 mg/L streptomycin 100 mg/L rifampicin and 10 mg/L chloramphenicol) was the selective media for yeast and filamentous fungi; (ii) nutrient agar (NA) amended with fungicide (Benomyl® 10 mg/L) was used to isolate bacteria; and (iii) water agar (WA) adjusted at pH of 10 was used to isolate Actinomycetes. An aliquot of 100 uL of a previously determined dilution was plated on each media and incubated at 24°C for 48 h. Colony forming units (CFUs) were estimated and values were expressed as CFUs per gram of potting mix (Graber et al., 2010).

3.5.3- Molecular microbial estimation in potting mix

Quantitative PCR (qPCR) assays were performed in the hope to correlate higher plant growth to higher PGPR DNA copy numbers per gram of soil. Roots, and rhizospheric and bulk

potting mix were recovered from -80°C storage and ground in liquid nitrogen for DNA extraction. DNA extraction from both types of soil (rhizospheric and bulk) was done using MO BIO laboratories™ Power Soil® extraction kits using 0.75 g of potting media per extraction. Three separate extractions from 3 subsamples were done per treatment. This yielded a total of 18 extractions per time point for each bulk and rhizospheric soil. Also, 200 mg of lyophilized root tissue was used for DNA extraction using a modified CTAB extraction method. Tissue sample was added with 600 µL of beta-mercarpoethanol and 5 µL of RNase to 15 mL of preheated extraction buffer [100 mM Tris-HCl pH 8.0; 2 M NaCl; 25 mM EDTA pH 8.0; 5% polyvinylpyyolidine (PVP) (w/v); 3% CTAB (hexadecyltrimethylammonium bromide) (w/v)]. The sample was votexed for 30 seconds and incubated at 70°C for one hour. The DNA was extracted by centrifugation at 3000 g for 10 minutes and purified using three rounds of chloroform : isoamyl alcohol (24:1). The aqueous phase (supernatant) was collected and 2/3 volume of isopropanol and 1/10 volume of 3 M sodium acetate pH 5.2 were added for precipitation of DNA. DNA was precipitate by incubation overnight at room temperature, and centrifugation at 5000 g for 10 minutes. The DNA pellet was rinsed in 2 mL of 70% ethanol and centrifuged at 5000 g for 10 minutes and dried at 37°C for 12 minutes. The DNA pellet was eluted in 100 µL of elution buffer containing 10 mM Tris-HCl; 0.5 mM EDTA at pH 7.0. The DNA quality was assessed by gel electrophoresis and quantified on a NanoDrop spectrometer (Thermo Scientific, Wilmington, USA).

Species specific primers for the bacteria *A. brasilense, M. ginsengisoli* and *V. paradoxus,* and also for the ErM fungi, *Sebacinaceae sp.* and *Phialocephaela sp* were developed by alignment of closely related species with ClustalW, designing the primers in unique regions using Primer3, and blasted to ensure no other known organisms would amplify (Table 3). The ErM genera were chosen as they were isolated from Quebec field grown cranberry roots by a collaborating research team led by Dr. Franz Lang from Université de Montréal. Cross-testing of the primers was done on pure culture DNA to

ensure that primers from one species would not amplify DNA from another species. To assess the presence or absence of the three rhizobacteria and the ErM fungi (*Sebacinaceae* sp. and *Phialocephaela* sp.) in the organic fertilizer Actisol®, potting mix and in roots of cranberry cuttings, prior to transplanting, DNA was extracted from the potting mixes using the CTAB method (see above) and quantitative PCR assays were performed with specific primers of the respective bacterial and fungal species.

Quantitative PCR (qPCR) using the designed primer sets (Table 3) was performed on bulk and rhizospehric potting mix substrate and also on roots to quantify the amount of bacterial and mychorrhizal fungi DNA copy numbers. To asses absolute copy numbers of DNA per gram of tissue or potting mix SYBR® Green (Agilent Technologies, Morrisville, NC, USA) qPCR assays were also performed on standard curves. Standard curves were composed of ten-fold serial dilutions of the species specific DNA ranging from 10^9 to 10^2 copy numbers. Each organism required a different standard curve, but all standard curves were designed using the same procedure. DNA was extracted from the bacteria of interest as previously described and from the mycorrhizal fungi using DNeasy Plant Mini Kit® (QIAGEN, Venlo, Netherlands). The DNA was PCR-amplified using specific primers (Table 3) under the following conditions: each amplification reaction contained 20 ng of template DNA, 1X PCR buffer (Thermo scientific, Ottawa, On, Canada), 0.2 mM of dNTP (New-England Biolab, Whiby, On, Canada), 2 mM of $MgCl_2$ (Thermo scientific, Ottawa, On, Canada), 0.16 μM of each primer (IDT, Coralville, IA, USA), 1 U of Taq polymerase (Thermo Scientific, Ottawa, On, Canada) in a total of 25 uL. All PCR reactions along with no template controls were run using a T-1000 thermal cycler (BioRad, Hercules, CA, USA) under the following conditions: one cycle of initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing temperatures were primer specific (see Table 3) all maintained for 45 s and extension at 72°C for 45 s. PCR products were separated on 1% agarose

gels and visualized using Gel Logic 200 Imaging system from (Kodak, Rochester, NY, USA) under UV light. The amplicons were gel excised using the QIAquick Gel Extraction Kit® from (QIAGEN, Venlo, Netherlands) to obtain purified products. The purified products were sequenced at Genome Quebec (Montreal, Quebec) and the resultant products were put through NCBI Genbank Basic Local Alignment Search Tool (BLAST) to ensure that the amplified DNA fragments belong to our target organisms. The sequences were ligated into pDrive (Qiagen, Venlo, Netherlands). Plasmid DNA was purified and sent for sequencing at Genome Quebec and blasted again to ensure the plasmid contained the proper sequence. Species-specific plasmids were serially diluted to obtain standard curves for qPCR reactions.

Quantitative real-time PCR (qPCR) was performed to quantify the amount of bacteria present in root plant tissues and in soil samples. Each 25 μL amplification mixture contained: 400 ng of template DNA, 1X SYBR II master mix (Agilent Technologies, Morrisville, NC, USA), 2.5 μM of each primer and 2 μM of ROX (Agilent Technologies, Morrisville, NC, USA) as a reference dye. To overcome the effects of inhibitors present in the root DNA, 2.5 mg/mL of bovine serium albumin (BSA) (Sigma, Oakville, On, Canada) and 3% of dimethyl sulfoxide (DMSO) (Fisher, Ottawa, On, Canada) were added to each reaction. Amplification was performed in a Stratagene Mx3005P real-time thermal cycler (Agilent Technologies, Morrisville, NC, USA) under the following conditions: one cycle of initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing temperature (see table 3) for 45 s and extension at 72°C for 45 s. Standard curves and no template controls were run with each sample. All samples were performed in duplicate technical runs. Amplification results were expressed as the threshold cycle value and converted to copy numbers by plotting the C_T values against the standard curve.

