The Isolation & Characterization of Endophytic Microorganisms from the Industrial Hemp Plant (*Cannabis sativa* L.)

by

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April 2016

A thesis submitted to McGill University in partial fulfillment of the requirements of the

Degree of Master of Science

Dedication:

I wish to acknowledge the role of my undergraduate thesis advisor Dr. Antonet Svircev had in promoting my continuing in academia past the undergraduate level; to take on and manage a project independently at the MSc level. It is through the techniques that I learned under the guidance of excellent technicians and senior students my previous labs, and especially during my BSc. Honours thesis, that allowed me to quickly adapt to the MSc. environment and get the most of my time. I would also like to note the supportive role of the entire Jabaji Lab, both longstanding and those who shared only a short period of time with me. I would like to especially highlight the support of our lab technician: Ms. Mamta Rani, a true friend during my MSc. tenure at McGill. Thank you all for helping me to grow in knowledge and confidence throughout my years at McGill.

I would like to dedicate this project most especially to my family, who constantly have my back. They have provided me with the core tools, values, and stimulation that has allowed me to explore my curiosity of the natural world from an early age. Their help has allowed me and by extension, this project, to continue and would not have been possible without them. Thank you all so much!

Table of Contents

The Isolation & Characterization of Endophytic Microorganisms from the Industrial Hemp Plant (*Cannabis sativa* L.) 1
Dedication: 2
Table of Contents 3
List of Tables and Figures: 6
Abstract: 7
Résume: 8
Acknowledgements: 10
List of Abbreviations: 11
Contribution of Authours: 12
Chapter 1: General Introduction 13
1.1 Project Objectives: 14
1.2 Hypotheses: 15
Chapter 2: Literature Review 16
2.1 Industrial Hemp 16
2.2 Microbial Endophytes 18
2.3 Endophyte Source and Host Colonization 20
2.4 Culture-based Endophyte Methods 22
2.5 Culture-Independent Endophyte Methods 23
2.6 Endophyte Characterization 24
2.7 Crop Improvement through Endophytes 26
2.8 Volatiles, IAA and ACC 27
2.9 Biological Control Agents (BCAs) 29
2.10 Nutrient mobilization 30
Chapter 3: Materials and Methods 33
3.1 Microbial Isolation & Characterization 33
3.1.1 Farm Site, Cultivars and Tissue Collection 33
3.1.2 Endophyte Isolation 34
3.1.3 Microbial Purification & Culturing 34

3.2 Molecular Identification and Characterization of Endophytes 35
3.3 Phenotypic and Chemical Characterization of Endophytes: 36
3.3.1 Cellulase and Phosphatase Solubilization 36
3.3.2 Siderophores 36
3.3.3 Hydrogen cyanide (HCN) 37
3.3.4 Antibiotic Resistance 37
3.3.5 Organic acid production (OAP) 37
3.3.6 Indole Acetic Acid Production 38
3.3.7 1-Aminocyclopropane-1-Carboxylate (ACC) Deaminase Activity 38
3.3.8 Antifungal Activity 38
3.4 Genetic Characterization: 39
3.5 Microbial Phylogenetic Tree Generation 39
3.6 Plant Response to Endophyte Colonization 40
3.6.1 Endophytic Bacterial Growth and Inoculum Preparation 40
3.6.2 Effect of Bacterial Volatiles on Tomato Seedlings 40
3.6.3 Detection and Colonization of Tomato Seedlings by Endophytic Bacteria 41
3.6.4 Plant Growth and Colonization Assessment following Re-introduction of Endophytes into C. sativa L. cv. Anka Seedlings 42
3.6.5 Assessment of endophytic colonization: 43
3.6.6 Culture Independent Colonization Confirmation: 44
Chapter 4: Results 45
4.1 Patterns of Isolation: 45
4.2 Isolate Identification: 46
4.3 Bacterial Screening, Selection, and Characterization: 47
4.4 Fungal Characterization: 48
4.5 Effect of Endophytic Bacterial Volatiles on Plant Growth 48
4.6 Re-inoculation of Plants with *Pseudomonad* Endophytes 49
4.6.1 Tomato Seedlings: 49
4.6.2 Hemp Seedling Re-Inoculation 49
Chapter 5: Discussion 51
5.1 Effects of Cultivar and Tissue Source on Endophyte Assemblage 51
5.2 Temporal Effects: 55
5.3 Characterization: 56

