The Plant Growth-Promoting Potential of Root-Associated Bacteria from Plants Growing in Stressed Environments

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ABSTRACT

Several studies have demonstrated the potential of plant growth-promoting bacteria, including their use as inoculants, in the contexts of both agriculture and enhanced phytoremediation. Despite their promise, there is still a need to characterize and identify plant growth-promoting bacteria amidst isolates cultured to date. Therefore the purpose of this study was to i) screen bacterial endophytes isolated from plants growing in both a chronically nutrient-deficient agricultural soil and a hydrocarbon-contaminated soil for plant growth-promoting potential *in vitro* and ii) assess the plant growth-promoting capabilities of isolates *in vivo*. Bacterial isolates belonging to genera common to both environments were screened for plant growth-promoting genotypes and phenotypes including ACC deaminase activity (*acds*), hydrocarbon degradation (*alkB* and *CYP153*), alkaline phosphatase activity (*phoD*), and nitrogen fixation (*nifH*). After screening, 28 isolates from 16 genera were subjected to further study *in vivo*. After using seed germination and root elongation screening in wheat (*Trictum aestivum)* and sweet clover (*Melilotus alba*), four isolates were selected for further study—A3 (*Pseudomonas sp.*) and A9 (*Delftia sp.*) in sweet clover and A12 (*Kluyvera sp.*) and B34 (*Luteimonas sp.*) in wheat—as they promoted early plant growth and development. Plant growth-promoting capabilities were then assessed by inoculating wheat and sweet clover seeds and growing plants in a growth chamber for 60 days in either a marginal agricultural soil or the same soil amended with diesel. Isolates A12 (*Kluyvera sp.*) and B34 (*Luteimonas sp.*) increased the acquisition of nitrogen (N) and phosphorous (P) by plants when growing in the marginal agricultural soil. However, plant growth-promoting effects were lost when diesel fuel was added. Further, no effect was observed in sweet clover in either soil condition. Initial screening for plant growth-promoting potential highlighted the importance of including functionality screening as the presence or absence of a plant growth-promoting genotype did not always indicate a positive phenotype. Further, this work exemplified the importance of *in vivo* screening assays to identify potential plant growth-promoting bacteria. Finally, results showed bacteria with plant growth-promotion potential can be isolated from stressed environments and may promote wheat growth in a marginal agricultural soil.

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DEDICATION

I dedicate this thesis to my parents — Carol Kohout[Mom], Stan Iglehart[Step Dad] and Ed Kohout[Dad]—and to my sisters—Jamie and Grace Iglehart and Lauren and Melanie Kohout—for providing me with the opportunity to pursue my dream of becoming a scientist but also for their never-ending love and support. I also dedicate this thesis to my oldest and dearest friends—Erin Deane, Lexi Cooper, Katherine Nager, Keili Maxon, Courtney Zavaleta—as well as my new friends here in Canada who were always there for me. All of these people never failed to keep me motivated, focused, and enjoying life throughout this process.

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LIST OF ABBREVIATIONS:

AAFC	Agriculture and Agri-Food Canada
ACC	1-Aminocyclopropane-1- Carboxylate
BPHS	Bitumount Provincial Historic Site
CFU	Colony Forming Units
Ctrl	Control Treatment
DI	De-ionized
DNA	Deoxyribonucleic Acid
FeMo	Molybdenum-Iron
g	Force of Gravity
h	Hour
HC	Hydrocarbon
HCS	Hydrocarbon-Amended Control Soil
IAA	Indole-3-Acetic Acid
min	Minute
MPP	Monopotassium Phosphate Buffer
N_{fert}	Nitrogen Fertilizer Treatment
NP_{fert}	Nitrogen and Phosphorous Fertilizer Treatment
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
P_{fert}	Phosphorous Fertilizer Treatment
RCBD	Randomized Complete Block Design
rt-PCR	reverse transcription PCR
sec	Second
T_0	Time Zero Control
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
UC	Uninoculated Control Plant

1. INTRODUCTION

The world population is projected to reach nine billion people within the next fifty years (Godfray et al. 2010). This increase in population will create a need for more food and land for agricultural production and habitation. However, much of the available land is unusable for either habitation or crop production due to factors such as contamination (World Health Organization 2013, Evangelou et al. 2015) or conditions unsuitable for plant growth (low nutrient content, salinity, water availability, etc.) (Alexandratos and Bruinsma 2012). As the effects of climate change become more apparent in coming years, it is projected that these problems will only be exacerbated (Lesk et al. 2016). This is anticipated to especially be the case in developing countries already considered to be more vulnerable due to their reliance on agriculture (Alexandratos and Bruinsma 2012). Although the use of chemical fertilizers and pesticides has made it possible for farmers to produce more food on the land that is currently available, this activity has been linked to environmental degradation. Negative environmental impacts from current agricultural practices include the release of greenhouse gases, pollution from run-off and leaching of excess nutrients and pesticides, water shortages, soil degradation, and loss of biodiversity from land conversion (Godfray et al. 2010). As such, current methods of crop production are considered to be unsustainable (Alexandratos and Bruinsma 2012).

In order to close the yield gap (the difference between actual plant productivity and the optimum achievable with current technology) without imparting any further environmental degradation, alternative or complementary technology must be developed. Though this is an incredibly huge task that must be completed in a relatively short amount of time, the ways one may go about accomplishing this goal are as vast as the problem itself. Considering this, it is important to remember oftentimes solutions to modern problems can be found in nature. Among these new alternatives is the exploitation of plant growth-promoting microorganisms found within the root microbiome.

Plants, much like humans, are colonized with a range of microorganisms. Just as recent studies demonstrate the role of the gut microbiome in proper function and disease prevention in humans (Gopal et al. 2013), the plant microbiome is known to contribute to plant health and fitness (Laslo et al. 2012). These plant growth-promoting effects can be direct–increased nutrient acquisition and alteration of plant hormones–or indirect–biological control of disease (Fallath et al. 2017). Microorganisms within the plant microbiome that perform these functions are often referred to as plant growth-promoting organisms. These organisms can be bacteria, fungi, protists, or archaea (Hardoim et al. 2015) that are acquired horizontally (from the environment surrounding the plant) as well as vertically (from the seed) (Shade et al. 2017) and are found in both above and belowground tissues (Bulgarelli et al. 2013).

Several studies demonstrate that it is possible to not only isolate and identify plant growth-promoting bacteria, but also to use these organisms as inoculants with positive effects on plant growth, stress tolerance and disease prevention/suppression. In fact, plant growth-promoting bacteria have been shown to alleviate drought stress (Timmusk et al. 2014, Chen et al. 2017), increase nutrient acquisition and plant growth (Ipek et al. 2014, Kumar et al. 2014), and reduce disease incidence and severity (Busby et al. 2016, Zahn and Amend 2017). As a result of the work and interest placed on plant growth-promoting bacteria in recent years, several commercial inoculants have been successfully developed and are currently being used in Canada, the United States, and Europe (Bashan et al. 2014, Owen et al. 2015, Huang et al. 2017).

One of the benefits of developing plant growth-promotion technology is that the applications are not strictly limited to agriculture. One of the areas of plant growth-promotion research that has been gaining traction focuses on utilizing plant growth-promoting bacteria to enhance phytoremediation. Phytoremediation is the use of green plants to remove pollutants from the environment, or to render them harmless, and has been shown to successfully remove recalcitrant contaminants such as heavy metals (Parmar and Singh 2015), petroleum hydrocarbons (Yavari et al. 2015), and other pollutants (Gerhardt et al. 2009). However, due to the toxicity of these contaminants, there are limitations to using phytoremediation (Thakur et al. 2016). Phytoremediation is reliant on the success of the plant; if the plant cannot survive then the contaminant cannot be removed. Studies have shown that, by taking advantage of the beneficial effects plant growth-promoting bacteria have on plant growth, it is possible to increase the success

of the plant and thereby the process of phytoremediation (Gurska et al. 2009, Hou et al. 2015, Kovalski Mitter 2018).

Although there are commercial products and several promising lab-based studies utilizing plant growth-promoting bacteria, experimental results are not always translated to the field (Owen et al. 2015). This has often been attributed to lack of rigorous field studies, the diversity of habitats (plant and soil), and the complexity of microbial interactions between inoculants and indigenous communities (Owen et al. 2015, Timmusk et al. 2017). Considering the abundance and diversity of bacteria in nature, it is likely that many plant growth-promoting bacteria have not yet been identified and tested. However, when the sheer number of bacterial isolates collected and cultured to date is considered, it becomes apparent that testing every isolate in the field simply isn't realistic. As such there is still a need to a) develop fast but efficient methods of screening for plant growth-promoting organisms amidst the abundance of plant-associated microbes collected to date and in the future and b) test those isolate *in vivo* under both controlled and field realistic conditions. In doing so, plant growth-promotion research will be able to not only better elucidate the mechanisms of plant growth-promotion, but also contribute to solving the problems faced by a changing environment.

The use of plant growth-promoting bacteria to improve crop production though sustainable intensification and phytoremediation through increased tolerance to contaminants is a large field of research with many applications that will become ever more valuable in coming years. The main objective of this study was to identify plant growth-promoting bacteria among collections of environmental isolates obtained in previous work. These two separate collections consisted of bacterial isolates cultured from the roots of plants growing at two field sites that have naturally-occurring and anthropogenically-imposed stresses: a chronically nutrient deficient agricultural site and a hydrocarbon-contaminated site. The following hypotheses were used to design and execute a series of experiments:

i. Root-associated bacteria isolated from different plants under stress will contain specific genes (*CYP153, alkB, phoD, nifH, acsds*) that encode proteins (hydrogenase, alkaline phosphatase, nitrogenase, ACC deaminase) associated with plant growth-promotion or stress tolerance.

ii. Plants inoculated with selected plant growth-promoting isolates will demonstrate increased growth in both hydrocarbon-contaminated and low nutrient soil.

To test these hypotheses, three study objectives were established:

(i) Test selected isolates for plant growth-promoting and hydrocarbon-degrading phenotypes such as nitrogen fixation, phosphorous solubilization and mineralization, ACC deaminase, and hydrogenase (hydrocarbon degradation) activity

(ii) Test selected isolates for plant growth-promoting and hydrocarbon-degrading genotypes associated with objective (i) including hydrocarbon-degrading genes (*CYP153*, *alkB*), stress tolerance genes (*acdS*), and genes involved in phosphorous (*phoD*) and nitrogen (*nifH*) availability

(iii) Assess plant growth-promoting capabilities of selected bacterial isolates in inoculated plants under controlled conditions.

Succeeding this introduction (Chapter 1) is a literature review (Chapter 2) which provides background information relevant to this thesis, followed by two research studies (Chapters 3 and 4) that address the above hypotheses. Chapter 3 presents the screening and selection process of bacterial isolates showing the greatest plant growth-promotion potential based on specific criteria. Chapter 4 evaluates the effects of select isolates on plant growth under different conditions. Chapter 5 integrates and summarizes research findings and provides suggestions for future work. The thesis is then concluded with a brief closing chapter (Chapter 6). The following research thesis is presented in a manuscript format. Thus, it is structured and written such that each chapter may stand alone as a peer reviewed article. Due to the nature of a manuscript style thesis some redundant information may occur.

2. LITERATURE REVIEW

2.1 Background

2.1.1 The Root Microbiome

Within the soil, plants and microbes interact extensively, each having the potential to affect the growth and success of the other (Lakshmanan et al. 2014). This is especially the case in the rhizosphere, or the area surrounding plant roots, where there is a greater abundance of microbes than the surrounding, bulk soil (Gaiero et al. 2013). This observed increased abundance is most likely as a result of the presence of plant exudates (e.g. sugars, amino acids). These low molecular weight compounds have been shown to not only enhance microbial abundance in the rhizosphere, but to also to act as a means of selecting for microbial partners or against microbial antagonists (Bais et al. 2006, Bakker et al. 2012, Quiza et al. 2015, Tkacz and Poole 2015, Pii et al. 2015). In fact, the interactions between plants and microbes in the area immediately around, on, and within the root have been demonstrated to have such important implications on plant growth that the term 'root microbiome' was coined.

Within the root microbiome, microorganisms have the potential to affect plant growth much in the same way that plants can select for microbial partners. Just as plants have the potential to alter microbial communities through the release of exudates, the activity of various microorganisms in the root microbiome has the potential to influence plant growth. Some organisms within the root microbiome act as pathogens and inhibit plant growth and function by producing toxic metabolites (Quiza et al. 2015, Pii et al. 2015). Therefore, these organisms are of interest in better understanding plant-microbe diseases. Other organisms found in the root microbiome have been shown to be beneficial for the success of the plant, and are often referred to as plant growth-promoting microorganisms (Rodríguez and Fraga 1999, Malboobi et al. 2009, Ji et al. 2014, Nosrati et al. 2014, Pankievicz et al. 2015, Tkacz and Poole 2015, Pii et al. 2015, Souza et al. 2015).

2.1.2 Plant Growth-Promoting Bacteria

Plant growth-promoting bacteria are identified in a wide variety of bacterial genera including but not limited to *Azospirillium*, *Bacillus*, *Pseudomonas*, *Rhizobium*, *Serratia*, *Stenotrophomonas*, *Streptomyces*, *Acetobacter*, *Burkholderia*, *Enterobacter*, *Gluconobacter*, and *Herbaspirillum* (Rodríguez and Fraga 1999, Holguin and Glick 2001, Vessey 2003, Richardson et al. 2009, Schlaeppi and Bulgarelli 2015). This phylogenetically diverse group of bacteria have an arsenal of plant growth-promotion strategies which can fall into three general categories: biofertilization, phytostimulation, and biocontrol (Bloemberg and Lugtenberg 2001).

Biofertilizing bacteria directly promote plant growth by increasing the availability and uptake of mineral nutrients (Vessey 2003). Most commonly, biofertilizing bacteria are selected based on their ability to increase nitrogen (N) and/or phosphorous (P) bioavailability. One of the best studied and understood mechanisms of increasing N is biological nitrogen fixation. In this case, bacteria use an enzyme called nitrogenase to convert, or fix, atmospheric N (N_2) into ammonium (NH_4^+), a form of N usable by plants (Dixon and Kahn 2004). In addition to increasing N availability, many biofertilizing plant growth-promoting bacteria are also able to influence the availability of P. This biofertilizing action has been demonstrated through a variety of mechanisms including the release of organic acids and the activity of different enzymes such as phytases and acid and alkaline phosphatases (Rodríguez and Fraga 1999).

Another common means of plant growth-promotion is phytostimulation. In this case plant growth-promoting bacteria affect plant physiology by either producing, releasing or altering the levels of phytohormones, such as auxins, cytokinins or giberellins, that promote plant growth (Bloemberg and Lugtenberg 2001). One commonly studied and well understood example of phytostimulation is the production of indole-3-acetic acid (IAA). Indole-3-acetic acid induced plant growth-promotion has been linked to the presence of tryptophan and other small molecules found in root exudates (Souza et al. 2015). When these precursors to IAA are detected by plant growth-promoting bacteria, they trigger the synthesis and release of bacterially produced IAA (Glick et al. 1998). Once released from the bacterium, both bacterial and plant produced IAA are taken up by the plant, which in turn leads to the stimulation of plant growth and/or elongation of roots. Another example of phytostimulation is the reduction of plant ethylene levels by the activity of bacterial enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase. This enzyme is produced by plant growth-promoting bacteria and works to lower levels of the phytohormone

ethylene which when present in high concentrations leads to decreased plant growth and productivity (Glick 2005). Also important in phytostimulation are the hormones jasmonic acid and salicylic acid. These phytohormones mediate defense signaling pathways and are key in controlling the growth of a wide range of plant pathogens (Kniskern et al. 2007). As such, these two phytohormones are also often studied in the context of biological control.

Biological control, or biocontrol, takes advantage of the ability of some bacteria to suppress and even prevent the establishment and proliferation of certain pathogens (Bulgarelli et al. 2013). In this case, plant growth-promoting bacteria are not directly providing plants with nutrients or hormones that will promote growth but rather mitigating the effects of plant pathogens. Like phytostimulation and biofertilization, there are several mechanisms of biocontrol. Some plant growth-promoting bacteria work as biological control agents by out-competing their pathogenic counterparts (Laslo et al. 2012). This phenomenon has been well documented in the case of iron (Fe), where plant growth-promoting bacteria use iron-chelating siderophores that bind to ferric iron (Fe^{3+}) so that it can be taken up and used by the cell thereby creating Fe deficient conditions for the pathogen (Kloepper et al. 1980, Lugtenberg and Kamilova 2009). Others work via antibiosis, and synthesize different antimicrobial compounds such phenazines, 2,4-DAPG, surfactants, and bacteriocins that pathogens may be susceptible to (Pérez-García et al. 2011, Bulgarelli et al. 2013). Others still are involved in the activation of two different plant defense responses known as induced systemic resistance and systemic acquired resistance which arise as the result of the production of the aforementioned jasmonic acid/ethylene and salicylic acid respectively (Pieterse and van Loon 1999). These two defense responses provide resistance to both above and belowground tissues of the plant, and are effective against a wide range of pathogens (van Loon et al. 1998).

Plant growth-promotion is suspected to occur as the result of a combination of these mechanisms. Though not all bacteria in the root microbiome possess the genetic potential for plant growth-promotion, many can be characterized by one or more plant growth-promotion associated phenotype. Thus, the plant growth-promotion potential of a microbial community is the sum of all plant growth-promoting activity. Because these phenotypes are generally associated with mitigating suboptimal growth conditions, the presence or absence of plant growth-promoting bacteria is most obvious when a plant is growing under stressed conditions (Glick 2010).

2.1.3 Endophytes

A unique subset of specialized bacteria found within the root microbiome and shown to have plant growth-promoting capabilities are the endophytes. Endophytes are defined as organisms that can be isolated from surface sterilized plant tissues which are able to colonize without damaging or prompting a strong defense response from the host plant (Hallmann et al. 1997) and include archaea, bacteria, and fungi (Reinhold-Hurek and Hurek 2011, Müller et al. 2015). Endophytes have been further defined as either obligate or facultative based on their dependence upon their plant host. Obligate endophytes spend the entirety of their lifecycle within the plant as they are unable to survive outside of their host (Hardoim et al. 2008). Alternatively, facultative endophytes have at least one stage in their life cycle where they exist outside of the host plant (Hardoim et al. 2008).

Obligate endophytes arise as a result of either vertical transmission (through the seed) (Shade et al. 2017) or via an insect vector (Hardoim et al. 2008). Facultative endophytes may arise in the same way as obligate endophytes. However, these organisms more often originate in the rhizosphere and enter the plant through cracks formed at lateral root junctions (Chi et al. 2005) or at root tips where new, undifferentiated tissues may be more vulnerable (Reinhold-Hurek and Hurek 1998). Though some of the colonization in the rhizosphere is passive, as occurs when entry is facilitated by root cracks, certain endophytes have adapted mechanisms to actively colonize the plant, such as the production of cell-wall degrading enzymes (Turner et al. 2013). It is important to note that though endophytes have been shown to colonize plants via entry in aboveground tissue, such as the stomata in leaves and wounds caused by bacterially produced enzymes, nematodes and other insects (Mercado-Blanco 2015), the majority of this colonization is recognized to occur in the rhizosphere (Hardoim et al. 2008, Reinhold-Hurek and Hurek 2011). Once they have entered the plant, endophytes are able to spread and colonize both above and belowground plant tissues (Guo et al. 2002).

Endophytes that successfully reach and attach to plant roots, outcompete other microorganisms at the interface, invade the plant, evade plant defenses, and establish themselves within the plant tissue are rewarded with several benefits. Within the plant endophytes enjoy both protection from soil conditions (pH, moisture content, and competition with other microorganisms) as well as an increased access to nutrients and water (Beattie 2007). However, because colonization requires the coordinated expression of several different genes, even once in the root interior not

many bacteria are able to successfully colonize and live as endophytes. The abundance and diversity of endophytes within the plant varies depending on a variety of factors such as plant age and genotype, host growth temperature and chemical changes, and competition with other endophytes (Beattie 2007, Turner et al. 2013). Unlike in the rhizosphere where cell densities can range from 10^8 to 10^{12} CFU·g^{-1} soil (Kennedy and de Luna 2005), in the endosphere cell densities rarely exceed 10^7 to 10^8 CFU·g^{-1} of root fresh $weight^{-1}$ (Hardoim et al. 2008, Turner et al. 2013). In addition to decreased abundance, endophyte diversity tends to be less than that observed among general rhizobacteria (Vandenkoornhuyse et al. 2015). This trend of decreased diversity and abundance continues moving up the plant and into the phyllosphere (Beattie 2007). In spite of their decreased abundance and diversity, endophytes are still key players in plant growth-promotion and may even provide more beneficial effects to their host than other rhizobacteria (Compant et al. 2010, Ma et al. 2011). Thus, identifying and studying plant growth-promoting endophytes is invaluable in the context of plant growth-promotion research.

2.1.4 Phytoremediation

As defined previously, phytoremediation is the use of green plants to remove or break down contaminants found in the soil, water, and air. It is generally considered to be preferable to alternative methods of clean up due to the fact that it is less expensive, less invasive, and offers several ecosystem services (Mahar et al. 2016). Depending on the plant, environment, and nature of the contaminant, there are different mechanisms for phytoremediation. Generally, these strategies fall into five different categories, each outlined briefly below:

- Phytoextraction: the translocation of pollutants to aboveground tissues via uptake through plant roots (Salt et al. 1998) most commonly used for the remediation of heavy metal contaminated sites (Ali et al. 2013).
- Phytostabilization: the immobilization of contaminants in the soil as a result of interactions with various enzymes and plant exudates (Behera 2014). Once immobilized, contaminants are no longer bioavailable and less likely to spread throughout the environment.
- Phytotransformation: the process by which various enzymes work to convert contaminants into more stable, less toxic forms (Behera 2014).

- Phytovolatilization: the conversion of toxic compounds to volatile forms that can then be released from either the plant or soil/water into the atmosphere (Salt et al. 1998).
- Rhizodegradation: the breakdown of organic pollutants in the rhizosphere that occur as a direct result of the stimulation of microbial communities by the plant (Behera 2014).

2.1.5 Plant Growth-Promoting Bacteria Assisted Phytoremediation

Though phytoremediation has been successfully implemented to clean up contaminated sites (Gerhardt et al. 2017), there are still several problems that have yet to be addressed. Among these is the low biomass and slow growth rate of many phytoremediating plants (Yavari et al. 2015, Mahar et al. 2016). Though some of these issues may be addressed by genetically modifying phytoremediating plants (Fasani et al. 2017), due to high cost and legislative constraints this application is limited (Beans 2017).

The importance of plant contributions in phytoremediation is clear. Still, it is important to keep in mind that plant-associated microbial communities are also highly relevant. As the relationships between different plants and plant growth-promoting bacteria become better understood, the opportunity for plant growth-promoting bacteria enhanced phytoremediation becomes more realistic. In fact, studies have already shown that some plant growth-promoting bacteria contribute to enhanced phytoremediation through the same plant growth-promotion strategies discussed previously. For example, a study by Dong et al. (2014) found that inoculating oat (*Avena sativa*) plants growing in petroleum contaminated soil with ACC deaminase producing plant growth-promoting bacteria *Serratia marcescens* and the fungi *Glomus intraradices* significantly increased both plant biomass and overall petroleum degradation compared to an untreated control. In another study by Islam et al. (2016), copper resistant *Providencia vermicola* possessing several plant growth-promoting traits (IAA, ACC deaminase, P solubilization and siderophore production) increased the growth, N and P uptake, and tolerance of lentil under copper concentrations ranging from 250 to 1000 ppm. Though these two examples show how partnering plant growth-promoting bacteria with phytoremediating plants can have positive effects, in this context an additional mechanism of plant growth-promotion is beginning to emerge: biodegradation.

As described previously, several bacteria within the rhizosphere possess biodegrading abilities (rhizodegradation). Studies have already shown that biodegrading bacteria can be isolated

from plants growing in contaminated environments (Daane et al. 2001, Siciliano et al. 2001, Phillips et al. 2008, Lumactud et al. 2016, Kovalski Mitter 2018), and as discussed previously there is strong evidence suggesting that plant growth-promoting bacteria can be used to enhance phytoremediation. Thus, by identifying biodegrading bacteria equipped with plant growth-promoting capabilities, the practical application of phytoremediation is further enhanced.

2.1.6 Bioprospection and Inoculants

Bioprospection is the search for valuable chemical products in natural biological systems (Millum 2010). In the past, this term has generally been used in the context of the pharmaceutical industry. However, the concept of bioprospection may not be limited to the realm of chemical products. As described, the root microbiome harbors several plant growth-promoting bacteria capable of promoting plant growth through a variety of biologically mediated processes. It is therefore reasonable to consider searching in subprime environments for bacteria capable of promoting plant growth as a type of bioprospection. In this case, however, the desired products are not valuable chemicals but rather enzymes produced by plant growth-promoting bacteria that enable processes which in turn promote plant growth. Because plant growth-promoting bacteria have the greatest effect in suboptimal environments where plants are under various types of stress, it could be hypothesized that the greatest diversity and abundance of plant growth-promoting bacteria would be found in plants growing within these environments. If this is the case, in order to isolate, identify, and study key plant growth-promoting bacteria which confer plant growth-promoting capabilities, the most interesting, and arguably valuable, environments will be those in which plants are experiencing a variety of different types of stress.

Endophytic plant growth-promoting bacteria are considered to be promising candidates for use as inoculants because of their ability to not only colonize plant roots but also to create an environment that is conducive for plant growth and function via the mechanisms of plant growth-promotion discussed previously (Souza et al. 2015). Additionally, endophytes tend to be less susceptible to inhibited growth and activity that can arise due to variations in soil conditions because they are housed within and protected by their plant host. To date, the use of rhizoplane and rhizosphere bacteria as inoculants comprise a serious area of study in the context of sustainable agricultural practices as a result of their potential to increase nutrient acquisition, growth, and yield (de Freitas et al. 1997, Vessey 2003, Cocking 2003, Richardson et al. 2009, Gaiero et al. 2013,

Chhabra et al. 2013, Farrar et al. 2014, Ji et al. 2014, Nosrati et al. 2014, Kandel et al. 2015, Schlaeppi and Bulgarelli 2015, Lowman et al. 2016). This is also true in terms of the use of bacterial inoculants to aid remediation and reclamation efforts (Phillips et al. 2006, 2008, Glick 2010, Bakker et al. 2012, Burns et al. 2013, Khan et al. 2013, Arslan et al. 2014). However, the use of endophytic plant growth-promoting bacteria has not been equally exploited in spite of their potential. There have been several studies looking at the different plant growth-promoting abilities and effects of endophytes on plant growth in a laboratory setting (Chen et al. 2010, Ji et al. 2014, Majeed et al. 2015, Oteino et al. 2015), but there are far fewer that actually look at plant-endophyte interactions based on variable, field realistic conditions (Hardoim et al. 2015). Thus, incorporating the concept of bioprospection and focusing on incorporating endophytes obtained from stressful environments into inoculant-based applications may identify bacteria that can be used to develop new plant growth-promoting inoculants.

2.2 Mechanisms of Plant Growth-Promotion

2.2.1 Biological Nitrogen Fixation: nifH and Nitrogenase

Nitrogen is required for the formation of several fundamental biological compounds such as amino acids, proteins, and nucleic acids, and is therefore essential for plant growth and function (Fisher and Newton 2002). On earth, N_2 accounts for over 70% of the earth's atmosphere. However in its atmospheric form, N_2 is highly stable and unusable by most biological organisms (Fisher and Newton 2002). In order to harness this massive pool of N for plant uptake, this N_2 must be fixed, or converted, to either ammonium (NH_4^+) or nitrate (NO_3^-) (Fisher and Newton 2002). As previously explained, biological nitrogen fixation is the conversion of N_2 gas to NH_4^+ by symbiotic, associative, and free-living bacteria. This process accounts for approximately 65% of global N fixation each year (Fisher and Newton 2002) and provides farmlands with about 40 Tg (23.5%) of biologically available N each year (Lowman et al. 2016). As such, this process is of significant importance to not only the environment but global agriculture as well.

The reduction of N_2 gas to NH_4^+ by microorganisms is catalyzed by the enzyme nitrogenase. This enzyme consists of two metalloproteins, the FeMo-protein and the Fe-protein (Souza et al. 2015), both of which are required for nitrogenase activity (Fisher and Newton 2002). The smaller Fe-protein component works as an ATP-dependent electron donor to the larger

molybdenum-iron (FeMo) component which contains the catalytic site (Benton et al. 2002, Dixon and Kahn 2004). Although all diazotrophic organisms contain an FeMo-protein, some are capable of synthesizing alternative nitrogenases under conditions where molybdenum is limited (Dixon and Kahn 2004). However, the majority of biochemical and biophysical studies focus on FeMo-containing nitrogenases (Benton et al. 2002).

The *nifH* gene encodes the nitrogenase reductase subunit which is an essential component in the reduction of N_2 gas as well as the formation of the Fe-Mo complex (Rubio and Ludden 2002). It is one of the most broadly sequenced nitrogenase genes, and has become a marker for studying the phylogeny, diversity, and abundance of nitrogenase-containing organisms (Gaby and Buckley 2012). Considered from a bioprospection perspective, nitrogenase is a valuable biological product. The requirement of *nifH* for the assembly and function of nitrogenase and the already existing knowledge gained about this gene and its products make it a strong candidate to be used in screening techniques for biological nitrogen fixation.

2.2.2 Phosphorous Solubilization: phoD and Alkaline Phosphatases

Like nitrogen, phosphorous is a critical element in biological organisms as it is contained in various biomolecules such as nucleic acids, phospholipids, lipopolysaccharides, and some proteins (Vershinina and Znamenskaya 2002). Unlike N, P is not interchanged between the soil and the atmosphere and as such it cannot be "fixed" from an atmospheric source (Rodríguez and Fraga 1999). Therefore, if there are insufficient P levels in the soil, plant growth will be severely limited. Luckily, some soils have been shown to contain a large pool of P in several organic and inorganic forms (Fraser et al. 2015). However, due to their insolubility, some of these phosphate compounds are not readily used by plants and microorganisms. In fact, only 0.1% of total soil P exists in a soluble form (Sharma et al. 2013).

As mentioned previously, specialized biofertilizing plant growth-promoting bacteria have evolved different mechanisms to aid in the mineralization and solubilization of inorganic and organic soil phosphorous (Rodríguez et al. 2007). In this case they convert insoluble organic and mineral phosphates into the biologically available form orthophosphate ($H_2PO_4^-$) (Fraser et al. 2015). The mechanism by which microorganisms convert unavailable soil P to $H_2PO_4^-$ depends on the type of soil P. For example, the solubilization of inorganic P, usually present as iron, aluminum, and calcium phosphate, is thought to occur through the production and release of organic acids

(Gupta et al. 1994, Rodríguez and Fraga 1999, Tkacz and Poole 2015, Pii et al. 2015). In contrast, organic P is made available through mineralization processes catalyzed by various different enzymes (Vershinina and Znamenskaya 2002, Sharma et al. 2013).

One relevant enzyme involved in the mineralization of organic phosphorous is alkaline phosphatase. In the soil, organic P can account for 30–50% of the total phosphorous (Rodríguez and Fraga 1999). Within this fraction phosphatases have the potential to hydrolyze up to 89% of that stored P (Sims et al. 2005, Ragot et al. 2016). Along with *phoA* and *phoX*, *phoD* has been identified to have a role in the coding of alkaline phosphatases (Kageyama et al. 2011, Fraser et al. 2015, Ragot et al. 2015, 2016). The gene *phoD* is found in both terrestrial and aquatic environments (Luo et al. 2009, Kageyama et al. 2011, Tan et al. 2013, Ragot et al. 2016) and is mainly produced by bacteria (Ragot et al. 2015). It is expressed in response to phosphorous starvation and among the phosphate starvation inducible genes contained within the phosphate starvation regulon (Vershinina and Znamenskaya 2002).

Among soil bacteria, *phoD* was the most frequently identified alkaline phosphatase gene in metagenomics datasets (Tan et al. 2013). The PhoD alkaline phosphatases possess both phosphomonoesterase and phosphodiesterase activities (Vershinina and Znamenskaya 2002), and catalyze the hydrolysis of ester-phosphate bonds. Thus, the PhoD alkaline phosphatases are a key player in the conversion of organic P to $H_2PO_4^-$ (Ragot et al. 2015). The abundance of *phoD* and importance of the PhoD alkaline phosphatase in the mineralization of soil organic P make it of particular interest for bioprospection.

2.2.3 Reduction of Plant Ethylene: acdS and ACC Deaminase

The production of ACC deaminase by plant growth-promoting bacteria has been shown to increase plant growth via phytostimulation (Glick et al. 1998, 2007, Penrose and Glick 2003, Glick 2005, Nikolic et al. 2011, Gaiero et al. 2013). All higher plants produce ethylene (Abeles et al. 1992). In plants, ethylene is considered to be a hormone (Abeles et al. 1992), and it plays a key role in several developmental processes as well as plant responses to stress (Burg 1973, Abeles et al. 1992). Although ethylene is an important plant hormone, if present in high levels it can be detrimental to plant growth. Some negative effects of elevated ethylene levels are the inhibition of cell division and DNA synthesis (senescence), inhibition of root/shoot growth, chlorosis, and leaf abscission (Burg 1973, Glick 2014).

The amount of ethylene produced by a plant at a given time is determined by a wide variety of factors such as plant life cycle, temperature, light, available nutrients, and the presence of various types of stress (Abeles et al. 1992). The production of "stress ethylene" is a normal plant response to the presence of metals, organic and inorganic chemicals, temperature extremes, flooding, drought, ultraviolet light, insect and nematode damage, pathogens, and mechanical wounding (Abeles et al. 1992). When stressed, plants will use the precursor to ethylene, 1-aminocyclopropane-1-carboxylate (ACC), present in the cell to produce a small amount of the hormone (Glick 2014). It is believed that this initial increase of ethylene and depletion of ACC initiates the production of plant defense/protection proteins (Glick 2014). However, as a part of the stress response additional ACC is produced causing the subsequent production of ethylene to be high enough to be detrimental to plant health and growth (Glick 2014). It has been proposed that any means of reducing the magnitude of the second peak of stress ethylene has the potential to minimize the damage caused to the plant by increased ethylene levels.

The enzyme ACC deaminase is encoded by the structural gene *acdS* (Holguin and Glick 2001), catalyzes the conversion of ACC to α-ketobutyrate and ammonia (NH_3) (Glick et al. 1998), and is relatively common among soil microorganisms (Blaha et al. 2006). More specifically, ACC deaminase is a sulfhydryl enzyme found in the cytoplasm of ACC deaminase producing microorganisms (Glick 2014), many of which have been shown to have plant growth-promoting capabilities (Wang et al. 2000, Penrose and Glick 2003, Glick 2005, 2010, 2014, Blaha et al. 2006, Nikolic et al. 2011, Khan et al. 2013, Souza et al. 2015). Due to the fact that ACC deaminase is not secreted but rather located within the cytoplasm, it has been proposed that endophytes can act as a sink for excess ACC (Glick 2005). In this mechanism, bacteria indirectly lower plant ethylene levels by taking up ACC exuded by plant tissues and convert it into forms of usable energy (Glick 2005).

By surveying bacterial populations for the *acdS* gene and ACC deaminase activity it is possible to identify bacteria that may have the potential to promote plant growth through the reduction of plant ACC (Wang et al. 2015) .

2.2.4 Hydrocarbon Degradation: alkB, CYP153 and Hydrogenases

In addition to their ability to contribute to plant growth-promotion through nutrient acquisition and regulation of plant hormones, endophytes can degrade toxic compounds such as

hydrocarbons (Siciliano et al. 2001, Phillips et al. 2008, Kukla et al. 2014, Lumactud et al. 2016). A key study by Siciliano et al. (2001) showed that plants have the ability to select for endophytes based on their ability to degrade pollutants present in the plant environment. In their study, *alkB*, an alkane monooxygenase, was one of four different genes used to assess the abundance of pollutant-degrading endophytes. This gene is found within the alkB gene cluster (Cappelletti et al. 2011) and encodes a membrane-bound alkane hydroxylase that requires two electron transfer proteins, rubredoxin (*alkG*) and rubredoxin reductase (*alkT*), to degrade medium (C_5) to long (C_{16}) chain alkanes (Van Beilen and Funhoff 2007, Cappelletti et al. 2011, Liang et al. 2016). This is one of several enzymes that catalyzes the terminal hydroxylation of alkanes to 1-alkanols, and enables microorganisms to break down and use alkanes as a sole carbon and energy source (Funhoff et al. 2006). The gene *alkB* is well conserved (Smith et al. 2013), and as such has been used in a wide variety of studies looking at the distribution, abundance, and diversity of hydrocarbon degraders in many different environments (Phillips et al. 2006, 2008, Koch et al. 2009, Andria et al. 2009, Yousaf et al. 2010, Smith et al. 2013, Kukla et al. 2014, Lumactud et al. 2016).

It has been noted that many alkane-degrading bacteria are able to grow on a variety of alkanes (van Beilen et al. 2006, Liang et al. 2016). This is most likely due to the fact that these bacteria tend to have genes that encode several different alkane hydroxylases with overlapping substrate ranges (Van Beilen and Funhoff 2007). In addition to AlkB, one of the commonly studied alkane hydroxylases is the heme-containing cytochrome P450 alkane hydroxylase of the CYP153 family (van Beilen et al. 2006). This enzyme is a soluble monooxygenase that transfers electrons from NAD(P)H to the cytrochrome using ferredoxin (*fdx*) and ferredoxin reductase (*fdxr*) (Nie et al. 2014, Liang et al. 2016) to catalyze that first critical step in the degradation of *n*-alkanes (Koch et al. 2009). Unlike AlkB which is able to degrade longer chain alkanes, CYP153 works best on alkanes containing less than 14 carbons (Nie et al. 2014). Together, AlkB and CYP153 are the main enxymes involved in medium chain alkane hydroxylation by cultivated bacteria (van Beilen et al. 2006).

3. AN ASSESSMENT OF BACTERIAL ENDOPHYTES ISOLATED FROM STRESSED ENVIRONMENTS FOR POTENTIAL USE AS PLANT GROWTH-PROMOTING, STRESS-REDUCING, AND HYDROCARBON-DEGRADING INOCULANTS

3.1 Preface

Studies have shown that plant growth-promoting endophytes can be used to increase nutrient acquisition, plant biomass and yield, as well as enhance the process of phytoremediation in hydrocarbon-contaminated soils. In most cases, these endophytes are obtained from environmental samples. However, due to the cost and time associated with testing these bacteria *in vivo*, it is unrealistic to blindly test every isolate cultured. As such, isolates must first be screened for indications of plant growth-promotion potential. In this study isolates collected and identified as a part of previously conducted studies were screened for plant growth-promoting genotypes and phenotypes in order to identify endophytes with high potential for plant growth-promotion. The results of the screening conducted herein will then be used to select candidates for further study *in vivo*.