3.6. Statistical analysis

All statistical analyses were performed by one-way ANOVA using the JMP 10.0 software (SAS Institute, Cary, NC, USA). For variables showing significant differences by ANOVA, two-way comparisons were determined via Student's *t*-test with a magnitude of the F-value ($P = 0.05$). In the cases of repeated experimental results Levene's test for equality of variance ($P = 0.05$) was done and samples were pooled if permitted.

3.7. List of tables and figures

Table 1. Elemental analysis and nutrient content of biochar

Element	ppm
Al	3117
As	< 2
Ba	339
Be	< 0.2
Bi	3.8
C	405666.7
Ca	158945
Cd	0.78
Co	1.29
Cr	4.5
Cu	20.6
Fe	2772
H	12333.33
Li	237
Mg	4172
Mn	3035
Mo	< 0.2
Na	776
Ni	6.5
Pb	4.3
Rb	16
Re	< 0,8
S	616
Sb	< 2
Sn	< 1.5
Sr	404
Ti	47
Zn	88
Zr	2.6
Nitrogen (mg/g)*	6.48 ± 0.07
Phosphorus (mg/g)*	2.52 ± 0.13
Potassium (mg/g)*	3.21 ± 0.07

Elemental composition of biochar powder was characterized in triplicates by inductive coupled plasma atomic emission spectroscopy
*Nutrient composition was performed on acidified biochar through acid digestion. Numbers represent the average of each element expressed as mg per g of acidified biochar of 3 replicates ± standard deviation.

Table 2. Treatment description

Treatment Number	Treatment Description#
1	Potting mix fortified with 100% rate of organic fertilizer
2	Potting mix fortified with 25% rate of organic fertilizer
3	Potting mix fortified with 25% rate of organic fertilizer and enriched with rhizobacteria
4	Potting mix fortified with 25% rate of organic fertilizer and amended with 1% biochar
5	Potting mix fortified with 25% rate of organic fertilizer, amended with 1% biochar, enriched with rhizobacteria

Potting mix consisted of 1 part sand and 1 part peat (v/v). The organic fertilizer was is the form of Actisol®. Each pot received either 15.6 g (100% dose rate) or 3.9g (25% dose rate) of Actisol®. Biochar was added at 10g/pot (1% w/w).

Table 3. List of bacteria used to enrich the biochar, and specific primers used in quantitative PCR assays

Organism	Primer name	Sequence (5'-3')	Amplicon size	Annealing T (°C)	Reference
Azospirilum brasilense	Azo494_F	GGCCYGWTYAGTCAGRAGTG	431	49	Lin et al., 2011
	Azo756_R	AAGTGCATGCACCCCRRCGTCTAG			
Variovorax paradoxus	VarioF	AGCTGTGCTAATACCGCATAA	423	54	Jones et al., 2011
	VarioR	GAGACTTTTCGTTCCGTAC			
Microbacterium ginsengisoli	Microbac_F	AGCTTGCTCTCTGGATCAGTGG	434	61	Current study
	microbac_R	TATTACCGCGGCTGCTGGCAC			
Sebacinaceae sp.	ITS1-FSeb	CTTGGTCATTTAGAGGAAGTAA	267	60	Current study
	ITS3Seb	GTGAGATTACAATGACACTCA			
Phialocephaela	CRF-1F	GCAGCGAAATGCGATAAGTAATG	281	60	Current study
	CRF-1R	GGACCC TATAGCGAGGAGATTTA			

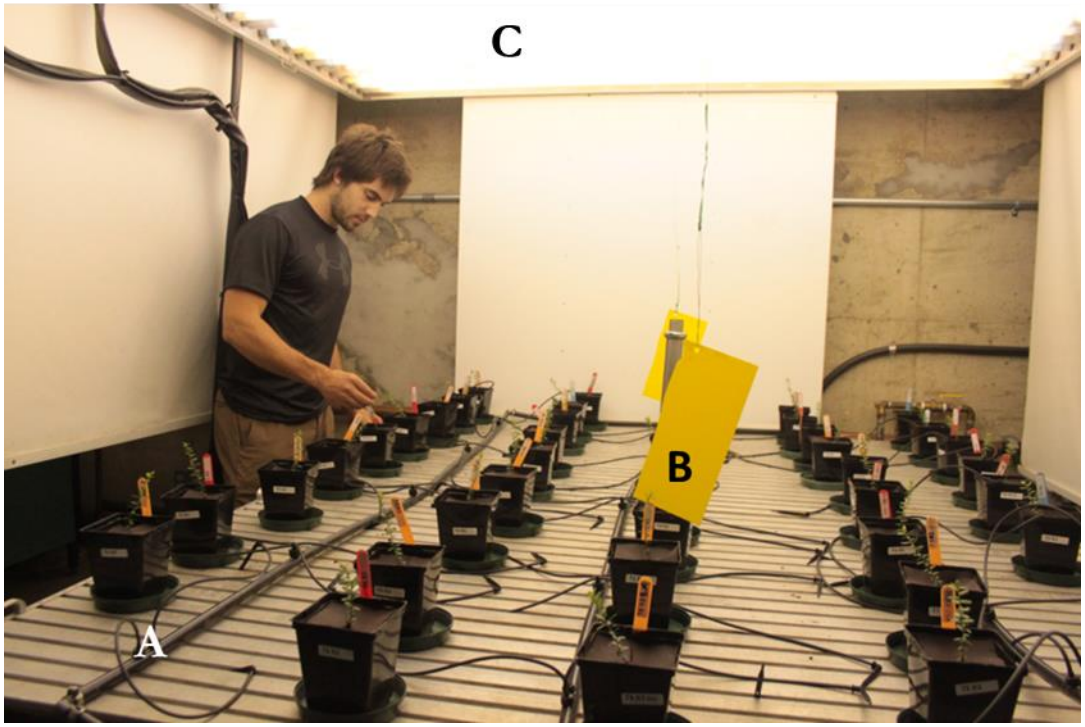
Figure 1. Maple bark feed stock and biochar production

A. Maple bark wood chip feedstock prior to pyrolysis at 700C.
B. Biochar after pyrolysis but prior to grinding.
C. Biochar ground and sieved to 1– 2 mm particles size

Figure 2. Standardized rooted cranberry cuttings from the cultivar Steven

A. Rooted cranberry cutting of the cultivar Stevens. Cranberry shoots with uniform height in the range of 8.5 to 11.0 cm were selected for further experiments

Figure 3. Growth bench with completely randomized experimental setup and drip irrigation system



A. Spaghetti type drip irrigation system. **B.** Pest insect control sticky trap.

C. The lighting was s a mix of incandescent and fluorescent light bulbs. Day/night temperatures was 22°C /18°C.

Chapter IV: Results

4.1. Plant parameters

4.1.1. Total length of cranberry uprights and runners

Cranberry seedlings grown in 25% fortified potting substrate amended with 1% biochar and enriched with bacteria (Treatment 5) had the highest length of cranberry uprights and runners compared to those in 100% fortified potting mix at 120 and 160 days after transplanting, respectively (Table 4). Additionally, the length of runners of cranberry seedlings grown in 25% fortified potting mix (Treatment 2) and also in 25% fortified potting mix but enriched with rhizospheric bacteria (Treatment 3) were also significantly longer than the control (Treatment 1) at 120 and 160 days after transplantation, respectively. There was no difference in total runner length of the remaining treatments compared to the control at both harvesting time periods.