5.4 Re-Introduction of Bacterial Endophytes to Plant Hosts: 59
Chapter 6: Conclusions 62
Chapter 7: Recommendations for Future Studies 63
Chapter 8: Literature Citations 64
Chapter 9: Tables 79
Chapter 10: Figures 90
Chapter 11: Appendix 97

List of Tables and Figures:

Table 1. GPS Co-ordinates of Hemp plants whose tissues where used for endophyte discovery ……79
Table 2. PCR primers used in this Study…….……...80
Table 3. Biochemical tests used for the characterization of endophytes isolated from Hemp tissues....……...81
Table 4. Cultivar and tissue origin of bacterial endophytes……...82
Table 5. Cultivar and tissue origin of fungal endophytes……...83
Table 6. Attributes of four endophytic *Pseudomonas* strains of interest…...…….84
Table 7. Inhibition of radial growth of test fungi in confrontation assays…...……...85
Table 8. Fungal isolate characterization….……86
Table 9. List of fungal endophytes of Hemp that possess positive sequences for Endophyael bacteria……87
Table 10. Effect of bacterial volatile compounds on the growth of Tomato seedlings……..88
Table 11. Growth parameters of re-inoculated Hemp seedlings grown under greenhouse conditions….…….89

Fig. 1 Distribution of bacterial and fungal endophytes..……...……..90
Fig. 2 Genera of microbes isolated from *C. sativa* L…….……91
Fig. 3 Temporal distribution of endophytic isolation……...……...……....92
Fig. 4 Maximum parsimony phylogeny of endophytic bacteria isolated from Hemp……...……..93
Fig. 5 Maximum parsimony phylogeny of endophytic fungi isolated from Hemp……...…..94
Fig. 6. Dynamics of *Pseudomonas* strains in a dicot model host plant…….……..95
Fig. 7 Re-colonization of Hemp seedlings by selected endophytes under greenhouse conditions…….96

Abstract:

Aim: To Identify, and characterize the cultivable endophytic microbiome of *Cannabis sativa* L. for Plant Growth Promoting (PGP) and Biological Control Agent (BCA) potential.

Methods and Results: One hundred thirty-seven bacterial and 53 fungal isolates were identified from the leaves, petioles and seeds of three field grown cultivars of hemp in the summer of 2013; Anka, CRS-1 and Yvonne. Eighteen bacterial, and thirteen fungal genera were recovered. Almost all fungal isolates (96 %) were members of the *Ascomycota* and all were dikarya. The most commonly identified bacterial isolates were *Pseudomonadaceae* (34 %), and *Enterobacteriaceae* (26 %). Leaves were heavily colonized by fungi (70 % of all isolates) and petioles were more often colonized by bacteria (67 %). Fungi were characterized for enzymatic activities and the presence of Endohyphael bacteria (EHB). Six of these fungi gave early indication of EHB infection. Biochemical and molecular assays were applied to characterize and screen bacterial isolates for top performing isolates for possible use as a bacterial inoculant. Four bacteria were selected for additional study based upon a promising suite of PGP attributes. Strains BTC6-3, BTC8-1, BTG8-5 and BT14-4 are all members of the *Pseudomonas* genus and showed good results in expanded PGP tests. Direct fungal growth inhibition was performed against 10 different fungi including important hemp pathogens *Botrytis cinerea* and *Sclerotinia sclerotiorum*. Three of four endophytic isolates were able to significantly reduce growth against some of the fungi used including *B. cinerea* and *S. sclerotiorum*. *In planta* testing confirmed competency in endophytic re-colonization under sterile growth chamber conditions using a Tomato host system, but demonstrated only epiphytic infection in greenhouse trials with the native host.

Conclusions: Genera from both fungal and bacterial endophytes were widespread, and diverse. Some fungi appear to possess EHB; select bacterial isolates possess known PGP and BCA characteristics and were shown to be able to re-colonize a dicot species. This study is the first description of endophytes in industrial hemp grown in NE North America.