3.2 Abstract

Plant growth-promoting bacteria have been identified across a variety of genera and have been shown to stimulate plant growth via the mechanisms of biofertilization (increase in available nutrients), biological control (prevention of disease), phytostimulation (alteration of plant hormones), and biodegradation (removal of toxic compounds). Though plant growth-promoting bacteria have been successfully used in a variety of applications, there is still a need to identify more, better plant growth-promoting bacteria among the wide diversity of bacteria naturally found in the environment. The goal of this study was to use an approach of genotype and phenotype screening to identify isolates with increased plant growth-promotion potential within two larger collections of environmental isolates. This was accomplished by first identifying genera common

to both a long-term nutrient deficient agricultural site and a hydrocarbon-contaminated site. To estimate plant growth-promotion potential, PCR was used to screen common isolates for plant growth-promotion associated genotypes (*nifH, phoD, acds, alkB, CYP153*) and *in vitro* biochemical assays were employed to test for related phenotypes (nitrogen fixation, alkaline phosphatase activity, ACC deaminase activity, and hydrocarbon degradation). Results of this study identified plant growth-promoting phenotypes and genotypes in bacteria isolated from both sites, with *alkB, nifH,* and *phoD* genotypes and hydrocarbon-degrading and alkaline phosphatase phenotypes being most common. Further, results indicated that mechanisms of plant growth-promotion, alternative to those studied here, may be responsible for observed phenotypes as several isolates positive for plant growth-promoting phenotypes were not always positive for selected genotypes. Nevertheless, results demonstrated that the combined approach of genotype and phenotype screening is a viable option for identifying isolates with increased plant growth-promotion potential among larger collections of environmental isolates. Further, several isolates were shown to have plant growth-promotion potential based on these results suggesting they are strong candidates for further testing.

3.3 Introduction

Plant growth-promoting bacteria are a specialized group of plant-associated microorganisms that are able to stimulate plant growth and development. Many plant growth-promoting bacteria identified to date have been isolated from the root microbiome, which consists of the microbiota inhabiting the area around plant roots (rhizosphere), on plant roots (rhizoplane), and within plant roots (endosphere) (Lareen et al. 2016). Endophytes are a specialized subset of plant growth-promoting bacteria found within the endosphere and are of particular interest not only for commercial application, but also for further understanding the role of the plant microbiome. These unique bacteria are able to colonize the internal tissues of the plant without causing damage or inducing a strong defense response from their host (Hallmann et al. 1997). Endophytes can be acquired vertically from the plant seed (Shade et al. 2017) or horizontally from the surrounding environment (Hardoim et al. 2008). Regardless of where they originate, once endophytes have colonized the internal tissues of the plant they receive protection, provided by the plant, as well as an increased access to essential resources such as nutrients and water (Beattie

2007). In exchange, endophytes stimulate plant growth and development via mechanisms such as biofertilization, phytostimulation, and biodegradation (Souza et al. 2015).

Biofertilizing bacteria promote plant growth by increasing the acquisition of essential nutrients, mainly nitrogen (N) and phosphorus (P), from the environment (Vessey 2003). One mechanism of biofertilization that is commonly studied and employed is biological nitrogen fixation. Here, specialized bacteria that possess the enzyme nitrogenase can convert the biologically unavailable atmospheric N (N_2) to the biologically available ammonium (NH_4^+) (Fisher and Newton 2002). Several different bacterial genera have been demonstrated to be involved in biological nitrogen fixation and include *Azoarcus* (Reinhold-Hurek and Hurek 1997), *Pantoea* (Feng et al. 2006), *Rhizobia* (O'Gara and Shanmugam 1976), *Azospirillum* (Boddey et al. 1986), *Herbaspirillum* (Elbeltagy et al. 2001), *Burkholderia* (Estrada-De Los Santos et al. 2001), *Enterobacter* (Neilson and Sparell 1976), and more (Franche et al. 2009, Muangthong et al. 2015).

Additional mechanisms of biofertilization involve liberating P that has been either fixed in the soil or stored in organic material. Studies have shown that there are several ways biofertilizing bacteria accomplish this, and the degree of activity will vary depending on the form of P and the conditions of the surrounding environment (Rodríguez and Fraga 1999). For example, in the case of inorganic phosphate, bacteria produce and release a suite of different organic acids (Chhabra et al. 2013). Alternatively, when the P is bound in organic material, alkaline and acid phosphatases hydrolyze bonds within organic compounds under different pH conditions (Nannipieri et al. 2011). Though the mechanisms of P liberation may vary, they generally involve a biologically unavailable form of P that is converted to the plant available orthophosphate ($H_2PO_4^-$, Pi) (Rodríguez and Fraga 1999).

Another commonly exploited method of plant growth-promotion is phytostimulation. Here, bacteria influence plant growth by altering plant hormone levels. In some cases, such as the production of indole-3-acetic acid (IAA), this involves the production and subsequent uptake of bacterially produced plant hormones directly involved in regulating plant growth (Glick et al. 1998). In others, such as the activity of 1-aminocyclopropane-1-carboxylate (ACC) deaminase, growth-promotion occurs via the reduction of plant hormones, such as ethylene, which when present in high levels can inhibit plant growth (Glick 2005). As ethylene is produced in response to various stressors such as drought, flooding, temperature extremes, high salt, metal and organic contaminants, insect and nematode predation, and both fungal and bacterial phytopathogens

(Olanrewaju et al. 2017), the use of ACC deaminase producing plant growth-promoting bacteria to increase stress tolerance in such environments has been well studied and documented in the literature (for review see Glick 2010, 2015).

A more indirect way endophytic bacteria may promote plant growth is via the process of biodegradation. Through this mechanism, bacteria are able to either eliminate, transform, or reduce toxic contaminants in the environment. These toxins can be present in a wide variety of forms including but not limited to heavy metals, hydrocarbons, explosives, volatile organic compounds, pesticides and more (McGuinness and Dowling 2009). Similar to the way ACC deaminase promotes plant growth by reducing ethylene levels, biodegradation stimulates plant growth by removing, reducing, or altering compounds that may inhibit plant development. In fact, studies have shown that plants not only select for biodegrading partners in contaminated environments (Siciliano et al. 2001), but that these plant-microbe interactions are vital in ensuring plant survival and success (Phillips et al. 2006, 2008, Mezzari et al. 2011, Khan et al. 2013, Fernet et al. 2016).

Successfully selecting and identifying plant growth-promoting bacteria amidst environmental isolates remains one the greatest challenges in plant growth-promoting bacteria research. Though there are several ways to achieve this, the methods to do so are often time consuming and inefficient. Therefore, there is a need to improve selection and screening methods in order to better detect plant growth-promoting bacteria. Considering that a) many mechanisms of plant growth-promotion aim to mitigate harmful effects on the plant and b) the level of intimacy in the plant-endopyte relationship enables endophytes to more strongly influence growth (Compant et al. 2010, Ma et al. 2011), it would be reasonable to suggest that screening endophytes common to environments where these conditions exist may identify plant growth-promoting bacteria.

The objective of this study was to screen root endophytes isolated from plants native to a hydrocarbon-contaminated site and wheat plants from long-term nutrient deficient agricultural site for plant growth-promoting genotypes and associated phenotypes selected based on their role in mitigating different types of stress encountered at both sites. To accomplish this, a study wherein it was hypothesized that root-associated bacteria isolated from different plants growing under stress will contain specific genes (*CYP153, alkB, phoD, nifH, acsds*) that encode proteins (hydrogenase, alkaline phosphatase, nitrogenase, ACC deaminase) associated with plant growth-promotion was conducted. In doing so, isolates with increased plant growth-promoting potential were identified making them strong candidates for additional *in vivo* studies.

3.4 Materials and Methods

3.4.1 Origin and Maintenance of Bacterial Cultures

All bacteria used for this work were derived from freezer stocks of cells collected by and maintained in the Soil Microbiology Laboratory at the University of Saskatchewan. More specifically, isolates consisted of endophytes previously isolated the roots of plants growing at two different field sites. The first site, Rotation ABC, is the location of a long-term crop fertility experiment that began in 1911 at the Agriculture and Agri-Food Canada (AAFC) Lethbridge Research and Development Center in Alberta. Wheat plants used in this study had been grown under a continuous wheat rotation as a part of a study initiated 1911 with the intention of evaluating the long-term impacts of various cropping systems on soil quality, productivity, and the broader environment (Smith et al. 2012). In 1972, four different fertilizer treatments were incorporated: no fertilizer (Control), P fertilizer only (P_{Fert}), N fertilizer only (N_{Fert}), and application of both N and P fertilizer (NP_{Fert}) (Smith et al. 2012). Isolates used in this study were originally obtained from roots collected in 2014 by members of the Soil Microbiology lab at the University of Saskatchewan as well as AAFC associates. After their collection roots were stored at –80 °C until processing.

Prior to the start of this work, culture-based techniques were used to isolate endophytes from samples. After collection, roots were washed in 195 mL phosphate-buffered saline (PBS) buffer (1.2 g of $Na_2HPO_4 \cdot L^{-1}$, 0.18 g of $NaH_2PO_4 \cdot L^{-1}$, 8.5 g of $NaCl \cdot L^{-1}$). Roots were then surface sterilized by shaking in 100 mL NaClO (1.05% v:v) on a rotary shaker (150 rpm) for 15 minutes (min). To remove any residual NaClO roots were washed ten times in sterile tap water. Sterility was confirmed by spreading 0.1 mL of the final wash onto $^1/_{10}$ strength tryptic soy agar (TSA) plates (per L: 3.0 g Bacto™ Tryptic Soy Broth mixed with 1.5% agar; TSB, BD Biosciences, NJ USA) and incubating for 3 days at 28°C. Sterile roots were then aseptically cut into smaller subsamples using a sterile razor blade. From this, 2.5 g of roots were macerated in 9 mL of sterile PBS using a sterile mortar and pestle. The buffer solution was then serially diluted onto $^1/_{10}$ TSA plates prepared with 0.1 $g \cdot L^{-1}$ of cycloheximide and incubated for 3 days at 28°C. Endophytic bacteria were then selected and streaked for isolation onto new $^1/_{10}$ TSA plates. Purified isolates were grown in 9 mL ½ strength tryptic soy broth (TSB) (per L: 15 g Bacto™ Tryptic Soy Broth).

Freezer stocks were prepared by mixing purified culture with 50% sterile glycerol and storing at –80°C. After DNA extraction, isolates were identified using 16S rRNA sequencing.

The second site, Bitumount Provincial Historic Site (BPHS), is the location of one of the world's first oil extraction and refining enterprises (Alberta Culture and Tourism), and is characterized by extensive natural and anthropogenic hydrocarbon contamination (Yergeau et al. 2012). In spite of government regulations, the land at BPHS has never been reclaimed and high hydrocarbon levels still persist. In spite of this contamination some plants have managed to recolonize the area (Blain et al. 2017). In 2016, Blain (2016) collected samples of six different native plants types from various locations at the site. Later, using culture-based techniques (described above), Blain (2016) isolated endophytes from these plant roots and identified them using 16S rRNA sequencing.

Working stocks of bacterial cells used in this study were obtained from previously prepared freezer stocks (–80°C; see above) and streaked onto $^{1}/_{10}$ TSA plates. Working stock plates were then incubated at 28°C for three days before being stored in the fridge (4°C). Fresh cells were streaked onto new plates every four weeks in order to keep fresh working stocks as needed.

3.4.2 Isolate Selection

Isolates previously isolated from plants sampled from either BPHS or Rotation ABC and identified using 16S rRNA sequencing were compiled into two lists (Appendix A). Isolates common to both lists at the genus level were identified and selected for further study. One isolate from each species was included in this study. Additionally, isolates from Rotation ABC that were present in the control (Ctrl), nitrogen (N_{fert}), or phosphorous (P_{fert}) treatments were included as those three locations present increased nutrient stress.

3.4.3 Gene Survey

3.4.3.1 DNA Extraction

Bacterial DNA was extracted using the Ultraclean Microbial DNA Isolation Kit (MoBio, USA) according to manufacturer instructions. Cells were grown for 24 hours (h) in $^{1}/_{2}$ strength TSB, transferred to a sterile microfuge tube, collected via centrifugation (10,000 x g for 30 seconds [sec]), resuspended in 300 µL of a MicroBead solution, and transferred to a MicroBead tube. Next,

50 µL of solution MD1 (containing SDS necessary for lysis) was added and samples heated at 65°C for 10 min (to aid in cell lysis). Cells were then horizontally vortexed at maximum speed for 10 min. Samples were centrifuged (10,000 x g for 30 sec) and the supernatant transferred to a new clean 2 mL collection tube. After adding 100 µL solution MD2 to supernatant and inverting gently to mix, samples were left to incubate at 4°C for 5 min. Samples were then centrifuged (10,000 x g for 1 min) and the supernatant transferred to another new, clean 2 mL collection tube. Solution MD3 (900 µL, salt solution to bind DNA to filter) was added to the sample. After vortexing, the solution was transferred to a spin filter, centrifuged (10,000 x g for 30 sec), and the flow through discarded. This was done several times until all of the solution had been passed through the filter. Samples were then washed by adding 300 µL solution MD4 (contains ethanol), centrifuging (10,000 x g for 30 sec), discarding flow through, then centrifuging again to remove any residual wash. Finally, the spin filter containing sample DNA was transferred to a new 2 mL collection tube and 50 µL solution MD5 (elution buffer) added directly to the filter. After allowing to incubate at room temperature for 1 min, the sample was centrifuged (10,000 x g for 30 sec) and DNA collected. After extraction, DNA concentrations were determined using a Nanodrop 2000 spectrophotometer (ThermoScientific). Working stocks of DNA were prepared using DNAse and RNAse free water to obtain final concentrations of 2 and 15 $ng \cdot \mu L^{-1}$. All diluted DNA working stock samples were temporarily stored at –20°C (according to MoBio recommendation) and original freezer stocks were stored at –80°C.

3.4.3.2 Polymerase Chain Reaction

Polymerase chain reaction (PCR) was used to 1) confirm the identify of isolates by amplifying the 16S rRNA gene (Fierer et al. 2005) and 2) screen isolates for five different functional genes: *nifH*, *phoD*, *alkB*, *CYP153*, and *acds*. For all reactions, the HotStarTaq Master Mix Kit (Qiagen) was added to equal half of the final concentration of the reaction (e.g. 10 µL of master mix in a 20 µL reaction). This resulted in a final concentration of 2.5 units HotStar Taq polymerase, 1x PCR buffer, 200 µM of each dNTP, 1.5 mM $MgCl_2$ for each reaction unless otherwise specified (Table 3.1). The final concentration of primers varied depending on which

Table 3.1: Primers and conditions used to screen isolates for functional genes.

Gene (amplicon size)	Forward Primer: (5'-3')	Reverse Primer: (5'-3')	Primers and Additives (µL)	Reaction Conditions	DNA (ng) per Reaction	Reaction Volume (µL)	Reference
16S rRNA (200 bp)	EUB 338: ACTCCTACGGGA GGCAGCAG	EUB 518: ATTACCGCGGCTGC TGG	Primers: 1.0; BSA 0.25	15 min at 95°C; 30 cycles of: 1 min at 95°C, 45 sec at 60°C , 1 min at 72°C; 10 min at 72°C	45	20	Fierer, 2005
phoD (380 bp)	phoD-F733: TGGGAYGATCAYGA RGT	phoD-R1083: CTGSGCSAKSACRTT CCA	Primers: 0.5 ; BSA: 0.25; MgCl2: 0.4	15 min at 95°C; 35 cycles of: 30 sec at 95°C, 30 sec at 58°C , 1 min at 72°C; 10 min at 72°C	2	20	Ragot, 2015
acds (750 bp)	DegACCf: GGBGGV AAYAARMYVMGSA AGCTYGA	DegACCr: TTDCCHKYRTANACB GGRTC	Primers: 0.8; BSA: 0.25 MgCl2: 0.4; DMSO: 1.00	15 min at 95°C; 35 cycles of: 30 sec at 95°C, 1 min at 46°C , 60 sec at 72°C; 10 min at 72°C	45	20	Nikolic, 2011
nifH (1st Round: 360 bp; 2nd Round: 314-317 bp)	PolF: TGCGAYCCSAARGC BGACTC; nifHFor: ACCCGCCTGATCCTG CACGCCAAGG	PolR: ATSGCCATCATYTCR CCGGA; nifHRev: ACGATGTAGATTTC CTGGGCCTTGTT	Primers: 0.75; BSA: 0.25; MgCl2: 0.5;	15 min at 95°C; 30 cycles of: 45 sec at 95°C, 45 sec at 55°C , 45 sec at 72°C; 10 min at 72°C	30	25	de Jesus Santos, 2014
CYP153 (320 bp)	P450fw1: GTSGGCGGCAACGA CACSAC	P450rv3: GCASCGGTGGATGC CGAAGCCRAA	Primers: 1.0; BSA: 0.625	15 min at 95°C; 25 cycles of: 45 sec at 95°C, 1 min at 58°C , 1 min at 72°C; 10 min at 72°C	45	20	Van Beilen et al., 2006
alkB (550 bp)	alkB-1f: AAYACIGCICAYGAR CTIGGICAYAA	alkB-1r: GCRTGRTGRTCIGA RTGICGYTG	Primers: 0.4; BSA: 0.25; MgCl2: 2.0	15 min at 95°C; 35 cycles of: 45 sec at 95°C, 1 min at 57°C , 45 sec at 72°C; 10 min at 72°C	45	20	Wallisch, 2014

primer pair was being used as specified in Table 3.1. Primers were always added from 25 µM working stocks except in the cases of *nifH* and *alkB* where 10 µM working stocks were used. In some cases, additives such as bovine serum albumin (BSA) (Ambion), PCR-grade dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, USA) at > 99.5% purity or additional $MgCl_2$ were used to increase PCR success and efficiency (Table 3.1). Working stocks of BSA and $MgCl_2$ were prepared using DNAse and RNAse free water at concentrations of 10 $mg \cdot mL^{-1}$ and 25 µM respectively and added to each reaction as specified in Table 3.1. In order to activate the HotStar Taq polymerase, all PCR cycles began with an initial heat activation step at 95°C for 15 min. Thermocycler conditions, amount of DNA used, final reaction volumes and amplicon sizes were dependent on the gene target and are specified in Table 3.1. For all reactions at least one negative control was included where water was added in place of template DNA. Positive controls for each reaction consisted of purified amplicons confirmed to be the gene target via sanger sequencing with the forward primer used for PCR (Macrogen Inc., Korea). In the case of 16S rRNA amplicons, all samples were sent for sequencing (Appendix A). In the event of non-specific binding, bands occurring in the target size range were excised using a sterile razor blade and sent for sequencing (Macrogen Inc., Korea). Sequencing results were then compared with the GenBank database on the National Center for Biotechnology Information website's Basic Local Alignment Search Tool, specifically blastn. Further, any sample that tested positive for a specific gene was tested three times to confirm consistent amplification.

3.4.4 Potential Plant Growth-Promoting Activity Assays

3.4.4.1 Alkaline Phosphatase Assay

Isolates were assayed for alkaline phosphatase activity as described by Malboobi et al. (2009). Isolates were streaked from working stocks onto Sperber media (per L: 10 g glucose, 0.5 g yeast extract, 0.1 g $CaCl_2$, 0.25 g $MgSO_4$, $7H_2O$, supplemented with 2.5 g of soluble KH_2PO_4, 1.5% agar, and pH adjusted to 7.2) plates containing 5-bromo-4-choloro-3-indolyl phosphate (BCIP) (50 $mg \cdot L^{-1}$) (Sigma-Aldrich, St. Louis, USA) and incubated in the dark at 28°C for one week. Following incubation, alkaline phosphatase activity was qualitatively determined by monitoring bacterial colonies for blue coloration indicating a positive result.

3.4.4.2 ACC Deaminase Assay

A method first described by Penrose and Glick (2003) was used to both promote and measure 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity. Isolates were grown in half strength TSB and then transferred to DF salts minimal medium (Dworkin and Foster, 1958). The DF salts minimal media was prepared in several steps. First a trace elements solution was prepared (per 100 mL distilled water: 10 mg H_3BO_3, 11.19 mg $MnSO_4 \cdot H_2O$, 124.6 mg $ZnSO_4 \cdot 7H_2O$, 78.22 mg $CuSO_4 \cdot 5H_2O$, and 10mg MoO_3, pH 7.2) and filter sterilized. Then 100 mg $FeSO_4 \cdot 7H_2O$ was dissolved in 10 mL distilled water and filter sterilized. Both the trace elements and $FeSO_4 \cdot 7H_2O$ solutions were stored at 4°C. In addition, a 0.5 M stock solution of ACC was prepared by dissolving 0.506 g ACC (Sigma-Aldrich, St. Louis, USA) in 10 mL distilled water and sterilized with a 0.2 µm filter. Finally, the DF media was prepared (per L of distilled water: 4.0 g KH_2PO_4, 6.0 g Na_2HPO_4, 0.2 g $MgSO_4 \cdot 7H2O$, 2.0 g glucose, 2.0 g gluconic acid, 2.0 g citric acid, and 2.0 g $(NH_4)_2SO_4$) and autoclaved (20 min sterilization). After autoclaving and cooling DF salts minimal media, 100 µL of the sterilized trace elements and $FeSO_4 \cdot 7H_2O$ solutions were added. In some cases, DF salts minimal media was prepared and autoclaved without $(NH_4)_2SO_4$ and after cooling sterile ACC (final concentration 3.0 mM) was added in its place.

Initially cells were transferred (1:50 dilution) from TSB to DF salts minimal medium containing 2.0 g $(NH_4)_2SO_4$ as the sole N source. Cells were incubated for 24 h in a shaking water bath at 200 rpm, 28°C. Following this growth period, cells were transferred to DF salts minimal media (1:50 dilution) containing ACC in place of $(NH_4)_2SO_4$. Following inoculation, cells were incubated in DF salts minimal media (containing ACC) in a shaking water bath at 200 rpm, 28°C for 1 h. During incubation, DF salts agar plates (DF salts minimal media without $(NH_4)_2SO_4$ as described above but autoclaved with 1.8% Bacto-Agar, known to have a very low N content) were spread with ACC (30 µmol•plate^{-1}; 0.250 mL of a 0.12 M ACC solution) and allowed to dry. After incubation in ACC supplemented media, 20 µL each culture was spread onto DF salts minimal media plates supplemented with ACC. Plates were then incubated at 28°C for one week. Plates showing bacterial growth after incubation were considered to be positive for ACC deaminase activity.

3.4.4.3 Acetylene Reduction Assay

Nitrogen-fixing potential was assessed via the acetylene reduction assay (ARA). The ARA was conducted using a slightly modified version of the assay as described by Lifshitz et al. (1986). Cultures (5 mL) of each isolate were grown overnight in half-strength TSB (in 20 mL flasks) at 120 rpm, 28°C. The following day, the OD of cells was checked on an Evolution 60S UV-Visible Spectrophotometer (Thermo Fisher Scientific) and adjusted to obtain a cell density of 10^9 colony forming units (CFU) per mL (OD_{600}=1). Cells were harvested and washed twice in 0.1 M $MgSO_4$ buffer and then resuspended in 0.1 M $MgSO_4$. Next, in triplicate, 100 µL of bacterial suspensions were added to 20 mL culture tubes containing 5 mL semisolid combined carbon media (CCM) (Rennie 1987). As a negative control, 100 µL of 0.1 M $MgSO_4$ was added CCM tubes and treated as described. Semisolid CCM was prepared as follows: solutions A and B autoclaved separately and mixed after cooling. Solution A: to 900 mL distilled water add 0.8 g of K_2HPO_4; 0.2g of KH_2PO_4; 0.1 g of NaCl; 0.028 g of $Na_2FeEDTA$; 0.025 g of $Na_2MoO_4 \cdot 2H_2O$; 0.100 g of yeast extract (Difco); 5.0 g of mannitol; 5.0 g of sucrose; 0.5 mL of 60% (vol/vol) sodium lactate; 2.0g agar; final pH of solution A adjusted to 7.0. Solution B: to 100 mL of distilled water add 0.2 g of $MgSO_4 \cdot 7H_2O$; 0.06 g of $CaCl_2 \cdot 2H_2O$. After mixing solutions A and B, filter-sterilized biotin and para-aminobenzoic acid (100 µL each) at final concentrations of 5 and 10 $\mu g \cdot L^{-1}$, respectively were added. Cultures were left to incubate in the dark at 28°C for five days. After incubation, tubes were sealed with sterilized rubber septa. Then, with a 10 mL syringe and 25-gauge needle, the septa was punctured and 10% of the tube atmosphere was removed and replaced with acetylene gas. Tubes were then left to incubate in the dark at 28°C for an additional 24 h. After incubation, ethylene concentrations were measured by removing 0.25 mL (1 mL syringe) of the tube atmosphere and running on a 5890A gas chromatograph (Hewlett-Packard) equipped with a six-foot Porapak R 80–100 mesh column. Carrier gas was hydrogen at 17 psi, oven temperature set to 80°C, and injection temperature 100°C.

3.4.4.4 Hydrocarbon Degradation Assay

Hydrocarbon-degrading capabilities were assessed using a method described by Phillips et al. (2006). Isolates were grown for 24 h in half strength TSB, harvested to obtain a cell density of 10^9 $CFU \cdot mL^{-1}$ (OD_{600}=1), washed twice in 1 mL of monopotassium phosphate (MPP) buffer (per L: 0.65g K_2HPO_4, 0.35 g KH_2PO_4, and 0.10 g $MgSO_4$), and finally resuspended in MPP buffer.

Autoclaved 2 mL tubes containing either 20 µL filter-sterilized (0.2 µm) n-octane (EMD Millipore Corporation, USA) or filter sterilized commercial diesel gasoline (Co-op, Saskatoon, Sk) and 720 µL Bushnell Haas mineral salts medium (per L: 0.2 g $MgSO_4 \cdot 7\ H_2O$, 0.02 g $CaCl_2 \cdot 2\ H_2O$, 1.0 g KH_2PO_4, 1.0 g $(NH_4)_2HPO_4$, 1.0 g KNO_3, 0.050 g $FeCl_3 \cdot 6\ H_2O$, and pH adjusted to 7.0) were inoculated with 80 µL of each bacterial suspension. Samples were then incubated in the dark for two weeks at room temperature. Two controls were included; an uninoculated Bushnell Haas mineral salts medium with filter sterilized n-octane, and uninoculated Bushnell Haas mineral salts medium with filter sterilized diesel. After two weeks, 200 µL sterile *p*-iodonitrotetrazolium violet ($3\ g \cdot L^{-1}$) (Sigma-Aldrich, St. Louis, USA) was added to each tube and incubated for one additional day. Tubes that exhibited a red color were determined to be positive for hydrogenase activity. This assay was repeated twice.

3.5 Results

3.5.1 Isolate Selection

After lists of bacterial isolates obtained from each site previous to the start of this study were compared and isolates selected based on common genera, 31 (53.4% of all isolates) isolates originated from BPHS and 27 (46.6% of all isolates) from Rotation ABC. Isolates common to both sites included: *Arthrobacter*, *Bacillus*, *Chryseobacterium*, *Leifsonia*, *Luteibacter*, *Microbacterium*, *Pedobacter*, *Pseudomonas*, *Rhizobium*, *Rhodococcus*, *Sphingomonas*, *Staphlococcus*, *and Xanthomonas* (Figure 3.1).

In addition to common isolates, organisms from the genus *Achromobacter* (Rotation ABC: P only treatment), *Acinetobacter* (Rotation ABC: N only treatment), *Delftia* (Rotation ABC: control and P only treatment), *Kluyvera* (Rotation ABC: N) were included as they were found specifically in locations where additional nutrient stress was present (Appendix A).

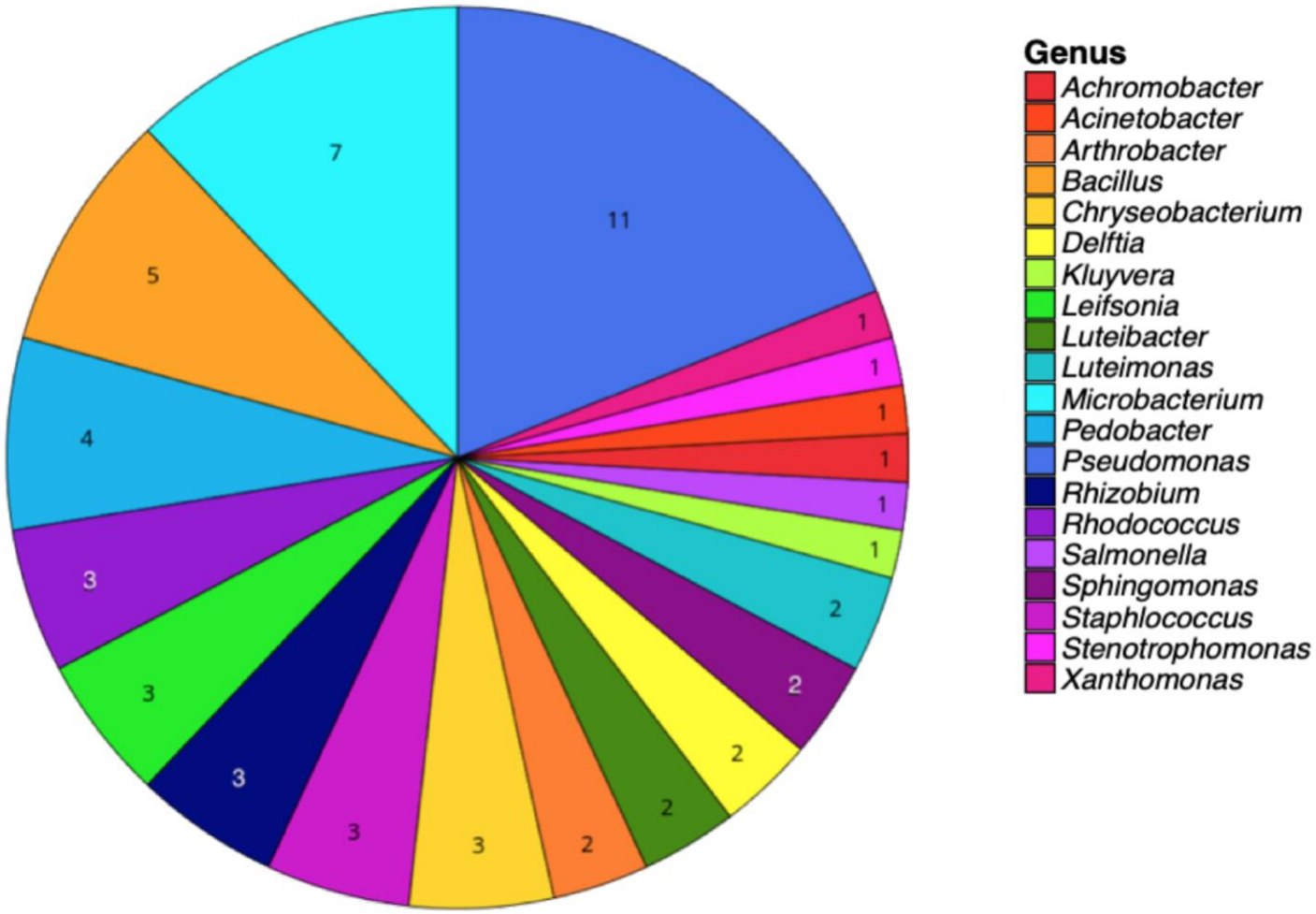

Figure 3.1: Pie chart of bacterial isolates obtained from Rotation ABC and Bitumount Provincial Historic Site belonging to genera identified at both locations. Each colour represents a different bacterial genera and numbers within each segment represent the total number of isolates belonging to that genera included for study. In total, 58 isolates were included.

3.5.2 Genotype Survey

Isolates were screened for five genes, *nifH, acds, phoD, alkB,* and *CYP153* associated with plant growth-promotion and hydrocarbon degradation. The most common gene was *alkB,* as15 out of 58 (26%) isolates tested positive (Figure 3.2). About the same number from Rotation ABC tested positive for *alkB* (7 out of 27; 26%) as at BPHS (8 out of 31; 26%) (Figure 3.3). Compared to other functional genes included in this study *CYP153* was the least abundant genotype as only 10 out of 58 (19%) isolates tested positive with 7 out of 10 positive isolates (70%,) originating from BPHS (Figure 3.3). After *CYP153,* the *acds* gene was least abundant as 12 out of all isolates (21%) tested positive (Figure 3.2). Eight out of the 12 positive isolates were originally isolated from BPHS and represented 26% (n = 31) of all BPHS isolates (Figure 3.3). In contrast, the four other isolates positive for this gene were from Rotation ABC (Figure 3.3).

For both biofertilization genes (*nifH* and *phoD*), 13 isolates tested positive (Figure 3.2). In both cases the majority of isolates positive for these two genes originated from BPHS with 8 of the 13 *nifH* positive isolates and 9 out of 13 *phoD* positive isolates originating from this location (Figure 3.3).

Based on the results of this genetic screen, genera positive for the most functional genes (at least four out of the five total) were *Pseudomonas, Rhodococcus,* and *Microbacterium* (Figure 3.2). Although psuedomonads collectively tested positive for all five genes, no single isolate screened positive for all five genes. However, isolate B19 contained all genes studied except *alkB* (Appendix B). *Microbacterium* and *Rhodococcus* isolates collectively tested positive for four out of all five functions (Figure 3.2). However, when considered individually, none of these isolates harbored more than two genes except for B13, a *Microbacterium*, which contained *phoD*, *alkB*, and *nifH* (Appendix B).

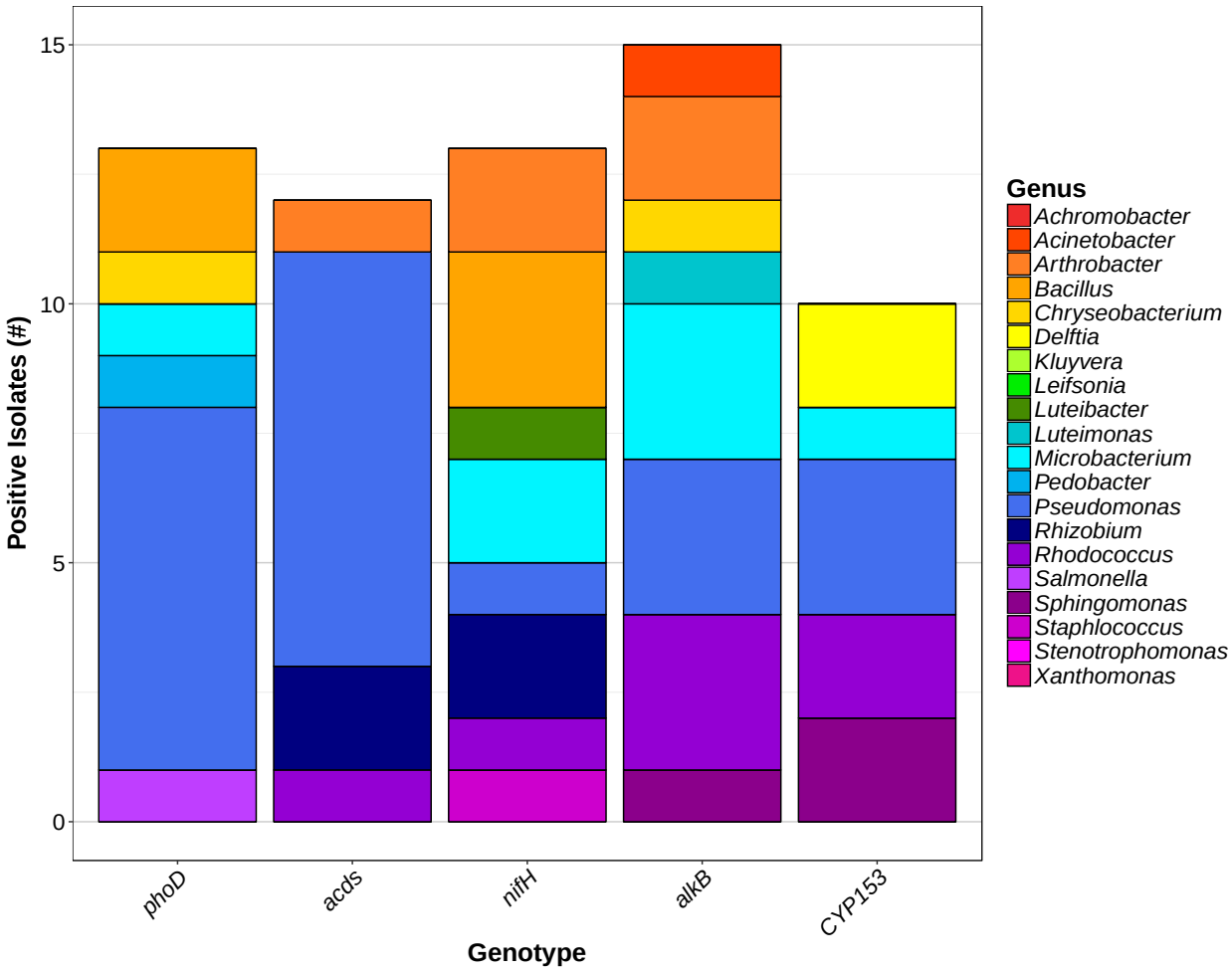

Figure 3.2: Number of isolates that contained *phoD* (alkaline phosphatase), *acds* (ACC deaminase), *nifH* (nitrogenase), *alkB* (hydrocarbon degradation), and *CYP153* (hydrocarbon degradation) genotypes for all isolates studied (n=58). Genera to which the isolates belong are represented by colored bars.