4.1.2. Cranberry shoots and roots dry mass

Generally at both harvesting days, the shoot and root mass of cranberry seedlings grown in 25% fortified potting mix that was amended with biochar and rhizospheric bacteria were significantly ($P < 0.05$) higher than the control treatment (Table 4). Depending on the age, shoot and root mass of plants grown in 25% fortified potting mix amended with biochar (Treatment 4) or in 25% fortified potting mix without biochar and enriched with bacteria

(Treatment 3) were significantly higher than the control. Shoot and root mass of all remaining treatments were not different from the control treatments at both harvesting time periods.

4.2. Leaf nutrient analysis

Generally, N, P, K contents of cranberry leaves were the highest ($P< 0.05$) in the control treatment (100% organic fertilizer dose) at both 120 and 160 days after transplanting compared to other treatments, in which biochar, or bacteria were added to the 25% fortified potting mix in various combinations (Table 5). Plants grown in 25% fortified potting mix amended with biochar and enriched with bacteria (Treatment 5) had the lowest nitrogen content after 120 days, but not at 160 days. At both days, leaves of plants grown at 100% fortified potting mix had the highest nitrogen content compared to other treatments. Significantly lower amounts of phosphorus and potassium were found in leaves of cranberry plants grown in 25% fortified potting mix amended with biochar (Treatment 4) or with biochar and bacteria (Treatment 5) compared to the control at 120 days. At 160 days after transplanting, addition of biochar or bacteria singly or in combination with 25% fortified potting mix had no effect on phosphorus (P) and potassium (K) concentrations (Table 5).

4.3. Microbial abundance in potting soil

To investigate whether the addition of biochar and rhizospheric PGPR to fortified potting mix has an effect on total microbial abundances in the rhizospheric and bulk soil of

cranberry seedlings, microbial communities abundances were calculated in all of the treatments at 120 and 160 days after transplanting.

4.3.1. Abundance of microbial communities in the rhizosphere of cranberry seedlings

Generally, irrespective of the treatment, the total counts expressed as CFUs of microbial communities in one gram of soil were substantially higher in the rhizosphere of cranberry seedlings harvested at 120 days than at 160 days (Table 6). The highest number of microbial counts was reported for bacteria followed by Actinomycetes and fungi in all treatments and at both harvesting days. The 25% fortified soil mix amended with biochar and enriched with bacteria (Treatment 5) supported significantly higher numbers of bacterial communities as compared to 25% fortified soil mix amended with biochar only (Treatment 4) or enriched with the bacteria only (Treatment 3) at 120 days. However, bacterial counts in Treatment 5 were not significantly different from those estimated in the rhizosphere of seedlings grown in 100% and 25% fortified soil mix, respectively (Table 6). In the case of Actinomycetes, significantly lower numbers were found in the rhizosphere of cranberry plants grown in 25% fortified soil mix amended with biochar and enriched with bacteria. There was no difference in the number of Actinomycetes in all other treatments. Fungal colony counts were not affected by any of the treatments. The rhizophere collected from cranberry plants harvested at 160 days after transplanting showed a different pattern of microbial communities. For example, total number of bacteria, Actinomycetes and fungi and the total number of all microbial taxonomic groups found in the rhizosphere of cranberry plants grown in 100%

fortified potting soil supported higher number than all other treatments (Table 6), which themselves had similar counts.

4.3.2. Abundance of microbial communities in bulk soil of cranberry seedlings

Similar to total abundance of microbial counts in rhizospheric soil, total counts as well as counts of specific taxonomic groups in bulk soil were drastically lower at 160 days compared to 120 days of all treatments (Table 7). Additionally, a similar trend as in rhizospheric soil showing that irrespective of the treatments and harvesting time period, bacterial abundance is highest followed by those of Actinomycetes and fungi.

Significantly higher Actinomycetes and fungi counts were found in bulk soil of treatments fortified with 25% organic fertilizer and amended with biochar and enriched or not with bacteria at 120 days as compared to the control. In contrast total bacterial counts in these treatments were similar to the control (Table 7).

Bulk soil collected from pots containing 25% organic fertilizer and amended with biochar and enriched with bacteria had significantly higher numbers of fungi, but not bacteria or Actinomycetes collected at 160 days. Bacteria CFUs were significantly higher in the control (Treatment 1) compared to all other treatments. Although not significant, total CFUs were highest for Treatments 1 and 5 compared to the remaining treatments.

Quantification by PCR using the species-specific primers for the rhizobacteria and ErM fungi confirmed only the presence of *Microbacterium* in the organic fertilizer and rhizospheric soil of the cranberry cuttings prior to inoculation and establishment of the greenhouse experiment.

The quantification by PCR (qPCR) of *A. brasilense, M. ginsengisoli* and *V. paradoxus* in rhizospheric potting mix and cranberry roots harvested at 120 and 160 days after transplanting provided varied results (Tables 8, 9 & 10). Irrespective of the treatment, higher DNA copies of *A. brasilense* were detected in roots than in rhizospheric soil (Table 8). Generally, *A. brasilense* DNA copy numbers were similar in rhizospheric soil of all treatments at both harvesting time points, while DNA copies were significantly higher only in roots of cranberry seedlings grown in 100% fortified potting soil and harvested at 120 days. However at 160 days, *A. brasilense* DNA copy numbers were high in all cranberry roots grown in control and all treatments except in roots grown in 25% fortified potting mix and enriched with PGPR (Table 8)

Generally, *M. ginsengisoli* copy numbers in soil and in roots increased with time in all treatments; however, higher numbers were found in roots than in rhizosphere soil (Table 9). Irrespective of the harvesting time period, DNA copy numbers of *M. ginsengisoli* were highest in rhizospeheric soil and roots of cranberry seedlings grown in 100% fortified potting mix (Table 9). Lower than the control treatment, but similar in copy numbers, *M. ginsengisoli* DNA copy numbers in rhizospheric soil and roots were detected in all 25% fortified potting mixes, amended or not with biochar and/or fortified or not with PGPR of plants harvested at 160 days. *M. ginsengisoli* copy numbers of roots and rhizospheric soil of plants grown in 25% fortified potting soil amended with biochar or enriched with PGPR were similar, and significantly lower than those detected in the control treatment harvested at 120 days.

Contrary to *M. ginsengisoli* and *A. brasilense, V. paradoxus* DNA copy numbers were highest in rhizospheric soil than in roots of plants grown in any of the treatments (Table 10). Rhizospheric soil of plants grown in 25% fortified potting mix that was amended or not with

biochar and fortified or not with PGPR had significantly higher DNA copie numbers than those detected in 100% and 25% of fortified potting mix at 160 days (Table 10).