Résume:

Le But : Cette étude a examiné la communauté microbienne endophytique du chanvre industriel (*Cannabis sativa* L) pour la présence de champignons et de bactéries qui possèdent des qualités de promotion de la croissance végétale (PCV) et antifongique.

Méthodes et Résultats : Les plantes de chanvre ont poussé au champ durant l'été 2013 et les feuilles, les pétioles et les grains des cultivars Anka, CRS-1 et Yvonne ont été évalués. Des bactéries de dix-huit genres parmi cent trente-sept isolats et des champignons de treize genres parmi cinquante-trois isolats ont été découverts. Presque tous les champignons isolés (96 %) sont membres d'*Ascomycota* et tous sont membres de dikarya. Les isolats de bactéries le plus fréquemment isolés sont des *Pseudomonadaceae* (34 %), et des *Enterobacteriaceae* (26 %). Les feuilles étaient plus colonisées par les champignons (68 % de champignons) ; alors que les bactéries étaient majoritaires dans les pétioles (67 %). Des tests biochimiques et moléculaires ont été utilisés pour caractériser et déterminer si les isolats obtenus peuvent servir d'inoculant chez leur hôte. Les champignons ont été évalués pour leurs activités enzymatiques et la présence des bactéries endohyphael (BEH). Six champignons ont donnés une indication préliminaire positive et possèdent un potentiel certain. Les isolats de bactéries BTC8-1, BTG8-5 et BT14-4 sont tous membres du genre *Pseudomonas* et ont bien performés lors des examens PCV. Dix champignons différents dont les pathogènes connues du chanvre industriel : *Botrytis cinerea* et *Sclerotinia sclerotiorum* ont été utilisés pour déterminer le potentiel antifongique des bactéries isolées. Trois des quatre isolats bactériens ont inhibé au moins un des dix champignons de façon significative. Les études *in planta* ont confirmé que des isolats avaient le potentiel de recoloniser des plants de tomates en conditions stériles. Par contre, ces isolats sont restés epiphytiques et non pas endophytiques lorsqu'appliqués sur des plants de chanvre cultivé dans des serres.

Conclusions : Des champignons et les bactéries endophytiques ont été découverts dans les tissus du chanvre industriel. Une partie de ces champignons possède des BEH et les isolats de bactéries possèdent des caractères PCV et antifongiques, mais il a été difficile de réintroduire ces endophytes dans leur milieu

naturel. Cette étude est la première qui examine la communauté endophytique du chanvre industriel cultivé dans le nord-est de l'Amérique.

Acknowledgements:

A work of this magnitude is not possible without the aid of many persons and supports. Firstly, I wish to thank funding sources from MAPAQ and NSERC operating grants provided by my supervisor Dr. Suha Jabaji and my co-supervisor Dr. Jean-Benoit Charron for allowing me to continue this project and for various supplies that were required for its completion. The NSERC's Michael Smith Foreign Study Supplement additionally allowed me to further my training on fungal endophytes, under the guidance of Dr. Arnold at the University of Arizona. The fungal identification and characterization would not have been possible without the support of Dr. Betsy Arnold and her gifted students' aid.

I would like to acknowledge the help of Ms. S. Thibaut who aided in the phenotypic and biochemical characterization of bacterial endophytes. Special thanks is also extended to Dr. F. Gagné-Bourque, whose advice and expertise with bacterial endophytes facilitated the initial design and sampling strategy used in this project. Ms. M. Aubin, who provided me with access to field grown help tissues and advice in the early stages of the project; Ms. M. Rani who provided technical aid, particularly throughout the last set of experiments of this project; and Ms. T. Copley who provided support and procedural knowledge necessary for its completion, all facilitated the progression of this work. All "Fun-lab" members, past and present, short or long term were important supports throughout this project, and certainly made its completion a more pleasant work than it could have otherwise been.