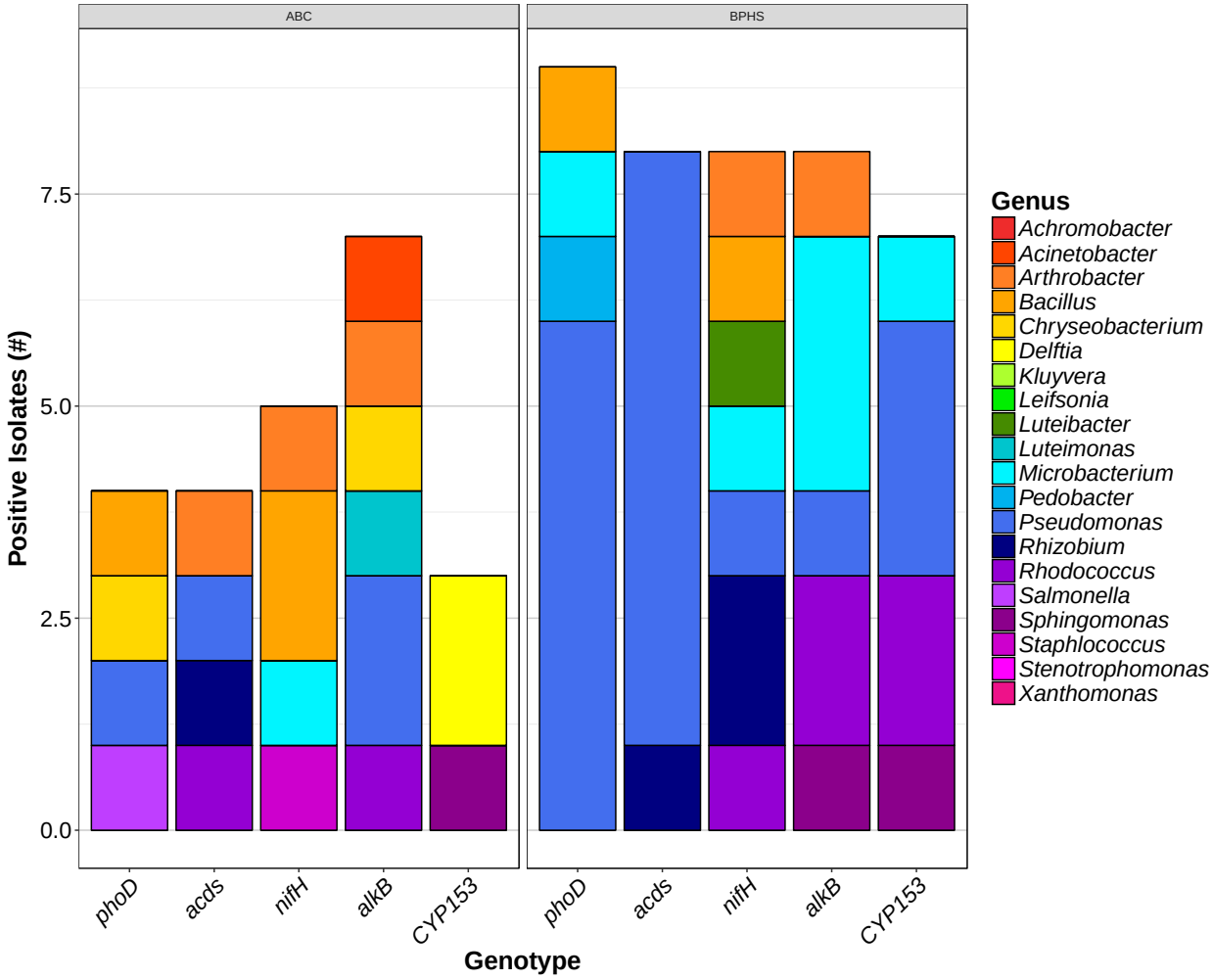

Figure 3.3: Number of isolates that contained *phoD* (alkaline phosphatase), *acds* (ACC deaminase), *nifH* (nitrogenase), *alkB* (hydrocarbon degradation), and *CYP153* (hydrocarbon degradation) genotypes for both Rotation ABC (n=27; Left Panel) and Bitumount Provincial Historic Site (n=31; Right Panel). Genera to which the isolates belong are represented by colored bars.

3.5.3 Phenotype Screening Assays

In addition to specific functional genes, isolates were screened for different phenotypes that are associated with plant growth-promotion. With the exception of the ARA, used to screen for nitrogenase activity, more than half of all isolates tested were positive for each function (Figure 3.4). Of the four phenotypes included, hydrocarbon degradation was the most common. In total, 44 out of 58 (76% of all isolates) were able to use diesel fuel as a sole carbon source, and 47 (81.0% of all isolates) were able to use n-octane (Figure 3.4). Among isolates positive for hydrocarbon degradation the majority—28 and 29 isolates for diesel and n-octane utilization respectively—were from BPHS with 93.6% and 90.3% of all BPHS isolates able to degrade octane and diesel respectively (Figure 3.5).

Thirty seven out of 58 isolates tested positive for alkaline phosphatase activity (Figure 3.4). Relative to the total number of isolates from each site, more of the BPHS isolates (n=22) tested positive than those from Rotation ABC (n=15) (Figure 3.5). When tested for ACC deaminase activity, 32 isolates tested positive (Figure 3.4). Additionally, when ACC deaminase activity was considered by site, more of the Rotation ABC isolates (18 out of 27) were positive for ACC deaminase activity than those from BPHS (14 out of 31) (Figure 3.5). As mentioned, nitrogenase activity (assessed using the ARA) was the least common trait among study isolates with only two isolates testing positive (Figure 3.4).

In total, eleven genera were identified as containing at least four of the five phenotypes. *Rhizobium* was the only genera that harbored all five phenotypes, though no one isolate screened positive for more than four plant growth-promoting phenotypes (Appendix B). Isolates positive for all phenotypes except nitrogenase activity included *Arthrobacter* (B35), *Kluyvera* (A12), *Luteimonas* (B34), *Pseudomonas* (B18, B19, B23), *Rhodococcus* (A23, B30, B31), *Salmonella* (A24), *Stenotrophomonas* (B38), and *Xanthomonas* (A29) (Appendix B). Though isolates from the genera *Bacillus* cumulatively tested positive for at least four of the five functions, no one isolate tested positive for more than three (Appendix B). Similarly, *Microbacteria* cumulatively tested positive for all phenotypes except ACC deaminase activity, but no one *Microbacteria* isolate was positive for more than two (Appendix B).

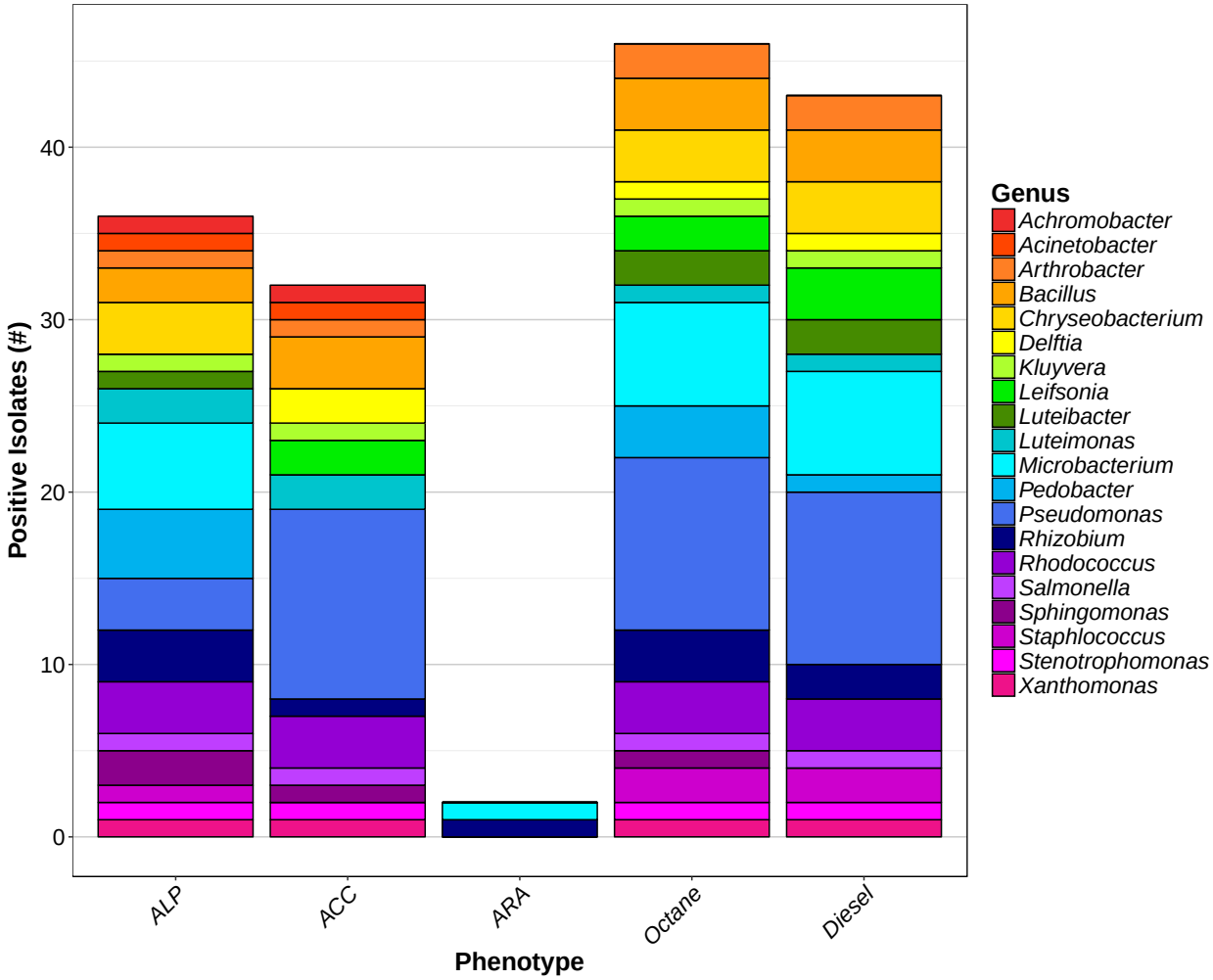

Figure 3.4: Number of isolates positive for alkaline phosphatase (ALP), ACC deaminase (ACC), acetylene reduction (ARA), octane degradation (Octane), and diesel degradation (Diesel) phenotypes for all isolates studied (n=58). Genera to which the isolates belong are represented by colored bars.

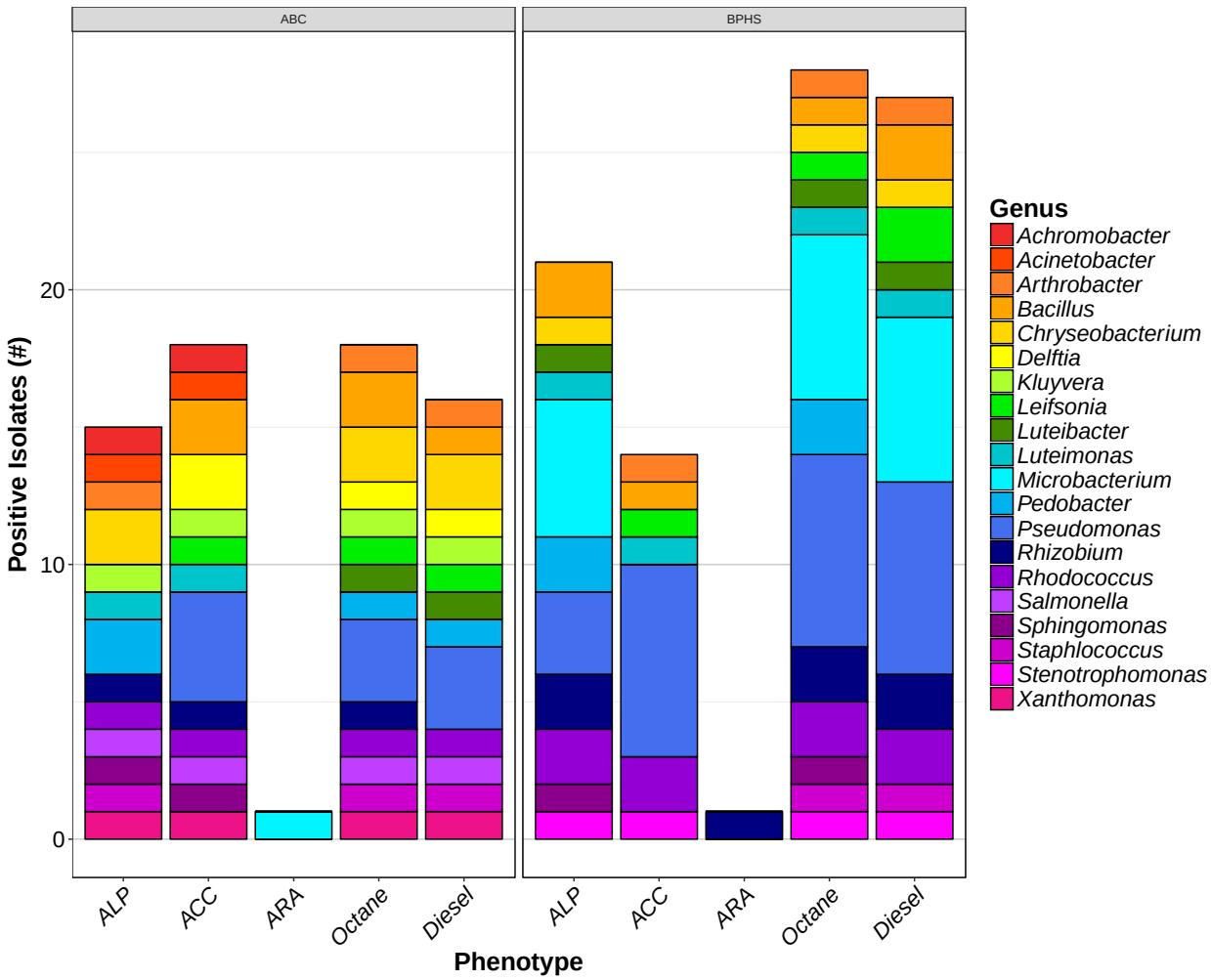

Figure 3.5: Number of isolates positive for alkaline phosphatase (ALP), ACC deaminase (ACC), acetylene reduction (ARA), octane degradation (Octane), and diesel degradation (Diesel) phenotypes for both Rotation ABC (n=27; Left Panel) and Bitumount Provincial Historic Site (n=31; Right Panel). Genera to which the isolates belong are represented by colored bars.

3.6 Discussion

3.6.1 Plant Growth-Promoting Phenotypes and Genotypes

This study aimed to develop a method of screening for plant growth-promoting genotypes and associated phenotypes in order to characterize endophytes from bacterial genera common to both a long-term nutrient-deficient agricultural soil (Rotation ABC) and a hydrocarbon-contaminated soil (BPHS) for plant growth-promotion potential. The functional genes *nifH* and *phoD* were selected as indicators for biofertilization as they have respectively been shown to be involved in nitrogen fixation (Gaby and Buckley 2012) and alkaline phosphatase activity (Ragot et al. 2015) among soil bacteria. Considering the role that ACC deaminase plays in mitigating the effects of different stressors by regulating plant ethylene levels (Glick et al. 1998), the ACC deaminase structural gene *acds* was included in the genotype survey (Glick et al. 2007). Finally, to help identify hydrocarbon-degrading bacteria, the *alkB* and *CYP153* genotypes were selected as the products of these two functional genes are key in the hydroxylation of medium chain length alkanes by bacteria (van Beilen et al. 2006, Funhoff et al. 2006, Koch et al. 2009). As hypothesized, root-associated bacteria common to both BPHS and Rotation ABC tested positive for these selected genotypes and phenotypes associated with plant growth-promotion, hydrocarbon degradation, and stress tolerance.

With the exception of *nifH*, functional genes were less common among bacterial isolates than associated phenotypes. This could be the result of a positive phenotype arising from a genotype not included in this study. For example, it was noted that although numerous isolates (>10) tested negative for the *phoD* genotype, they tested positive for the alkaline phosphatase phenotype. Although *phoD* is a common alkaline phosphatase gene among soil bacteria (Tan et al. 2013), it is not the sole alkaline phosphatase gene (Luo et al. 2009, Kageyama et al. 2011). In fact, Monds et al. (2006) employed a mixed gene knockout and reverse transcription PCR (*rt*-PCR) method in order to demonstrate that another gene, *phoX*, was not only a member of the Pho regulon but a key player in phosphate starvation induced alkaline phosphatase activity when *phoD* is absent. Thus, this observed activity in the absence of *phoD* could be due to the presence and successful activation of another alkaline phosphatase, such as the PhoX alkaline phosphatase, as opposed to PhoD.

Another example of how a negative genotype and positive phenotype may arise due to alternative mechanisms is illustrated by considering the relationship of n-octane and diesel degrading phenotypes to the two genotypes selected here. In some cases, hydrocarbon utilization was observed though only one gene was present. Whilst they are both key enzymes in medium chain alkane hydroxylation, they produce different products that function in slightly different ways (Van Beilen and Funhoff 2007). Thus, a hydrocarbon-degrading phenotype observed in the presence of only one genotype can be attributed to the activity of that gene product and not the other. In cases such as these, had the genotype survey only included the absent gene, the hydrocarbon-degrading phenotype would be observed despite the absence of the selected functional gene. Further, the degradation of hydrocarbons does not occur explicitly via the activity of the AlkB and CYP153 alkane monooxygenases. In fact, several bacterial enzymes exist that are capable of degrading hydrocarbons of different lengths and structures. Among these are the soluble methane monooxygenases which degrade C_1-C_8 alkanes, alkenes, and cycloalkanes (McDonald et al. 2006) and the dioxygenases which degrade C_{10}-C_{30} alkanes (Maeng et al. 1996). Thus, a positive phenotype for hydrocarbon degradation could have also arisen as a result of the activity of any of the additional catabolic enzymes present in nature.

3.6.2 Degenerate Primers and Specificity

Degenerate primers are useful when screening a large diversity of organisms (performed herein) because in specific positions where there is sequence variability among species degenerate primers have several different bases (Telenius et al. 1992). In this way, it becomes possible to use the same set of primers on different organisms without a strong bias towards certain iterations of the genetic code (Telenius et al. 1992). Though degenerate primers are more effective when screening a larger diversity of organisms, their degeneracy also leads to a reduction in specificity (Linhart and Shamir 2005). In fact, one of the largest challenges in the design of degenerate primers is creating an efficient way to balance diversity recovery and specificity (Linhart and Shamir 2005). This reduced specificity in exchange for increased diversity recovery becomes relevant in this study as it is possible that some isolates, that screened positive for a phenotype, were missed in the screening method. Non-specific binding, which results when specificity is reduced, was a significant problem encountered with the *alkB*, *CYP153* and *acds* primers. In order to reduce this non-specific binding so that pertinent bands could be excised and sequenced, some modifications

to the PCR methods previously published were made (e.g. addition of DMSO, increase annealing temperature). Though these changes would have increased specificity and reduced non-specific binding, this would also lead to a decrease in diversity recovery and increase in type-II error. This situation could help explain why several isolates positive for ACC deaminase activity were not positive for the *acds* genotype despite its requisite for enzyme production (Glick et al. 2007) as non-specific binding was greatest in this case.

3.6.3 Plant Growth-Promotion Pathways

Alternative to the aforementioned scenarios, isolates that tested positive for functional genes did not always test positive for associated phenotypes. One possible reason for this result is that the biochemical pathway is broken. For example, the gene *nifH* is one of several genes included in the nif operon (Rubio and Ludden 2002). Though *nifH* is commonly used to screen for N fixation because it encodes the nitrogenase reductase subunit—an essential component in the reduction of inert N_2—there are other essential functional genes, such as *nifD*, *nifK*, *nifN*, *nifM* and *nifB*, that must also be present for proper assembly and activity of the complete nitrogenase protein (Dixon and Kahn 2004, Rubio and Ludden 2005, 2008, Franche et al. 2009). If any one of these genes are missing or disrupted then no activity will be observed (Franche et al. 2009).

A similar logic can be used to explain the disconnect between the presence of *phoD* and alkaline phosphatase activity. The alkaline phosphatase gene *phoD* is one of many genes found in the Pho-regulon and requires conditions of H_2PO_4 starvation for induction (Santos-Beneit 2015). Due to the complexity of this regulated response, *phoD* derived alkaline phosphatase activity is reliant on the coordinated expression of several other genes (Santos-Beneit 2015). The PhoD alkaline phosphatase will only be active when this expression occurs successfully for all regulatory genes (Wanner 1993). Furthermore, in a study by Summers et al. (1998), a Δ*phoB* mutant was unable to induce expression of several *pho* genes previously linked to H_2PO_4 stress including the gene *phoD*. The importance of *phoB* in the Pho regulatory response was further demonstrated by (Monds et al. 2006) where a Δ*phoB* mutant had significantly reduced phosphatase activity compared to the wild type. Thus, if *phoB* or any other key regulatory gene is missing or disrupted, alkaline phosphatase activity will not be observed.

3.6.4 Prevalence of Plant Growth-Promoting Attributes by Field Site

Nearly the same number of isolates from Rotation ABC screened positive for *alkB* as those at BPHS. Studies have previously shown that *alkB* is not specific to contaminated soils. In fact, Whyte et al. (2002) found that *alkB* was nearly always equally abundant in pristine soils as contaminated soils. This observation was further supported by a study by Kloos et al. (2006) which looked at the abundance and diversity of bacterial *alkB* in soils with no known history of anthropogenic hydrocarbon contamination. Again, *alkB* bacteria were identified in these soils further demonstrating that alkane degradation is a common trait among soil bacteria regardless of the presence of a contaminant.

The prevalence of alkane-degrading bacteria among environmental microorganisms has been attributed to the production of alkanes by plants, insects and other organisms in the form of pheromones or metabolic byproducts (Afzal et al. 2013). As a result, microorganisms, such as bacteria, living in association with organisms producing such compounds would derive a competitive advantage by being able to make use of this resource (Van Beilen and Funhoff 2007). This explains why alkane-degrading bacteria were identified within the rhizosphere, an environment where plant-produced alkanes may be found, even without any known history of hydrocarbon contamination.

While nearly an equal number of isolates contained the *alkB* genotype at Rotation ABC as at BPHS, the effects of increased selective pressure for degrading bacteria in the presence of a contaminant can still be demonstrated by considering the number of isolates positive for both *alkB* and *CYP153* as well as the prevalence of hydrocarbon-degrading phenotypes. It has been previously reported that in contaminated sites, the selection for organisms with multiple degradation pathways will be increased (Liu et al. 2011, Nie et al. 2014, Liang et al. 2016). A study by Siciliano et al. (2001) also demonstrated that this selection for organisms with degradation potential is even further enhanced when plants are selecting for endophytic partners in the presence of contaminants. These two considerations support the observation that whereas none of the isolates from Rotation ABC tested positive for both *alkB* and *CYP153*, several of the pseudomonads from BPHS did. Although *alkB* and *CYP153* exhibit overlapping alkane substrate ranges, they are not identical. For example, AlkB (the enzyme produced by translating the *alkB* gene) has been shown to degrade propane, *n*-butane, and C_5-C_{13} alkanes (Rojo 2009). Alternatively, the CYP153 alkane hydroxylase has been shown to degrade C_6-C_{11} alkanes as well

as 1-alkanols (Funhoff et al. 2006). Further, nearly all (>90%) BPHS isolates tested positive for at least one hydrocarbon-degrading phenotype and more from this site were able to use both n-octane *and* diesel as a carbon source. Here, another an example of how organisms capable of using a wide range of compounds present in the environment to confer a competitive advantage will be selected against those that cannot.

Bacteria positive for alkaline phosphatase activity were more common among BPHS isolates than Rotation ABC isolates. In hydrocarbon-contaminated soils, degradation, and survival in general, is often limited by the availability of N and P (Boopathy 2000, Bais et al. 2006). Thus, in most cases the stimulation of plant growth and microbial activity requires increasing available N and P. Considering that plants and environmental conditions play a large role in selecting for microbial communities (as discussed previously), it could be that organisms capable of liberating any organic P found at BPHS would be more desirable considering the deficit of this key nutrient.

Although alkaline phosphatase activity at BPHS appears to be preferentially selected for, the effect of the P deficiencies at Rotation ABC are not insignificant as more than 50% of all isolates tested were positive for this phenotype. However, it could be that at Rotation ABC the competitive advantage that would arise from alkaline phosphatase activity is less than at BPHS. Recall that at Rotation ABC one of the treatments receive P fertilizer applications (P_{Fert}). Though P fertilizer can provide plants with additional, necessary P, only 30% of what is applied actually is taken up by the plant (Sharma et al. 2013). This remaining P becomes fixed in the soil as either Al, Fe of Ca PO_4^{3-}, and is inaccessible to plants. This form of inaccessible P can be liberated my microorganisms, but not via alkaline phosphatase activity. In this case the P can be converted to H_2PO_4 by the release of organic acids instead (Rodríguez and Fraga 1999). Thus, perhaps at Rotation ABC bacteria capable or solubilizing mineral P through the release of organic acids are more important players than those that can mineralize organic P.

3.6.5 Isolates Identified as Potential Plant Growth-Promoting Bacteria

Of the fifty-eight original isolates belonging to bacterial genera common to both sites, 22 tested positive for at least 40% of plant growth-promoting genotypes and/or phenotypes. At the threshold of 40% at least two of the plant growth-promoting phenotypes were present. As might be expected many of these specific isolates belonged to genera previously reported to be associated with plant growth-promotion in a variety of different plant types. For example, *Bacillus* plant

growth-promoting bacteria have not only been used in biological control of plant diseases (for review see Kloepper et al. 2004), but also to promote plant growth. For example, Zaidi et al. (2006) found that when Indian mustard plant (*Brassia juncea*) growing in nickel-contaminated soils is inoculated with *Bacillus subtilus* both plant growth and nickel accumulation is increased. Additionally, Cheng et al. (2007) found that inoculating canola with *Pseudomonas putida UW4* improved growth and increase tolerance to salinity. Further, Belimov et al. (2001) showed that several different ACC deaminase containing pseudomonads and one *Rhodococcus* isolate were able to promote the growth of pea (*Pisum sativum*). In the same study, multiple pseudomonads were able to promote the growth of rape (*Brassica napus*) in cadmium contaminated soils under greenhouse conditions, with one (*Pseudomonas brassicacearum*) common to both pot studies.

3.7 Conclusions

This study highlights the importance of including functionality screening in the process of selecting plant growth-promoting bacteria and illustrates that the presence or absence of a known plant growth-promotion gene cannot be used as a sole indicator of plant growth-promoting potential. Moreover, results indicate that when using this approach including several key regulatory and functional genes is imperative. Though *in vivo* studies have yet to be discussed, the aforementioned results indicate that this screening method does successfully identify bacteria with high plant growth-promoting potential and may identify better plant growth-promoting bacteria candidates among a larger collection to be included in *in vivo* studies. Future work will aim to test this hypothesis and confirm plant growth-promoting capabilities of these isolates.

4. *IN VIVO* TESTING OF BACTERIAL ISOLATES OBTAINED FROM STRESSED ENVIRONMENTS FOR USE AS PLANT GROWTH-PROMOTING INOCULANTS IN NUTRIENT DEFICIENT AND HYDROCARBON-CONTAMINATED SOILS

4.1 Preface

In the previous chapter, bacterial isolates belonging to genera common to Rotation ABC and Bitumount Provincial Historic Site were screened for plant growth-promoting genotypes and associated phenotypes. Results showed that several isolates were positive for more than one genotype and/or phenotype, indicating potential for use as a plant growth-promoting inoculant. Although these results indicated plant growth-promotion potential, effects on plant growth cannot be resolved without also assessing the impact of inoculation on plant growth. Therefore, the purpose of this chapter is to test bacterial isolates as inoculants *in vivo* to determine if plant growth-promotion potential confers an actual effect on plant growth.

4.2 Abstract

The use of plant growth-promoting bacteria has become an attractive new tool in both agricultural production and the restoration of degraded environmental sites. The goal of this study was to determine the plant growth-promoting capabilities of bacterial endophytes previously identified to have genetic and physiological potential for plant growth-promotion. This was accomplished by first selecting candidates based on the results of various *in vitro* tests followed by a series of *in vivo* assays that identified bacterial candidates as potential plant growth-promoting inoculants in wheat and sweet clover plants. Initial seed germination and root elongation studies demonstrated the importance of testing for plant-endophyte compatibility as several candidates were inhibitory. Nonetheless, promotion of root elongation was observed in several treatments in both plant types. Based on results from the *in vivo* tests, two candidates demonstrating the greatest positive effect on growth in each plant type were used as inoculants in a pot study where plants were grown for 60 days in a marginal agricultural soil with and without diesel fuel.

No promotion of plant growth was observed in sweet clover plants or in wheat plants growing in hydrocarbon-amended soil (HCS). Further, no significant degradation of hydrocarbons was detected in any inoculated treatments. However, in wheat plants growing in the marginal soil, inoculation with *Kluyvera* isolate A12 and *Luteimonas* isolate B34 increased both nitrogen (N) and phosphorus (P) uptake. It can be concluded that two isolates identified herein may have potential as biofertilizers in wheat plants, and a positive plant growth-promoting genotype and/or phenotype does not always confer plant growth-promotion in the environment.

4.3 Introduction

As the need for food and land increases with the continued rise in human population, newer alternatives to current methods of crop production are ever more vital (Alexandratos and Bruinsma 2012). Further, as population continues to rise and the production and release of industrial contaminants continue to degrade vital land resources, efficient and sustainable means of remediation must be developed (Glick 2010). Although the use of chemical fertilizers, high yielding cultivars, pesticides, and irrigation has helped to provide sufficient food production to date (Foley 2005, Kumar et al. 2014) and methods exist to clean up degraded land (Khan et al. 2004), these approaches often lead to further environmental degradation including (but not limited to) contaminated water sources and reduced soil quality. Thus, one of the greatest global challenges in coming years is altering current approaches to land use such that negative impacts on the environment are minimized without a subsequent reduction in production while simultaneously finding ways to mitigate damage caused thus far.

One alternative and/or complement to the aforementioned methods of enhanced crop production and environmental clean-up is the use of plant growth-promoting bacteria. Plant growth-promoting bacteria can be isolated from the rhizosphere (area around the roots), rhizoplane (surface of the roots), and endosphere (within the plant) of plants (Reinhold-Hurek et al. 2015), and promote plant growth through a variety of mechanisms. These mechanisms generally involve the processes of biofertilization (increased nutrient acquisition), phytostimulation (production and/or alteration of plant hormones), biological control (control of pathogens), and biodegradation (reduction and/or removal of harmful contaminants) (Bloemberg and Lugtenberg 2001, Thijs et al. 2016). Several studies have demonstrated the capacity of plant growth-promoting bacteria to not only promote plant growth and increase yield of valuable food products (Montañez et al. 2012,

Pérez-Montaño et al. 2014, Kumar et al. 2014, Majeed et al. 2015) but also enhance the process of phytoremediation (Ma et al. 2011, Hou et al. 2015, Islam et al. 2016, Kovalski Mitter 2018). In fact, several commercial bacterial inoculants have already been developed, sold, and used in Europe, North and South America, and Australia (Berg 2009, Bashan et al. 2014, Owen et al. 2015) and a larger focus is being placed on better incorporating plant growth-promoting bacteria into the practice of phytoremediation (McGuinness and Dowling 2009, Jha and Jha 2015).

One relevant characteristic of plant growth-promoting bacteria is biofertilization (Pii et al. 2015). Nitrogen and P are vital plant nutrients as they are required for the synthesis of biomolecules (Fisher and Newton 2002, Vershinina and Znamenskaya 2002). Without sufficient access to either of these fundamental nutrients, plant growth and yield will be severely diminished. Though the atmosphere is a vast reservoir of N and some soils have a generous supply of P, these are present in forms unavailable to plants (Fisher and Newton 2002, Sharma et al. 2013). Thus, the capacity of plant growth-promoting bacteria to increase N by converting atmospheric N_2 to plant available ammonium (NH_4^+) via the process of nitrogen fixation, and fixed P to the biologically relevant orthophosphate (H_2PO_4) via the processes of solubilization/mineralization is of great value.

As mentioned, the ability to alter and produce plant hormones via the process of phytostimulation is another well studied mechanism of plant growth-promotion. One bacterially produced enzyme that plays a large role in this process is 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which reduces the amount of ethylene present in the plant (Glick et al. 2007). Ethylene is an essential plant hormone involved in regulating a variety of developmental processes including root hair formation, ripening, flowering, leaf and petal senescence, and stress responses (Dugardeyn and Van Der Straeten 2008). Although ethylene can lead to a stimulation of plant growth, if high levels persists for an extended period of time growth is negatively impacted (Pierik et al. 2006). Bacterial ACC deaminase promotes plant growth by breaking down the molecule ACC, a precursor to plant ethylene, into ammonia (NH_3) and α-ketobutyrate thereby reducing the levels of ethylene (Glick et al. 1998). Thus, bacteria that produce ACC deaminase not only help their plant host by reducing ethylene but receive a competitive advantage from this activity because it provides them with an additional source of N (Glick 2005).

Finally, when considering a plant growth-promoting bacterium for use as an inoculant to enhance phytoremediation it is important to first consider the capacity of that bacterium to tolerate and degrade the contaminant present. In bacteria, there are several different mechanisms to degrade

harmful contaminants (Scoma et al. 2015). Different hydrocarbon-degrading enzymes vary mostly on the type and length of molecule they can degrade (Afzal et al. 2013). Among these are the alkane hydroxylases which are involved in the aerobic alkane degradation of oil, chlorinated hydrocarbons, fuel additives and more (Van Beilen and Funhoff 2007). Some bacteria are involved in the degradation of several compounds of interest (Glick 2010). Therefore, identifying organisms with a broad range of catabolic enzymes capable of degrading a variety of hydrocarbons is advantageous when selecting plant growth-promoting bacteria to enhance phytoremediation because they will be applicable among a wider assortment of contaminants.

Development of new bacterial inoculants generally begins in a research facility where bacteria are isolated and identified from environmental samples, screened for plant growth-promoting characteristics, tested in a growth chamber/greenhouse, and then tested in a small field trial (Bashan et al. 2014). In an effort to better identify plant growth-promoting bacteria, *in vitro* assessment of plant growth-promoting characteristics such as N fixation, P mineralization/solubilization, ACC deaminase activity, and biodegradative capacity are completed followed by *in vivo* testing of potential plant growth-promoting bacteria (Chen et al. 2010, Baig et al. 2012, Ipek et al. 2014, Kumar et al. 2014, Majeed et al. 2015, Islam et al. 2016). Considering this, the objective of this study was to determine the actual plant growth-promoting capabilities of environmental isolates previously identified to be strong plant growth-promoting bacteria candidates based on a series of *in vitro* studies. To accomplish this, a series of studies were conducted wherein it was hypothesized that plants inoculated with bacterial isolates identified as positive for plant growth-promoting phenotypes and genotypes will demonstrate increased growth in both hydrocarbon-contaminated and low nutrient soil.

4.4 Materials and Methods

4.4.1 Isolate Selection

Bacteria to be used in for *in vivo* testing were selected based on the results presented in Chapter 3. Isolates were scored according to the number of genotypes and phenotypes they tested positive for (Appendix B). A plant growth-promoting genotype conferred each study isolate one point, and a plant growth-promoting phenotype gave two points. Phenotypes were weighted more in an effort to ensure isolates exhibiting activity, even in the absence of a genotype, were included

in *in* vivo studies. Isolates were rated based the sum of all points for each isolate over the total possible (13) and converted to a percent. Because both hydrocarbon degradation assays measured the same function but with different carbon sources, a positive result for hydrocarbon degradation was counted once (i.e. an isolate positive for both n-octane and diesel degradation would receive 2 points not 4). This was done to prevent creating a bias towards hydrocarbon degradation. Isolates positive for greater than 40% of all genotypes/phenotypes (the threshold at which two or more plant growth-promotion functions were represented) were included for further study in plants. Isolates positive for less than 40% but greater than 35% of all plant growth-promotion traits were only included if ACC deaminase activity was present to ensure that isolates positive for ACC deaminase activity, along with other functional genes and plant growth-promoting functions, were tested.

4.4.2 Plant Growth-Promoting Capacity in vivo

4.4.2.1 Preparation of Bacterial Cultures

Bacterial isolates selected for further study were grown in 250 mL ½ concentration Bacto™ Tryptic Soy Broth (TSB) (15 $g \cdot L^{-1}$; BD Biosciences, NJ USA) (28°C for 48 h at 150 rpm) until a cell density of 10^9 colony forming units (CFU)$\cdot mL^{-1}$ (OD_{600}=1) was reached. Cells were harvested using a Sorvall RC 6+ Centrifuge (Thermo Scientific) (5000 x g, 15 min). Any remaining media was removed by triple washing cells in 150 mL sterile phosphate-buffered saline (PBS) buffer (1.2 g of $Na_2HPO_4 \cdot L^{-1}$, 0.18 g of $NaH_2PO_4 \cdot L^{-1}$, and 8.5 g of $NaCl \cdot L^{-1}$). After the final wash, cells were re-suspended in 15 mL sterile PBS buffer to obtain an inoculum with a cell density of 10^{10} CFU$\cdot mL^{-1}$. In addition to isolates of interest, both autoclaved cells and PBS were prepared as described and used as controls.

4.4.2.2 Surface Sterilization and Inoculation of Seeds

Wheat (*Trictum aestivum* cv 'CDC Waskeda') and sweet clover (*Melilotus albus;* cultivar information unavailable; seeds sourced from Nutrient Agriculture Solutions, SK, Canada) seeds were acquired and used for all *in vivo* studies. All seeds were surface sterilized by first soaking in ethanol (65% $w \cdot v^1$) for 3 min, then in a sodium hypochlorite (NaClO) solution (10% $v \cdot v^1$) for 5 min, followed by 10 rinses in sterile tap water (de Freitas et al., 1997). An aliquot (100 µL) from

the final wash was spread onto $^{1}/_{10}$ tryptic soy agar (TSA) (3.0 g TSB per liter with 1.5% agar) plates and incubated at 28°C for three days to ensure sterility. Surface sterilized seeds were then suspended in 10 mL bacterial suspension and incubated for 2 h at room temperature on a rotary shaker (150 rpm). After incubation, the cell and seed mixture was poured into a petri dish to make subsequent seed transfers easier. Using autoclaved tweezers, one seed from each treatment was added to 10 mL sterile distilled water (dH_2O). The mixture was then vortexed, 10 µL of water spread onto $^{1}/_{10}$ TSA plates, and incubated in the dark at 28°C to estimate/ monitor the number of adhered cells.

4.4.2.3 Seed Germination

The germination rate of inoculated seeds was assessed to a) ensure that the inoculant did not inhibit early seed germination and b) to identify any inoculants that may promote germination. Immediately following inoculation, seeds were aseptically transferred to a petri dish containing sterile filter paper that had been moistened with five mL sterile distilled water. For each inoculant five replicates were prepared, each containing 10 seeds. Plates were sealed with parafilm and left to incubate in the dark. The total number of germinated seeds was recorded one, three, and seven days after inoculation. Seeds were considered to have germinated when the radicle was 1 mm in length. Seeds treated with autoclaved cells and PBS alone were included as controls. Seed germination was calculated as:

$$[(\#\ \text{seeds germinated 7 days after treatment})/\ (\text{Total}\ \#\ \text{seeds})\ ] * 100 \qquad \text{(Equation 4.1)}$$

4.4.2.4 Root Elongation

Root elongation assays were conducted using CYG™ seed germination pouches (Mega International, MN, USA). Twenty mL of dilute (1:5) sterile Hoagland solution (Hoagland and Arnon 1950) was prepared and added to each pouch. Pouches were grouped by treatment in sets of five, enclosed in aluminum foil, and autoclaved on L20 cycle (20 min at 120°C) prior to seeding. After cooling, 15 and 10 seeds were transferred to each root pouch for wheat and sweet clover respectively. Pouches were then transferred to a growth chamber with a 16 h/ 25°C day (1500 $\mu mol \cdot m^{-2}$) and 8 h/15°C night cycle (Appendix E). Throughout the growth period, plants were watered with sterile dH_2O such that an approximate volume of 20 mL was maintained. Plants were

rotated within their racks daily so as to minimize the effects of light and air flow variations on the growth of plants positioned at the ends of each set of replicates. After one day, the top of the foil enclosure was opened to allow direct light. After two days, seeds were thinned to five per pouch. Growth periods were designated for each plant based on the amount of time required for roots to reach the bottom of the growth pouch. These were seven and ten days for wheat and sweet clover respectively. Upon the completion of each growth period, plants were removed from the growth chamber, leaves and stem trimmed, and roots removed from the pouches for analysis. Roots were scanned using an Epson (Perfection V00) scanner and processed using WinRhizo image analysis software 2013e (Regent Instruments, Canada).