4.3.3. Ericaceous fungi colonization of cranberry roots and DNA copy number

Roots sampled from young cranberry cuttings prior to transplanting and after transplanting at 120 or 160 days showed exhibited typical colonization patterns and structures of ericoid mycorrhiza (Fig. 4) with extra-radical hyphae surrounding the roots (Fig. 4A). Dense intracellular coils (Figs 4B-D) as well as intracellular hyphae (Fig. 4C) were distributed in epidermal and cortical cells.

Species-specific primers successfully detected *Sebacinecea* species in rhizospheric soils of cranberry plants grown in all treatments and harvested at 120 and 160 days (Table 11). Compared to the control treatment, highest DNA copy numbers of *Sebacinecea* sp. were detected in rhizospheric soil of plants grown in 25% fortified soil mix enriched with bacteria and amended or not with biochar and harvested at 120 days (Table 11). Both soil of plants harvested at 160 days and contained 25% fortified soil mix amended with biochar had the highest numbers of *Sebacinecea* sp. In contrast, *Phialocephala* sp. failed to show detectable DNA copy numbers using qPCR. Cycle threshold values were below the standard curve minimum referral point.

4.4 List of Tables and Figures

Table 4. Plant growth parameters of cranberry plants harvested at 120 days and 160 days after transplanting.

Harvesting time	Treatment No.	Treatment description	Total shoot length (cm)	Shoot dry weight (g)	Root dry weight (g)
120 days	1	100% fortified potting mix*	34.90 ± 10.87^{B}	0.32 ± 0.09^{D}	0.09 ± 0.03^{B}
	2	25% fortified potting mix	93.99 ± 72.56^{A}	0.84 ± 0.61^{BC}	0.30 ± 0.21^{AB}
	3	25% fortified potting mix + bacteria&	68.20 ± 34.77^{AB}	0.47 ± 0.19^{CD}	0.17 ± 0.08^{B}
	4	25% fortified potting mix +1% biochar	87.77 ± 63.60^{AB}	0.95 ± 0.53^{AB}	0.38 ± 0.28^{A}
	5	25% fortified potting mix + 1% biochar + bacteria	118.08 ± 51.65^{A}	1.35 ± 0.59^{A}	0.44 ± 0.30^{A}
Harvesting time	Treatment No.	Treatment description	Total shoot length (cm)	Shoot dry weight (g)	Root dry weight (g)
160 days	1	100% fortified potting mix*	36.72 ± 15.66^{B}	0.39 ± 0.20^{B}	0.26 ± 0.16^{B}
	2	25% fortified potting mix	85.80 ± 50.31^{AB}	0.94 ± 0.44^{AB}	0.70 ± 0.53^{AB}
	3	25% fortified potting mix + bacteria&	121.72 ± 82.89^{A}	1.30 ± 1.06^{A}	0.77 ± 0.44^{A}
	4	25% fortified potting mix +1% biochar	77.61 ± 36.61^{AB}	0.81 ± 0.43^{AB}	0.65 ± 0.40^{AB}
	5	25% fortified potting mix + 1% biochar + bacteria	113.04 ± 88.68^{A}	1.19 ± 0.80^{A}	0.79 ± 0.44^{A}

Numbers represent the average length or weight of 7 replicates ± standard deviation
Capital letters within the same column represent significant differences based on Student's *t*-test $P = 0.05$
*Potting mix fortified with 100% or 25% the standard dose of organic fertilizer, Actisol®
&The concentration of 10^6 CFUs/mL of each rhizobacteria species was used

Table 5. Nutrient analysis of cranberry leaves of plants harvested at 120 days and 160 days after transplanting.

Harvesting time	Treatment No.	Treatment description	Nitrogen (mg/g)	Phosphorus (mg/g)	Potassium (mg/g)
120 days	1	100% fortified potting mix*	11.65 ± 0.41^{A}	1.38 ± 0.10^{A}	13.64 ± 0.34^{A}
	2	25% fortified potting mix	7.63 ± 0.24^{B}	1.04 ± 0.01^{B}	10.93 ± 0.38^{B}
	3	25% fortified potting mix + bacteria&	7.38 ± 0.33^{B}	0.92 ± 0.05^{C}	9.42 ± 0.54^{C}
	4	25% fortified potting mix +1% biochar	7.54 ± 1.30^{B}	0.82 ± 0.07^{D}	8.22 ± 0.43^{D}
	5	25% fortified potting mix + 1% biochar + bacteria	5.64 ± 0.21^{C}	0.72 ± 0.01^{D}	8.54 ± 0.10^{D}
Harvesting time	Treatment No.	Treatment description	Nitrogen (mg/g)	Phosphorus (mg/g)	Potassium (mg/g)
160 days	1	100% fortified potting mix*	13.97 ± 0.74^{A}	1.32 ± 0.06^{A}	12.54 ± 0.91^{A}
	2	25% fortified potting mix	7.51 ± 0.28^{B}	0.92 ± 0.12^{B}	9.04 ± 0.72^{B}
	3	25% fortified potting mix + bacteria&	6.24 ± 0.50^{C}	0.81 ± 0.07^{B}	7.63 ± 0.11^{C}
	4	25% fortified potting mix +1% biochar	7.67 ± 0.16^{B}	0.96 ± 0.08^{B}	9.20 ± 0.13^{B}
	5	25% fortified potting mix + 1% biochar + bacteria	8.44 ± 0.18^{B}	0.98 ± 0.10^{B}	8.83 ± 0.30^{BC}

Numbers represent the average of each element expressed as mg per gram of dry leaf tissue of 3 replicates ± standard deviation
Letters within the same column represent significant differences based on Student's *t*-test $P = 0.05$
*Potting mix fortified with 100% or 25% the standard dose of organic fertilizer, Actisol®
&The concentration of 10^6 CFUs/mL of each rhizobacteria species was used

Table 6. Colony forming unit (CFU/g) of rhizospheric soil of cranberry plants harvested at 120 days and 160 days after transplanting