My Supervisors Dr. S. Jabaji and Dr. J-B. Charron were a vital source of direction and support throughout the planning, and execution of the project, in addition to their aid in the correction of draft writings of this manuscript. Thanks to Dr. P. Seguin, who provided advice for hemp physiology and growth, Dr. V. Gravel who kindly provided statistical aid and informative design discussion, and technician Mr. G. Rimmer who was instrumental facilitating quick access to and continued care of greenhouse and tissue chambers for these experiments. Lastly, I wish to thanks to all members of the Plant Science Department who supported me during my years at McGill, a work of this magnitude is only accomplished with the support of many unnamed persons in the backing foundation.

List of Abbreviations:

ACC-1-Aminocyclopropane-1-Carboxylate

BCA- Biological Control Agent

CTAB- Cetyl trimethylammonium bromide

HCN- Hydrogen Cyanide

HIA- Hemp Industries Association

IAA- Indole-3-Acetic Acid

ITS- Internal Transcribed Spacer

LSU- Large Ribosomal Subunit

NS- Non-sterile

PGP- Plant Growth Promotion

ROS- Reactive Oxygen Species

rDNA- Ribosomal DNA

rRNA- Ribosomal RNA

S- Sterile

THC- Δ-9-tetrahydrocannabinol

VOC- volatile organic compound

Contribution of Authours:

This work has been written in the traditional thesis format as per the Department of Plant Science and Graduate studies at McGill University according to “Guidelines for Thesis Preparation and Submission”. The work herein was done under the co-supervision and co-authourship of Drs. Jabaji and Charron and with financial support from Le ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec (MAPAQ), and the Natural Sciences and Engineering Research Council of Canada (NSERC). The candidate conducted the field collection, isolation and subsequent characterization studies. Data compilation, and analysis was also done by the candidate. This thesis was written by the candidate under the supervision of Dr. Jabaji, with special aid from Dr. Charron for the French abstract, and is composed of 11 chapters.

The author, Maryanne Scott, performed the experiments detailed below, data analysis and manuscript writing. Dr. Charron and Dr. Jabaji provided funds and support for the research, including supervisory support and manuscript review. Dr. Arnold provided research materials and support for fungal characterization studies.

Chapter 1: General Introduction

There is evidence that many parts of the world are reaching their productive maximum for agricultural land use (OECD-FAO, 2012). The negative ramifications from the use of unsustainable inputs, such as chemical pesticides, stresses the ecosystem, and endangers future yields (OECD-FAO, 2012). As public opinion and advances in biotechnology turn toward more sustainable means to meet food and product needs, research interests are responding. Future yield increases will be brought about through multiple means such as closing waste gaps, use of previously non-agrarian environments in creative ways, and intensification of plant growth itself. These goals can be achieved, sustainably, through harnessing the natural, biodiverse microbiota from multiple sources, including the interior of agricultural and industrial crops.

Endophytes are organisms that live within the tissues of healthy plants and belong to all domains of microbial life (Hallmann et al., 1997; Muller et al., 2015; Rodriguez et al., 2009). They are ubiquitous in natural plant life, arising from both horizontal infections from the plants' environment and from vertical transmission between generations in seed tissues. Many of these microbes have been found to confer growth or adaptation benefits to their host, especially under stressful conditions (Sun, 2014; Ali, 2014). As the global climate becomes increasingly irregular and we continue to search for sustainable means to help agriculture adapt, endophytes have become one of the prime targets of research.

Diversification of crops is one approach to meet more needs and improve soil and pest problems. One multiuse crop that has recently played an increasing role in Canadian agriculture, especially in the Prairie Provinces, is the industrial hemp plant (*Cannabis sativa* L.). Hemp is a fast-growing, renewable resource that can be grown for both its fiber and seed; it has been frequently processed into a variety of surprising products (Health Canada, 2013). There is rising international interest in hemp and hemp products (Callaway & Pate, 2009). While Canada is not the most prolific grower of hemp; Canadian hemp is considered a source of excellent, food quality hemp seed and downstream products (Callaway & Pate, 2009). Canadian hemp farmers' largest demand comes from the United States; where the Hemp Industries

Association (HIA) has estimated hemp and hemp products' total value to have been 500 million dollars in 2013 (AgMRC, 2015). In 2010, ten million dollars of hemp seed and oilcakes alone were exported to the US, so the crop is of clear value, despite comprising less than 3 % of Canadian crop production (Johnson, 2012; Government of Alberta, 2012; Aubin, 2014).