4.4.2.5 Statistical Analyses

Data was analyzed using RStudio (Version 1.1.383) statistical software. Both seed germination and root lengths were analyzed with an ANOVA followed by Fisher's Least Significant Difference (LSD) test (Agricolae package) when appropriate. Figures were generated using the R package ggplot2 (Wickham 2009).

4.4.3 Inoculation and Assessment of Plant Growth Over 60 Days

4.4.3.1 Experimental Setup

Inoculated plants were grown in a growth chamber (16 h/ 25°C day (1500 $\mu mol \cdot m^{-2}$); 8 h/15°C night cycle) under two soil conditions to assess the plant growth-promotion of selected isolates in a) marginal soil and b) marginal soil amended with diesel fuel (5000 $mg \cdot kg^{-1}$) (Appendix E). In both cases plants were grown in pots containing 1.5 kg of a marginal agricultural soil classified as a sandy loam that had been collected in Central Butte, SK (50 43 52 N and 106 25 0 W) in late October, 2017. Soil was sieved using a 4 mm sieve and homogenized prior to nutrient analysis. Soil properties were analyzed (ALS Laboratory Group, Saskatoon, Canada) and were as follows: available nitrate-N: 2.9 $mg \cdot kg^{-1}$, available sulfate-S: 5.4 $mg \cdot kg^{-1}$, available phosphate-P: 11.4 $mg \cdot kg^{-1}$, available potassium: 339 $mg \cdot kg^{-1}$, total organic carbon (Walkley Black): 0.8 %, organic matter (Walkley Black): 1.36%. Prior to planting, pots were arranged in a fully randomized complete block design (RCBD) and sterile distilled water was added to reach 50% gravimetric water content. Simultaneously, the soil used to determine plant

growth-promotion in hydrocarbon-contaminated soil was amended with commercial diesel fuel (5000 $mg \cdot kg^{-1}$) (Coop Saskatoon, SK, Canada) by mixing diesel and sterile distilled water and pouring evenly across the soil. After the addition of water/water + diesel and prior to seeding, pots were covered with aluminum foil and left to incubate in the growth chamber for ten days to rejuvenate the activity of native soil microbial communities in the previously air-dried soils.

After ten days, ten seeds were inoculated (as described in section 4.4.2.2) and planted into each pot. Also included were two controls 1) an uninoculated control plant (UC) and 2) hydrocarbon-amended soil (HCS) unplanted and uninoculated. All treatments and controls were replicated five times. For the duration of the study, pots were rotated (daily) and watered to maintain 50% gravimetric water content. After an initial trial, the number of sweet clover seeds planted into each pot of diesel amended soil was increased to 15 due to low survival rates. After seven days, germination rates were recorded, and pots were thinned to two plants per pot. Plants were harvested after 60 days (at approximately flowering) and the number of secondary shoots (sweet clover), plant height (both), and fresh weight of the aboveground biomass (both) and heads (wheat) were measured. All aboveground biomass was then dried, reweighed, ground and analyzed for NH_4^+ and phosphate (PO_4^{3-}). Soils were homogenized and collected from diesel amended pots as well as HCS pots to be analyzed for F2-F3 hydrocarbon fractions. Soil samples were stored at –20°C until analysis.

4.4.3.2 Plant Nutrient Analyses

Plant material was digested and analyzed for NH_4^+ and PO_4^{3-} as described by Thomas et al. (1967). Immediately after harvesting plants, aboveground plant matter was dried and ground to a fine powder using a ball grinder. For analysis, 0.25 g of each sample was weighed into glass digestion tubes. As controls, 0.03 g of apple leaf (NIST SRM 1515, Gaithersburg, MD) and spinach leaf (NIST SRM 1570a, Gaithersburg, MD) standards were included as well as two tubes without any plant material. Five milliliters of 36N sulfuric acid (H_2SO_4) was added to each digestion tube. Samples were then vortexed and placed on a digestion block set to 360°C for 30 min. Tubes were then removed from the block and left to cool for approximately 20 min. After cooling, 5 mL hydrogen peroxide (H_2O_2) was added to each tube. Tubes were vortexed and placed back on the digestion block (360°C) for 30 min. Cooling and subsequent H_2O_2 additions were repeated four times until the solution appeared clear. After the final H_2O_2 addition, tubes were

heated for 60 min to ensure all H_2O_2 had been removed. Samples were left to cool for 20 min and then approximately 55 mL de-ionized (DI) water was added to each tube. This reaction is exothermic, so samples were then left to cool overnight. The following morning, DI water was added until a final volume of exactly 75 mL was reached. Samples were then capped and inverted several times to mix. Samples were then transferred to clean vials and analyzed via a Technicon autoanalyzer for NH_4^+ and PO_4^{3-}. Uptake was calculated as:

$$U = C_{N,P} \times m_{dry} \quad \text{(Equation 4.2)}$$

Where U is the uptake of N and P (mg nitrogen or phosphorous per plant), $C_{N,P}$ is the concentration of N or P (mg nutrient per g dry sample digested) and m_{dry} is the average dry biomass for each pot (g). Uptake of N and P was calculated on the basis of average dry biomass for each pot to account for the size of plants as well as the number of plants in each pot.

4.4.3.3 Soil Hydrocarbon Analyses

Soils were analyzed for F2 and F3 fractions using a method described by Schwab et al. (1999) and Siddique et al. (2006). After measuring soil water content, the equivalent of 2.0 g dry soil was weighed out and added to clean, Teflon lined centrifuge tubes. Additionally, several quality control samples were prepared and included: a method blank (uncontaminated sand and solvent), blank spike (uncontaminated sand spiked with 5000 $mg \cdot kg^{-1}$ diesel fuel and solvent), matrix spike (sample spiked with 5000 $mg \cdot kg^{-1}$ diesel fuel and solvent), a duplicate, and a reagent blank (hexane alone). For use as a time zero (T_0) reference, 2.0 g soil was spiked with 5000 $mg \cdot kg^{-1}$ diesel fuel by mixing water and commercial diesel in a 2 mL microfuge tube. The diesel/water mixture was then poured into a Teflon tube containing 2.0 g of the same soil used for growth study. To ensure water was removed 2.0 g anhydrous sodium sulfate (previously oven dried at 110°C for 24 h) was added to each tube. After gently tapping tubes to mix soil and sodium sulfate, 30 mL of 1:1 hexane to acetone solution was added to each tube. Samples were then shaken at 120 rpm for 2 h. After shaking, samples were removed from the shaker and left to settle overnight. The following day, extracts were transferred to 60 mL amber vials. If samples were cloudy and did not settle overnight, they were centrifuged for 10 min at 180 g prior to transfer. Samples were then dried under N flow to approximately 0.5 mL. Condensed extracts were then transferred to 2 mL

GC vials. To maximize recovery of the extract, 60 mL vials were rinsed with 0.6 mL hexane twice. Each time the rinse was then transferred to the GC vial until a final volume between 1.5 and 2 mL was reached. Rather than measuring the exact volume of hexane added to each sample, the volume of hexane was calculated as follows:

$$(M_f - M_e)/d_{hexane} \quad \text{(Equation 4.3)}$$

where M_f is the mass of the full GC vial, M_e is the mass of the empty GC vial, and d_{hexane} is the density of hexane. Quantification of F2 and F3 fractions was completed by ALS Laboratory Group (Saskatoon, SK, Canada) using a gas chromatograph equipped with a flame ionization detector.

4.4.3.4 Statistical Analyses

Data was analyzed using RStudio (Version 1.1.383) statistical software. Both seed germination and various indicators of plant growth (dry biomass, height, etc.) were first analyzed with a one-way analysis of variance (ANOVA). If the ANOVA indicated significant differences, either Dunnett's test (using the package DescTools) was used to compare treatments within the same soil treatment to the control or a Tukey test (TukeyHSD in base R) was used to compare all treatments regardless of soil treatment. Figures were generated using the package ggplot2 (Wickham 2009).

4.5 Results

4.5.1 Isolate Selection for Preliminary in vivo Assays

Results from genotype and phenotype screening (Chapter 3) were pooled and converted to a percentage of positive attributes out of 13 total to determine which isolates showed the greatest plant growth-promoting potential (Appendix B). In total, 23 out of 58 (39.7%) isolates tested positive for 40% or more genes and/or phenotypes. After including isolates positive for between 35% and 40% and also tested positive or ACC deaminase activity, 13 isolates from Rotation ABC and 15 BPHS were selected for additional study. This represented 48.3% of all bacterial isolates.

4.5.2 Seed Germination

Seed germination tests were used to identify the effect of treating wheat and sweet clover seeds with bacterial isolates on germination rates. None of the treatments stimulated seed germination in wheat. However, 13 isolates belonging to the genera *Acinetobacter, Arthrobacter, Bacillus, Chryseobacterium, Delftia, Pedobacter, Pseudomonas, Rhodococcus, and Sphingomonas* significantly (ANOVA, $p = 1.3 \times 10^{-4}$; Fischer's LSD) inhibited seed germination after seven days (Figure 4.1).

In sweet clover, significant (ANOVA, $p = 1.6 \times 10^{-4}$; Fischer's LSD) stimulation and inhibition of seed germination was observed. Seven treatments with isolates belonging to the genera *Delftia, Kluyvera, Luteimonas, Pseudomonas, Rhodococcus, Salmonella, and Sphingomonas* promoted seed germination, and one pseudomonad was inhibitory (Figure 4.2).

4.5.3 Root Elongation

To assess the effect of bacterial isolates on root growth, roots of inoculated plants were measured seven and ten days after treatment for wheat and sweet clover respectively. When analyzing root length in wheat, twelve isolates significantly (ANOVA, $p = 1.3 \times 10^{-4}$); Fischer's LSD) promoted root elongation (Figure 4.3). More specifically, these were *Acinetobacter* isolate A2, *Chryseobacterium* isolate A7, *Delftia* isolate A9, *Kluyvera* isolate A12, *Rhizobium* isolate A22, *Rhodococcus* isolate A23, *Sphingomonas* isolate A25, *Rhodococcus* isolate B30, *Rhodococcus* isolate B31, *Sphingomonas* isolate B32, *Luteimonas* isolate B34, and *Stenotrophomonas* isolate B38. In wheat, no inhibition of root growth by any of the treatments was observed (Figure 4.3).

In sweet clover, significant (ANOVA, $p = 7.4 \times 10^{-6}$) promotion and inhibition of root growth was observed. Specifically, three bacteria—*Pseudomonas* isolate A3, *Delftia* isolate A9, and *Rhodococcus* isolate A23—promoted root elongation and two bacteria — *Sphingomonas* isolate A25 and *Pseudomonas* isolate B23— inhibited root elongation (Fischer's protected LSD) (Figure 4.4)

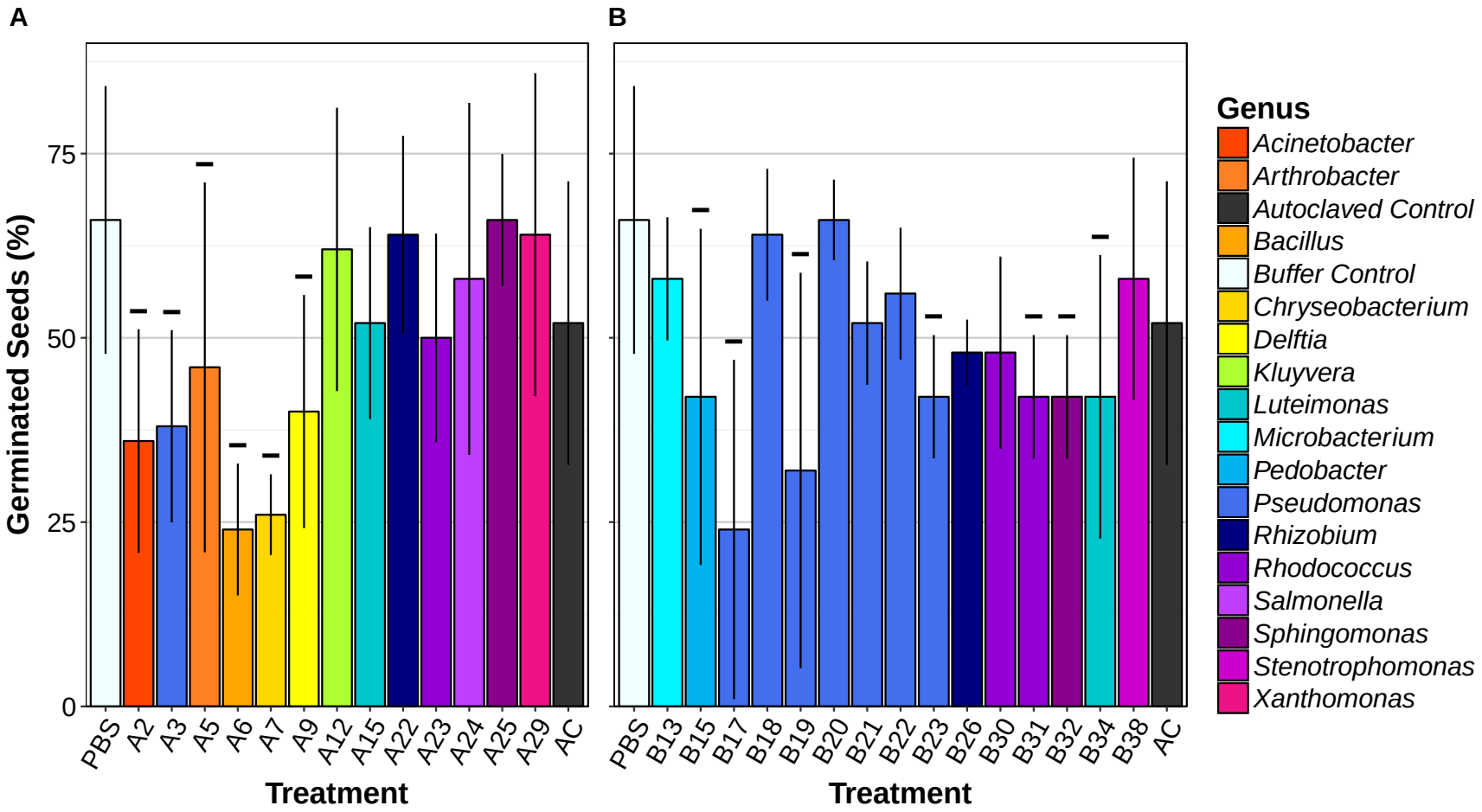

Figure 4.1. Wheat seed germination after inoculation with bacteria obtained from Rotation ABC (A) and Bitumount Provincial Historic Site (B). Error bars represent ± 1 standard deviation. Each colour represents the genus of the corresponding treatment (x-axis). The buffer control (PBS) and autoclaved control (AC) shown in panels A and B are the same. A minus sign (–) represents a significant decrease at in germination compared to the buffer control at $\alpha = 0.05$ (Fischer's LSD)

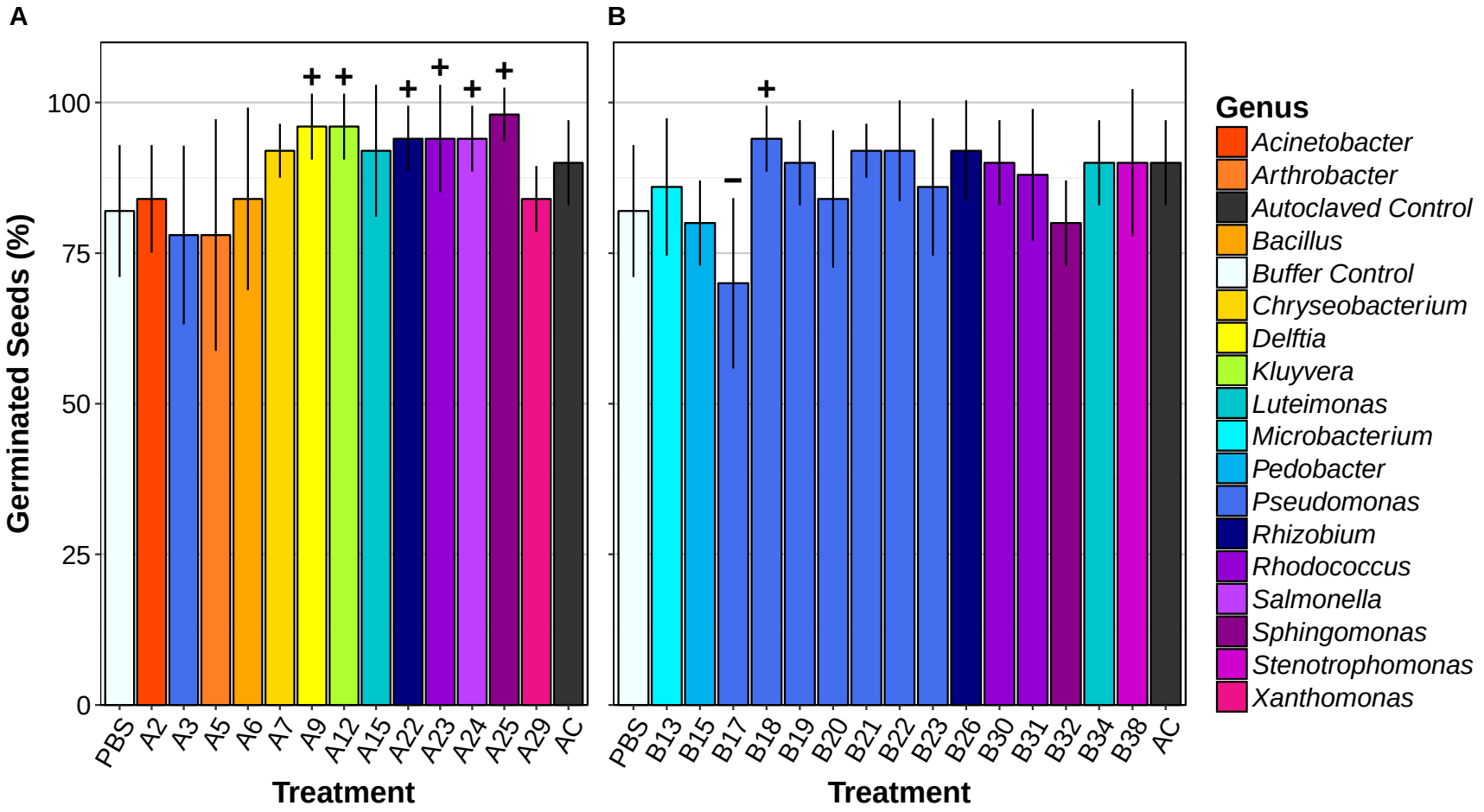

Figure 4.2: Sweet clover seed germination after inoculation with bacteria obtained from Rotation ABC (A) and Bitumount Provincial Historic Site (B). Error bars represent ± 1 standard deviation. Each colour represents the genus of the corresponding treatment (x-axis). The buffer control (PBS) and autoclaved control (AC) shown in panels A and B are the same. A plus sign (+) denotes a significant increase and a minus sign (–) a significant decrease in seed germination compared to the buffer control at $\alpha = 0.05$ (Fischer's LSD).

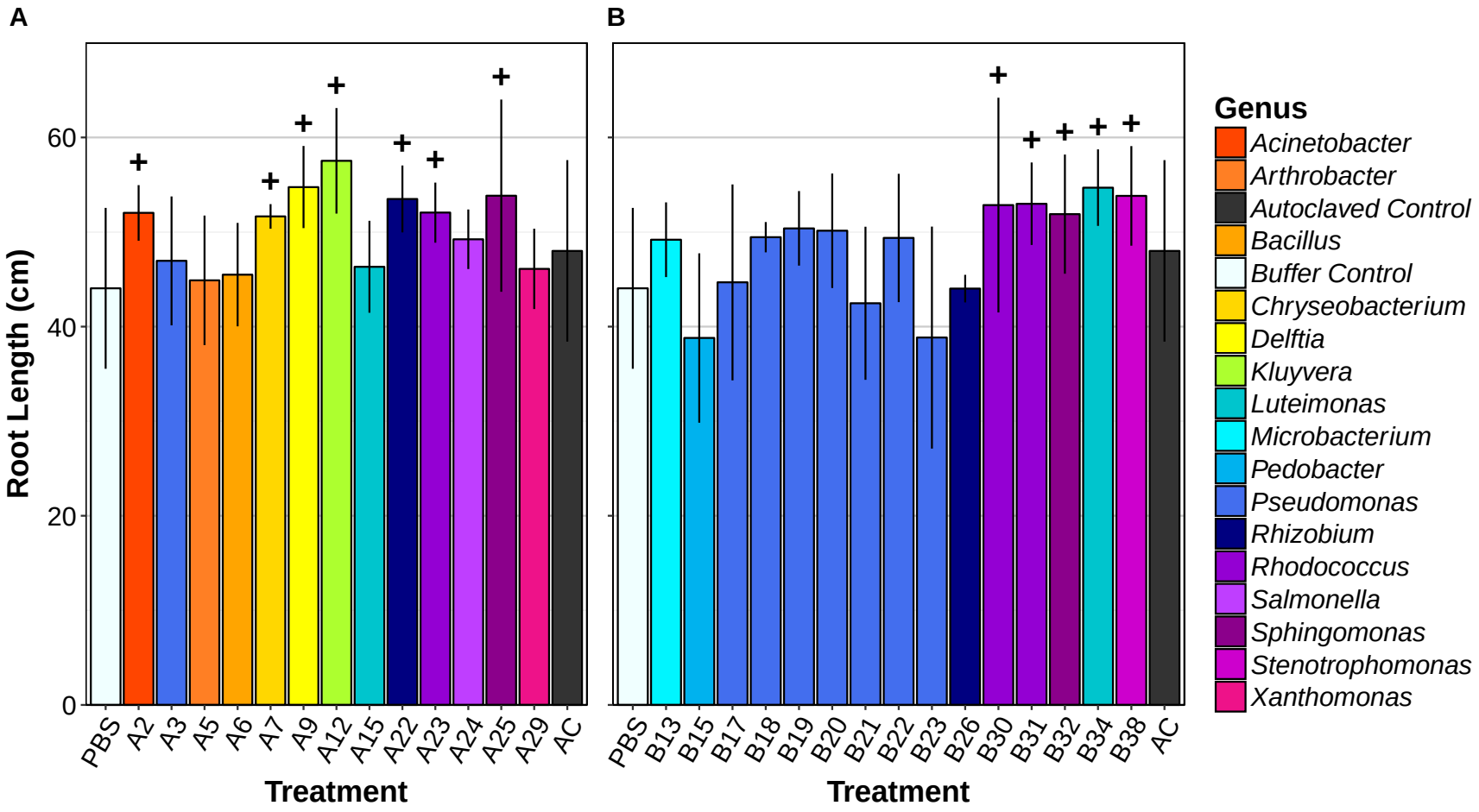

Figure 4.3: Wheat root lengths seven days after treatment with bacteria from Rotation ABC (A) or Bitumount Provincial Historic Site (B). Error bars represent ± 1 standard deviation. Each colour represents the genus of the corresponding treatment (x-axis). The buffer control (PBS) and autoclaved control (AC) shown in panels A and B are the same. A plus sign (+) denotes a significant increase compared to a buffer control at $\alpha = 0.05$ (Fischer's LSD).

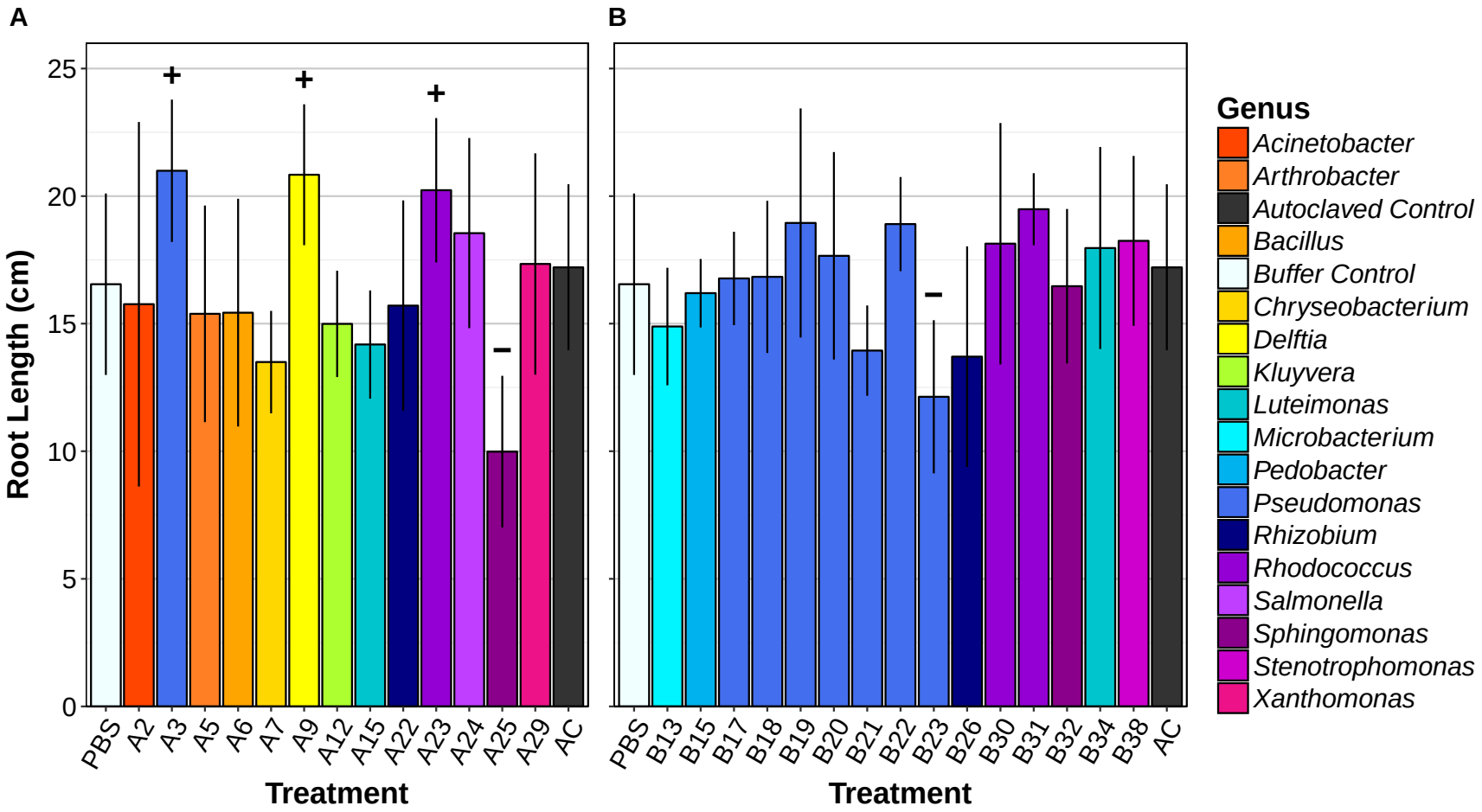

Figure 4.4: Sweet clover root lengths 10 days after treatment with bacteria from Rotation ABC (A) or Bitumount Provincial Historic Site (B). Error bars represent ± 1 standard deviation. Each colour represents the genus of the corresponding treatment (x-axis). The buffer control (PBS) and autoclaved control (AC) shown in panels A and B are the same. A plus sign (+) denotes a significant increase and a minus sign (–) a significant decrease in root length compared to a buffer control at $\alpha = 0.05$ (Fischer's LSD).

4.5.4 Response of Wheat and Sweet Clover to Inoculation with Bacteria in Soil With and Without Hydrocarbons

4.5.4.1 Wheat

Based on the results of seed germination and root elongation studies, *Kluyvera* isolate A12 and *Luteimonas* isolate B34 were selected for further study in wheat. Emergence was monitored for plants treated with either one of the two inoculants and PBS as a control after planting in either a marginal agricultural or hydrocarbon-amended soil.

No significant differences in emergence rates were found between seed treatments irrespective of soil treatment (ANOVA, $p = 0.306$) (Appendix C). No mortality was observed over the 60 day growth period in wheat plants grown in the marginal soil (Figure 4.5). Although some mortality was observed among wheat plants grown in hydrocarbon-amended soil, no significant differences were detected (ANOVA, $p = 0.552$) (Appendix C). However, due to mortality, only 3 replicates of B34 and Ctrl plants were viable and included in analysis. Additionally, due to decreased emergence in the presence of hydrocarbons and some mortality, several pots only had one plant instead of two at the end of the growth period. Interesting to note was that plants growing in pots with one plant generally had improved growth with biomass, height, and yield greater than individual plants growing in pots with two plants (Figure 4.6) indicating an effect from competition.

No significant differences were detected in the mean height of treated wheat plants grown in either the marginal or hydrocarbon-amended soil ($ANOVA^{Marginal}$, $p = 0.345$) and ($ANOVA^{HC}$, $p = 0.545$; Figure 4.7a). However, the mean heights of plants grown in the presence of hydrocarbons were significantly different from plants grown the same soil without diesel (ANOVA, $p = 2.2 \times 10^{-5}$; Tukey, $\alpha = 0.05$; Figure 4.7a). Further, there were no significant differences in the mean dry weights of either plants grown in the presence of diesel ($ANOVA^{HC}$, $p = 0.644$; Figure 4.7b) or plants grown in the same soil without the addition of hydrocarbons ($ANOVA^{Marginal}$, $p = 0.088$; Figure 4.7b).

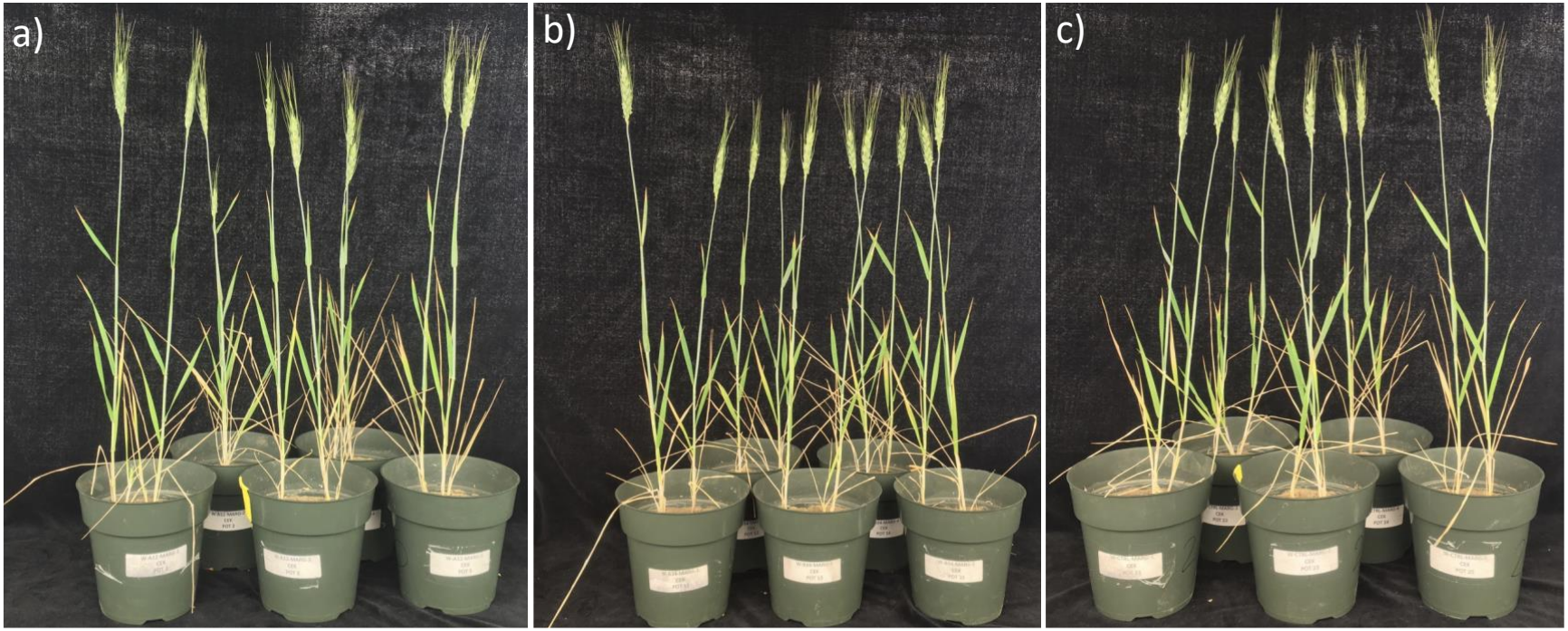

Figure 4.5: Wheat plants treated with isolate A12 (a), B34 (b), and no inoculant (c) grown in a marginal agricultural soil 60 days after planting.

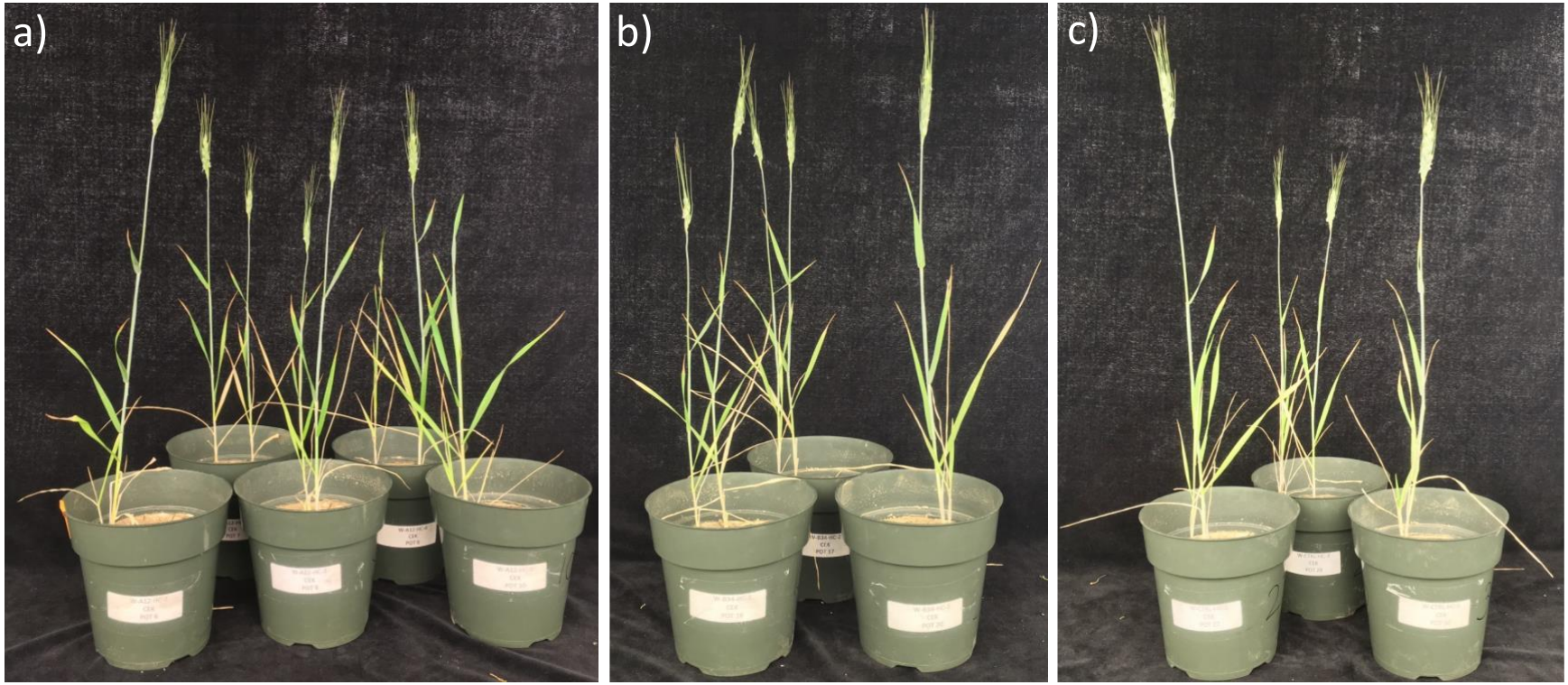

Figure 4.6: Wheat plants treated with isolate A12 (a), B34 (b), and no inoculant (c) grown in a marginal agricultural soil amended with diesel (5000 $mg \cdot kg^{-1}$) 60 days after planting.

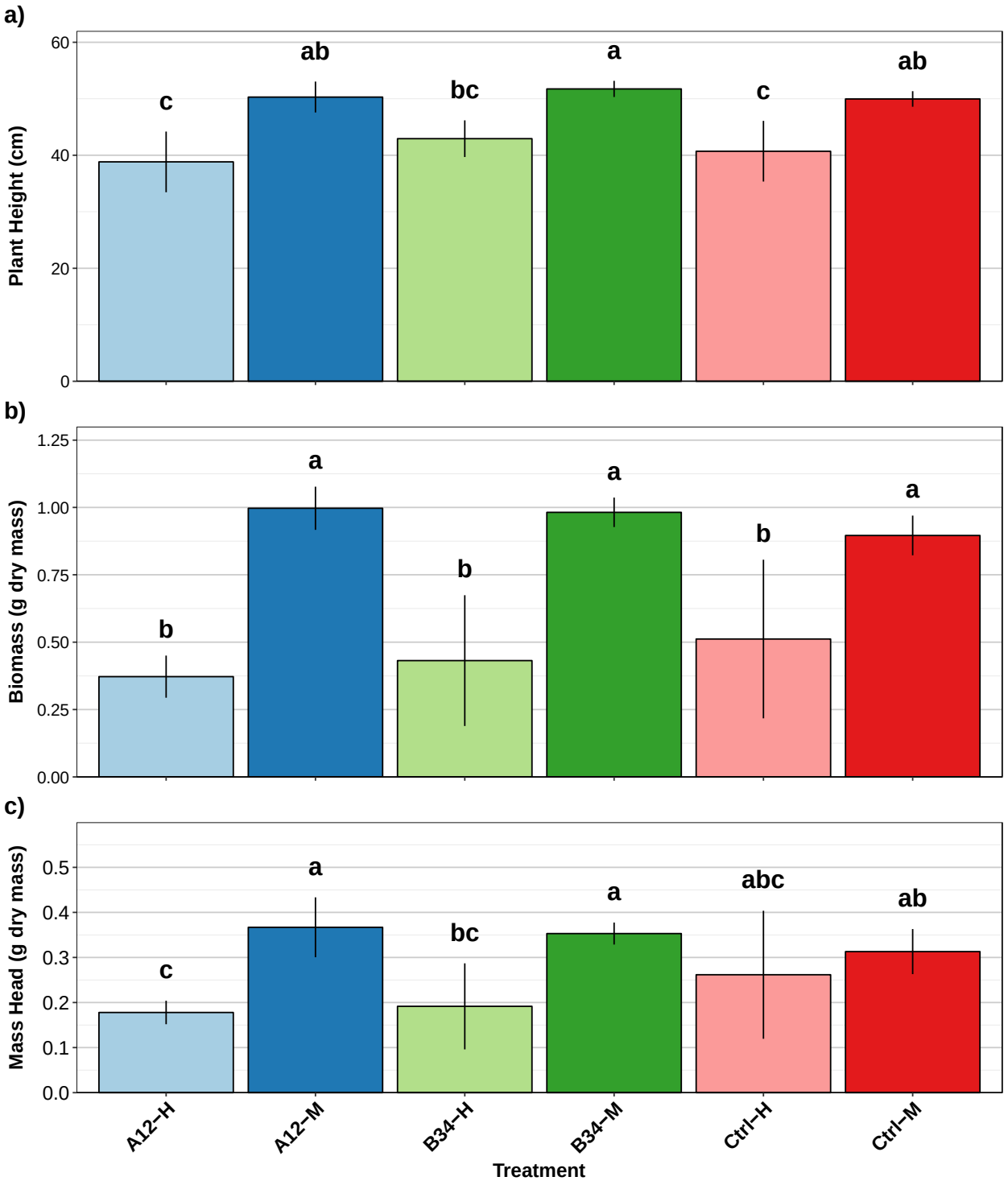

Figure 4.7: Plant height (a), dry biomass, (b) and potential yield (c) mean values in treated wheat plants 60 days after planting in soil with (dark shaded bars) and without (light shaded bars) diesel (5000 mg·kg^{-1}) and inoculated with bacteria. Error bars represent ± 1 standard deviation. Different letters denote a significant difference (Tukey HSD; $\alpha = 0.05$).