Harvesting time	Treatment No.	Treatment description	Bacteria#	Actinomycetes#	Fungi#	Total#
160 days	1	100% fortified potting mix*	363 ± 20^{A}	126 ± 46^{A}	0.57 ± 0.15^{A}	490 ± 25^{A}
	2	25% fortified potting mix	189 ± 22^{B}	94 ± 31^{AB}	0.20 ± 0.17^{B}	284 ± 65^{B}
	3	25% fortified potting mix + bacteria&	164 ± 38^{B}	66 ± 21^{B}	0.40 ± 0.30^{AB}	230 ± 33^{B}
	4	25% fortified potting mix +1% biochar	196 ± 19^{B}	45 ± 18^{B}	0.20 ± 0.10^{B}	241 ± 31^{B}
	5	25% fortified potting mix + 1% biochar + bacteria	203 ± 29^{B}	62 ± 15^{B}	0.23 ± 0.11^{B}	266 ± 47^{B}
Harvesting time	Treatment No.	Treatment description	Bacteria#	Actinomycetes#	Fungi#	Total#
120 days	1	100% fortified potting mix*	1360 ± 122^{AB}	390 ± 96^{A}	20.0 ± 35^{AB}	1770 ± 216^{AB}
	2	25% fortified potting mix	1220 ± 407^{AB}	277 ± 104^{AB}	6.7 ± 6^{B}	1503 ± 347^{AB}
	3	25% fortified potting mix + bacteria&	490 ± 100^{C}	290 ± 115^{AB}	34.7 ± 58^{AB}	815 ± 38^{C}
	4	25% fortified potting mix +1% biochar	1007 ± 415^{BC}	220 ± 95^{AB}	25.0 ± 53^{AB}	1252 ± 423^{BC}
	5	25% fortified potting mix + 1% biochar + bacteria	1823 ± 586^{A}	173 ± 61^{B}	46.3 ± 40^{A}	2043 ± 581^{A}

Numbers represent the average of CFU/gram of soil of 3 biological replicates ± standard deviation
Capital letters within the same column represent significant differences based on Student's *t*-test *P =0.05*
*Potting mix fortified with 100% or 25% the standard dose of organic fertilizer, Actisol®
#Numbers in the column represent CFUs X 1000
& The concentration of 10^6 CFUs/mL of each rhizobacteria strain was used

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Table 7. Colony forming unit (CFU/g) of bulk soil of cranberry plants harvested 120 days and 160 days after transplanting

Harvesting time	Treatment No.	Treatment description	Bacteria[#]	Actinomycetes[#]	Fungi[#]	Total[#]
120 days	1	100% fortified potting mix*	1840 ± 260[#]	463 ± 119[C]	5.3 ± 1.50[C]	2309 ± 190[B]
	2	25% fortified potting mix	2043 ± 261[A]	310 ± 79[C]	1.0 ± 1.00[C]	2354 ± 310[AB]
	3	25% fortified potting mix + bacteria[&]	1640 ± 684[A]	413 ± 124[C]	1.0 ± 1.00[C]	2054 ± 752[B]
	4	25% fortified potting mix +1% biochar	2383 ± 748[A]	950 ± 249[B]	20 ± 5.60[B]	3353 ± 802[A]
	5	25% fortified potting mix + 1% biochar + bacteria	1913 ± 344[A]	1413 ± 142[A]	26 ± 3.60[A]	3353 ± 461[A]
Harvesting time	Treatment No.	Treatment description	Bacteria[#]	Actinomycetes[#]	Fungi[#]	Total[#]
160 days	1	100% fortified potting mix*	391 ±1[A]	156 ± 87[AB]	0.38 ± 0.58[B]	547 ± 87[A]
	2	25% fortified potting mix	203 ± 53[BC]	98 ± 90[AB]	0.20 ± 0.10[B]	301 ± 114[C]
	3	25% fortified potting mix + bacteria[&]	156 ± 13[C]	52 ± 10[B]	0.20 ± 0.17[B]	208 ± 22[C]
	4	25% fortified potting mix +1% biochar	246 ± 86[B]	80 ± 58[B]	0.43 ± 0.21[B]	327 ± 143[BC]
	5	25% fortified potting mix + 1% biochar + bacteria	265 ± 16[B]	215 ±73[A]	2.10 ± 0.44[A]	482 ± 87[AB]

Numbers represent the average of DNA copy number per gram of bulk soil of 6 replicates (3 biological & 2 technical replicates) ± standard deviation

*Potting mix fortified with 100% or 25% the standard dose of organic fertilizer, Actisol®

[#]Numbers in the column represent CFUs X 100 000

[&] The concentration of 10^6 CFUs/mL of each rhizobacteria strains was used

Capital letters within the same column represent significant differences based on Student's *t*-test *P =0.05*

Table 8. DNA copy numbers of the rhizobacterium *Azospirulium brasilense* in the rhizospheric soil and roots of cranberry plants harvested at 120 and 160 days after transplanting

Treatment No.	Treatment description	Soil 120 days#	Soil 160 days#	Root 120 days#	Root 160 days#
1	100% fortified potting mix*	1.68 ± 0.10^{A}	1.29 ± 0.43^{A}	36.28 ± 3.68^{A}	13.49 ± 7.23^{A}
2	25% fortified potting mix	1.41 ± 0.34^{A}	1.05 ± 0.56^{A}	4.01 ± 0.74^{B}	14.36 ± 2.12^{A}
3	25% fortified potting mix + bacteria&	0.56 ± 0.13^{A}	2.68 ± 0.51^{A}	3.23 ± 0.07^{B}	3.84 ± 1.15^{B}
4	25% fortified potting mix +1% biochar	0.71 ± 0.12^{A}	2.60 ± 0.19^{A}	4.23 ± 1.59^{B}	11.15 ± 1.31^{A}
5	25% fortified potting mix + 1% biochar + bacteria	0.95 ± 0.23^{A}	3.07 ± 0.32^{A}	6.01 ± 0.18^{B}	10.90 ± 1.23^{A}

Numbers represent the average of DNA copy number per gram of rhizosphere soil or root tissue of 6 replicates (3 biological & 2 technical replicates) ± standard deviation

#Numbers in the column represent DNA copy numbers X 100 000 measured by qPCR

*Potting mix fortified with 100% or 25% the standard dose of organic fertilizer, Actisol®

& The concentration of 10^6 CFUs/mL of each rhizobacteria strain was used

Capital letters within the same column represent significant differences based on Student's *t*-test *P =0.05*

Table 9. DNA copy numbers of the rhizobacterium *Microbacterium ginsengisoli* in the rhizosphere and roots of cranberry plants at 120 and 160 days after transplanting

Treatment No.	Treatment description	Soil 120 days[#]	Soil 160 days[#]	Root 120 days[#]	Root 160 days[#]
1	100% fortified potting mix*	134 ± 7^{A}	55 ± 2^{A}	503 ± 26^{A}	208 ± 70^{A}
2	25% fortified potting mix	20 ± 8^{C}	25 ± 7^{B}	75 ± 30^{C}	95 ± 27^{B}
3	25% fortified potting mix + bacteria[&]	21 ± 4^{C}	25 ± 4^{B}	81 ± 14^{C}	95 ± 16^{B}
4	25% fortified potting mix +1% biochar	54 ± 2^{B}	37 ± 5^{B}	201 ± 60^{B}	141 ± 19^{B}
5	25% fortified potting mix + 1% biochar + bacteria	40 ± 6^{B}	23 ± 2^{B}	148 ± 21^{B}	86 ± 7^{B}

Numbers represent the average of DNA copy number per gram of rhizosphere or root tissue of 6 replicates (3 biological & 2 technical replicates) ± standard deviation

[*]Potting mix fortified with 100% or 25% the standard dose of organic fertilizer, Actisol®