Nationally and locally, interest in hemp for both home use and as an export commodity is driving a search to better understand the plant itself. While international support and Canadian acreage dedicated to hemp seems to be stable or increasing (Franz-Warkentin, 2013), farmer adoption of this crop within Québec has been slow. This may be due to insufficient and conflicting information available to farmers on this crop. It was not until very recently that regulations for optimal growth of hemp in Québec were written (Aubin, 2014). To better understand the biology of this crop, this project sought to improve knowledge of the endophytic microbial community present within industrial hemp and, following their identification, to characterize endophytes for plant growth promoting (PGP) and biological control agent (BCA) attributes. Select endophytic bacteria were studied more closely for potential agricultural application using both hemp and tomato seedlings. This study provides a valuable contribution to the scientific community's collective knowledge of endophytic micro-organisms and in the long-term, is hoped to improve the quality and yield of the Québec and Canadian hemp crop.

1.1 Project Objectives:

1. Isolation of endophytic bacteria and fungi from multiple varieties and tissues of *Cannabis sativa* L.
2. Phenotypic, molecular and biochemical characterization of microbial endophytes of hemp
3. Study the effect of select endophytes on the growth of tomato seedlings
4. Evaluate the plant growth promoting abilities of selected isolates to enhance hemp seedling growth

1.2 Hypotheses:

1. Endophytes belonging to different taxonomic groups are present in tissues and organs of hemp cultivars
2. Both culture-dependent and culture-independent methods will successfully isolate and characterize the endophytes of industrial hemp. A variety of characteristics will be present in isolated endophytes
3. A subset of the isolates is able to re-colonize hemp and tomato seedlings
4. Introduction of the endophytes to young hemp seedlings will lead to improved growth outcomes; overall improvements in yield quantity and quality

Chapter 2: Literature Review

2.1 Industrial Hemp

Originating from the Himalayas, industrial hemp has been grown for many centuries (Clarke, 1999). Hemp was grown in Canada from the 1800s to 1930s; and more recently since 1998. The intervening years saw a ban on its growth throughout Canada and the United States; where it is still in effect. This legislation was largely due to public association of the crop with its medicinal relative: marijuana and the negative social stigma associated with marijuana and its use (Health Canada, 2012; Clarke, 1999; Queen, 2014).

Fibrous hemp has uses ranging from the traditional: paper, rope, and textiles manufacture, to more recent and revolutionary innovations; its use in the construction of homes in the UK and building of electric car components in Canada (Callaway & Pate, 2009; Minor, 2010; Canadian Hemp Trade Alliance, 2010). Hemp seeds and concomitant oils are well known as having a very high health profile and are a large part of its insurgent popularity. Hemp seeds are approximately 36 % oil; 80% of which are healthier, unsaturated fats (Hemp Oil Canada, 2013). In addition, the ratio of omega fatty acids present in the oil is considered close to ideal for human health (Callaway & Pate, 2009; Canada Hemp Foods, 2015). The high amount of protein (25 %) found in the seed makes hemp a powerful non-meat protein source when compared to other non-meat sources such as soy bean (32 %) and egg white (11 %) (Callaway & Pate, 2009; Canada Hemp Foods, 2015). Hemp oil, like the plant itself, is multi-use; being found in human foods, dressings, and health food products, and as an additive in industrial grade agents, including cleaners and paints (Canadian Hemp Trade Alliance, 2010).

Following its re-legalization, the economic benefit of hemp growth in Canada has become evident through its increasing use as a rotation crop in many regions of Canada, and the increasing acreage given to hemp (Health Canada, 2012). Across Canada; 66,700 acres were grown in 2013, a record number that was a 24 % increase from the previous year's 54,000 acres (Franz-Warkentin, 2013).