For potential yield, determined by comparing the dry weight of the head of the wheat plant, no significant differences were found between treated and control plants in either the hydrocarbon-amended ($ANOVA^{HC}$, $p = 0.444$) or marginal soil ($ANOVA^{Marginal}$, $p = 0.248$; Figure 4.7c). However, when analyzed together, there were differences detected between plants grown in marginal and hydrocarbon-amended soil (ANOVA, $p = 1.08 \times 10^{-3}$; Figure 4.7c).

Interesting to note is that although the potential yield of treated plants grown in hydrocarbon-amended soil was always significantly lower than their marginal counterpart, this was not the case for control plants (Figure 4.7c) which were the same in soils with or without added hydrocarbons (TukeyHSD, $p = 0.899$).

Significant differences in both NH_4^+ and PO_4^{3-} ($mg \cdot plant^{-1}$) were present in wheat plants growing in the marginal soil (ANOVA, $p = 0.00691$ for NH_4^+ and ANOVA, $p = 0.0472$ for PO_4^{3-}) (Figure 4.8). More specifically, NH_4^+ content in B34 plants growing in the marginal soil increased by 15% when compared to control plants (Dunnett's, $p = 0.0042$) (Figure 4.8a) and PO_4^{3-} increased by 10% (Dunnett's, $p = 0.0744$) (Figure 4.8b). In A12-M plants, NH_4^+ increased by 10% compared to control plants (Dunnett's, $p = 0.0441$; Figure 4.8a) and PO_4^{3-} content increased by 12% (Dunnett's, $p = 0.0434$) (Figure 4.8b).

Among wheat plants grown in hydrocarbon-amended soil, no significant effect was observed for either NH_4^+ (ANOVA, $p = 0.671$) or PO_4^{3-} (ANOVA, $p = 0.578$) content (Figure 4.8). Further, no significant difference in the degradation of F2 (ANOVA, $p = 0.202$) or F3 (ANOVA, $p = 0.386$) fractions of diesel fuel were detected among any of the treatments, HCS or T_0 control (Figure 4.9).

Important to note is that in plants grown in hydrocarbon-amended soil, a greater concentration of N and P was detected per gram of plant tissue (Appendix D). However, after accounting for the generally smaller size of these plants and adjusting for dilution (by converting to mg N/P per plant as opposed to mg N/P per g dry plant material) it is clear that N and P acquisition was greater in plants growing in soil free of hydrocarbons.

4.5.4.2 Sweet Clover

Survival rates of sweet clover plants grown in hydrocarbon-amended soil were especially low, leading to the loss of a majority of study replicates. Before omitting this soil treatment from the study, a second trial was conducted where planting method and the height of the lights were

adjusted. Then emergence and mortality rates were compared from the two trials. Though no significant differences were found between plants grown in a marginal agricultural soil from trial 1 and 2, in both cases treatment A9 promoted seed emergence (94% Trial 1 and 96% Trial 2) when compared to the control (54% Trial 1 and 56% Trial 2) (ANOVA, $p = 1.30 \times 10^{-4}$); Tukey HSD, $p < 0.05$) (Figure 4.10a).

In contrast to plants grown in unamended soil, significant differences in mean emergence rates were detected between trials for plants grown in hydrocarbon-amended soil (ANOVA, $p = 0.0856$) (Figure 4.10a). The emergence rate of A9 treated plants grown in the same soil amended with diesel from the first trial (78%) was significantly greater (Tukey HSD, $p = 0.0467$) than those in the second trial (38%) (Figure 4.10a). Although A9 emergence rates in the hydrocarbon-amended soil from the first trial were significantly greater than A9 plants in the second trial, they were not significantly different from either the control or A3 plants grown in the hydrocarbon-amended soil in either trial (Figure 4.10a).

Survival results indicate that hydrocarbons influenced sweet clover mortality. Mean survival rates among plants grown in marginal soil were all above 70%, and only marginally significant differences were detected between treatments (ANOVA, $p = 0.0867$) (Figure 4.10b and Figure 4.11). In hydrocarbon-amended soil, mean survival rates were below 30% and again no significant differences (ANOVA, $p = 0.878$) were detected between trials or subjects (Figure 4.10b and Figure 4.12).

No significant promotion of plant growth was observed in any of the treatments for plants grown in a marginal agricultural soil: plant height (ANOVA, $p = 0.213$), dry biomass (ANOVA, $p = 0.393$), of number of secondary shoots (ANOVA, $p = 0.936$) (Figure 4.13). Note that an analysis of treated plants in hydrocarbon-amended soil was omitted due to an insufficient number of replicates (Figure 4.12).

Considering nutrient uptake, no significant increase in NH_4^+ (ANOVA, $p = 0.843$) or PO_4^{3-} (ANOVA, $p = 0.856$) content was detected (Figure 4.14). Again, note than an analysis of treated plants in hydrocarbon-amended soil was omitted due to an insufficient number of replicates.

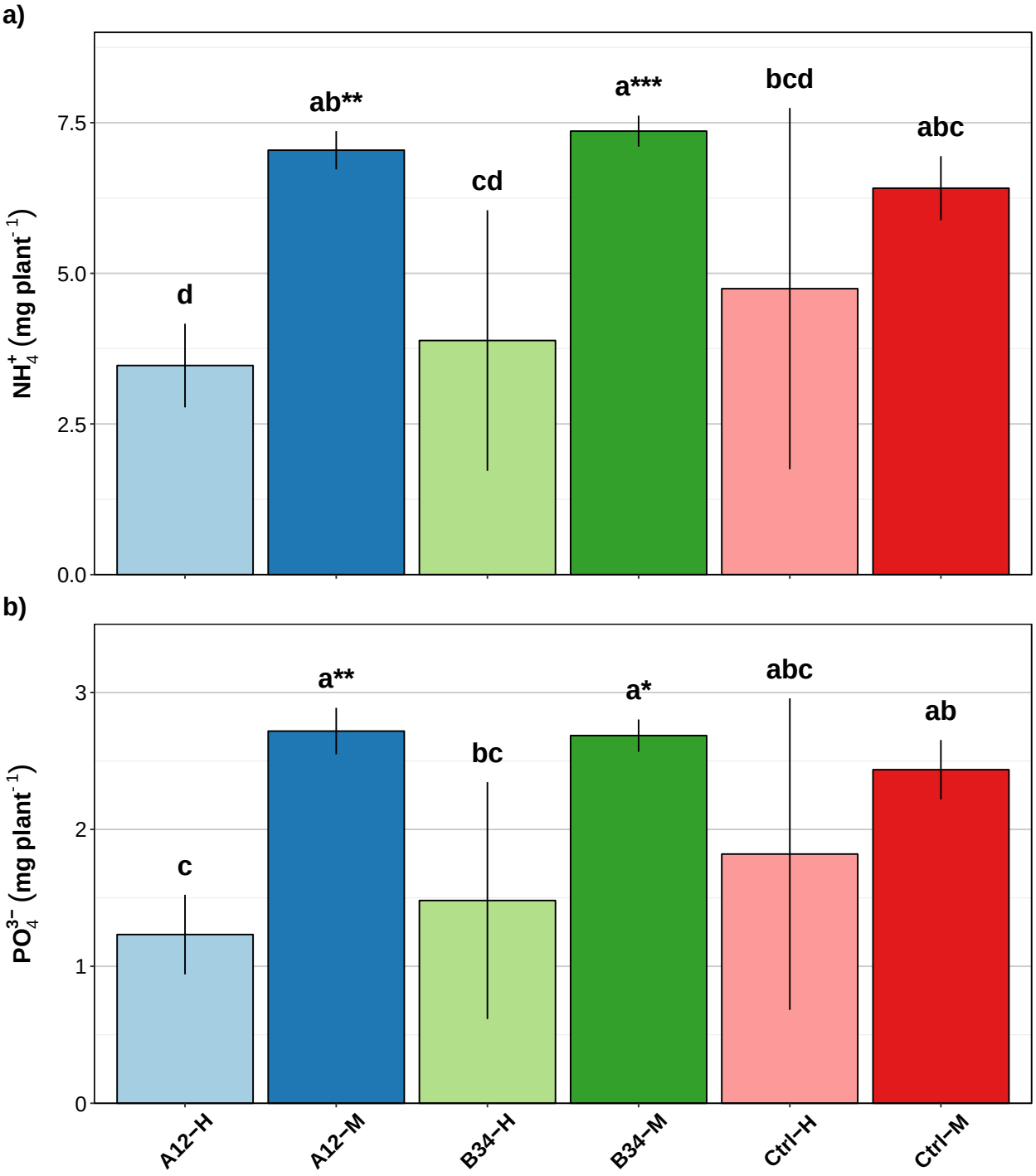

Figure 4.8: Concentration of NH_4^+ (a) and PO_4^{3-} (b) mean values in treated wheat plants 60 days after planting in soil with (dark shaded bars) and without (light shaded bars) diesel (5000 mg·kg^{-1}) and inoculated with bacteria. Error bars represent ± 1 standard deviation. Different letters denote a significant difference (Tukey HSD; a = 0.05) and asterisks denote a significant difference in plant growth compared to the uninoculated control (Dunnett's; * at α=0.1, ** at 0.05 and *** at .001).

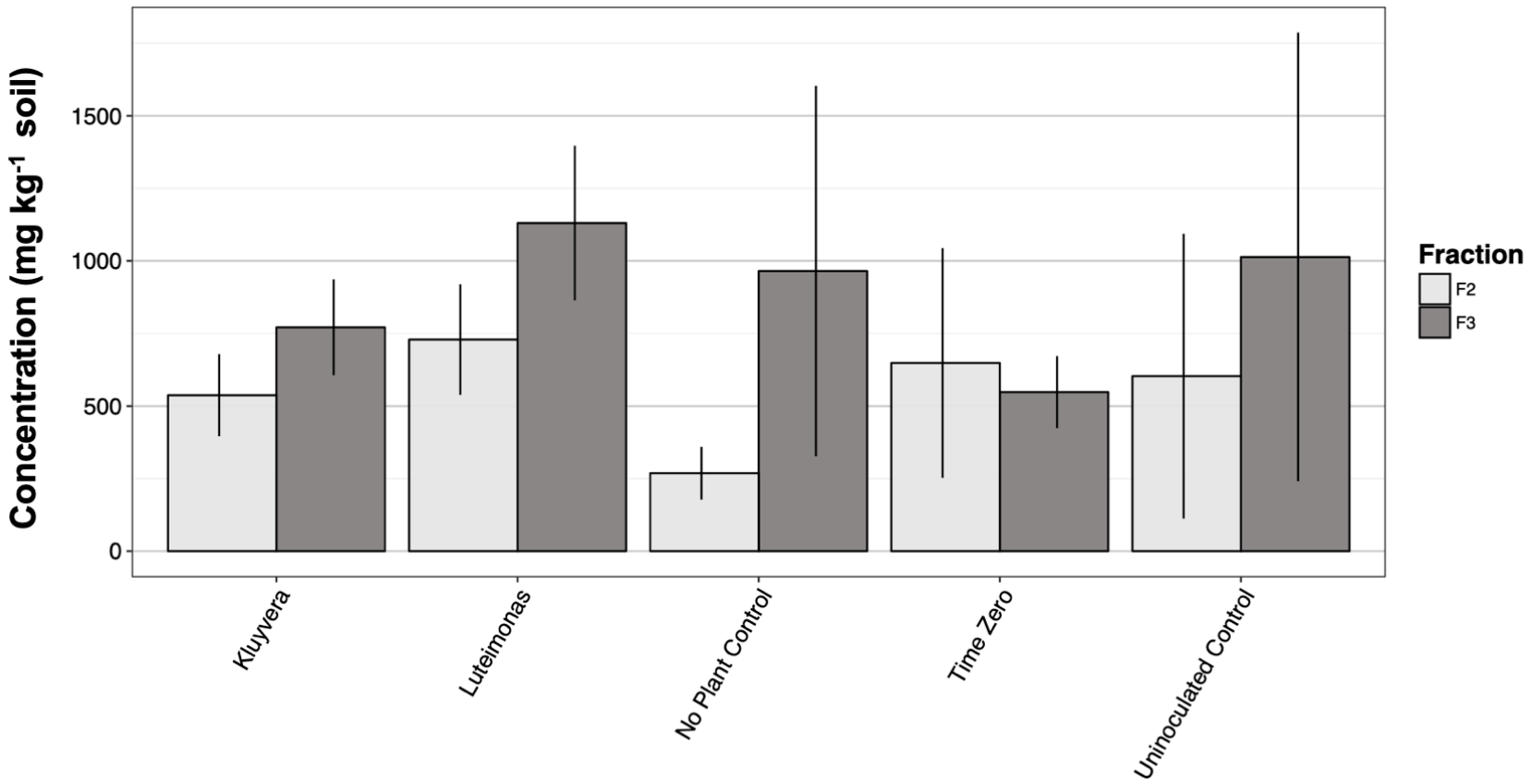

Figure 4.9: Concentration of F2 (light gray bars) and F3 (dark gray bars) fractions at harvest (60 days after planting, 70 days after addition of diesel). Error bars represent ± 1 standard deviation. No significant differences in degradation were detected in either the F2 or F3 fractions (ANOVA, $\alpha = 0.05$).

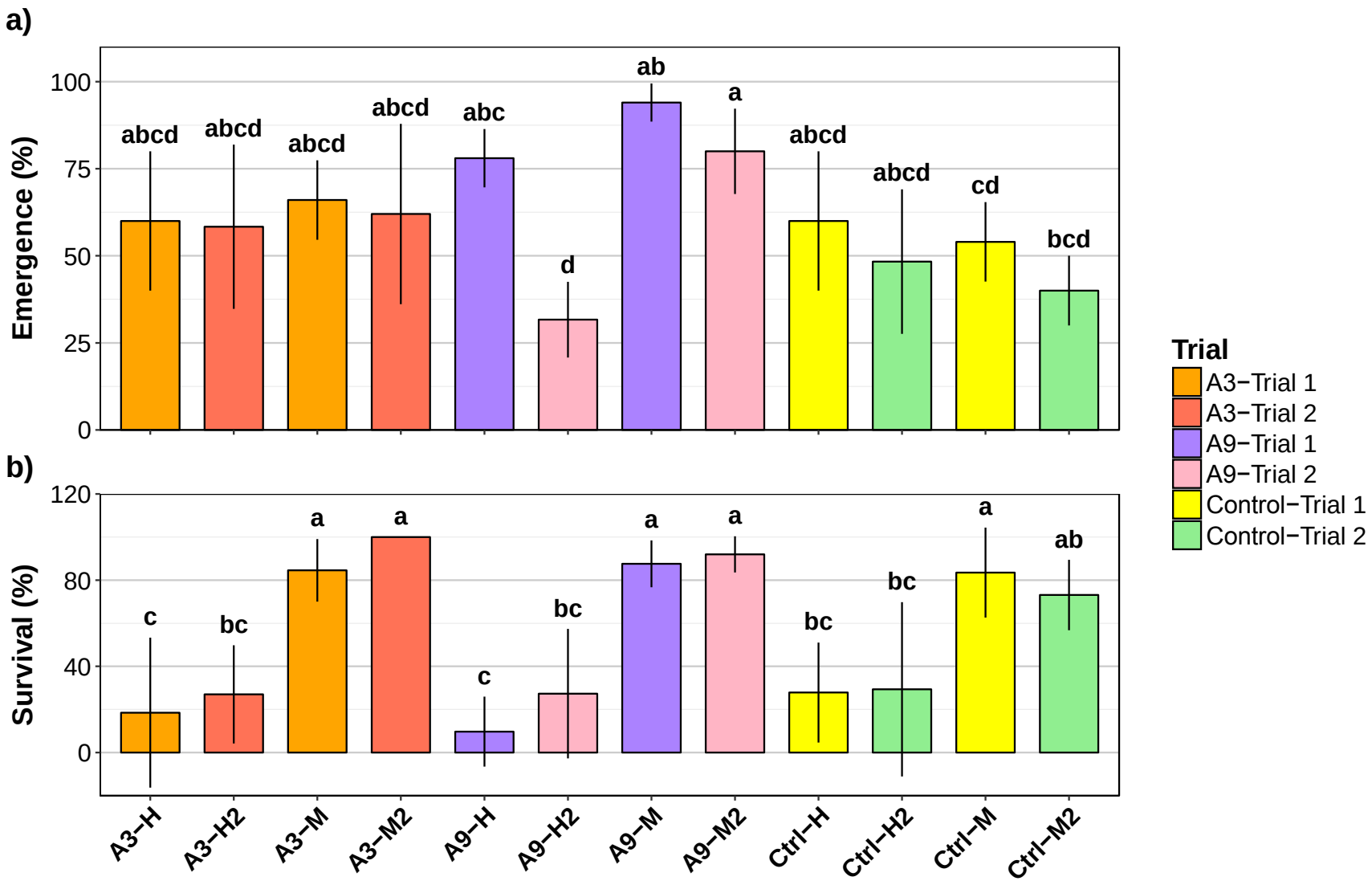

Figure 4.10: Sweet clover emergence (a) and survival (b) rates seven days after planting in diesel amended soil (5000 mg·kg^{-1}). Error bars represent ± 1 standard deviation. Colours are grouped by treatment and trial. Letters of significance were determined using Tukey HSD at $\alpha = 0.05$.

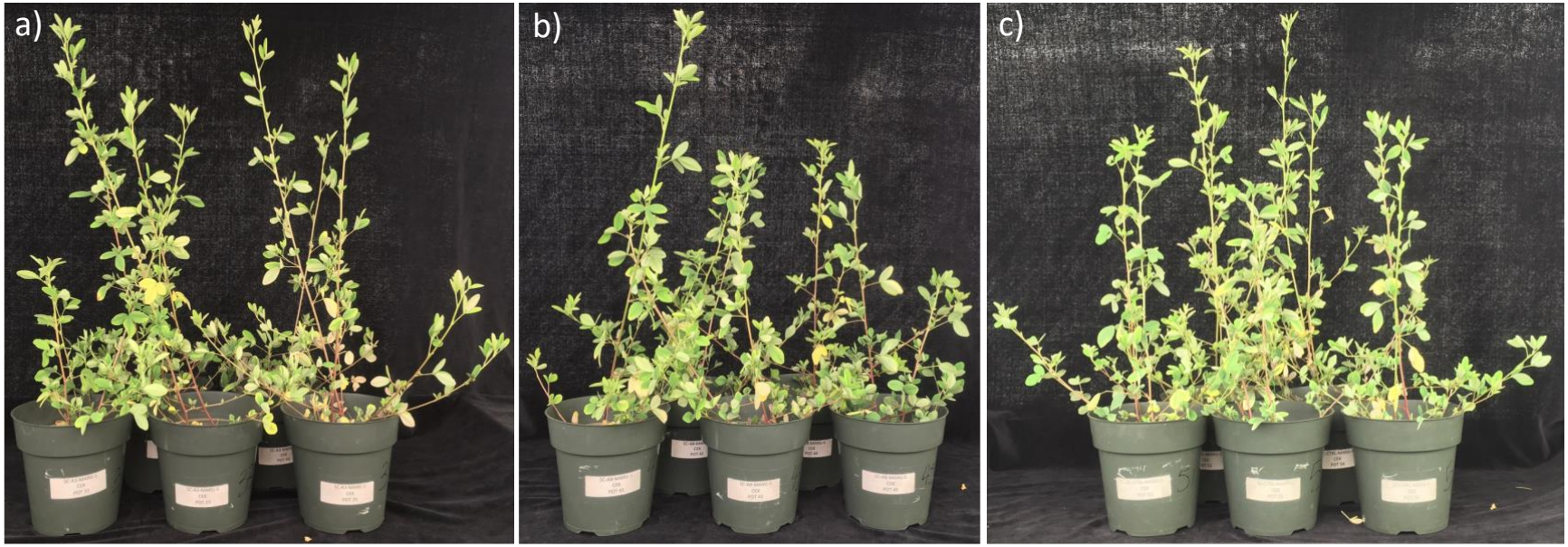

Figure 4.11: Sweet clover plants at 60 days after planting, grown in marginal soil and inoculated with bacterial isolate A3 (a), A9 (b) compared to an uninoculated control (c).

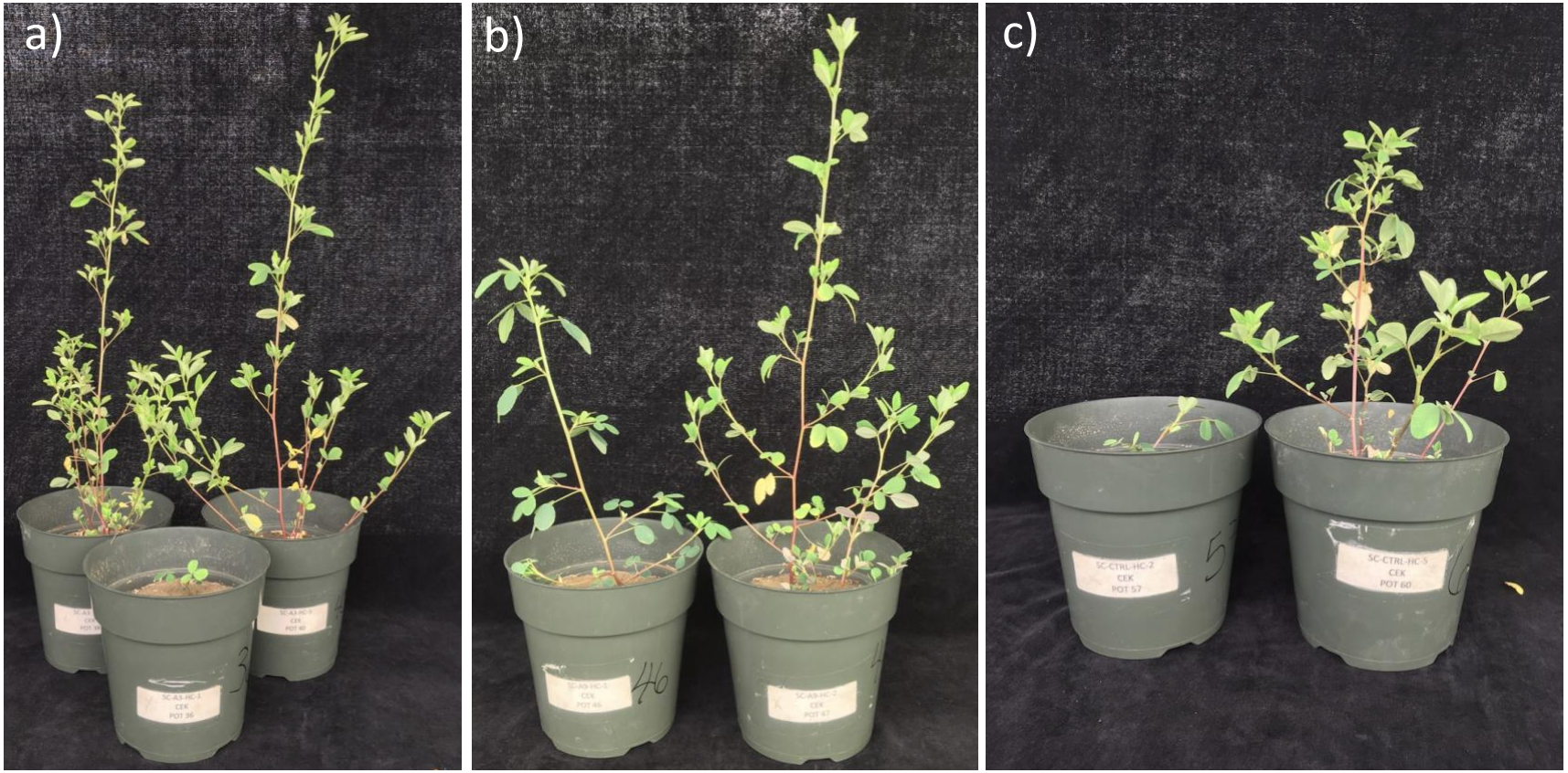

Figure 4.12: Sweet clover plants at 60 days after planting, grown in marginal soil amended with diesel (5000 mg·kg^{-1}) and inoculated with bacterial isolate A3 (a), A9 (b) compared to an uninoculated control (c).

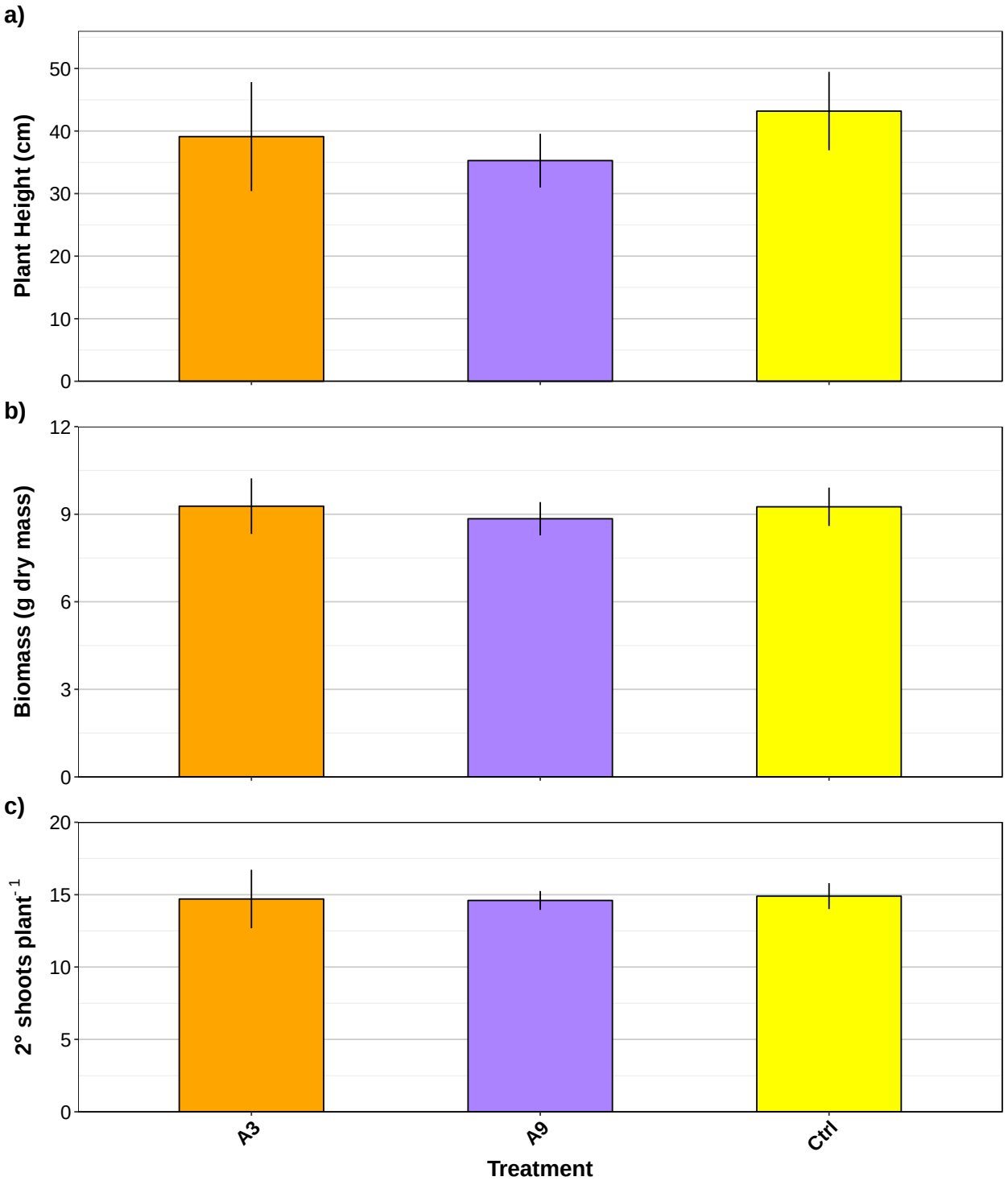

Figure 4.13: Plant height (a), dry biomass (b), and the number of secondary shoots (c) mean values of inoculated sweet clover plants 60 days after planting. Error bars represent ± 1 standard deviation. No significant differences were detected between treatments for plant height, dry biomass, or the number of secondary shoots (ANOVA, $\alpha = 0.05$).

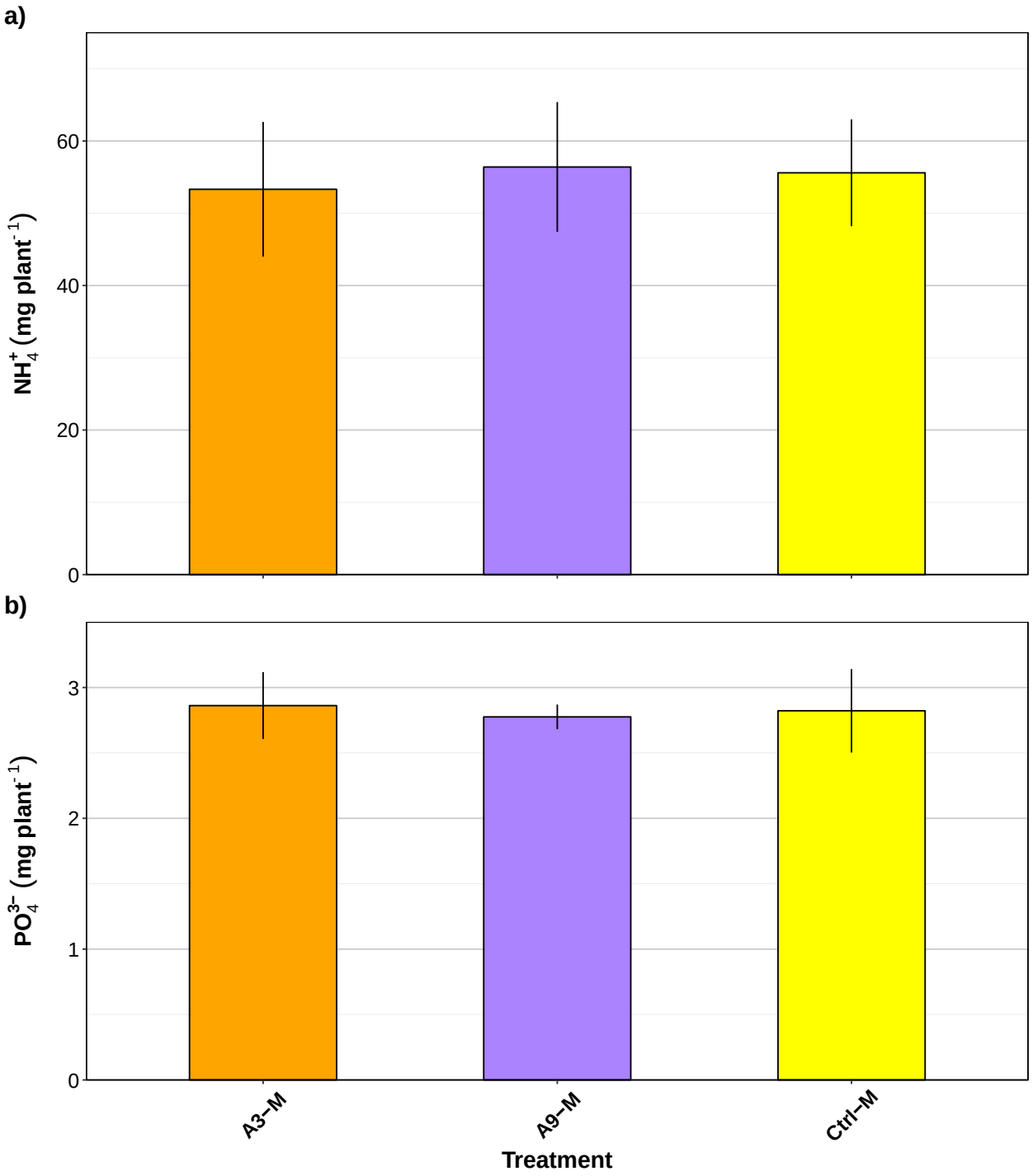

Figure 4.14: Concentration of NH_4^+ (a) and PO_4^{3-} (b) mean values in sweet clover plants. Error bars represent ± 1 standard deviation. No significant differences were detected between treatments for either NH_4^+ or PO_4^{3-} concentrations (ANOVA, $\alpha = 0.05$).

4.6 Discussion

This work assessed the plant growth-promoting capabilities of endophytes previously shown to have plant growth-promoting genotypes and or phenotypes with the aim of identifying new bacterial inoculants for use with plants grown in stressed environments. Wheat and sweet clover were chosen for testing because nearly half of the endophytes assessed originated from wheat and previous studies have suggested that leguminous plants, such as sweet clover, may be good candidates for use in the phytoremediation of hydrocarbon-contaminated soils (Adam and Duncan 2003, Hall et al. 2011, Kovalski Mitter 2018). Using *in vivo* assays as a screening tool, four isolates, two per plant type, were selected from those identified with increased plant growth-promotion potential (Chapter 3) for testing as inoculants in order to determine effects on plant growth and development under two soil conditions.

Several studies have used root elongation and seed germination as a measure of plant-endophyte compatibility and overall plant growth-promotion (Kloepper and Schroth 1981, Burd et al. 1998, Wang et al. 2000, Smyth et al. 2011, Rana et al. 2011, Montañez et al. 2012, Baig et al. 2012). Here, stimulation and inhibition of plant growth varied depending on the plant type, suggesting different plant-endophyte compatibility which is consistent with what has been previously reported (Rosenblueth and Martínez-Romero 2006, Hol et al. 2013, Hardoim et al. 2015). Further, isolates from the genera *Pseudomonas* had opposing effects on seed germination in sweet clover; *Pseudomonas fluorsecens* stimulated seed germination and *Pseudomonas brassicacearum* was inhibitory. This is in agreement with what has previously been reported among *Pseudomonas* species as several studies have shown some *Pseudomomas* species to promote plant growth (Glick et al. 1997, Vyas and Gulati 2009, Park et al. 2015, Oteino et al. 2015) while others studies have shown that some species of *Pseudomonas* cause disease (Buyer and Leongg 1986, Nomura et al. 2006, Nowell et al. 2016) .

The observation of opposing effects on plant growth by closely related species of bacteria is not limited to the genera *Pseudomonas* as effects on plant growth have been shown to be dependent on a variety of factors including but not limited to nutrient availability, plant species, and which bacterial species or even which strain was used (Hol et al. 2013). Thus, these results agree with previous work indicating that the effect of inoculation on plant growth will vary depending on the species. Further, these results serve to highlight the importance of plant-inoculant

compatibility testing as even closely related bacteria will sometimes have contrasting effects on plant growth.

Nearly all of the isolates identified to promote root elongation belong to genera—*Acinetobacter, Chryseobacterium*, *Delftia*, *Kluyvera*, *Pseudomonas, Rhizobium*, *Rhodococcus*, *Sphingomonas*, *Luteimonas*, and *Stenotrophomonas*—previously shown to contain plant growth-promoting bacteria (Burd et al. 1998, Sessitsch et al. 2002, Ryan et al. 2009, Marques et al. 2010, Rokhbakhsh-Zamin et al. 2011, Khan et al. 2014). That being said, the goal of the seed germination and root elongation studies was to identify isolates that will stimulate plant growth. Only the top two candidates in each plant type were considered for use in a pot-study experiment. In wheat, these isolates were *Kluyvera* isolate A12 and *Luteimonas* isolate B34 and in sweet clover *Pseudomonas* isolate A3 and *Delftia* isolate A9. These isolates were chosen because they all stimulated root growth and did not strongly inhibit seed germination. Of these isolates, only *Luteimonas sp.* has not been previously linked to plant growth-promotion. Still, bacteria belonging to the genera *Luteimonas* have been previously isolated from both the soil (Zhang et al. 2010) and plants (Dombrowski et al. 2017), indicating that they can be found within the plant-soil environment. Unlike *Luteimonas sp.*, studies have previously identified bacteria belonging to the genera *Kluyvera*, *Delftia,* and *Pseudomonas* as plant growth-promoting bacteria. For example, in a study by Burd et al. (1998), *Kluyvera ascorbata* was shown to increase tolerance to nickel toxicity as well as promote the growth of pearl millett seedlings. Further, Han et al. (2005) identified *Delftia tsuruhatensis* as a plant growth-promoting bacterium that works via mechanisms of biological control and increased N acquisition.

The efficacy of an inoculant can decrease in the field due to changes in pH, moisture, and interactions with native soil bacteria (Hol et al. 2013, Botta et al. 2013, Owen et al. 2015, Souza et al. 2015, Bacilio et al. 2017). In wheat plants grown in the absence of hydrocarbons, no significant increase in height or yield was detected. However, plants treated with isolates A12 and B34 had a significant increase in P uptake when compared to an uninoculated control. Many studies have demonstrated that plant growth-promoting bacteria can increase P uptake in plants (for review see Zaidi et al. 2009). This is accomplished through a variety of mechanisms including inorganic P solubilization via organic acid secretion (Rodríguez and Fraga 1999) and organic P breakdown via phosphatase and phytase activity (Nannipieri et al. 2011). Though previous work has shown *Kluyvera sp.* to be capable of P solubilization (Vazquez et al. 2000), the same does not

apply for *Luteimonas*. However, as demonstrated in Chapter 3, both isolates tested positive for alkaline phosphatase activity, suggesting that under P starvation these isolates can mineralize organic P (Nannipieri et al. 2011). The alkaline phosphatases have phosphomonoesterase and phosphodiesterase activity (Kageyama et al. 2011), which works to liberate soluble $H_2PO_4^-$ from organic compounds. Thus, the increase in available P via alkaline phosphatase activity may have contributed to the increased P uptake observed in inoculated wheat plants.