[#]Numbers in the column represent DNA copy numbers X 100 000 measured by qPCR

[&] The concentration of 10^6 CFUs/ml of each rhizobacteria strain was used

Capital letters within the same column represent significant differences based on Student's *t*-test *P =0.05*

Table 10. DNA copy numbers of the rhizobacterium *Variovorax paradoxus* per gram of sample in the rhizosphere and roots of cranberry plants at 120 and 160 days after transplanting

Treatment No.	Treatment description	Soil 120 days[#]	Soil 160 days[#]	Root 120 days[#]	Root 160 days[#]
1	100% fortified potting mix*	4166 ±1507^{BC}	16557 ± 5102^{B}	0.30 ± 0.03^{A}	0.66 ± 0.30^{B}
2	25% fortified potting mix	9584 ± 2219^{A}	3142 ± 274^{B}	0.25 ± 0.02^{B}	0.53 ± 0.10^{B}
3	25% fortified potting mix + bacteria[&]	2129 ± 383^{C}	13971 ± 347^{A}	0.15 ± 0.04^{B}	1.57 ± 0.28^{A}
4	25% fortified potting mix +1% biochar	3495 ± 1276^{C}	12411 ± 2543^{A}	0.16 ± 0.03^{A}	0.40 ±0.12^{B}
5	25% fortified potting mix + 1% biochar + bacteria	6779 ± 2458^{AB}	12718 ± 1426^{A}	0.31 ± 0.07^{A}	0.32 ± 0.03^{B}

Numbers represent the average of DNA copy number per gram of rhizosphere or root tissue of 6 replicates (3 biological & 2 technical replicates) ± standard deviation

[*]Potting mix fortified with 100% or 25% the standard dose of organic fertilizer, Actisol®

[#]Numbers in the column represent DNA copy numbers X 100 000 measured by qPCR

[&] The concentration of 10^6 CFUs/mL of each rhizobacteria strain was used

Capital letters within the same column represent significant differences based on Student's *t*-test *P =0.05*

Table 11. DNA copy numbers of the ErM fungus, *Sebacinecea* sp. in the rhizospheric soil of cranberry plants harvested at 120 and 160 days after transplanting

Treatment No.	Treatment description	120 days[#]	160 days[#]
1	100% fortified potting mix*	0.77 ± 0.16 [C]	176.45 ± 143.27 [BC]
2	25% fortified potting mix	15.46 ± 0.94 [B]	42.22 ± 2.58 [D]
3	25% fortified potting mix + bacteria[&]	38.07 ± 9.34 [A]	224.12 ± 48.54 [AB]
4	25% fortified potting mix +1% biochar	3.17 ± 1.68 [BC]	307.27 ± 24.14 [A]
5	25% fortified potting mix + 1% biochar + bacteria	33.12 ± 13.27 [A]	93.85 ± 14.23 [CD]

Numbers represent the average of DNA copy number per gram of rhizosphere of 6 replicates (3 biological & 2 technical replicates) ± standard deviation

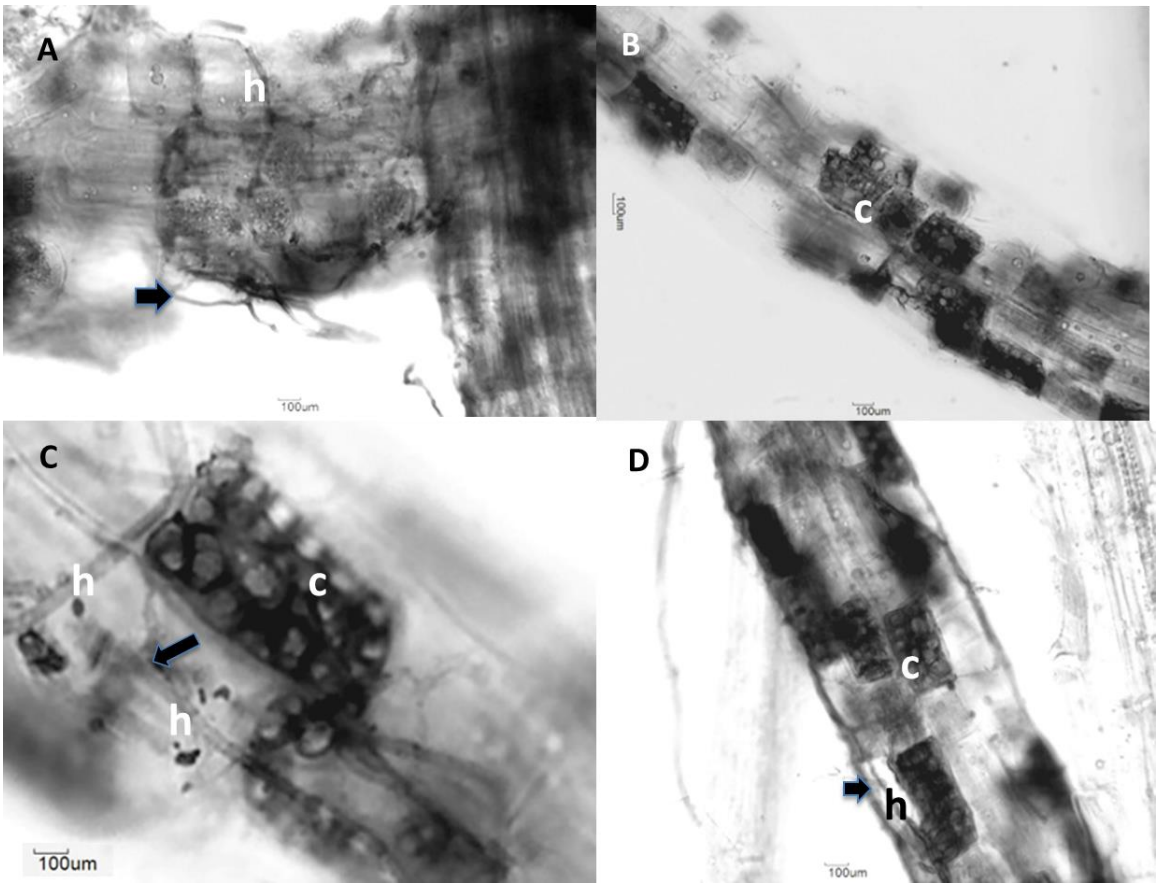
[*]Potting mix fortified with 100% or 25% the standard dose of organic fertilizer, Actisol®

[#] Numbers in the column represent DNA copy numbers X 100 000 measured by qPCR

[&] The concentration of 10^6 CFUs/mL of each rhizobacteria strain was used

Capital letters within the same column represent significant differences based on Student's *t*-test *P =0.05*

Figure 4. Mycorrhizal fungi (ErM) in roots of cranberry cultivar Stevens



A. Overview of cleared roots showing external hyphae (**h**). **B**. Intracellular complex hypha in the form of coils (**C**) found only in cortical cells. Stele is devoid of fungal structures. **C** and **D**. Intracellular hyphae (**h**) in cortical cells (arrows).