Both medicinal and hemp varieties are members of the *Cannabis sativa* species, however industrial hemp cultivars have long been bred for their fiber, oil and seed aspects, and possess very low narcotic value. In Canada varieties are mandated to have under 0.3 % of Δ-9-tetrahydrocannabinol (THC), a chemical found at its most concentrated in the plants' top leaves and flowering heads. THC acts as the primary cause of drug effects found in marijuana varieties (Health Canada, 2012). Medicinal *Cannabis* seized in England was found to possess THC content between 1.15 to 23.17%, with a median value of 13.9% (Potter et al., 2008); while concentrations of THC in American street marijuana were over 8 % (Mehmedic et al., 2010). There is ongoing research into the chemical pathways that differentiate hemp from its narcotic cousin. Both the medicinal and hemp varieties of *C. sativa* have been sequenced, and will shed light on differences between these cultivar groups in the future (van Bakel et al., 2011; Minor, 2010).

Hemp is a fairly robust crop. It has a reputation as being generally pest free (McPartland, 1999), it is responsive to traditional manure fertilizer or more synthetic additions, and it is tolerant of variation in the quantity of water and therefore the quality of irrigation (Montford, 1999). Hemp has long been known as a weed suppressant due to its physiological characteristics such as fast establishment and extensive canopy (Clarke, 1999). It shows allopathic activity against both monocot and dicot species, reducing neighbouring, competitor plant growth (Pudelko et al., 2014). Research has also found growth benefits of over 9 %, in the three years following an intervening year of hemp growth in an otherwise continuous soybean cropping situation, thought to be a result of anti-nematode effects of hemp planting (Liu et al., 2012).

While it is true that hemp is pest tolerant, it does have some pests. Insects such as stem borers (*Grapholita delineana*) and flea beetles (*Psylliodes attenuata*) can cause stem and leaf damages respectively, and fungal diseases such as grey mold and damping off, caused by *Botrytis cinerea* or stalk cankers due to *Sclerotinia sclerotiorum* are also problematic (McPartland, 1999). There are few pathogens that could be termed truly destructive for hemp, and no pesticides, herbicides, or fungicides are

currently licensed for grain quality hemp grown in Canada. As the crop can be grown chemical free, it can be considered environmentally friendly, which improves its sustainability profile when compared to alternative crops (Hemp Oil Canada, 2015). Since much of the hemp industry has evolved more recently than other crops and thanks to its natural fortitude, much of Canadian hemp is certified organic (Hemp Trade Alliance, 2013) which aids in marketing of hemp products for human consumption.

Health Canada reviews and approves the varieties for growth in Canada annually; there were 39 varieties approved in 2012. Some of the mostly widely cultivated varieties were CRS, Finola, Alyssa and Anka (Health Canada, 2012; Canadian Hemp Trade Organization, 2015). Varieties grown in Canada are generally grain or multi-use; where both the seeds and stalk find an end market. Fiber/ multi-use varieties are typically grown for a shorter period of time, 60-90 days in Canada, and can grow up to 4 m in height. Seed varieties are grown for approximately 30 days longer and are usually below 2 m in height (Manitoba Agriculture, 2013; Callaway & Pate, 2009). In Québec, farmers produced hemp on 290 hectares in 2011; a fairly small commitment in comparison to the prairie provinces, which had planted 15,056 hectares in the same year (Government of Alberta, 2012). This means that within the Québec market there is a great potential for growth and profitability once farmers become familiar with the plant. Misinformation about the growth and the yield potential of the crop is compounded with lacking local research to make farmers hesitant in trying industrial hemp. Much of the Québec growing climate is similar to Northern Ontario which has yielded an average crop of 6.1 tons per hectare (Ontario Ministry of Agriculture and Food, 2011). Considering that demand appears to be continuing on a positive trend, improving knowledge of hemp locally could yield great benefits. Recent research is working to better inform the farming community and generate the level of interest appropriate for this important crop (Aubin, 2014).