In addition to increased P uptake, inoculated wheat plants had significantly higher levels of N when compared to the uninoculated control. As shown in Chapter 3, neither of the isolates used in this study tested positive for N-fixation. Although biological nitrogen fixation is commonly used by plant growth-promoting bacteria to fix atmospheric N (Fisher and Newton 2002), it is not the only mechanism of increasing plant available N. Plants can use two forms of N, nitrate (NO_3^-) and NH_4^+ (Kraiser et al. 2011). Thus, processes that result in the increased availability of either of these forms of N will increase N availability.

Nitrogen, like P, exists in the soil in organic forms such as peptides and polymers of various amino sugars (Mengel 1996). Through a sequence of enzymatic processes, mineralizing microbes use enzymes such as proteases, deaminases, O-glycosidases, and acetyl hydrolases (Mengel 1996) to break down organic N into plant available forms such as NH_4^+ (Laughlin 2011). Nitrifying microbes take NH_4^+ and further convert it to nitrite (NO_2^-) and NO_3^- (Boyd 2015), a form of N that may be preferable to plants (Zhang et al. 2013). Although the isolates in this study were not tested for their ability to mineralize organic N or reduce nitrate, their involvement in these processes could lead to an increase in available soil N. In fact, a study by Zhang et al. (2010) found that *Luteimonas terricola*, a bacterium isolated from soil, was positive for nitrate reduction. Further, Zhang et al. (2015) found evidence of two nitrate reductases in the genome of *Luteimonas abyssi XH031*^{T}. This suggests that isolates belonging to this genus may be involved in N cycling and could help to explain the observed increase in N uptake by plants treated with *Luteimonas* isolate B34.

It is relevant to also consider how an increase in root length and surface area would impact plant growth. A stimulation of root growth and increase in the total area of the root surface interacting with the soil could potentially increase access to vital nutrients such as N and P (Kraiser et al. 2011). Both A12 and B34 promoted root growth. Therefore, although root length was not measured at the end of the 60 day growth period, it is conceivable that inoculation lead to a

promotion in root growth and development. In fact, a similar effect was observed by Bertrand et al. (2000) where inoculation of oilseed rape (*Brassica napus*) with *Achromobacter* increased root hair development and uptake of NO_3^-.

Unlike wheat, no significant effect on plant growth was detected in inoculated sweet clover plants grown in the marginal agricultural soil. Other studies have also observed that isolates with plant growth-promotion potential do not significantly promote plant growth when placed in a more complex environment (e.g. soil, the field). For example, in study by Rana et al. (2011) only three out of ten isolates selected based on the presence of four or more plant growth-promoting traits and positive effects on seed germination led to a promotion of wheat plant growth in a pot study. Further, Bacilio et al. (2017) tested the correlation of positive results from *in vitro* experiments with those obtained under greenhouse and field conditions. They found that the beneficial effect of plant growth-promoting bacteria was reduced in the greenhouse compared to *in vitro* testing, and even further reduced in the field. This work, combined with previous studies serves to reinforce the importance of rigorous *in vivo* testing under conditions that most closely reflect what will be encountered in the field.

Another reason the plant growth-promoting effect of an inoculant may be reduced when moving from *in vitro* and simple vs complex *in vivo* studies may be reduced activity, colonization, and/or survival. In order for plant growth-promoting bacteria to have an effect on plant growth, it must be able to survive within the plant-soil environment (Lugtenberg and Kamilova 2009). In fact, Smyth et al. (2011) further acknowledge the limitation of using *in vitro* analyses to select for plant growth-promoting bacteria in a study modeled similar to that executed herein. They found that although several bacterial candidates did well in *in vitro* testing, only one out of eighteen promoted plant growth in a glasshouse study. Furthermore, the expression of plant growth-promoting phenotypes and subsequent production of plant growth-promotion related compounds can be affected by interactions with the plant and other indigenous bacteria in the soil environment (Bashan et al. 2004, Hol et al. 2013). Botta et al. (2013), also observed that in a more complex environment, inoculants are sometimes unable survive, colonize, and proliferate sufficiently. Therefore, it is possible that *Pseudomonas* isolate A3 and *Delftia* isolate A9 were unable to maintain plant growth-promoting effects previously observed due to reduced plant growth-promoting activity and/or diminished survival.

Inoculated plants growing in diesel amended soil showed no significant promotion of growth compared to the uninoculated control in any of the plant growth-promotion indicators measured here. Further, no significant degradation of either the F2 or F3 fraction was detected in the hydrocarbon-contaminated soils growing inoculated wheat plants. Although many of the factors discussed previously can help explain why inoculation had no impact on plant growth or degradation, it is important to consider how the presence of hydrocarbons would have influenced the growth, success, and activity of the inoculants. In order for an inoculant to successfully promote plant growth, it needs to not only survive and proliferate but also maintain its plant growth-promoting capacity. Despite the conclusion that the beneficial effect of plant growth-promoting bacteria is increased as stress is increased (Glick 2010), this is only the case if these bacteria can survive and maintain their functionality. A study by Afzal et al. (2011) found that sandy soils with low organic matter content were the least conducive to not only plant growth but also the success of an inoculant. Further, Davis and Madsen (1996) found that biodegradation of toluene was much lower in sandy soils with low organic content (0.8%) compared to loam and clay soils with high (4.0 and 5.5% respectively) organic content. In both cases, the authors hypothesized that these observed decreases in degradation were because sandy soils tend to be drier, more porous, warmer, and less fertile than soils with a finer texture. These conditions are generally less favorable for both plant growth and microbial activity (Davis and Madsen 1996, Afzal et al. 2011). Compounded with the toxicity and reduced water availability caused by the presence of hydrocarbons, this strongly limits both plant and microbial growth and activity (Davis and Madsen 1996, Kirk et al. 2005). The soil used in this study was a sandy loam with low organic matter content (0.8%). Therefore, the diminished plant growth-promotion in wheat and sweet clover growing in hydrocarbon-amended soil can be attributed to the reduced ability of inoculants to not only survive in spite of increased toxicity but also colonize the plant.

The presence of hydrocarbons is also relevant when considering the interactions between a plant and an inoculant because the addition of diesel leads to a significant increase bioavailable carbon (C), which in turn alters the ratio of C to N present in the soil. Xu and Johnson (1997) demonstrated that in a hydrocarbon-contaminated soil, microbial activity is stimulated which in turn leads to the immobilization of available N in microbial biomass. This in turn leads to a reduction in the amount of N available for plant uptake (Xu and Johnson 1997, Adam and Duncan

2003). Consequently, it becomes possible for the presence of a hydrocarbon-adapted plant growth-promoting bacteria to become detrimental to the plant as competition for N is increased.

4.7 Conclusion

In an effort to identify novel plant growth-promoting bacteria with potential for use in both agriculture and phytoremediation, this work investigated the plant growth-promoting effect of various bacterial isolates on wheat and sweet clover plants in a marginal agricultural and hydrocarbon-contaminated soil. *In vivo* testing demonstrated that several candidates led to a decrease in seed germination and root elongation indicating poor plant compatibility. Nevertheless, several isolates did promote root elongation and were selected for testing in a long-term growth chamber experiment. Not all isolates selected promoted plant growth; none of the treatments promoted sweet clover growth and in hydrocarbon-contaminated soil plant growth was strongly inhibited with no significant degradation detected. However, plant growth-promotion in wheat was detected by *Kluyvera* isolate A12 and *Luteimonas* isolate B34 as they increased both N and P when compared to an uninoculated control. Therefore, although none of the isolates tested here indicate potential for use in enhanced phytoremediation, isolates A12 and B34 may be candidates for use as biofertilizers in an agricultural context.

5. GENERAL DISCUSSION

The use of plant growth-promoting bacteria is a natural, sustainable approach to dealing with a wide array of problems. Not only can these bacteria be used to increase crop production, but they can also take land that was formerly unsuitable for plant growth due to factors such as salinity and drought and make them more tolerable. Further, the use of biodegrading bacteria with plant growth-promotion capabilities has been used to enhance the process of phytoremediation, thereby restoring degraded, contaminated land for human use. Although work has been done to identify plant growth-promoting bacteria that can be used as inoculants, there is still a great need to identify more, better plant growth-promoting bacteria with different applications in a wide variety of plants and soils. Plant growth-promoting bacteria are commonly selected for by plants living in stressful environments (Siciliano et al. 2001). Seeking out plant growth-promoting bacteria living in association with plants under such conditions is an approach to identify newer, better, plant growth-promoting bacteria. Here, plants growing at two field sites, Rotation ABC and BPHS, provide a potential reservoir for plant growth-promoting bacteria because these locations are characterized by a wide variety of stressors including severe nutrient stress, degraded soil quality, and the presence of harmful contaminants.

The first objective of this work (Chapter 3) was to identify root endophytes from genera common to a long-term nutrient-deficient agricultural site (Rotation ABC) and a hydrocarbon-contaminated site (BPHS) and screen them for specific plant growth-promoting genotypes and associated phenotypes. Genotypes were selected for study based on previous work that has related them to specific plant growth-promoting activities. In this study, the main areas of focus were increased nutrient acquisition via biofertilization (*nifH* and nitrogen fixation; *phoD* and phosphorous acquisition), stress reduction via phytostimulation (*acds* and ACC deaminase), and hydrocarbon degradation (*alkB* and *CYP153* involved in the production of two different alkane hydroxylases) because these processes are all involved in mitigating the stressors present at

Rotation ABC and BPHS (Van Beilen and Funhoff 2007, Kageyama et al. 2011, Nikolic et al. 2011, Gaby and Buckley 2012). To date, several studies screen for plant growth-promoting bacteria based on phenotypes (Marques et al. 2010, Montañez et al. 2012, Laslo et al. 2012, Ipek et al. 2014, Ji et al. 2014, Kumar et al. 2014, Majeed et al. 2015). Still, far fewer have investigated the potential of screening for these organisms based on the relationship of plant growth-promotion phenotypes to associated genotypes. By using a combined method of genotype (PCR) and phenotype (biochemical assays) screening, several isolates belonging to genera present at both locations were shown to have the genetic and/or physiological potential for plant growth-promotion. Although the selected genotypes and associated phenotypes are well studied in the context of plant growth-promotion, the results presented in Chapter 3 demonstrated that there may be additional molecular pathways that will still result in a plant growth-promoting phenotype because isolates negative for a selected plant growth-promoting genotype were commonly positive for an associated phenotype. This most likely occurred as a result of the expression of an alternative plant growth-promotion gene as other mechanisms have been elucidated (Monds et al. 2006, Van Beilen and Funhoff 2007). In addition, this chapter demonstrated that the presence of a plant growth-promoting genotype does not always mean that an isolate harbors the full genetic potential to induce expression. Although the genes selected in this study are key functional genes for enzymes involved in plant growth-promotion, there are several other genes that must be present for complete assembly and expression (Vershinina and Znamenskaya 2002, Monds et al. 2006, Rubio and Ludden 2008, Nikolic et al. 2011). Therefore, if any of these genes are missing or disrupted, then the plant growth-promotion potential of that isolate is lost.

The second objective of this study was to assess the actual plant growth-promoting capabilities of isolates identified to have increased plant growth-promotion potential based on the genotype/phenotype screening conducted in Chapter 3. As such, in Chapter 4 a greater focus was placed on *in vivo* testing of isolates selected according to indications of increased plant growth-promotion potential (i.e. the presence of multiple plant growth-promoting phenotypes and genotypes). Based on preliminary seed germination and root elongation studies, conducted to ensure inoculation did not negatively impact early plant growth, several isolates were eliminated from further study as either no or an inhibitory effect was observed. This was consistent with what has been previously reported where isolates, regardless of plant growth-promotion characteristics, had no impact on plant growth when used as inoculants (Chen et al. 2010, Kumar et al. 2014), and

serves to highlight the importance of plant-endophyte compatibility testing. Nevertheless, two isolates per plant type—*Psuedomonas* isolate A3 and *Delftia* isolate A9 for sweet clover and *Kluyvera* isolate A12 and *Luteimonas* isolate B34 for wheat—were identified to stimulate early plant growth in preliminary analyses and were selected for further study. Of these, three out of the four isolates belonged to genera previously shown to be involved in plant growth-promotion (Burd et al. 1998, Han et al. 2005, Hol et al. 2013), though not in either of the plant types considered here. Further, neither had been previously tested in a hydrocarbon-contaminated soil. To assess how isolates would function in a more complex soil environment, a growth chamber experiment was conducted wherein inoculated wheat and sweet clover plants were grown in both a marginal agricultural soil and the same soil amended with diesel fuel. Here, no growth-promotion was observed in treated sweet clover plants in either soil treatment. Additionally, in both wheat and sweet clover plants growing in hydrocarbon-amended soil, no significant degradation was detected. Although preliminary results were promising for these candidates, this phenomena of decreased efficacy with increased complexity has been observed previously (Smyth et al. 2011, Botta et al. 2013, Bacilio et al. 2017). In fact several studies discuss the limitations of using *in vitro* analyses to test for plant growth-promotion, and emphasize that more focus should be placed on better understanding the interactions that occur between inoculants and indigenous microorganisms (in the plant and soil)(Smyth et al. 2011, Rana et al. 2011, Botta et al. 2013, Bacilio et al. 2017).

Although no promotion was observed in sweet clover plants or in either plant type in hydrocarbon-contaminated soil, inoculated wheat plants growing in a marginal agricultural soil had significantly greater N and P uptake. These observations can be directly linked back to the results obtained in Chapter 3 because these two isolates were positive for alkaline phosphatase activity and ACC deaminase activity. Alkaline phosphatase activity would contribute to increased P acquisition via the breakdown of organic P (Nannipieri et al. 2011). Further, ACC deaminase activity would impact both N and P acquisition by lowering ethylene levels, which in turn may lead to an increase in root growth and the total area of roots interacting with the soil thereby increasing access to essential nutrients (Kraiser et al. 2011). While neither A12 nor B34 tested positive for N fixation, there is evidence that B34 may be involved in the N cycle (Zhang et al. 2010), and as such may have contributed to an increase in N acquisition by increasing available N

and NO_3^- in the soil. This when considered with the increased root growth and development, an effect demonstrated in Chapter 4, can help explain increased N acquisition.

In all, these results are consistent with the conclusions of Baig et al. (2012), which found that plant growth-promoting bacteria with more than one plant growth-promoting characteristic can have a greater impact on plant growth than plant growth-promoting bacteria with only one growth-promoting characteristic. This in turn suggests that isolates with more than one plant growth-promoting characteristic are more likely to behave as a superior inoculant when compared to isolates with just one trait. However, as demonstrated in Chapter 4, the effect of plant growth-promoting bacteria is also critically dependent on the survival and success of an inoculant in a plant/soil environment (Lugtenberg and Kamilova 2009). Therefore, work that identifies isolates able to survive and colonize within their environment while possessing several plant growth-promoting characteristics will be key in identifying plant growth-promoting bacteria for use as inoculants.

6. CONCLUSIONS AND FUTURE WORK

In the next 30 years, the needs of the global community are going to change as resources such as food and land become more limited. Although the application of chemical fertilizers has made it possible to provide for human needs to date, these methods have been shown to result in negative environmental consequences and are generally considered to be unsustainable. Further, current methods used to increase crop production do little to address the problem of contaminated land, which will become ever more necessary for both habitation and crop production. Thus, is it imperative that these issues are addressed proactively, with a concentration on both current and future interests. The focus of this work was to survey root endophytes isolated from stressful environments for plant growth-promoting capabilities. Based on the results from Chapter 3, it can be concluded endophytes belonging to genera common to both Rotation ABC and BPHS harbor the genetic and physiological potential for plant growth-promotion. Further, among these isolates several demonstrated increased plant growth-promotion potential as they tested positive for multiple plant growth-promoting genotypes and/or associated phenotypes. The results of Chapter 4 lead to the conclusion that although screening for plant growth-promoting phenotypes and genotypes may help to identify plant growth-promoting bacteria among a larger collection of environmental isolates the efficacy of bacterial seed treatment will vary *in vivo*; this is not only from one plant type to another, as demonstrated in early seed germination and root elongation studies, but also in different environments, as demonstrated in the long-term growth chamber experiment. In all, this work serves to emphasize the importance of rigorous *in vivo* testing. Even with phenotype and genotype screening *and* preliminary root elongation and seed germination testing only two out of the four isolates tested in a long-term growth chamber experiment demonstrated any plant growth-promoting effect, and none of the isolates contributed to the degradation of hydrocarbons.

The observations and conclusions of this thesis suggest several possibilities for future work. One area worth considering is testing *Kluyvera* isolate A12 and *Luteimonas* isolate B34 under more variable, field-realistic conditions. Although the goal is to identify inoculants that can be used in the field, further testing starting in a greenhouse would make it possible to see if variable light and temperature diminish the plant growth-promoting effects observed in Chapter 4. If those

effects are maintained, then moving forward with the more time and cost intensive task of conducting a field trial would be essential.

Another opportunity for future work would be incorporating the concept of a consortia rather than the more common single inoculant approach. In this application, several plant growth-promoting bacteria shown to have multiple plant growth-promoting traits are used as a mixed inoculant (Rana et al. 2012, Thijs et al. 2014, Inostroza et al. 2017). This increases the likelihood of at least one inoculant surviving and thriving thereby increasing overall potential for plant growth-promotion. For example, an inoculant that contains both *Kluyvera* isolate A12 and *Luteimonas* isolate B34 could result in a greater promotion of plant growth and nutrient acquisition as the biofertilizing effect could be increased through the combined action of each isolate. Although this type of work will require a better understanding of not only how inoculants behave and compete with each other but also how a mixed inoculant would interact with the plant/soil microbiome, it provides an attractive avenue for future research.

Despite the fact that there is still not a strong understanding of how isolates within a mixed inoculant may interact with each other, no less the microbiomes of the plant and soil they will be influencing, future work utilizing consortias in hydrocarbon-contaminated soils may be more effective at developing inoculants. Here it was hypothesized that the plant growth-promoting effect of isolates A12 and B34 were lost when seeds were planted in the diesel amended soil due to reduced activity, colonization, and/or survival. However, when utilizing a consortium the probability of survival increases as well as the potential for a cooperative effect. Therefore, one project could focus on testing the effect of a mixed inoculant containing *Kluyvera* isolate A12 and *Luteimonas* isolate B34 in a diesel amended soil.

The final, and perhaps most important, area for future work should aim to develop a more efficient, effective, and lasting method of seed treatment. In order for an inoculant to become commercialized, treatment must yield seeds that are shelf-stable, thoroughly colonized, and able to maintain functionality over time and changing conditions (temperature, moisture, etc.). Although the approach used in this work has been shown to be effective in other studies, better methods need to be established in protocols so that results can be readily translated to an industrial scale.

Broadly speaking, this work found that potential inoculants can be identified among environmental isolates belonging to bacterial genera common to two different stressed

environments. The conclusions discussed here have provided direction for several areas of future work. By combining the development and implementation of a better seed treatment with broader, more expansive approaches to inoculant testing (e.g. consortia, colonization studies), a better method of delivery and application of bacterially treated seeds can be further elucidated. Furthermore, as an enhanced understanding of both the rhizosphere and endosphere is developed it will become easier to anticipate how inoculants will not only interact with indigenous microorganisms but also with the surrounding environment. In doing so, the potential to identify more effective and reliable inoculants will be increased. Thus, in time the results of this and future work can be successfully applied to either promote plant growth and yield in an agricultural context and mitigate the negative effect of stress in contaminated sites for enhanced phytoremediation.

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REFERENCES

Abeles, F.B., Morgan, P.W., and Saltveit, M.E. (Mikal E. 1992. Ethylene in plant biology. Academic Press.

Adam, G., and Duncan, H. 2003. The effect of diesel fuel on common vetch (Vicia Sativa L.) plants. Environ. Geochem. Health **25**(1): 123–130. Kluwer Academic Publishers. doi:10.1023/A:1021228327540.

Afzal, M., Yousaf, S., Reichenauer, T.G., Kuffner, M., and Sessitsch, A. 2011. Soil type affects plant colonization, activity and catabolic gene expression of inoculated bacterial strains during phytoremediation of diesel. J. Hazard. Mater. **186**(2–3): 1568–1575. Elsevier. doi:10.1016/J.JHAZMAT.2010.12.040.

Afzal, M., Yousaf, S., Reichenauer, T.G., and Sessitsch, A. 2013. Ecology of alkane-degrading bacteria and their interaction with the plant. *In* Microbial Ecology of the Rhizosphere.

Alberta Culture and Tourism. (n.d.). Bitumount - Alberta's Energy Heritage. Available from http://history.alberta.ca/energyheritage/bitumount/Default.aspx [accessed 8 March 2017].

Alexandratos, N., and Bruinsma, J. 2012. World agriculture towards 2030/2050: the 2012 revision. Rome. Available from www.fao.org/economic/esa [accessed 14 July 2018].

Ali, H., Khan, E., and Sajad, M.A. 2013. Phytoremediation of heavy metals—Concepts and applications. doi:10.1016/j.chemosphere.2013.01.075.

Andria, V., Reichenauer, T.G., and Sessitsch, A. 2009. Expression of alkane monooxygenase (alkB) genes by plant-associated bacteria in the rhizosphere and endosphere of Italian ryegrass (Lolium multiflorum L.) grown in diesel contaminated soil. Environ. Pollut. **157**(12): 3347–3350. doi:10.1016/j.envpol.2009.08.023.

Arslan, M., Afzal, M., Amin, I., Iqbal, S., and Khan, Q.M. 2014. Nutrients can enhance the abundance and expression of alkane hydroxylase CYP153 gene in the rhizosphere of ryegrass planted in hydrocarbon-polluted soil. PLoS One **9**(10): e111208. Public Library of Science. doi:10.1371/journal.pone.0111208.

Bacilio, M., Moreno, M., Lopez-Aguilar, D.R., and Bashan, Y. 2017. Scaling from the growth chamber to the greenhouse to the field: demonstration of diminishing effects of mitigation of salinity in peppers inoculated with plant growth-promoting bacterium and humic acids. Appl. Soil Ecol. **119**: 327–338. Elsevier. doi:10.1016/J.APSOIL.2017.07.002.

Baig, K.S., Arshad, M., Shaharoona, B., Khalid, A., and Ahmed, I. 2012. Comparative effectiveness of Bacillus spp. possessing either dual or single growth-promoting traits for improving phosphorus uptake, growth and yield of wheat (Triticum aestivum L.). Ann. Microbiol. **62**(3): 1109–1119. Springer-Verlag. doi:10.1007/s13213-011-0352-0.

Bais, H.P., Weir, T.L., Perry, L.G., Gilroy, S., and Vivanco, J.M. 2006. The role of root exudates in rhizosphere interactions with plants and other organisms. Annu. Rev. Plant Biol. **57**(1): 233–266. Annual Reviews. doi:10.1146/annurev.arplant.57.032905.105159.

Bakker, M.G., Manter, D.K., Sheflin, A.M., Weir, T.L., and Vivanco, J.M. 2012. Harnessing the rhizosphere microbiome through plant breeding and agricultural management. Plant Soil **360**(1–2): 1–13. Springer Netherlands. doi:10.1007/s11104-012-1361-x.

Bashan, Y., De-Bashan, L.E., Prabhu, S.R., and Hernandez, J.P. 2014. Advances in plant growth-promoting bacterial inoculant technology: formulations and practical perspectives (1998-2013). doi:10.1007/s11104-013-1956-x.

Bashan, Y., Holguin, G., and De-Bashan, L.E. 2004. Azospirillum -plant relationships: physiological, molecular, agricultural, and environmental advances (1997-2003). Can. J. Microbiol. **50**(8): 521–577. NRC Research Press Ottawa, Canada. doi:10.1139/w04-035.

Beans, C. 2017. Core Concept: Phytoremediation advances in the lab but lags in the field. Proc. Natl. Acad. Sci. U. S. A. **114**(29): 7475–7477. National Academy of Sciences. doi:10.1073/pnas.1707883114.

Beattie, G.A. 2007. Plant-associated bacteria: survey, molecular phylogeny, genomics and recent advances. *In* Plant-Associated Bacteria. Springer Netherlands, Dordrecht. pp. 1–56. doi:10.1007/978-1-4020-4538-7_1.

Behera, K.K. 2014. Phytoremediation, transgenic plants and microbes. Springer, Cham. pp. 65–85. doi:10.1007/978-3-319-00915-5_4.

Van Beilen, J.B., and Funhoff, E.G. 2007. Alkane hydroxylases involved in microbial alkane degradation. Appl. Microbiol. Biotechnol. **74**(1): 13–21. doi:10.1007/s00253-006-0748-0.

van Beilen, J.B., Funhoff, E.G., van Loon, A., Just, A., Kaysser, L., Bouza, M., Holtackers, R., Röthlisberger, M., Li, Z., and Witholt, B. 2006. Cytochrome P450 alkane hydroxylases of the CYP153 family are common in alkane-degrading eubacteria lacking integral membrane alkane hydroxylases. Appl. Environ. Microbiol. **72**(1): 59–65. American Society for Microbiology. doi:10.1128/AEM.72.1.59-65.2006.

Belimov, A.A., Safronova, V.I., Sergeyeva, T.A., Egorova, T.N., Matveyeva, V.A., Tsyganov, V.E., Borisov, A.Y., Tikhonovich, I.A., Kluge, C., Preisfeld, A., Dietz, K.-J., and Stepanok, V. V. 2001. Characterization of plant growth promoting rhizobacteria isolated from polluted soils and containing 1-aminocyclopropane-1-carboxylate deaminase. Can. J. Microbiol. **47**(7): 642–652. doi:10.1139/w01-062.

Benton, P.M.C., Sen, S., and Peters, J.W. 2002. Nitrogenase structure. *In* Nitrogen Fixation at the Millennium. Elsevier. pp. 35–71. doi:10.1016/B978-044450965-9/50002-1.

Berg, G. 2009. Plant–microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. Appl. Microbiol. Biotechnol. **84**(1): 11–18. Springer-Verlag. doi:10.1007/s00253-009-2092-7.

Bertrand, H., Plassard, C., Pinochet, X., Touraine, B., Normand, P., and Cleyet-Marel, J.C. 2000. Stimulation of the ionic transport system in Brassica napus by a plant growth-promoting rhizobacterium (Achromobacter sp.). Can. J. Microbiol. **46**(3): 229–236. NRC Research Press Ottawa, Canada. doi:10.1139/w99-137.

Blaha, D., Prigent-Combaret, C., Mirza, M.S., and Moënne-Loccoz, Y. 2006. Phylogeny of the 1-aminocyclopropane-1-carboxylic acid deaminase-encoding gene acdS in phytobeneficial and pathogenic Proteobacteria and relation with strain biogeography. FEMS Microbiol. Ecol. **56**(3).

Blain, N. 2016. A Survey of the Bacterial Root Endophytes Associated With the Natural Vegetation At the Bitumount Provincial Historic Site , Alberta , Canada. University of Saskatchewan. Available from https://ecommons.usask.ca/handle/10388/7537.

Blain, N.P., Helgason, B., and Germida, J.J. 2017. Endophytic root bacteria associated with the natural vegetation growing at the hydrocarbon contaminated Bitumount Provincial Historic site. Can. J. Microbiol.: cjm-2017-0039. doi:10.1139/cjm-2017-0039.

Bloemberg, G. V, and Lugtenberg, B.J.. 2001. Molecular basis of plant growth promotion and biocontrol by rhizobacteria. Curr. Opin. Plant Biol. **4**(4): 343–350. doi:10.1016/S1369-5266(00)00183-7.

Boddey, R.M., Baldani, V.L.D., Baldani, J.I., and Döbereiner, J. 1986. Effect of inoculation ofAzospirillum spp. on nitrogen accumulation by field-grown wheat. Plant Soil **95**(1): 109–121. Kluwer Academic Publishers. doi:10.1007/BF02378857.

Boopathy, R. 2000. Factors limiting bioremediation technologies. Bioresour. Technol. **74**(1): 63–

67. Elsevier. doi:10.1016/S0960-8524(99)00144-3.

Botta, A.L., Santacecilia, A., Ercole, C., Cacchio, P., and Del Gallo, M. 2013. In vitro and in vivo inoculation of four endophytic bacteria on Lycopersicon esculentum. N. Biotechnol. **30**(6): 666–674. Elsevier. doi:10.1016/J.NBT.2013.01.001.

Boyd, C.E. 2015. Nitrogen. *In* Water Quality. Springer International Publishing, Cham. pp. 223–241. doi:10.1007/978-3-319-17446-4_11.

Bulgarelli, D., Schlaeppi, K., Spaepen, S., Ver Loren van Themaat, E., and Schulze-Lefert, P. 2013. Structure and functions of the bacterial microbiota of plants. Annu. Rev. Plant Biol. **64**. doi:10.1146/annurev-arplant-050312-120106.

Burd, G.I., Dixon, D.G., and Glick, B.R. 1998. A plant growth-promoting bacterium that decreases nickel toxicity in seedlings. Appl. Environ. Microbiol. **64**(10): 3663–8. American Society for Microbiology.

Burg, S.P. 1973. Ethylene in plant growth. Proc. Natl. Acad. Sci. U. S. A. **70**(2): 591–7. National Academy of Sciences.

Burns, R.G., DeForest, J.L., Marxsen, J., Sinsabaugh, R.L., Stromberger, M.E., Wallenstein, M.D., Weintraub, M.N., and Zoppini, A. 2013. Soil enzymes in a changing environment: Current knowledge and future directions. Soil Biol. Biochem. **58**: 216–234. doi:10.1016/j.soilbio.2012.11.009.

Busby, P.E., Peay, K.G., and Newcombe, G. 2016. Common foliar fungi of *Populus trichocarpa* modify *Melampsora* rust disease severity. New Phytol. **209**(4): 1681–1692. doi:10.1111/nph.13742.

Buyer, J.S., and Leongg, J. 1986. Iron transport-mediated antagonism between plant growth-promoting and plant-deleterious pseudomonas strains*. *In* The Journal of Biological Chemistry.

Cappelletti, M., Fedi, S., Frascari, D., Ohtake, H., Turner, R.J., and Zannoni, D. 2011. Analyses of both the alkB gene transcriptional start site and alkB promoter-inducing properties of Rhodococcus sp. strain BCP1 grown on n-alkanes. Appl. Environ. Microbiol. **77**(5): 1619–27. American Society for Microbiology (ASM). doi:10.1128/AEM.01987-10.

Chen, C., Xin, K., Liu, H., Cheng, J., Shen, X., Wang, Y., and Zhang, L. 2017. Pantoea alhagi, a novel endophytic bacterium with ability to improve growth and drought tolerance in wheat. Sci. Rep. **7**(1): 41564. Nature Publishing Group. doi:10.1038/srep41564.

Chen, L., Luo, S., Xiao, X., Guo, H., Chen, J., Wan, Y., Li, B., Xu, T., Xi, Q., Rao, C., Liu, C., and Zeng, G. 2010. Application of plant growth-promoting endophytes (PGPE) isolated from Solanum nigrum L. for phytoextraction of Cd-polluted soils. Appl. Soil Ecol. **46**(3): 383–389. doi:10.1016/j.apsoil.2010.10.003.

Cheng, Z., Park, E., and Glick, B.R. 2007. 1-Aminocyclopropane-1-carboxylate deaminase from Pseudomonas putida UW4 facilitates the growth of canola in the presence of salt. Can. J. Microbiol. **53**(7): 912–918. doi:10.1139/W07-050.

Chhabra, S., Brazil, D., Morrissey, J., Burke, J.I., O'Gara, F., and N Dowling, D. 2013. Characterization of mineral phosphate solubilization traits from a barley rhizosphere soil functional metagenome. Microbiologyopen **2**(5): 717–24. Wiley-Blackwell. doi:10.1002/mbo3.110.

Chi, F., Shen, S.-H., Cheng, H.-P., Jing, Y.-X., Yanni, Y.G., and Dazzo, F.B. 2005. Ascending migration of endophytic rhizobia, from roots to leaves, inside rice plants and assessment of benefits to rice growth physiology. Appl. Environ. Microbiol. **71**(11): 7271–8. American Society for Microbiology (ASM). doi:10.1128/AEM.71.11.7271-7278.2005.

Cocking, E.C. 2003. Endophytic colonization of plant roots by nitrogen-fixing bacteria. Plant Soil **252**(1): 169–175. Kluwer Academic Publishers. doi:10.1023/A:1024106605806.

Compant, S., Clément, C., and Sessitsch, A. 2010. Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. Soil Biol. Biochem. **42**(5): 669–678. Pergamon. doi:10.1016/J.SOILBIO.2009.11.024.

Daane, L.L., Harjono, I., Zylstra, G.J., and Häggblom, M.M. 2001. Isolation and characterization of polycyclic aromatic hydrocarbon-degrading bacteria associated with the rhizosphere of salt marsh plants. Appl. Environ. Microbiol. **67**(6): 2683–91. American Society for Microbiology. doi:10.1128/AEM.67.6.2683-2691.2001.

Davis, J.W., and Madsen, S. 1996. Factors affecting the biodegradation of toluene in soil. Chemosphere **33**(1): 107–137.

Dixon, R., and Kahn, D. 2004. Genetic regulation of biological nitrogen fixation. Nat. Rev. Microbiol. **2**(8): 621–631. Nature Publishing Group. doi:10.1038/nrmicro954.

Dombrowski, J.E., Hollenbeck, V.G., and Martin, R.C. 2017. Isolation and identification of bacterial endophytes from grasses along the Oregon coast. Am. J. Plant Sci. **8**: 574–601.

doi:10.4236/ajps.2017.83040.

Dong, R., Gu, L., Guo, C., Xun, F., and Liu, J. 2014. Effect of PGPR Serratia marcescens BC-3 and AMF Glomus intraradices on phytoremediation of petroleum contaminated soil. Ecotoxicology **23**(4): 674–680. Springer US. doi:10.1007/s10646-014-1200-3.

Dugardeyn, J., and Van Der Straeten, D. 2008. Ethylene: Fine-tuning plant growth and development by stimulation and inhibition of elongation. Plant Sci. **175**(1–2): 59–70. doi:10.1016/j.plantsci.2008.02.003.

Elbeltagy, A., Nishioka, K., Sato, T., Suzuki, H., Ye, B., Hamada, T., Isawa, T., Mitsui, H., and Minamisawa, K. 2001. Endophytic colonization and in planta nitrogen fixation by a Herbaspirillum sp. isolated from wild rice species. Appl. Environ. Microbiol. **67**(11): 5285–93. American Society for Microbiology. doi:10.1128/AEM.67.11.5285-5293.2001.

Estrada-De Los Santos, P., Bustillos-Cristales, R., and Caballero-Mellado, J. 2001. Burkholderia, a genus rich in plant-associated nitrogen fixers with wide environmental and geographic distribution. Appl. Environ. Microbiol. **67**(6): 2790–8. American Society for Microbiology. doi:10.1128/AEM.67.6.2790-2798.2001.

Evangelou, M.W.H., Papazoglou, E.G., Robinson, B.H., and Schulin, R. 2015. Phytomanagement: Phytoremediation and the production of biomass for economic revenue on contaminated land. *In* Phytoremediation. Springer International Publishing, Cham. pp. 115–132. doi:10.1007/978-3-319-10395-2_9.

Fallath, T., Rosli, A. Bin, Kidd, B., Carvalhais, L.C., and Schenk, P.M. 2017. Toward plant defense mechanisms against root pathogens. *In* Agriculturally Important Microbes for Sustainable Agriculture. Springer Singapore, Singapore. pp. 293–313. doi:10.1007/978-981-10-5343-6_10.

Farrar, K., Bryant, D., and Cope-Selby, N. 2014. Understanding and engineering beneficial plant-microbe interactions: Plant growth promotion in energy crops. doi:10.1111/pbi.12279.

Fasani, E., Manara, A., Martini, F., Furini, A., and DalCorso, G. 2017. The potential of genetic engineering of plants for the remediation of soils contaminated with heavy metals. Plant. Cell Environ. doi:10.1111/pce.12963.

Feng, Y., Shen, D., and Song, W. 2006. Rice endophyte Pantoea agglomerans YS19 promotes host plant growth and affects allocations of host photosynthates. J. Appl. Microbiol. **100**(5): 938–945. doi:10.1111/j.1365-2672.2006.02843.x.

Fernet, J.L., Lawrence, J.R., and Germida, J.J. 2016. Naturally occurring phenanthrene degrading bacteria associated with seeds of various plant species. Int. J. Phytoremediation **18**(4): 423–425. Taylor & Francis. doi:10.1080/15226514.2015.1109593.

Fierer, N., Jackson, J.A., Vilgalys, R., and Jackson, R.B. 2005. Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. Appl. Environ. Microbiol. **71**(7): 4117–4120. doi:10.1128/AEM.71.7.4117-4120.2005.

Fisher, K., and Newton, W.E. 2002. Nitrogen fixation – a general overview. *In* Nitrogen Fixation at the Millennium. Elsevier. pp. 1–34. doi:10.1016/B978-044450965-9/50001-X.

Foley, J.A. 2005. Global Consequences of Land Use. Science (80-.). **309**(5734): 570–574. doi:10.1126/science.1111772.

Franche, C., Lindström, K., and Elmerich, C. 2009. Nitrogen-fixing bacteria associated with leguminous and non-leguminous plants. Plant Soil **321**(1–2): 35–59. Springer Netherlands. doi:10.1007/s11104-008-9833-8.

Fraser, T.D., Lynch, D.H., Bent, E., Entz, M.H., and Dunfield, K.E. 2015. Soil bacterial phoD gene abundance and expression in response to applied phosphorus and long-term management. Soil Biol. Biochem. **88**: 137–147. doi:10.1016/j.soilbio.2015.04.014.

de Freitas, J.R., Banerjee, M.R., and Germida, J.J. 1997. Phosphate-solubilizing rhizobacteria enhance the growth and yield but not phosphorus uptake of canola (Brassica napus L.). Biol. Fertil. Soils **24**(4): 358–364. Springer-Verlag. doi:10.1007/s003740050258.

Funhoff, E.G., Bauer, U., García-Rubio, I., Witholt, B., and van Beilen, J.B. 2006. CYP153A6, a soluble P450 oxygenase catalyzing terminal-alkane hydroxylation. J. Bacteriol. **188**(14): 5220–7. American Society for Microbiology. doi:10.1128/JB.00286-06.

Gaby, J.C., and Buckley, D.H. 2012. A comprehensive evaluation of PCR primers to smplify the nifH gene of nitrogenase. PLoS One **7**(7): e42149. Public Library of Science. doi:10.1371/journal.pone.0042149.

Gaiero, J.R., McCall, C.A., Thompson, K.A., Day, N.J., Best, A.S., and Dunfield, K.E. 2013. Inside the root microbiome: bacterial root endophytes and plant growth promotion. Am. J. Bot. **100**(9): 1738–50. Botanical Society of America. doi:10.3732/ajb.1200572.

Gerhardt, K.E., Gerwing, P.D., and Greenberg, B.M. 2017. Opinion: Taking phytoremediation from proven technology to accepted practice. Plant Sci. **256**: 170–185. doi:10.1016/j.plantsci.2016.11.016.

Gerhardt, K.E., Huang, X.-D., Glick, B.R., and Greenberg, B.M. 2009. Phytoremediation and rhizoremediation of organic soil contaminants: Potential and challenges. Plant Sci. **176**(1): 20–30. doi:10.1016/j.plantsci.2008.09.014.