Chapter V: Discussion

Biochar has proven to have positive effects on nutrient retention (Glaser et al. 2002; Major et al., 2010), cation-exchange capacity (Hibbett et al., 2007), water-holding capacity (Glaser et al., 2002), soil microbial and mycorrhizal activity (Warnock et al., 2007; Thies et al., 2009), soil acidity (Chan et al., 2009), and electric conductivity when applied to soils (Asai et al., 2009) all of which improve soil fertility and thereby plant growth (Lehmann et al., 2003; Rondon et al., 2007; Novotny et al., 2009).

In order to assess the full interaction between biochar and PGPR on organic cranberry establishment, field experiments are essential. However, as the overarching goal of the project is to study whether a combination of biochar and microorganisms, when applied as a soil amendment, would decrease fertilizer requirements and/or enhance the growth of cranberry cuttings of the cultivar Stevens, leading to a faster establishments, we consider that the results of our study, conducted on growth benches under controlled conditions, allowed the isolation of parameters of interest needed for the investigation of the hypothesis.

5.1. Plant growth promoting rhizobacteria

Recent reports have shown that biochar can help increase the overall growth of plants and health status by improving the development and maintenance of beneficial soil microbe communities and mycorrhizal fungi (Kolb et al., 2009; Solaiman et al., 2010), and increasing soil microbial content (Graber et al., 2010) and mycorrhizal colonization (Makoto et al., 2010). PGPR have also been reported to have positive impacts on plant development and maintenance through a variety of mechanisms. PGPR are living microorganisms which, when applied to seed, plant surfaces

or soil, colonizes the rhizosphere or the interior of the plant and promote growth by a wide variety of mechanisms such as phosphate solubulization, siderophore production, nitrogen fixation, induction of systemic resistance, and promoting the symbiosis with other beneficial microorganisms are only some examples (Bhattacharya, 2012). In our study, the application of *A. brasilens*, *M. ginsengisoli and V. paradoxus* as the microbes of choice was dictated by the fact that these bacteria play an important role in nutrient mobilization and protection against pathogens (Lecomte et al., 2011; Steenhoudt, 2000)

5.2. Organic fertilizers

The addition of full dose of the organic fertilizer, Actisol® caused a significant decrease in shoot dry weights and total length of cranberry upright runners, although leaf nutrient content of N, P, K of these cuttings was the highest compared to all other treatments. These results suggest that high dosage of Actisol® could impede cranberry growth even if N, P, K concentrations in leaves were high. The reasons for this observation are not clear, however it may be related to physiological factors: it has been shown that cranberry grown under saline conditions exhibit low photosynthetic activity leading to reduced growth and productivity (Jeranyama et al., 2009). Although, we did not attempt to measure the potting mix salinity, it is known that the form of potassium in chicken manure can be responsible for salt stress (Liebhardt et al., 1974). A high dose of Actisol® applied once in pots might have increased soil salinity leading to underdeveloped cranberry runners. Taken together, these results point out that the reduced growth observed in Treatment 1 is not due to nutrient limitation, underpinning the salt stress hypothesis supported by Jeranyama and DeMoranville (2009).

Statistically, no significant increases in cranberry growth parameters at both harvesting dates were seen when biochar was applied concurrently with a reduced rate of Actisol® to the potting mix as compared when reduced rate of Actisol® was added to the potting mix. This is contrary to specific recommendations in the literature, advocating fertilizer addition in order to maximize the positive impacts of biochar application to soil (Asai et al., 2009; Yamato et al., 2006).

5.3. Cranberry growth parameters

Increased crop yield is a commonly reported benefit of adding biochar to soils (Lehmann et al., 2003; Rondon et al., 2007; Novotny et al., 2009). However, experimental results on the application of biochar are reported to be variable and dependent on the experimental set-up, crop type, soil properties and conditions, while causative mechanisms are yet to be fully elucidated (Jeffrey et al 2011). In our study, significant differences in total length of uprights and runners and total dry weights of shoots and roots of cranberry seedlings grown in 25% fortified potting mix and amended with biochar and bacteria were observed at both harvesting dates as compared to those grown in potting mix fortified with a full dose of Actisol®. However, no differences in cranberry growth parameters at both dates were observed when biochar not amended with bacteria was used. These results indicate that biochar alone, PGPR alone, or combination of both applied together as a soil amendment with reduced dose of Actisol® did not statistically increase plant growth parameters. These results are not surprising and are in accordance with what has recently been reported. For example, statistically significant increases in crop productivity were found to occur in both radishes (Chan et al., 2009) and soybean (Van Zwieten et al., 2010) upon addition of biochar to soil ($P < 0.05$), while the opposite was observed in ryegrass (Wisnubroto et al., 2010).

5.4. Effect of biochar on microbial population

Plant root exudates mediate a multitude of rhizospheric interactions at the species level. It has been shown that root exudates play an important role in mediating interactions with plants and microbes (Bais et al., 2006). Root exudates of tea (*Camellia sinensis),* sweet basil (*Ocimum basilicum*), rice (*oriza sativa)* and *Lithospermum erythrorhizon* have been found to be suppressive to some microorganisms (Bais et al., 2006; Brigham et al., 1999; Pandey & Palni, 1996). In our study, total microbial abundance numbers expressed in CFUs per gram of soil were lower in the rhizosphere than those reported for bulk soil at both harvesting dates. These results indicate that cranberry root exudates are able to influence the microbial population of their rhizosphere either by suppressing microbe numbers or modulating the type of microbial interaction it favors. Although no information is available on root exudates of ericaceous plants, previous studies suggested that most plants select and attract specific microbes and, therefore, alter the composition and diversity of microbial communities in the rhizosphere in a plant-specific manner (Broeckling et al., 2008).

The addition of biochar alone or enriched with selected PGPR and added to potting soil containing reduced rates of Actisol® had no effect on the microbial abundance numbers of specific taxonomic groups nor on the total microbial abundance numbers. However, total microbial abundance numbers were the highest in the rhizosphere of cranberry plants grown with a full dose of Actisol® without the addition of biochar. These results are in agreement with the findings that microbial diversity is closely correlated to soil fertility especially with respect to organic carbon and total nitrogen (Yao et al., 2000).

A different trend was observed with microbe abundance numbers estimated in bulk soil as a result of biochar addition to the potting mix. Total number of microbes in bulk soil of cranberry plants harvested at 120 and 160 days were statistically high ($P< 0.05$) and so are those of fungal and

Actinonmycete taxonomic groups at 120 and 160 days after harvesting. These results support other reports on the positive impact on bulk soil CFU counts by biochar (Graber et al., 2010; Pietikäinen et al., 2000).