2.2 Microbial Endophytes

As an emerging and sustainable approach, the utilization of beneficial microbes to protect against disease, and to improve both plant growth and crop yield, will increasingly play a role in the future of

agriculture. In contrast to genetically modified organisms, the use of non-modified biological organisms or their products falls within the basic tenants of organic agriculture, a growing sector of today's commercial market (National Standard of Canada, 2006), and is more likely to meet general consumer approval. Endophyte research has joined mycorrhizal, pathogenic, epiphytic and saprotrophic research domains in helping to understand the plant-microbe biome (Porras-Alfaro & Bayman, 2011). The re-classification of previously understood to be exclusively saprophytic and pathogenic organisms as endophytes, reveals gaps in our collective knowledge of the complexity of this interaction, and is a fascinating field of study.

The traditional concept of endophytes is of organisms isolated from within a tissue or from surface sterilized tissues, which do not cause any overt disease symptoms in the host plant (Hallmann et al., 1997). Endophytic research has been undertaken for over a century, however it was concurrent with the common belief that the presence of a bacteria or fungi following tissue sterilization was a result of poor technique or difficult tissues; healthy plants were believed internally sterile (Smith, 1911; Hallmann et al., 1997). While there was some early fungal endophyte research (Bacon et al., 1977), it is only in the last two decades that research interest has intensified, and the diversity of microbes that make their home within the plant has been acknowledged (Hallmann et al., 1997; Rodriguez et al., 2009; Partida-Martinez & Heil; 2011). Endophyte categorization schemes suggested by various authors have been based upon the tissue of isolation, extent of colonization of the plant host, mode of transmission, inter- vs intra cellular localization, and effects exerted on the host (Rosenblueth & Martinez-Romero, 2006; Pineda et al., 2013; Rodriguez et al., 2009). Current technologies have allowed for the detection of non-cultivable members of the endophytic community, generating even greater interest. Assemblages of endophytes can differ greatly from the cultivated assemblage of microbes; and non-cultivable technologies are thus allowing a greater appreciation for the putative ecological functions and important traits of these microbes (Sessitsch et al., 2013; Arnold et al. 2007).

2.3 Endophyte Source and Host Colonization

Phylogenetic studies have shown that endophytes are more-often closely related to saprophytes and pathogens rather than biotrophs (Delaye et al., 2013); there are conflicting reports of potential origins of endophytes and how they relate with the external microbial fauna. Many endophytes are members of the rhizospheric microbiome and colonize the plant through the roots; using natural openings such as breaks in the epidermal tissues, injury though abiotic or biotic factors, or entry via root tips or hairs (Compant et al., 2008; Mano et al., 2006; Hallmann et al., 2001; Ji et al., 2008). Frequently isolated root endophytes include common soil residents such as *Burkholderia*, *Rhizobium,* and *Trichoderma* species (Mano & Morisaki, 2008; Chadha et al., 2014). Roots are by no means the exclusive method of entry. Certainly for both leaf and seed endophytes this may be the origin of a minority of endophytes, although endophytic movement from the roots or seeds to distal tissues has been demonstrated, and soil members may be found within these distal tissues (Compant et al., 2008; Gagne-Bourque et al., 2015). Colonization theories have suggested that movement through vascular tissue allows the movement of endophytes which arise from rhizosphere microflora to colonize above ground tissues (Lodewyckx et al., 2002; Truyens et al., 2014). Air spores, insect vectors, and vertical transmission from one generation to the seeds of the next are all known sources of endophytes (Petrini, 1991; Faeth & Hammon, 1997; Compant et al., 2011; Gagne-Bourque et al., 2015), and for aerial tissues may be how the majority of endophytes arise. For example; leaf endophytes include a variety of previously well-known epiphytic and plant associated genera encompassing both pathogenic and beneficial members; including *Pseudomonas, Bacillus, Alternaria*, *Dothideomycetes* and *Sordariomycetes* (Mercado-Blanco & Bakker, 2007; Mano et al., 2006; Arnold et al., 2007).

Many important crop plants have been explored for endophytic microbes. This has been done as much to understand prevalence and possible roles of these microbes in the lifecycle and health of the plant host, as to create microbial formulations to improve net plant growth or quality or to discover novel new compounds for unrelated fields. Contrasting studies of isolates across different latitudes, in differing

environments and with different histories have also been done (Arnold & Lutzoni, 2007, Huang et al., 2015; Sicilano et al., 2001).