Glick, B.R. 2005. Modulation of plant ethylene levels by the bacterial enzyme ACC deaminase. FEMS Microbiol. Lett. **251**(1).

Glick, B.R. 2010. Using soil bacteria to facilitate phytoremediation. Biotechnol. Adv. **28**: 367–374. doi:10.1016/j.biotechadv.2010.02.001.

Glick, B.R. 2014. Bacteria with ACC deaminase can promote plant growth and help to feed the world. Microbiol. Res. **169**(1). doi:10.1016/j.micres.2013.09.009.

Glick, B.R. 2015. Environmental interactions. *In* Beneficial Plant-Bacterial Interactions. Springer, Cham. pp. 159–190. doi:10.1007/978-3-319-13921-0_6.

Glick, B.R., Cheng, Z., Czarny, J., and Duan, J. 2007. Promotion of plant growth by ACC deaminase-producing soil bacteria. *In* New Perspectives and Approaches in Plant Growth-Promoting Rhizobacteria Research. Springer Netherlands, Dordrecht. pp. 329–339. doi:10.1007/978-1-4020-6776-1_8.

Glick, B.R., Liu, C., Ghosh, S., and Dumbroff, E.B. 1997. Early development of canola seedlings in the presence of the plant growth-promoting rhizobacterium Pseudomonas putida GR12-2. Soil Biol. Biochem. **29**(8): 1233–1239. Pergamon. doi:10.1016/S0038-0717(97)00026-6.

Glick, B.R., Penrose, D.M., and Li, J. 1998. A model for the lowering of plant ethylene concentrations by plant growth-promoting bacteria. J. Theor. Biol. **190**(1): 63–68. doi:10.1006/jtbi.1997.0532.

Godfray, H.C.J., Beddington, J.R., Crute, I.R., Haddad, L., Lawrence, D., Muir, J.F., Pretty, J., Robinson, S., Thomas, S.M., and Toulmin, C. 2010. Food security: the challenge of feeding 9 billion people. Science (80-.). **327**(5967).

Gopal, M., Gupta, A., and Thomas, G. V. 2013. Bespoke microbiome therapy to manage plant diseases. Front. Microbiol. **4**: 355. Frontiers Media SA. doi:10.3389/fmicb.2013.00355.

Guo, X., van Iersel, M.W., Chen, J., Brackett, R.E., and Beuchat, L.R. 2002. Evidence of association of salmonellae with tomato plants grown hydroponically in inoculated nutrient solution. Appl. Environ. Microbiol. **68**(7): 3639–43.

Gupta, R., Singal, R., Shankar, A., Kuhad, R.C., and Saxena, R.K. 1994. A modified plate assay for screening phosphate solubilizing microorganisms. J. Gen. Appl. Microbiol. **40**(3): 255–

260. Applied Microbiology, Molecular and Cellular Biosciences Research Foundation. doi:10.2323/jgam.40.255.

Gurska, J., Wang, W., Gerhardt, K.E., Khalid, A.M., Isherwood, D.M., Huang, X.-D., Glick, B.R., and Greenberg, B.M. 2009. Three year field test of a plant growth promoting rhizobacteria enhanced phytoremediation system at a land farm for treatment of hydrocarbon waste. Environ. Sci. Technol. **43**(12): 4472–4479. American Chemical Society. doi:10.1021/es801540h.

Hall, J., Soole, K., and Bentham, R. 2011. Hydrocarbon phytoremediation in the family Fabacea — a review. Int. J. Phytoremediation **13**(4): 317–332. Taylor & Francis Group. doi:10.1080/15226514.2010.495143.

Hallmann, J., Quadt-Hallmann, A., Mahaffee, W.F., and Kloepper, J.W. 1997. Bacterial endophytes in agricultural crops. Can. J. Microbiol. **43**(10): 895–914. NRC Research Press Ottawa, Canada. doi:10.1139/m97-131.

Han, J., Sun, L., Dong, X., Cai, Z., Sun, X., Yang, H., Wang, Y., and Song, W. 2005. Characterization of a novel plant growth-promoting bacteria strain Delftia tsuruhatensis HR4 both as a diazotroph and a potential biocontrol agent against various plant pathogens. Syst. Appl. Microbiol. **28**(1): 66–76. Urban & Fischer. doi:10.1016/J.SYAPM.2004.09.003.

Hardoim, P.R., Overbeek, L.S. van, Berg, G., Pirttilä, A.M., Compant, S., Campisano, A., Döring, M., and Sessitsch, A. 2015. The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. Microbiol. Mol. Biol. Rev. **79**(3). doi:10.1128/MMBR.00050-14.

Hardoim, P.R., van Overbeek, L.S., and Elsas, J.D. van. 2008. Properties of bacterial endophytes and their proposed role in plant growth. Trends Microbiol. **16**(10): 463–471. doi:10.1016/j.tim.2008.07.008.

Hoagland, D.R., and Arnon, D.I. 1950. The water-culture method for growing plants without soil. Circ. Calif. Agric. Exp. Stn. **347**(2nd edit).

Hol, W.H.G., Bezemer, T.M., and Biere, A. 2013. Getting the ecology into interactions between plants and the plant growth-promoting bacterium Pseudomonas fluorescens. Front. Plant Sci. **4**: 81. Frontiers Media SA. doi:10.3389/fpls.2013.00081.

Holguin, G., and Glick, B.R. 2001. Expression of the ACC deaminase gene from enterobacter cloacae UW4 in Azospirillum brasilense. Microb. Ecol. **41**(3): 281–288. Springer-Verlag.

doi:10.1007/s002480000040.

Hou, J., Liu, W., Wang, B., Wang, Q., Luo, Y., and Franks, A.E. 2015. PGPR enhanced phytoremediation of petroleum contaminated soil and rhizosphere microbial community response. Chemosphere **138**: 592–598. Pergamon. doi:10.1016/J.CHEMOSPHERE.2015.07.025.

Huang, P., de-Bashan, L., Crocker, T., Kloepper, J.W., and Bashan, Y. 2017. Evidence that fresh weight measurement is imprecise for reporting the effect of plant growth-promoting (rhizo)bacteria on growth promotion of crop plants. Biol. Fertil. Soils **53**(2): 199–208. Springer Berlin Heidelberg. doi:10.1007/s00374-016-1160-2.

Inostroza, N.G., Barra, P.J., Wick, L.Y., Mora, M.L., and Jorquera, M.A. 2017. Effect of rhizobacterial consortia from undisturbed arid- and agro-ecosystems on wheat growth under different conditions. Lett. Appl. Microbiol. **64**(2): 158–163. Wiley/Blackwell (10.1111). doi:10.1111/lam.12697.

Ipek, M., Pirlak, L., Esitken, A., Figen Dönmez, M., Turan, M., and Sahin, F. 2014. Plant growth-promoting rhizobacteria (pgpr) increase yield, growth and nutrition of strawberry under high-calcareous soil conditions. J. Plant Nutr. **37**(7): 990–1001. doi:10.1080/01904167.2014.881857org/10.1080/01904167.2014.881857.

Islam, F., Yasmeen, T., Ali, Q., Mubin, M., Ali, S., Arif, M.S., Hussain, S., Riaz, M., and Abbas, F. 2016. Copper-resistant bacteria reduces oxidative stress and uptake of copper in lentil plants: potential for bacterial bioremediation. Environ. Sci. Pollut. Res. **23**(1): 220–233. Springer Berlin Heidelberg. doi:10.1007/s11356-015-5354-1.

Jha, P., and Jha, P.N. 2015. Plant-microbe partnerships for enhanced biodegradation of polychlorinated biphenyls. *In* Plant Microbes Symbiosis: Applied Facets. Springer India, New Delhi. pp. 95–110. doi:10.1007/978-81-322-2068-8_4.

Ji, S.H., Gururani, M.A., and Chun, S.-C. 2014. Isolation and characterization of plant growth promoting endophytic diazotrophic bacteria from Korean rice cultivars. Microbiol. Res. **169**(1): 83–98. doi:10.1016/j.micres.2013.06.003.

Kageyama, H., Tripathi, K., Rai, A.K., Cha-Um, S., Waditee-Sirisattha, R., and Takabe, T. 2011. An alkaline phosphatase/phosphodiesterase, PhoD, induced by salt stress and secreted out of the cells of Aphanothece halophytica, a halotolerant cyanobacterium. Appl. Environ. Microbiol. **77**(15): 5178–83. American Society for Microbiology. doi:10.1128/AEM.00667-

11.

Kandel, S.L., Herschberger, N., Kim, S.H., and Doty, S.L. 2015. Diazotrophic endophytes of poplar and willow for growth promotion of rice plants in nitrogen-limited conditions. Crop Sci. **55**(4): 1765. The Crop Science Society of America, Inc. doi:10.2135/cropsci2014.08.0570.

Kennedy, A.C., and de Luna, L.Z. 2005. Rhizosphere. Encycl. Soils Environ.: 399–406. Elsevier. doi:10.1016/B0-12-348530-4/00163-6.

Khan, A.L., Waqas, M., Kang, S.-M., Al-Harrasi, A., Hussain, J., Al-Rawahi, A., Al-Khiziri, S., Ullah, I., Ali, L., Jung, H.-Y., and Lee, I.-J. 2014. Bacterial endophyte Sphingomonas sp. LK11 produces gibberellins and IAA and promotes tomato plant growth. J. Microbiol. **52**(8): 689–695. The Microbiological Society of Korea. doi:10.1007/s12275-014-4002-7.

Khan, F.I., Husain, T., and Hejazi, R. 2004. An overview and analysis of site remediation technologies. J. Environ. Manage. **71**(2): 95–122. Academic Press. doi:10.1016/J.JENVMAN.2004.02.003.

Khan, S., Afzal, M., Iqbal, S., and Khan, Q.M. 2013. Plant–bacteria partnerships for the remediation of hydrocarbon contaminated soils. Chemosphere **90**(4): 1317–1332. doi:10.1016/j.chemosphere.2012.09.045.

Kirk, J.L., Klironomos, J.N., Lee, H., and Trevors, J.T. 2005. The effects of perennial ryegrass and alfalfa on microbial abundance and diversity in petroleum contaminated soil. Environ. Pollut. **133**(3): 455–465. doi:10.1016/j.envpol.2004.06.002.

Kloepper, J.W., Leong, J., Teintze, M., and Schroth, M.N. 1980. Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. Nature **286**(5776): 885–886. Nature Publishing Group. doi:10.1038/286885a0.

Kloepper, J.W., Ryu, C.-M., and Zhang, S. 2004. Induced systemic resistance and promotion of plant growth by Bacillus spp. Phytopathology **94**(11): 1259–1266. The American Phytopathological Society. doi:10.1094/PHYTO.2004.94.11.1259.

Kloepper, J.W., and Schroth, M.N. 1981. Plant growth promoting rhizobacteria and plant growth under gnotobiotic conditions. Phytopathology **71**(642–644).

Kloos, K., Munch, J.C., and Schloter, M. 2006. A new method for the detection of alkane-monooxygenase homologous genes (alkB) in soils based on PCR-hybridization. doi:10.1016/j.mimet.2006.01.014.

Kniskern, J.M., Traw, M.B., and Bergelson, J. 2007. Salicylic acid and jasmonic acid signaling defense pathways reduce natural bacterial diversity on Arabidopsis thaliana. Mol. Plant-Microbe Interact. MPMI **20**(12): 1512–1522. doi:10.1094.

Koch, D.J., Chen, M.M., van Beilen, J.B., and Arnold, F.H. 2009. In vivo evolution of butane oxidation by terminal alkane hydroxylases AlkB and CYP153A6. Appl. Environ. Microbiol. **75**(2): 337–44. American Society for Microbiology. doi:10.1128/AEM.01758-08.

Kovalski Mitter, E. 2018. Bacterial endophytes of plants used for soil reclamation. University of Saskatchewan. Available from https://ecommons.usask.ca/handle/10388/8439 [accessed 14 July 2018].

Kraiser, T., Gras, D.E., Gutierrez, A.G., Gonzalez, B., and Gutierrez, R.A. 2011. A holistic view of nitrogen acquisition in plants. J. Exp. Bot. **62**(4): 1455–1466. Oxford University Press. doi:10.1093/jxb/erq425.

Kukla, M., Płociniczak, T., and Piotrowska-Seget, Z. 2014. Diversity of endophytic bacteria in Lolium perenne and their potential to degrade petroleum hydrocarbons and promote plant growth. Chemosphere **117**: 40–46. doi:10.1016/j.chemosphere.2014.05.055.

Kumar, A., Maurya, B.R., and Raghuwanshi, R. 2014. Isolation and characterization of PGPR and their effect on growth, yield and nutrient content in wheat (Triticum aestivum L.). Biocatal. Agric. Biotechnol. **3**(4): 121–128. Elsevier. doi:10.1016/J.BCAB.2014.08.003.

Lakshmanan, V., Selvaraj, G., and Bais, H.P. 2014. Functional soil microbiome: belowground solutions to an aboveground problem. Plant Physiol. **166**(2): 689–700. American Society of Plant Biologists. doi:10.1104/pp.114.245811.

Lareen, A., Burton, F., and Schäfer, P. 2016. Plant root-microbe communication in shaping root microbiomes. doi:10.1007/s11103-015-0417-8.

Laslo, É., György, É., Mara, G., Tamás, É., Ábrahám, B., and Lányi, S. 2012. Screening of plant growth promoting rhizobacteria as potential microbial inoculants. Crop Prot. **40**: 43–48. Elsevier. doi:10.1016/J.CROPRO.2012.05.002.

Laughlin, D.C. 2011. Nitrification is linked to dominant leaf traits rather than functional diversity. J. Ecol. **99**(5): 1091–1099. Wiley/Blackwell (10.1111). doi:10.1111/j.1365-2745.2011.01856.x.

Lesk, C., Rowhani, P., and Ramankutty, N. 2016. Influence of extreme weather disasters on global crop production. Nature **529**(7584): 84–87. Nature Publishing Group.

doi:10.1038/nature16467.

Liang, J.-L., JiangYang, J.-H., Nie, Y., and Wu, X.-L. 2016. Regulation of the alkane hydroxylase CYP153 gene in a gram-positive alkane-degrading bacterium, Dietzia sp. strain DQ12-45-1b. Appl. Environ. Microbiol. **82**(2): 608–619. doi:10.1128/AEM.02811-15.

Lifshitz, R., Kloepper, J.W., Scher, F.M., Tipping, E.M., and Laliberté, M. 1986. Nitrogen-fixing pseudomonads isolated from roots of plants grown in the canadian high arctic. Appl. Environ. Microbiol. **51**(2): 251–5. American Society for Microbiology.

Linhart, C., and Shamir, R. 2005. The degenerate primer design problem: theory and applications. J. Comput. Biol. **12**(4): 431–456. Mary Ann Liebert, Inc. 2 Madison Avenue Larchmont, NY 10538 USA. doi:10.1089/cmb.2005.12.431.

Liu, C., Wang, W., Wu, Y., Zhou, Z., Lai, Q., and Shao, Z. 2011. Multiple alkane hydroxylase systems in a marine alkane degrader, Alcanivorax dieselolei B-5. Environ. Microbiol. **13**(5): 1168–1178. Wiley/Blackwell (10.1111). doi:10.1111/j.1462-2920.2010.02416.x.

van Loon, L.C., Bakker, P.A.H.M., and Pieterse, C.M.J. 1998. Systemic resistance induced by rhizosphere bacteria. Annu. Rev. Phytopathol. **36**(1): 453–483. Annual Reviews 4139 El Camino Way, P.O. Box 10139, Palo Alto, CA 94303-0139, USA. doi:10.1146/annurev.phyto.36.1.453.

Lowman, S., Kim-Dura, S., Mei, C., and Nowak, J. 2016. Strategies for enhancement of switchgrass (Panicum virgatum L.) performance under limited nitrogen supply based on utilization of N-fixing bacterial endophytes. Plant Soil **405**(1–2): 47–63. Springer International Publishing. doi:10.1007/s11104-015-2640-0.

Lugtenberg, B., and Kamilova, F. 2009. Plant-growth-promoting rhizobacteria. doi:10.1146/annurev.micro.62.081307.162918.

Lumactud, R., Shen, S.Y., Lau, M., and Fulthorpe, R. 2016. Bacterial endophytes isolated from plants in natural oil seep soils with chronic hydrocarbon contamination. Front. Microbiol. **7**: 755. Frontiers Media SA. doi:10.3389/fmicb.2016.00755.

Luo, H., Benner, R., Long, R.A., and Hu, J. 2009. Subcellular localization of marine bacterial alkaline phosphatases. Proc. Natl. Acad. Sci. U. S. A. **106**(50): 21219–23. National Academy of Sciences. doi:10.1073/pnas.0907586106.

Ma, Y., Prasad, M.N.V., Rajkumar, M., and Freitas, H. 2011. Plant growth promoting rhizobacteria and endophytes accelerate phytoremediation of metalliferous soils. Biotechnol. Adv. **29**(2):

248–258. Elsevier. doi:10.1016/J.BIOTECHADV.2010.12.001.

Maeng, J.H., Sakai, Y., Tani, Y., and Kato, N. 1996. Isolation and characterization of a novel oxygenase that catalyzes the first step of n-alkane oxidation in Acinetobacter sp. strain M-1. J. Bacteriol. **178**(13): 3695–700. American Society for Microbiology Journals. doi:10.1128/JB.178.13.3695-3700.1996.

Mahar, A., Wang, P., Ali, A., Awasthi, M.K., Lahori, A.H., Wang, Q., Li, R., and Zhang, Z. 2016. Challenges and opportunities in the phytoremediation of heavy metals contaminated soils: A review. (126): 111–121. doi:10.1016/j.ecoenv.2015.12.023.

Majeed, A., Abbasi, M.K., Hameed, S., Imran, A., and Rahim, N. 2015. Isolation and characterization of plant growth-promoting rhizobacteria from wheat rhizosphere and their effect on plant growth promotion. Front. Microbiol. **6**: 198. Frontiers Media SA. doi:10.3389/fmicb.2015.00198.

Malboobi, M.A., Owlia, P., Behbahani, M., Sarokhani, E., Moradi, S., Yakhchali, B., Deljou, A., and Morabbi Heravi, K. 2009. Solubilization of organic and inorganic phosphates by three highly efficient soil bacterial isolates. World J. Microbiol. Biotechnol. **25**(8): 1471–1477. Springer Netherlands. doi:10.1007/s11274-009-0037-z.

Marques, A.P.G.C., Pires, C., Moreira, H., Rangel, A.O.S.S., and Castro, P.M.L. 2010. Assessment of the plant growth promotion abilities of six bacterial isolates using Zea mays as indicator plant. Soil Biol. Biochem. **42**(8): 1229–1235. Pergamon. doi:10.1016/J.SOILBIO.2010.04.014.

McDonald, I.R., Miguez, C.B., Rogge, G., Bourque, D., Wendlandt, K.D., Groleau, D., and Murrell, J.C. 2006. Diversity of soluble methane monooxygenase-containing methanotrophs isolated from polluted environments. FEMS Microbiol. Lett. **255**(2): 225–232. Oxford University Press. doi:10.1111/j.1574-6968.2005.00090.x.

McGuinness, M., and Dowling, D. 2009. Plant-associated bacterial degradation of toxic organic compounds in soil. Int. J. Environ. Res. Public Health **6**(8): 2226–2247. Molecular Diversity Preservation International. doi:10.3390/ijerph6082226.

Mengel, K. 1996. Turnover of organic nitrogen in soils and its availability to crops. Plant Soil **181**(1): 83–93. Kluwer Academic Publishers. doi:10.1007/BF00011295.

Mercado-Blanco, J. 2015. Life of Microbes Inside the Plant. *In* Principles of Plant-Microbe Interactions. Springer International Publishing, Cham. pp. 25–32. doi:10.1007/978-3-319-

08575-3_5.

Mezzari, M.P., Zimermann, D.M.H., Corseuil, H.X., and Nogueira, A.V. 2011. Potential of grasses and rhizosphere bacteria for bioremediation of diesel-contaminated soils. Rev. Bras. Ciência do Solo **35**(6): 2227–2236. Sociedade Brasileira de Ciência do Solo. doi:10.1590/S0100-06832011000600038.

Millum, J. 2010. How should the benefits of bioprospecting be shared? Hastings Cent. Rep. **40**(1): 24–33. NIH Public Access. [accessed 28 November 2016].

Monds, R.D., Newell, P.D., Schwartzman, J.A., and O'Toole, G.A. 2006. Conservation of the Pho regulon in Pseudomonas fluorescens Pf0-1. Appl. Environ. Microbiol. **72**(3): 1910–24. American Society for Microbiology. doi:10.1128/AEM.72.3.1910-1924.2006.

Montañez, A., Blanco, A.R., Barlocco, C., Beracochea, M., and Sicardi, M. 2012. Characterization of cultivable putative endophytic plant growth promoting bacteria associated with maize cultivars (Zea mays L.) and their inoculation effects in vitro. Appl. Soil Ecol. **58**: 21–28. doi:10.1016/j.apsoil.2012.02.009.

Muangthong, A., Youpensuk, S., and Rerkasem, B. 2015. Isolation and characterisation of endophytic nitrogen fixing bacteria in sugarcane. Trop. Life Sci. Res. **26**(1): 41–51. [accessed 16 March 2017].

Müller, H., Berg, C., Landa, B.B., Auerbach, A., Moissl-Eichinger, C., and Berg, G. 2015. Plant genotype-specific archaeal and bacterial endophytes but similar Bacillus antagonists colonize Mediterranean olive trees. Front. Microbiol. **6**: 138. Frontiers Media SA. doi:10.3389/fmicb.2015.00138.

Nannipieri, P., Giagnoni, L., Landi, L., and Renella, G. 2011. Role of phosphatase enzymes in soil. Springer Berlin Heidelberg. pp. 215–243. doi:10.1007/978-3-642-15271-9_9.

Neilson, A.H., and Sparell, L. 1976. Acetylene reduction (nitrogen fixation) by enterobacteriaceae isolated from paper mill process waters. Appl. Environ. Microbiol. **32**(2): 197–205. American Society for Microbiology (ASM).

Nie, Y., Liang, J.-L., Fang, H., Tang, Y.-Q., and Wu, X.-L. 2014. Characterization of a CYP153 alkane hydroxylase gene in a gram-positive Dietzia sp. DQ12-45-1b and its "team role" with alkW1 in alkane degradation. Appl. Microbiol. Biotechnol. **98**(1): 163–173. doi:10.1007/s00253-013-4821-1.

Nikolic, B., Schwab, H., and Sessitsch, A. 2011. Metagenomic analysis of the 1-

aminocyclopropane-1-carboxylate deaminase gene (acdS) operon of an uncultured bacterial endophyte colonizing Solanum tuberosum L. Arch. Microbiol. **193**(9): 665–76. doi:10.1007/s00203-011-0703-z.

Nomura, K., DebRoy, S., Hoon Lee, Y., Pumplin, N., Jones, J., and Yang He, S. 2006. A bacterial virulence protein suppresses host innate immunity to cause plant disease. Science (80-.). **313**: 220–223. doi:10.1126/science.1126548.

Nosrati, R., Owlia, P., Saderi, H., Rasooli, I., and Ali Malboobi, M. 2014. Phosphate solubilization characteristics of efficient nitrogen fixing soil Azotobacter strains. Iran. J. Microbiol. **6**(4): 285–95. Tehran University of Medical Sciences.

Nowell, R.W., Laue, B.E., Sharp, P.M., and Green, S. 2016. Comparative genomics reveals genes significantly associated with woody hosts in the plant pathogen Pseudomonas syringae. Mol. Plant Pathol. **17**(9): 1409–1424. Wiley/Blackwell (10.1111). doi:10.1111/mpp.12423.

O'Gara, F., and Shanmugam, K.T. 1976. Regulation of nitrogen fixation by Rhizobia export of fixed N2 as NH4+. Biochim. Biophys. Acta - Gen. Subj. **437**(2): 313–321. Elsevier. doi:10.1016/0304-4165(76)90001-5.

Olanrewaju, O.S., Glick, B.R., and Babalola, O.O. 2017. Mechanisms of action of plant growth promoting bacteria. World J. Microbiol. Biotechnol. **33**(11): 197. Springer Netherlands. doi:10.1007/s11274-017-2364-9.

Oteino, N., Lally, R.D., Kiwanuka, S., Lloyd, A., Ryan, D., Germaine, K.J., and Dowling, D.N. 2015. Plant growth promotion induced by phosphate solubilizing endophytic Pseudomonas isolates. Front. Microbiol. **6**: 745. Frontiers. doi:10.3389/fmicb.2015.00745.

Owen, D., Williams, A.P., Griffith, G.W., and Withers, P.J.A. 2015. Use of commercial bio-inoculants to increase agricultural production through improved phosphrous acquisition. Appl. Soil Ecol. **86**: 41–54. Elsevier. doi:10.1016/J.APSOIL.2014.09.012.

Pankievicz, V.C.S., do Amaral, F.P., Santos, K.F.D.N., Agtuca, B., Xu, Y., Schueller, M.J., Arisi, A.C.M., Steffens, M.B.R., de Souza, E.M., Pedrosa, F.O., Stacey, G., and Ferrieri, R.A. 2015. Robust biological nitrogen fixation in a model grass-bacterial association. Plant J. **81**(6): 907–919. doi:10.1111/tpj.12777.

Park, Y.-S., Dutta, S., Ann, M., Raaijmakers, J.M., and Park, K. 2015. Promotion of plant growth by Pseudomonas fluorescens strain SS101 via novel volatile organic compounds. Biochem. Biophys. Res. Commun. **461**(2): 361–365. Academic Press.

doi:10.1016/J.BBRC.2015.04.039.

Parmar, S., and Singh, V. 2015. Phytoremediation approaches for heavy metal pollution: a review. J. Plant Sci. Res. **2**(2): 2. Open Science Publications.

Penrose, D.M., and Glick, B.R. 2003. Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria. Physiol. Plant. **118**(1): 10–15. Blackwell Science Ltd. doi:10.1034/j.1399-3054.2003.00086.x.

Pérez-García, A., Romero, D., and de Vicente, A. 2011. Plant protection and growth stimulation by microorganisms: biotechnological applications of Bacilli in agriculture. Curr. Opin. Biotechnol. **22**(2): 187–193. doi:10.1016/j.copbio.2010.12.003.

Pérez-Montaño, F., Alías-Villegas, C., Bellogín, R.A., Del Cerro, P., Espuny, M.R., Jiménez-Guerrero, I., López-Baena, F.J., Ollero, F.J., and Cubo, T. 2014. Plant growth promotion in cereal and leguminous agricultural important plants: From microorganism capacities to crop production. doi:10.1016/j.micres.2013.09.011.

Phillips, L., Germida, J., Farrell, R., and Greer, C. 2008. Hydrocarbon degradation potential and activity of endophytic bacteria associated with prairie plants. Soil Biol. Biochem. **40**(12): 3054–3064. doi:10.1016/j.soilbio.2008.09.006.

Phillips, L.A., Greer, C.W., and Germida, J.J. 2006. Culture-based and culture-independent assessment of the impact of mixed and single plant treatments on rhizosphere microbial communities in hydrocarbon contaminated flare-pit soil. Soil Biol. Biochem. **38**(9): 2823–2833. doi:10.1016/j.soilbio.2006.04.038.

Pierik, R., Tholen, D., Poorter, H., Visser, E.J.W., and Voesenek, L.A.C.J. 2006. The Janus face of ethylene: growth inhibition and stimulation. Trends Plant Sci. **11**(4): 176–183. doi:10.1016/j.tplants.2006.02.006.

Pieterse, C.M.., and van Loon, L.C. 1999. Salicylic acid-independent plant defence pathways. Trends Plant Sci. **4**(2): 52–58. Elsevier Current Trends. doi:10.1016/S1360-1385(98)01364-8.

Pii, Y., Mimmo, T., Tomasi, N., Terzano, R., Cesco, S., and Crecchio, C. 2015. Microbial interactions in the rhizosphere: beneficial influences of plant growth-promoting rhizobacteria on nutrient acquisition process. A review. Biol. Fertil. Soils **51**(4): 403–415. Springer Berlin Heidelberg. doi:10.1007/s00374-015-0996-1.

Quiza, L., St-Arnaud, M., and Yergeau, E. 2015. Harnessing phytomicrobiome signaling for

rhizosphere microbiome engineering. Front. Plant Sci. **6**: 507. Frontiers Media SA. doi:10.3389/fpls.2015.00507.

Ragot, S.A., Huguenin-Elie, O., Kertesz, M.A., Frossard, E., and Bünemann, E.K. 2016. Total and active microbial communities and phoD as affected by phosphate depletion and pH in soil. Plant Soil **408**(1–2): 15–30. Springer International Publishing. doi:10.1007/s11104-016-2902-5.

Ragot, S.A., Kertesz, M.A., and Bünemann, E.K. 2015. phoD alkaline phosphatase gene diversity in soil. Appl. Environ. Microbiol. **81**(20): 7281–9. American Society for Microbiology (ASM). doi:10.1128/AEM.01823-15.

Rana, A., Saharan, B., Joshi, M., Prasanna, R., Kumar, K., and Nain, L. 2011. Identification of multi-trait PGPR isolates and evaluating their potential as inoculants for wheat. Ann. Microbiol. **61**(4): 893–900. Springer-Verlag. doi:10.1007/s13213-011-0211-z.

Rana, A., Saharan, B., Nain, L., Prasanna, R., and Shivay, Y.S. 2012. Enhancing micronutrient uptake and yield of wheat through bacterial PGPR consortia Enhancing micronutrient uptake and yield of wheat through bacterial PGPR consortia. Soil Sci. Plant Nutr. **58**: 573–582. doi:10.1080/00380768.2012.716750.

Reinhold-Hurek, B., Bünger, W., Burbano, C.S., Sabale, M., and Hurek, T. 2015. Roots shaping their microbiome: global hotspots for microbial activity. Annu. Rev. Phytopathol. **53**(1). doi:10.1146/annurev-phyto-082712-102342.

Reinhold-Hurek, B., and Hurek, T. 1997. Azoarcus spp. and their interactions with grass roots. Plant Soil **194**(1/2): 57–64. Kluwer Academic Publishers. doi:10.1023/A:1004216507507.

Reinhold-Hurek, B., and Hurek, T. 1998. Life in grasses: diazotrophic endophytes. Trends Microbiol. **6**(4): 139–144. Elsevier Current Trends. doi:10.1016/S0966-842X(98)01229-3.

Reinhold-Hurek, B., and Hurek, T. 2011. Living inside plants: bacterial endophytes. Curr. Opin. Plant Biol. **14**(4): 435–443. doi:10.1016/j.pbi.2011.04.004.

Rennie, R.J. 1987. A single medium for the isolation of acetylene-reducing (dinitrogen-fixing) bacteria from soils. Can. J. Microbiol. **27**(1): 8–14.

Richardson, A.E., Barea, J.-M., McNeill, A.M., and Prigent-Combaret, C. 2009. Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. Plant Soil **321**(1–2): 305–3399. Springer Netherlands. doi:10.1007/s11104-009-9895-2.

Rodríguez, H., Fraga, R., Gonzalez, T., and Bashan, Y. 2007. Genetics of phosphate solubilization

and its potential applications for improving plant growth-promoting bacteria. *In* First International Meeting on Microbial Phosphate Solubilization. Springer Netherlands, Dordrecht. pp. 15–21. doi:10.1007/978-1-4020-5765-6_2.

Rodríguez, H., and Fraga, R. 1999. Phosphate solubilizing bacteria and their role in plant growth promotion. Biotechnol. Adv. **17**(4): 319–339. doi:10.1016/S0734-9750(99)00014-2.

Rojo, F. 2009. Degradation of alkanes by bacteria. Environ. Microbiol. **11**(10): 2477–2490. doi:10.1111/j.1462-2920.2009.01948.x.

Rokhbakhsh-Zamin, F., Sachdev, D., Kazemi-Pour, N., Engineer, A., Pardesi, K.R., Zinjarde, S., Dhakephalkar, P.K., and Chopade, B.A. 2011. Characterization of plant-growth-promoting traits of Acinetobacter species isolated from rhizosphere of Pennisetum glaucum. J. Microbiol. Biotechnol. **21**(6): 556–66.

Rosenblueth, M., and Martínez-Romero, E. 2006. Bacterial endophytes and their interactions with hosts. Mol. Plant-Microbe Interact. **19**(8): 827–837. The American Phytopathological Society. doi:10.1094/MPMI-19-0827.

Rubio, L.M., and Ludden, P.W. 2002. The gene products of the nif regulon. *In* Nitrogen Fixation at the Millennium. Elsevier. pp. 101–136. doi:10.1016/B978-044450965-9/50004-5.

Rubio, L.M., and Ludden, P.W. 2005. Maturation of nitrogenase: a biochemical puzzle. J. Bacteriol. **187**(2): 405–14. American Society for Microbiology. doi:10.1128/JB.187.2.405-414.2005.

Rubio, L.M., and Ludden, P.W. 2008. Biosynthesis of the iron-molybdenum cofactor of nitrogenase. Annu. Rev. Microbiol. **62**(1): 93–111. Annual Reviews. doi:10.1146/annurev.micro.62.081307.162737.

Ryan, R.P., Monchy, S., Cardinale, M., Taghavi, S., Crossman, L., Avison, M.B., Berg, G., van der Lelie, D., and Dow, J.M. 2009. The versatility and adaptation of bacteria from the genus Stenotrophomonas. Nat. Rev. Microbiol. **7**(7): 514–525. Nature Publishing Group. doi:10.1038/nrmicro2163.

Salt, D.E., Smith, R.D., and Raskin, I. 1998. Phytoremediation. Annu. Rev. Plant Physiol. Plant Mol. Biol **49**: 643–68. [accessed 1 November 2017].

Santos-Beneit, F. 2015. The Pho regulon: a huge regulatory network in bacteria. Front. Microbiol. **6**: 402. Frontiers Media SA. doi:10.3389/fmicb.2015.00402.

Schlaeppi, K., and Bulgarelli, D. 2015. The plant microbiome at work. Mol. Plant. Microbe.

Interact. **28**(3): 212–7. doi:10.1094/MPMI-10-14-0334-FI.

Schwab, A.P., Su, J., Wetzel, S., Pekarek, S., and Banks, M.K. 1999. Extraction of petroleum hydrocarbons from soil by mechanical shaking. Environ. Sci. Technol. **33**(11): 1940–1945. American Chemical Society. doi:10.1021/ES9809758.

Scoma, A., Hernandez-Sanabria, E., Lacoere, T., Junca, H., Boon, N., Pieper, D.H., and Vilchez-Vargas, R. 2015. Primers: bacterial genes encoding enzymes for aerobic hydrocarbon degradation. *In* Hydrocarbon and Lipid Microbiology Protocols. Springer, Berlin, Heidelberg. pp. 23–37. doi:10.1007/8623_2015_140.

Sessitsch, A., Howieson, J.G., Perret, X., Antoun, H., and Martínez-Romero, E. 2002. Advances in rhizobium research. CRC. Crit. Rev. Plant Sci. **21**(4): 323–378. Taylor & Francis Group. doi:10.1080/0735-260291044278.

Shade, A., Jacques, M.-A., and Barret, M. 2017. Ecological patterns of seed microbiome diversity, transmission, and assembly. Curr. Opin. Microbiol. **37**: 15–22. Elsevier Current Trends. doi:10.1016/J.MIB.2017.03.010.

Sharma, S.B., Sayyed, R.Z., Trivedi, M.H., and Gobi, T.A. 2013. Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils. Springerplus **2**: 587.

Siciliano, S.D., Fortin, N., Mihoc, A., Wisse, G., Labelle, S., Beaumier, D., Ouellette, D., Roy, R., Whyte, L.G., Banks, M.K., Schwab, P., Lee, K., and Greer, C.W. 2001. Selection of specific endophytic bacterial genotypes by plants in response to soil contamination. Appl. Environ. Microbiol. **67**(6): 2469–75. American Society for Microbiology. doi:10.1128/AEM.67.6.2469-2475.2001.

Siddique, T., Rutherford, P.M., Arocena, J.M., and Thring, R.W. 2006. A proposed method for rapid and economical extraction of petroleum hydrocarbons from contaminated soils. Can. J. Soil Sci. **86**(4): 725–728. NRC Research Press Ottawa, Canada. doi:10.4141/S05-075.

Sigma-Aldrich. 2017. Iodonitrotetrazolium chloride. [accessed 17 March 2017].

Sims, J.T., Sharpley, A.N., Condron, L.M., Turner, B.L., and Cade-Menun, B.J. 2005. Chemistry and dynamics of soil organic phosphorus. *In* Phosphorus: agriculture and the environment. American Society of Agronomy, Crop Science Society of America, and Soil Science Society of America. pp. 87–121. doi:10.2134/agronmonogr46.c4.

Smith, C.B., Tolar, B.B., Hollibaugh, J.T., and King, G.M. 2013. Alkane hydroxylase gene (alkB)

phylotype composition and diversity in northern Gulf of Mexico bacterioplankton. Front. Microbiol. **4**: 370. Frontiers Media SA. doi:10.3389/fmicb.2013.00370.

Smith, E.G., Ellert, B.H., Janzen, H., and Nakonechny, D.J. 2012. Rotation ABC - Lethbridge, Alberta. Prairie Soils Crop. J. **5**: 147–154. [accessed 8 March 2017].

Smyth, E.M., McCarthy, J., Nevin, R., Khan, M.R., Dow, J.M., O'Gara, F., and Doohan, F.M. 2011. In vitro analyses are not reliable predictors of the plant growth promotion capability of bacteria; a Pseudomonas fluorescens strain that promotes the growth and yield of wheat. J. Appl. Microbiol. **111**(3): 683–692. Wiley/Blackwell (10.1111). doi:10.1111/j.1365-2672.2011.05079.x.

Souza, R. de, Ambrosini, A., and Passaglia, L.M.P. 2015. Plant growth-promoting bacteria as inoculants in agricultural soils. Genet. Mol. Biol. **38**(4): 401–19. Sociedade Brasileira de Genética. doi:10.1590/S1415-475738420150053.

Summers, M.L., Elkins, J.G., Elliott, B.A., and McDermott, T.R. 1998. Expression and regulation of phosphate stress inducible genes in Sinorhizobium meliloti. Mol. Plant-Microbe Interact. **11**(11): 1094–1101. doi:10.1094/MPMI.1998.11.11.1094.

Tan, H., Barret, M., Mooij, M.J., Rice, O., Morrissey, J.P., Dobson, A., Griffiths, B., and O'Gara, F. 2013. Long-term phosphorus fertilisation increased the diversity of the total bacterial community and the phoD phosphorus mineraliser group in pasture soils. Biol. Fertil. Soils **49**(6): 661–672. Springer Berlin Heidelberg. doi:10.1007/s00374-012-0755-5.

Telenius, H., Carter, N.P., Bebb, C.E., Nordenskjo¨ld, M., Ponder, B.A.J., and Tunnacliffe, A. 1992. Degenerate oligonucleotide-primed PCR: General amplification of target DNA by a single degenerate primer. Genomics **13**(3): 718–725. Academic Press. doi:10.1016/0888-7543(92)90147-K.