Interestingly, abundance numbers belonging to fungal taxonomic groups were significantly and consistently higher in bulk soil amended with biochar and PGPR at both harvesting dates. Although we did not attempt to characterize the different fungal phyla, evidence points out that mycorrhizal fungi respond more positively to biochar additions than to additions of other types of organic material added as control (Harvey et al. 1976; Ishii et al., 1994; Wornock et al. 2007). It is highly possible that the positive responses shown by mycorrhizal fungi are determined in part by the amount of carbon in the material being added to the soil, with the expectation that the biochar is more carbon-rich than the organic matter. ErM could have been responsible for the increase in fungal CFUs as it been shown in many reports that the availability N may affect colonization rates of ErM in cranberry beds, as high N availability can suppress ErM colonization of cranberry (Leake et al., 1990; Read, 1996).

5.5 Effect of bichar on the presence of PGPR rhizobacteria

The quantification by qPCR of the different PGPR demonstrated the highest copy numbers of *A. brazilens* in roots. This was expected as *A. brasilense* is known to colonize and binds to the surface of the roots (Bashan et al., 1991). The absence of difference in copy numbers in bulk soil among treatments over time can be explained by the composition of the potting medium, which was made of 80% sand to support cranberry growth. Coarse and fine sand have a highly negative effect on *A. brasiliens* viability and survival over time (Bashan et al., 1995). This could have created an unfavourable environment for the bacterium, which in turn led to the averaging of the population to

similar levels across treatments. Copy numbers of *A. brasilense* detected in roots of plants grown with a full dose of Actisol® were significantly higher at 120 days, but not at 160 days. This could be due to freely available nutrients at this time period.

A. brasiliens, like the two other PGPR, was detected in roots and bulk soil in all treatments even in those treatments in which it was not introduced (Treatments 1, 2 and 4), suggesting that cross contamination may be a factor although careful measures, such as the placement of a geotextile layer in the bottom and on the surface of every pot, were taken during the experimental design to prevent cross contamination. It has been reported that *A. brasiliens* is capable of horizontal and vertical spread of 160 and 50 cm, respectively in field and greenhouse studies (Bashan et al., 1995; Bashan & Levanony, 1987).

Compared to all other treatments, DNA copy numbers of *M. ginsengisoli* were present in substantial numbers in bulk soil and roots of plants grown with a full dose of Actisol® after 120 and 160 days of transplantating, but with slighter higher numbers at 120 days. This is not surprising since *M. ginsengisoli* was present in the Actisol® and in the rhizospheric soil of cranberry cuttings prior to transplantating and multiplied in the presence of a full dose of Actisol®. Additionally, DNA copie numbers of *M. ginsengisoli* increased in treatments in which biochar was added to the potting mix, which also paralleled with the increase in cranberry root biomass. Our results are in agreement with other reports demonstrating that biochar can support microbial communities, which in turn support plant biomass (Graber et al., 2010; Pietikäinen et al., 2000). Additionally, evidence points out that the *Microbacterium* genus benefits from biochar incorporation and is known to be NO_3 reducers (Anderson et al. 2011).

V. paradoxus DNA copy numbers were clearly more abundant in rhizospheric soil than in roots. This confirms previous descriptions of this bacterium habitat and biology (Daviset al., 1970; Willems et al., 1991). Similar to *A. brasilense*, the presence of *V. paradoxus* in all treatments is due to cross

contamination. Unlike *A. brasilense* and *M. ginsengisoli*, soil fertility did not seem to influence the abundance of this bacterium. Copy numbers increased over time suggesting a constant increase in population of *V. pararadoxus*. Although *V. pararadoxus* was the most abundant PGPR detected in the qPCR assays, no link could be traced to other measured parameters (i.e. CFUs per gram of soil, plant growth parameter). The mechanism by which *V. pararadoxus* positively affects plant growth is by the production of 1-aminocyclopropane-1-carboxylate deaminase (Felici et al. 2008), which consequently helps the plant to resist stress by reducing ethylene levels (Belimov et al., 2009). The cranberry cuttings were kept under moist soil conditions throughout the entire experiment duration, and therefore the beneficial effect of *V. paradoxus* could not have been observed since no drought stress occurred.

The only ErM fungal genus detected in the root rhizosphere was the *Sebacineceae* genus. Copy numbers of *Sebacinacea* sp. varied substantially across all treatments and time points, however, increased copy numbers of *Sebacinacea* sp. over time seems to have evolved independently of the different treatments. The absence of literature on the effect of biochar on ericoid mycorrhiza makes it difficult to draw sound conclusions. The addition of activated charcoal has been shown to have a positive impact on root colonization by ErM of blueberries *in vitro* (Duclos et al., 1983). Many other reports have shown the beneficial impact of biochar on mycorrhizal fungi in several crops (Solaiman et al., 2010; Warnock et al., 2007).

The inability to detect the most commonly ErM genus, *Phialocephala,* in cranberry roots might be due to the fact that the rooted cranberry cuttings had never been exposed to *Phialocephalea* sp., which is known to thrive naturally in fields of cultivated cranberry plants in Québec. The team of Dr. Franz Lang, University of Montreal, had isolated several *Phialocephalea* sp. from roots of several cranberry cultivars including Stevens that were cultivated under field conditions in Quebec. Although the unrooted cuttings used in our study originated from the same field site, rooting of the

cuttings was performed under greenhouse conditions that are not conducive to the colonization or the development of *Phialocephalea*.

Chapter VI: Conclusions

This study was undertaken to test whether biochar derived from maple bark and applied as an amendment with selective rhizobacteria can facilitate the development and growth of cranberry cuttings and whether biochar can be used as part of the growing medium for the establishment of organic cranberry cuttings.

Statistical differences in total length of uprights and runners and total dry weights of shoots and roots of cranberry cuttings grown in 25% fortified potting mix with Actisol® and amended with biochar and bacteria were observed at both harvesting dates as compared to those grown in potting mix fortified with a full dose of Actisol®. While growth parameters of cranberry cuttings grown in potting mix with reduced rates of Actisol® but amended with biochar or PGPR were similar. These results reinforce the views of what has been reported by others (Jeffrey et al. 2010) that experimental results dealing with crop productivity as a result of biochar amendments are variable and dependent on the experimental set-up, soil properties and conditions, while causative mechanisms are yet to be fully elucidated.

The biological properties of soil are also influenced by biochar amendments. Khodadad et al. (2011) reported that microbial community composition in field soil is influenced by biochar amendments. Biochar composition can be more conducive to growth of certain microorganisms, while limiting the growth of others. Results of this study indicate that biochar amendment was conducive to certain microbial taxonomic groups in bulk and rhizospheric soils, but not to others.

The only ErM fungal genus detected in the rhizospheric soil was the *Sebacineceae* genus with variable DNA copy numbers across all treatments and with substantial increases over time. Future directions of research could look at the influence of biochar amendment with *Sebacineceae* species in rooting experiments of cranberry grown in soilless substrate.

In conclusion, the results of this study demonstrate that biochar amendment with or without selected bacteria yielded variable results over control treatments. Such high variation likely stems from the suitability of biochar derived from maple wood for cranberry growth parameters, its reaction with organic fertilizers and the choice of microorganisms added. Future experiments should focus on the effects of the biofertilizers and biochar as soil amendement during the propagation stage (i.e. at root initiation of the cranberry cuttings).

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