Thakur, S., Singh, L., Wahid, Z.A., Siddiqui, M.F., Atnaw, S.M., and Din, M.F.M. 2016. Plant-driven removal of heavy metals from soil: uptake, translocation, tolerance mechanism, challenges, and future perspectives. Environ. Monit. Assess. **188**(4): 206. Springer International Publishing. doi:10.1007/s10661-016-5211-9.

Thijs, S., Sillen, W., Rineau, F., Weyens, N., and Vangronsveld, J. 2016. Towards an enhanced understanding of plant-microbiome interactions to improve phytoremediation: engineering the metaorganism. Front. Microbiol. **7**: 341. Frontiers Media SA. doi:10.3389/fmicb.2016.00341.

Thijs, S., Weyens, N., Sillen, W., Gkorezis, P., Carleer, R., and Vangronsveld, J. 2014. Potential for plant growth promotion by a consortium of stress-tolerant 2,4-dinitrotoluene-degrading bacteria: isolation and characterization of a military soil. Microb. Biotechnol. **7**(4): 294–306. Wiley-Blackwell. doi:10.1111/1751-7915.12111.

Thomas, R.L., Sheard, R.W., and Moyer, J.R. 1967. Comparison of conventional and automated procedures for nitrogen, phosphorus, and potassium analysis of plant material using a single digestion. Agron. J. **59**(3): 240. American Society of Agronomy. doi:10.2134/agronj1967.00021962005900030010x.

Timmusk, S., Abd El-Daim, I.A., Copolovici, L., Tanilas, T., Kännaste, A., Behers, L., Nevo, E., Seisenbaeva, G., Stenström, E., and Niinemets, Ü. 2014. Drought-tolerance of wheat improved by rhizosphere bacteria from harsh environments: enhanced biomass production and reduced emissions of stress volatiles. PLoS One **9**(5): e96086. Public Library of Science. doi:10.1371/journal.pone.0096086.

Timmusk, S., Behers, L., Muthoni, J., Muraya, A., and Aronsson, A.-C. 2017. Perspectives and challenges of microbial application for crop improvement. Front. Plant Sci. **8**: 49. Frontiers Media SA. doi:10.3389/fpls.2017.00049.

Tkacz, A., and Poole, P. 2015. Role of root microbiota in plant productivity. J. Exp. Bot. **66**(8): 2167–75. Oxford University Press. doi:10.1093/jxb/erv157.

Turner, T.R., James, E.K., and Poole, P.S. 2013. The plant microbiome. Genome Biol. **14**(6): 209. BioMed Central. doi:10.1186/gb-2013-14-6-209.

Vandenkoornhuyse, P., Quaiser, A., Duhamel, M., Le Van, A., and Dufresne, A. 2015. The importance of the microbiome of the plant holobiont. New Phytol. **206**(4): 1196–1206. doi:10.1111/nph.13312.

Vazquez, P., Holguin, G., Puente, M.E., Lopez-Cortes, A., and Bashan, Y. 2000. Phosphate-solubilizing microorganisms associated with the rhizosphere of mangroves in a semiarid coastal lagoon. *In* Biol Fertil Soils. Springer-Verlag.

Vershinina, O.A., and Znamenskaya, L. V. 2002. The pho regulons of bacteria. Microbiology **71**(5): 497–511. Kluwer Academic Publishers-Plenum Publishers. doi:10.1023/A:1020547616096.

Vessey, J.K. 2003. Plant growth promoting rhizobacteria as biofertilizers. Plant Soil **255**(2): 571–586. Kluwer Academic Publishers. doi:10.1023/A:1026037216893.

Vyas, P., and Gulati, A. 2009. Organic acid production in vitro and plant growth promotion in maize under controlled environment by phosphate-solubilizing fluorescent Pseudomonas. BMC Microbiol. **9**(1): 174. BioMed Central. doi:10.1186/1471-2180-9-174.

Wang, C., Knill, E., Glick, B.R., and Défago, G. 2000. Effect of transferring 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase genes into *Pseudomonas fluorescens* strain CHA0 and its *gac* A derivative CHA96 on their growth-promoting and disease-suppressive capacities. Can. J. Microbiol. **46**(10): 898–907. NRC Research Press Ottawa, Canada . doi:10.1139/w00-071.

Wang, X., Mavrodi, D. V, Ke, L., Mavrodi, O. V, Yang, M., Thomashow, L.S., Zheng, N., Weller, D.M., and Zhang, J. 2015. Biocontrol and plant growth-promoting activity of rhizobacteria from Chinese fields with contaminated soils. Microb. Biotechnol. **8**(3): 404–18. Wiley-Blackwell. doi:10.1111/1751-7915.12158.

Wanner, B.L. 1993. Gene regulation by phosphate in enteric bacteria. J. Cell. Biochem. **51**(1): 47–54. doi:10.1002/jcb.240510110.

Whyte, L.G., Schultz, A., Beilen, J.B., Luz, A.P., Pellizari, V., Labba, D., and Greer, C.W. 2002. Prevalence of alkane monooxygenase genes in Arctic and Antarctic hydrocarbon-contaminated and pristine soils. FEMS Microbiol. Ecol. **41**(2): 141–150. Oxford University Press. doi:10.1111/j.1574-6941.2002.tb00975.x.

Wickham, H. 2009. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag, New York.

World Health Organization. 2013. Contaminated sites and health. Copenhagen . Available from http://www.euro.who.int/pubrequest [accessed 14 July 2018].

Xu, J.G., and Johnson, R.L. 1997. Nitrogen dynamics in soils with different hydrocarbon contents planted to barley and field pea. Can. J. Soil Sci. **77**(3): 453–458. NRC Research Press Ottawa, Canada. doi:10.4141/S96-046.

Yavari, S., Malakahmad, A., and Sapari, N.B. 2015. A review on phytoremediation of crude oil spills. Water, Air, Soil Pollut. **226**(8): 279. Springer International Publishing. doi:10.1007/s11270-015-2550-z.

Yergeau, E., Lawrence, J.R., Sanschagrin, S., Waiser, M.J., Korber, D.R., and Greer, C.W. 2012. Next-generation sequencing of microbial communities in the Athabasca River and its tributaries in relation to oil sands mining activities. Appl. Environ. Microbiol. **78**(21): 7626–37. American Society for Microbiology. doi:10.1128/AEM.02036-12.

Yousaf, S., Ripka, K., Reichenauer, T.G., Andria, V., Afzal, M., and Sessitsch, A. 2010. Hydrocarbon degradation and plant colonization by selected bacterial strains isolated from Italian ryegrass and birdsfoot trefoil. J. Appl. Microbiol. **109**(4): 1389–1401. Blackwell Publishing Ltd. doi:10.1111/j.1365-2672.2010.04768.x.

Zahn, G., and Amend, A.S. 2017. Foliar microbiome transplants confer disease resistance in a critically-endangered plant. PeerJ **5**: e4020. PeerJ, Inc. doi:10.7717/peerj.4020.

Zaidi, A., Khan, M.S., Ahemad, M., Oves, M., and Wani, P.A. 2009. Recent advances in plant growth promotion by phosphate-solubilizing microbes. *In* Microbial Strategies for Crop Improvement. Springer Berlin Heidelberg, Berlin, Heidelberg. pp. 23–50. doi:10.1007/978-3-642-01979-1_2.

Zaidi, S., Usmani, S., Singh, B.R., and Musarrat, J. 2006. Significance of Bacillus subtilis strain SJ-101 as a bioinoculant for concurrent plant growth promotion and nickel accumulation in Brassica juncea. Chemosphere **64**(6): 991–997. Pergamon. doi:10.1016/J.CHEMOSPHERE.2005.12.057.

Zhang, D.-C., Liu, H.-C., Xin, Y.-H., Zhou, Y.-G., Schinner, F., and Margesin, R. 2010. Luteimonas terricola sp. nov., a psychrophilic bacterium isolated from soil. Int. J. Syst. Evol. Microbiol. **60**(7): 1581–1584. doi:10.1099/ijs.0.015537-0.

Zhang, L., Wang, X., Yu, M., Qiao, Y., and Zhang, X.-H. 2015. Genomic analysis of Luteimonas abyssi XH031(T): insights into its adaption to the subseafloor environment of South Pacific Gyre and ecological role in biogeochemical cycle. BMC Genomics **16**: 1092. BioMed Central. doi:10.1186/s12864-015-2326-2.

Zhang, X., Liu, W., Schloter, M., Zhang, G., Chen, Q., Huang, J., Li, L., Elser, J.J., and Han, X. 2013. Response of the abundance of key soil microbial nitrogen-cycling genes to multi-factorial global changes. PLoS One **8**(10): e76500. Public Library of Science. doi:10.1371/journal.pone.0076500.

APPENDIX

APPENDIX A: Information on bacteria isolated from Rotation ABC and Bitumount Provincial Historic Site

Table A.1: Studied isolates obtained from Bitumount Provincial Historic Site. New code indicates the abbreviation to be used during this project, and old code indicates the designation used during isolations from various plants collected from Bitumount Provincial Historic Site (Blain 2016). The identity is presented as the genus and species of the nearest relative identified in the NCBI database along with the accession number and % similarity of that sequence to the 16S rRNA sequence obtained in Chapter 3. Location indicates the location as well as the plant from which isolates were obtained (See Table A2).

New Code	**Old Code**	**Identity (Accession/ % similarity)**	**Location**
B1	L11-16	*Bacillus aryabhattai* (KY316449.1 / 99%)	L1, L11
B2	L11-45	*Bacillus megaterium* (MF101170.1 / 100%)	L10, L11
B3	L1-58	*Bacillus cereus* (NR_074540.1 / 99%)	L1
B4	L10-20	*Chryseobacterium indologenes* (CP022058.2 / 100%)	L10
B5	L9-30	*Leifsonia lichenia* (MH881510.1 / 100%)	L9, L12
B6	L11-5	*Leifsonia psychrotolerans* (KY476619.1 / 100%)	L2-1, L4, L8, L9, L11, L12
B8	L7-23	*Luteibacter rhizovicinus* (KX036584.1 / 99%)	L6, L7, L8
B9	L12-70	*Microbacterium deminutum* (KY753262.1 / 99%)	L2-2, L5, L12
B10	L9-47	*Microbacterium lacus* (MF179571.1 / 99%)	L4, L9
B11	L9-51	*Microbacterium mangrovi.* (NR_126283.1 / 100%)	L9
B12	L8-64	*Microbacterium deminutum* (KY753262.1 / 99%)	L8
B13	L12-76	*Microbacterium oxydans* (HQ425662.2 / 100%)	L2-1, L12
B14	L10-31	*Microbacterium hydrothermale* (MF405748.1 / 99%)	L10
B15	L9-20	*Pedobacter alluvionis* (HF936841.1 / 98%)	L4, L8, L9, L12
B16	L8-4	*Pedobacter aquatilus* (KX611476.1 / 98%)	L8
B17	L1-35	*Pseudomonas brassicacearum.* (MK424302.1 / 100%)	L1, L2

Table A.1 continued

New Code	Old Code	Identity	Location
B18	L10-2	*Pseudomonas fluorescens* (MK235236.1 / 100%)	L10
B19	L7-31	*Pseudomonas frederiksbergensis* (NR_028906.1 / 100%)	L2-1, L4, L7
B20	L2-2-18	*Pseudomonas baetica* (NR_116899.1 / 100%)	L2-2, L12
B21	L2-1-54	*Pseudomonas lini* (MG569813.1 / 100%)	L2-1
B22	L10-25	*Pseudomonas mediterranea* (MG516162.1 / 100%)	L10, L12
B23	L11-7	*Pseudomonas poae* (MG972911.1 / 100%)	L5, L8, L9, L11
B25	L1-8	*Rhizobium galgae* (KC462450.1 / 99%)	L1, L7, L5, L6
B26	L7-10	*Rhizobium herbae* (MH917338.1 / 100%)	L4, L6, L7, L8, L9, L12
B30	L9-45	*Rhodococcus cerastii* (MF777046.1 / 98%)	L9
B31	L10-1	*Rhodococcus globerulus* (NR_026184.1 / 100%)	L10
B32	L9-52	*Sphingomonas glacialis* (NR_117270.1 / 100%)	L2-2, L9
B34	L8-22	*Xanthomonas fuscans* (NR_104958.1 / 100%)	L5, L6, L8
B35	L11-48	*Arthrobacter subterraneus* (NR_074770.1 / 98%)	L2, L10, L11, L12
B38	L11-46	*Stenotrophomonas pavanii* (NR_118008.1 / 96%)	L8, L11
B40	L11-56	*Staphylococcus epidermidis* (NR_074995.1 / 98%)	L10, L11

Table A.2: Location key for isolates from Bitumount Provincial Historic Site. Location code indicates the abbreviation to be used denoting where plant roots were collected (on site location) as well as the plant identity.

Location Code	On Site Locaiton	Plant
L1	River Bank	Wheatgrass
L2-1	River Bank	Smooth Brome
L2-2	River Bank	Horsetail
L4	Pathway	Kentucky Bluegrass
L5	Pathway	Smooth Brome
L6	Quarry Border	Wild Strawberry
L7	Quarry	Pea Family
L8	Processing Area	Smooth Brome
L9	Processing Area	Slender Wheatgrass
L10	Processing Area	Horsetail
L11	Entrance	Slender Wheatgrass
L12	Entrance	Smooth Brome

Table A.3: List of project isolates obtained from Rotation ABC. New code indicates the abbreviation to be used during this project, and old code indicates the designation used during isolations from wheat roots (unpublished work, Soil Microbiology Laboratory at the University of Saskatchewan). The identity is presented as the genus of the nearest relative identified in the NCBI database along with the accession number and % similarity of that sequence to the 16S rRNA sequence obtained in Chapter 3. Location indicates the treatments these isolates were obtained from: control treatment (Ctrl), nitrogen fertilizer (N_{fert}), phosphorous fertilizer (P_{fert}), and nitrogen and phosphorous fertilizer (NP_{fert}) applications. Isolates in bold were selected not because they were common to Rotation ABC and Bitumount but rather because they were unique to a fertilizer treatment where additional nutrient stress was present.

New Code	Old Code	Identity (Accession/ % similarity)	Location
A1	**P34**	***Achromobacter spanius*** (NR_025686 / 100%)	**P_{fert}**
A2	**N30**	***Acinetobacter baumanni*** (NR_026206 / 99%)	**N_{fert}**
A3	P5	*Pseudomonas flourescens* (CP012830.1 / 100%)	P_{fert}
A4	P41	*Bacillus halosaccharovoians* (NR_109116.1 / 99%)	P_{fert}
A5	P9	*Arthrobacter oryzae* (NR_041545.1 / 100%)	P_{fert}
A6	C2	*Bacillus pumillus* (AB741856 / 99%)	Ctrl, P_{fert}, N_{fert}, NP_{fert}
A7	C25	*Chryseobacterium indoltheticum* (KX249602 / 99%)	Ctrl, NP_{fert}
A8	C18	*Chryseobacterium oncorhynchi.* (MH542627.1 / 99%)	Ctrl, NP_{fert}
A9	**C30**	***Delftia acidovorans*** (MF156902.1 / 99%)	**Ctrl, P_{fert}**
A10	**C16**	***Delftia tsuruhatensis*** (CP017420.1 / 99%)	**Ctrl, P_{fert}**
A12	**N35**	***Kluyvera intermedia*** **(**KX417293.1 / 99%)	**N_{fert}, NP_{fery}**
A13	C29	*Leifsonia xyli* (KX881442 / 99%)	Ctrl, P_{fert}
A14	NP33	*Luteibacter rhizovicinus* (CP017480.1 / 99%)	NP_{fert}
A15	**C43**	***Luteimonas composti*** (NR_043983.1 / 99%)	**Ctrl, P_{fert}**
A16	C40	*Microbacterium lacticum* (MF101157 / 98%)	Ctrl
A17	NP26	*Pedobacter alluvionis* (HF936841 / 97%)	NP_{fert}

Table A.3 continued:

New Code	Old Code	Identity	Location
A18	P33	*Pedobacter steynii* (KX981359.1 / 98%)	Pfert
A20	NP12	*Pseudomonas vancouverensis* (KY753376.1 / 99%)	Nfert, NPfert
A21	NP14	*Pseudomonas brassicacearum* (KT695818.1 / 99%)	NPfert
A22	C23	*Rhizobium lemnae* (NR_126174.1 / 100%)	Ctrl,Nfert, NPfert
A23	P36	*Rhodococcus cerastii* (KY775501.1 / 99%)	Pfert
A24	P26	*Salmonella enterica* (CP009102.1 / 95%)	Pfert
A25	C19	*Sphingomonas wittichii* (MK318617 / 100%)	Ctrl
A26	P15	*Pseudomonas rhodesiae* (KX657792.1 / 99%)	P_{fert}
A27	C37	*Staphylococcus pasteuri* (MG966355 / 99%)	Ctrl
A28	NP16	*Staphylococcus epidermidis* (KX648542.1 / 99%)	NP_{fert}
A29	C33	*Xanthomonas campestris* (MF150306 / 99%)	Ctrl, P_{fert}, N_{fert},NP_{fert}

APPENDIX B: Results from genotype/phenotype screening.

Table B.1: Summary of results for gene survey and plant growth-promoting and hydrocarbon degradation activity assays. Isolates are given one point for each positive genotype and two points for each positive phenotype denoted by Y (+) and N (–). The total score for each isolate is tallied and shown.

I.D	Gene: *phoD*	ALP Assay	Gene: *alkB*	Gene: *CYP153*	Octane Utilization	Diesel Utilization	Gene: *acds*	ACC Deaminse	Gene: *nifH*	ARA	Total (%)
A27	–	N	–	–	N	N	–	N	–	N	0.00
B5	–	N	–	–		Y	–	N	–	N	15.38
B40	–	N	–	–	Y	Y	–	N	–	N	15.38
A14	–	N	–	–	Y	Y	–	N	–	N	15.38
A17	–	Y	–	–	N	N	–	N	–	N	15.38
B1	+	N	–	–	Y	Y	–	N	–	N	23.08
B12	–	N	–	+	Y	Y	–	N	–	N	23.08
A10	–	N	–	+	N	N	–	Y	–	N	23.08
A16	–	N	–	–	N	N	–	N	+	Y	23.08
A4	–	N	–	–	Y	Y	–	N	+	N	23.1
B2	–	Y	–	–	N	Y	–	N	+	N	30.77
B3	–	Y	–	–	–	–	–	Y	–	N	30.77
B4	–	Y	–	–	Y	Y	–	N	–	N	30.77
B6	–	N	–	–	Y	Y	–	Y	–	N	30.77
B10	–	Y	–	–	Y	Y	–	N	–	N	30.77
B14	–	Y	–	–	Y	Y	–	N	–	N	30.77

Table B.1 continued

I.D	Gene: *phoD*	ALP Assay	Gene: *alkB*	Gene: *CYP153*	Octane Utilization	Diesel Utilization	Gene: *acds*	ACC Deaminse	Gene: *nifH*	ARA	Total (%)
B16	–	Y	–	–	Y	N	–	N	–	N	30.77
B35	–	N	+	–	Y	Y	–	N	+	N	30.77
A1	–	Y	–	–	N	N	–	Y	–	N	30.77
A8	–	Y	–	–	Y	Y	–	N	–	N	30.77
A13	–	N	–	–	Y	Y	–	Y	–	N	30.77
A18	–	Y	–	–	Y	Y	–	N	–	N	30.77
A20	–	N	–	–	Y	Y	–	Y	–	N	30.77
A21	–	N	–	–	Y	Y	–	Y	–	N	30.77
A26	+	N	+	–	N	N	–	Y	–	N	30.77
B8	–	Y	–	–	Y	Y	–	N	+	N	38.46
B9	–	Y	+	–	Y	Y	–	N	–	N	38.46
B11	–	Y	+	–	Y	Y	–	N	–	N	38.46
B15	+	Y	–	–	Y	N	–	N	–	N	38.46
B20	–	N	–	–	Y	Y	+	Y	–	N	38.46
B26	–	Y	–	–	Y	Y	–	N	+	N	38.46
B33	–	Y	–	+	Y	Y	–	N	–	N	38.46
A2	–	Y	+	–	N	N	–	Y	–	N	38.46
A9	–	N	–	+	Y	Y	–	Y	–	N	38.46

Table B.1 continued

I.D	Gene: *phoD*	ALP Assay	Gene: *alkB*	Gene: *CYP153*	Octane Utilization	Diesel Utilization	Gene: *acds*	ACC Deaminse	Gene: *nifH*	ARA	Total (%)
A15	–	Y	+	–	N	N	–	Y	–	N	38.46
A25	–	Y	–	+	N	N	–	Y	–	N	38.46
A28	–	Y	–	–	Y	Y	–	N	+	N	38.46
B17	+	N	–	–	Y	Y	+	Y	–	N	46.15
B32	–	Y	+	+	Y	N	–	N	–	N	46.15
B34	–	Y	–	–	Y	Y	–	Y	–	N	46.15
B38	–	Y	–	–	Y	Y	–	Y	–	N	46.15
A3	–	N	+	–	Y	Y	+	Y	–	N	46.15
A6	+	N	–	–	Y	N	–	Y	+	N	46.15
A7	+	Y	+	–	Y	Y	–	N	–	N	46.15
A12	–	Y	–	–	Y	Y	–	Y	–	N	46.15
A29	–	Y	–	–	Y	Y	–	Y	–	N	46.15
B13	+	Y	+	–	Y	Y	–	N	+	N	53.85
B21	+	N	–	+	Y	Y	+	Y	–	N	53.85
B22	+	N	+	–	Y	Y	+	Y	–	N	53.85
A5	–	Y	+	–	Y	Y	+	N	+	N	53.85
A22	–	Y	–	–	Y	N	+	Y	–	N	53.85
A24	+	Y	–	–	Y	Y	–	Y	–	N	53.85

Table B.1 continued

I.D	Gene: *phoD*	ALP Assay	Gene: *alkB*	Gene: *CYP153*	Octane Utilization	Diesel Utilization	Gene: *acds*	ACC Deaminse	Gene: *nifH*	ARA	Total (%)
B18	+	Y	–	–	Y	Y	+	Y	–	N	61.54
B25	–	Y	–	–	Y	Y	+	N	+	Y	61.54
B30	–	Y	+	+	Y	Y	–	Y	+	N	61.54
B31	–	Y	+	+	Y	Y	–	Y	–	N	61.54
A23	–	Y	+	–	Y	Y	+	Y	–	N	61.54
B23	+	Y	–	+	Y	Y	+	Y	–	N	69.23
B19	+	Y	–	+	Y	Y	+	Y	+	N	76.92

APPENDIX C: Mean emergence and survival rates of wheat plants in growth chamber experiments.

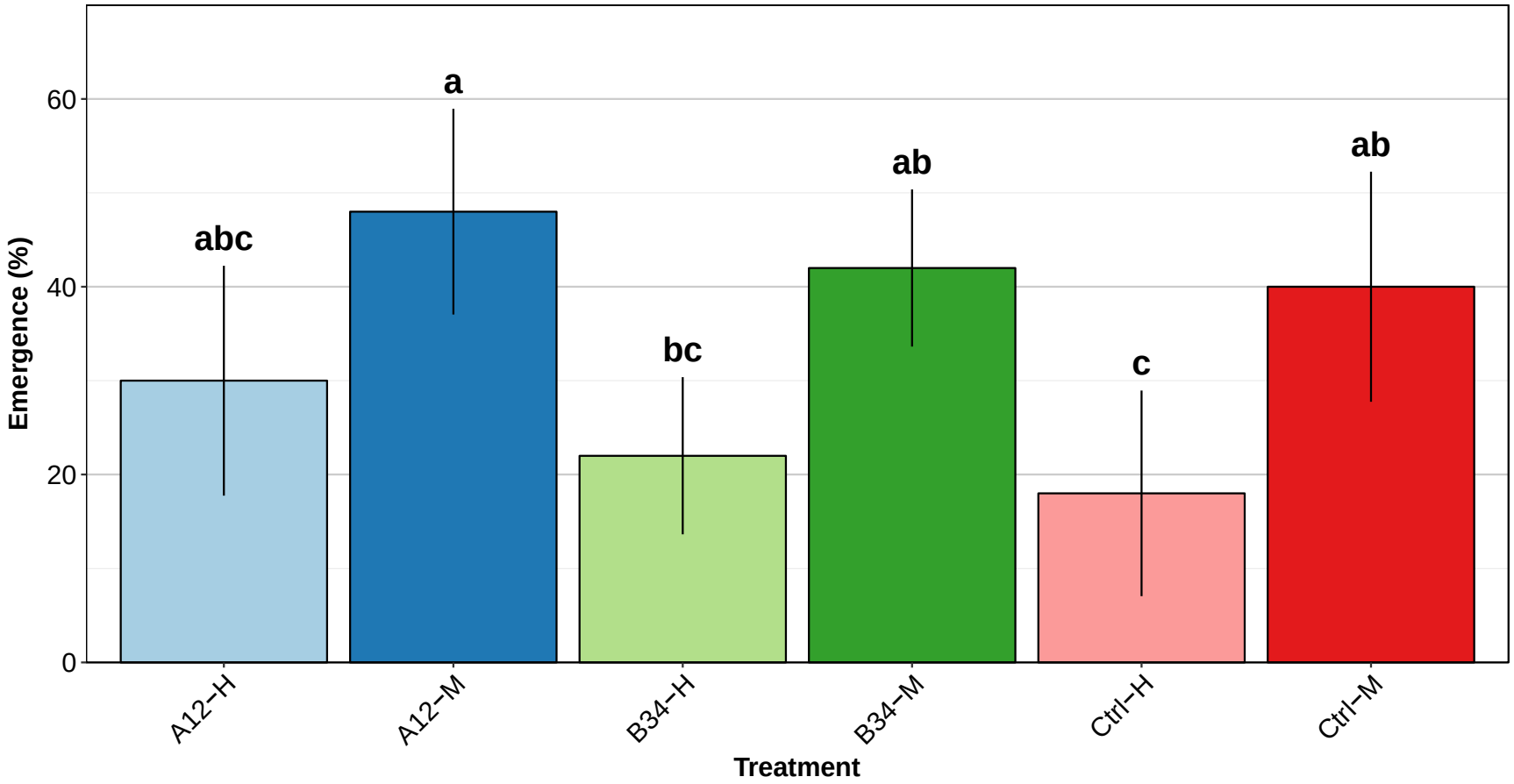

Figure C.1: Emergence of treated wheat plants 7 days after planting in soil with (dark shaded bars) and without (light shaded bars) diesel (5000 mg·kg^{-1}) and inoculated with bacteria. Error bars represent ± 1 standard deviation. Letter of significance were determined using Tukey HSD at $\alpha = 0.05$.

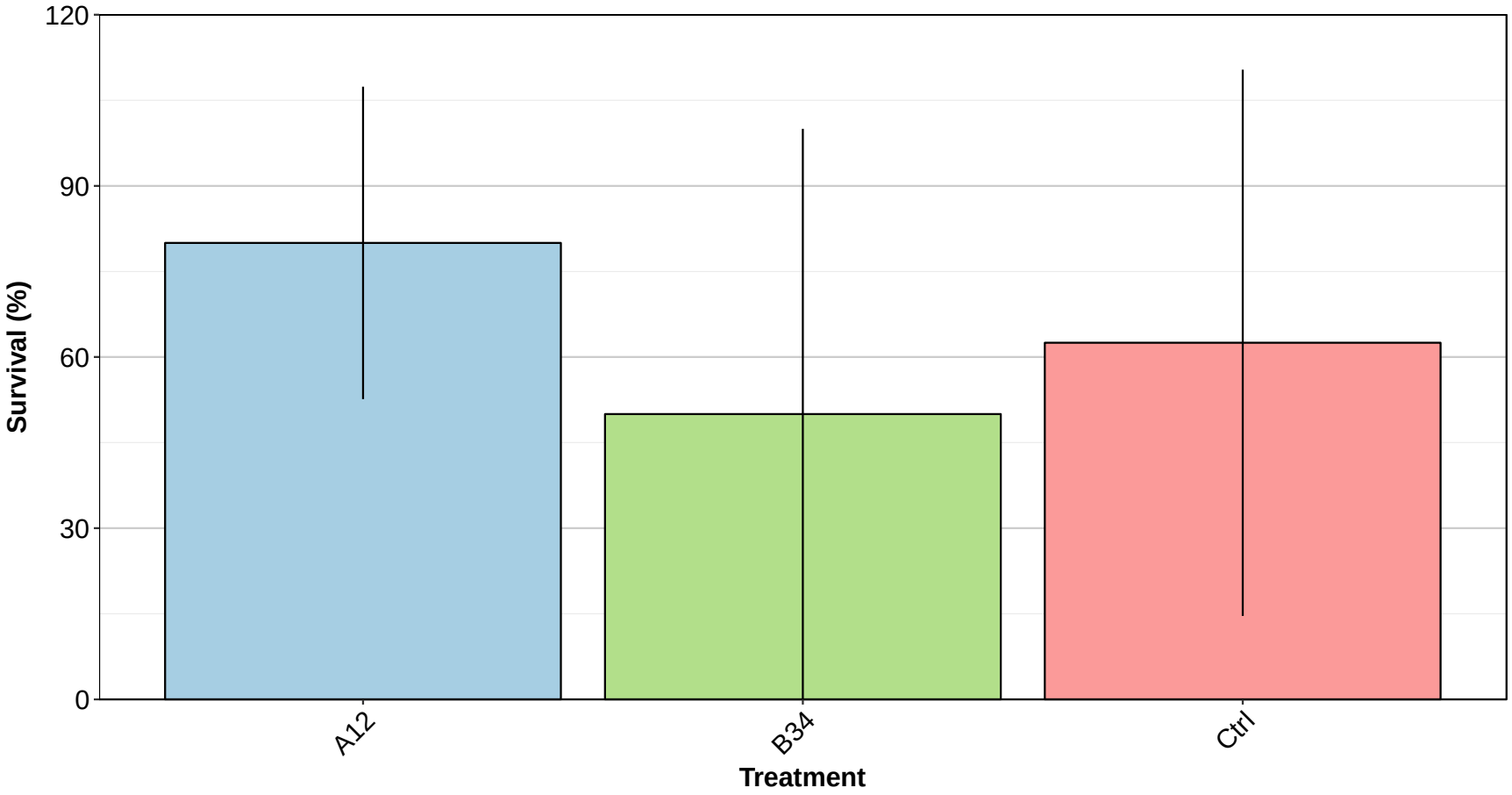

Figure C.2: Mean survival rates (± 1 s.d) of treated wheat plants grown in soil amended with diesel (5000 $mg \cdot kg^{-1}$) at harvest (60 days after planting). No significant difference in survival was detected among treatments (ANOVA, $\alpha = 0.05$).

APPENDIX D: Mean values (± s.d) NH_4^+ (a) and PO_4^{3-} (b) in wheat plants (mg nutrient g^{-1} dry plant material)

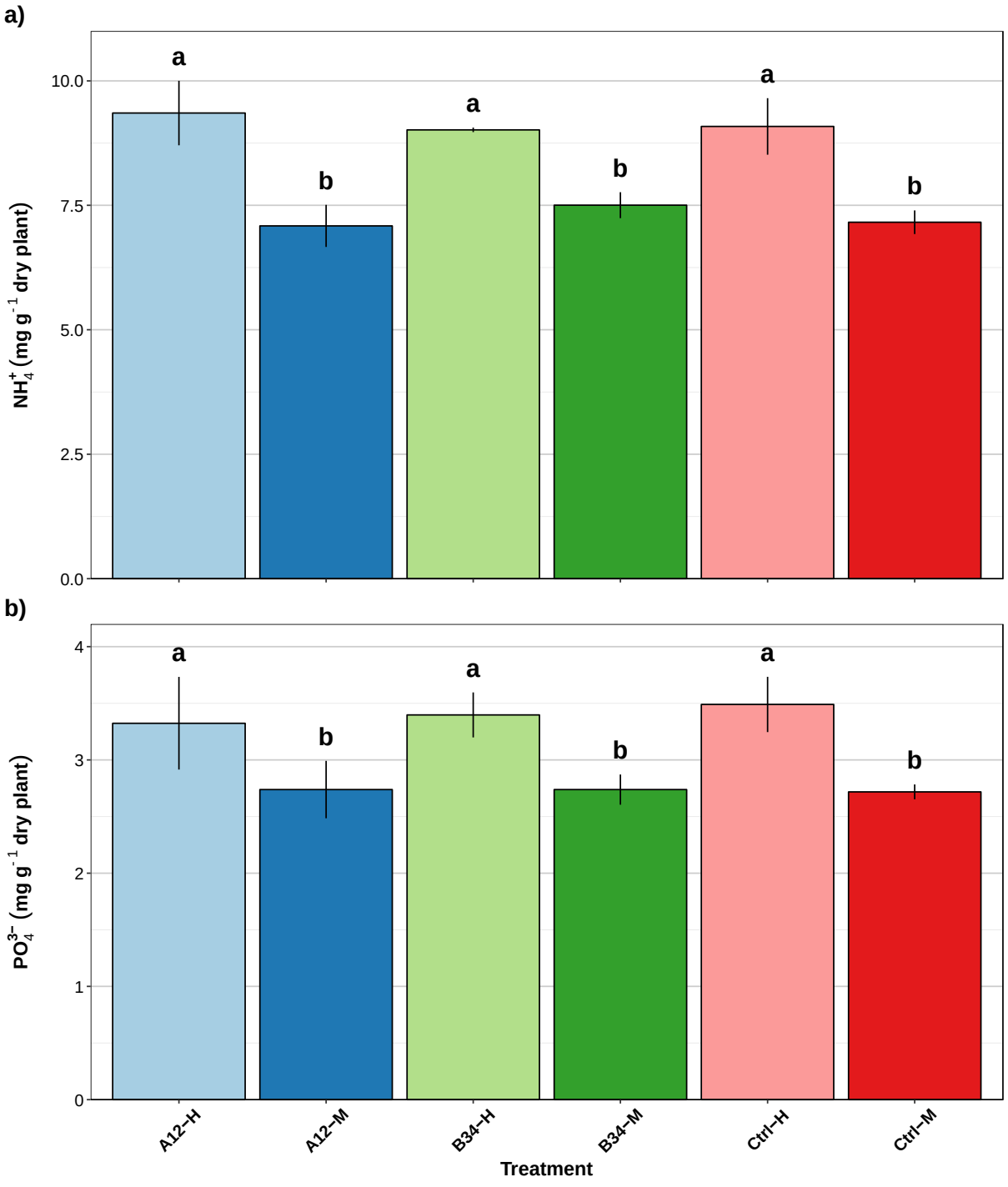

Figure D.1: Concentration of NH_4^+ (a) and PO_4^{3-} (b) in wheat plants in soil with (dark shaded bars) and without (light shaded bars) diesel (5000 mg·kg^{-1}) and inoculated with bacteria. Error bars represent ± 1 standard deviation. Letter of significance were determined using Tukey HSD at α = 0. 05.

APPENDIX E: Photos of plants in growth chamber

Figure E.1: Treated wheat plants after growing in root pouches seven days after inoculation.

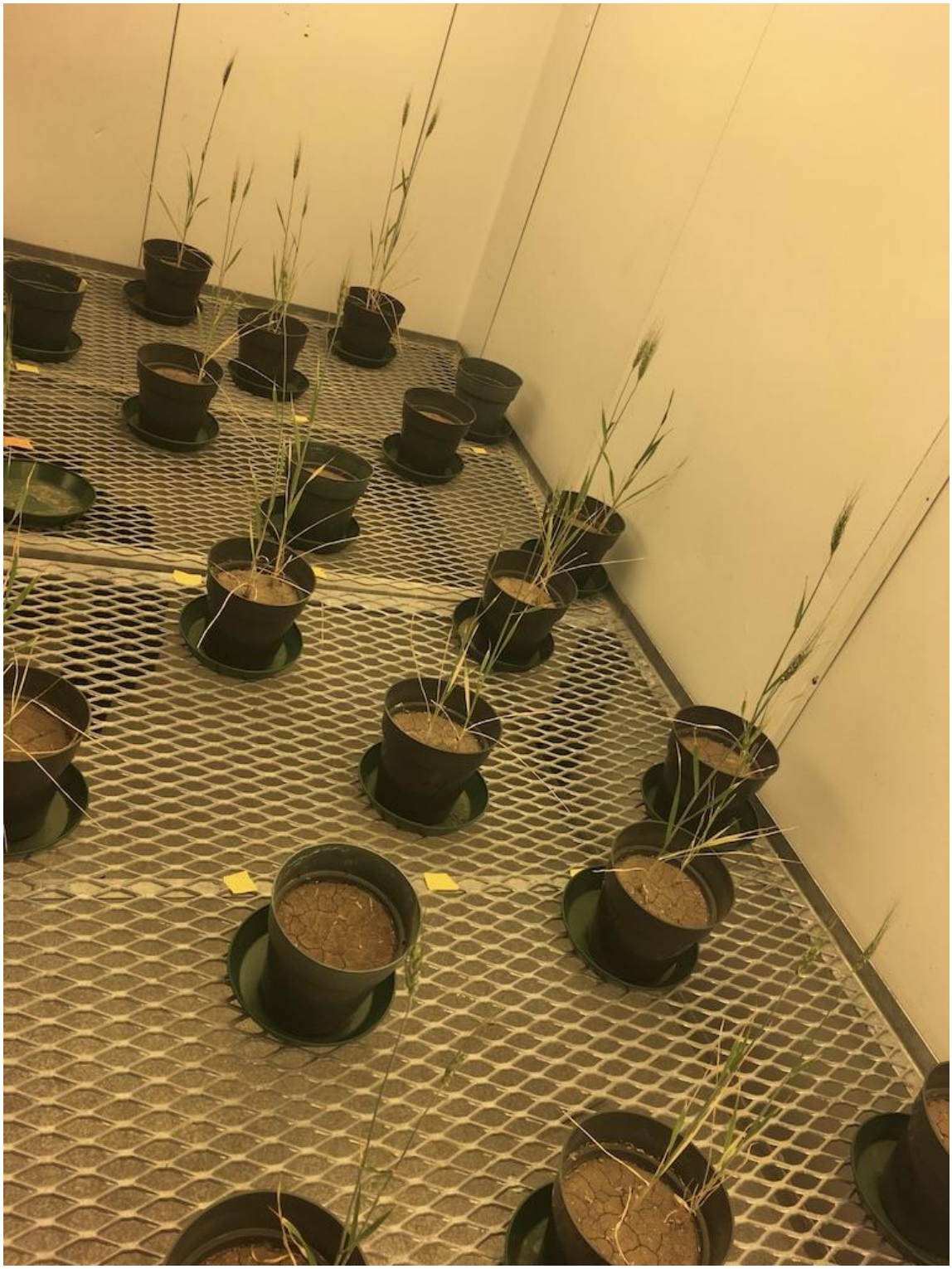

Figure E.2: Inoculated wheat plants 60 days after planting and immediately prior to harvest organized in RCBD in growth chamber

Figure E.3: Inoculated sweet clover plants 60 days after planting and immediately prior to harvest organized in RCBD in growth chamber.