Endophytes are most often found as intercellular colonies in the apoplast between plant cells, although intracellular colonies have also been noted (Rosenblueth & Martinez-Romero, 2006; Gagne-Bourque et al., 2015). Habitation within the host affords endophytes protection from uncertain environmental conditions found in soil or air, although they must deal with plant defense mechanisms (Rodriguez et al., 2009; Gagne-Bourque et al., 2015; Petrini, 1991). It has been suggested that this population must have gone through some form of host selection during colonization of the inner tissues when compared to similar rhizosphere microbial populations (Strobel & Daisy, 2003; Conn & Franco, 2004; Hallmann et al., 1997) and that they should therefore confer a benefit to their host. This benefit may only manifest under stressful conditions, such as drought; at other times, even if not directly pathogenic, infected plants may show negative effects as a result of hosting the microbes (Arnold & Engelbrecht, 2007; DuPont et al., 2015).

Vertical transmission, where an endophyte arises via previous incorporation into seed tissue during its development in the parent plant, is a microbial means to colonize future generations of the same plant species (Clay, 1988) and suggests a functional role of the microbe in the life of the plant host. Positive host impacts would be anticipated for vertically transmitted microbes because the mother plant has actively conferred a starter microbiome to the next generation, or the microbe has successfully evaded plant defenses. Fungal endophytes of grasses (*Poaceae*) and forbes are well known (Clay & Schardl, 2002; Hodgson et al., 2013), and tend to be a single, vertically transmitted isolate per grass host (Wille et al., 1999). Bacterial endophytes have similarly been seen in seed tissues, and vertical transmission has been demonstrated in a variety of plant families (Kremer, 1987; Rosenblueth et al., 2012; Liu et al., 2012; Ferreira et al., 2008). Examples of vertically transmitted endophytes include *Acremonium*, *Pantoea*, *Pseudomonas,* and *Bacillus* (Clay, 1988; Mano & Morisaki, 2008; Compant et al., 2011; Gagne-Bourque et al., 2013; Gagne-Bourque et al. 2015).

Regardless of their source, endophytes must possess the appropriate tools to enter, colonize and create a relationship with the host plant. A recent metagenomic analysis has found that when compared to other plant associated communities, bacterial endophytes were enriched in elements that might be expected to aid in this process such as flagella, genes for detoxification of reactive oxygen species (ROS), plant polymer degradative enzymes, and quorum sensing (Sessitsch et al., 2013; De Weger et al., 1987). Numerous studies support the importance of these degradative enzymes in particular for both bacterial and fungal endophytes in initial plant establishment and internal movement (Gagne-Bourgue et al., 2013; Compant et al., 2005; Petrini, 1991). Quorum and ROS management are well known in non-endophytic members of frequently isolated genera such as *Bacillus* and *Pantoea* (Giedroc, 2009; von Bodman et al., 1998).

The stable balance between plant and microbe is likely a result of selection by the plant in order for the relationship to be so persistent and ubiquitous. There have been demonstrated examples of positive growth effects from vertically transmitted endophytes (Puente et al., 2009; Clay, 1988; Faeth et al., 2006; Rodriguez et al., 2009) however; horizontally transmitted isolates represent a much larger consortium of endophytes discovered to date. They have been shown to confer similar benefits in growth, defense or stress tolerances as vertically transmitted isolates (Hoffman et al., 2013; Sun et al., 2014; Compant et al., 2013).

2.4 Culture-based Endophyte Methods

Various methods have been employed for the surface sterilization of plant tissues in culture dependent endophyte isolation. Techniques depend chiefly on the plant and tissue type being used, with more delicate plants and tissues such as leaves and fruits of herbaceous plants receiving less harsh treatments than tougher stem and root tissues. Among the most common sterilization materials are varying concentrations of ethanol, sodium hypochlorite, hydrogen peroxide or mixes thereof in cycles or washes (Mishagi & Donndelinger, 1990; Kaga et al., 2009; Fürnkranz et al., 2012; Hallmann et al., 1997). The treatment chosen must balance between removal of surface microbes and the death of all organisms

residing on and within the tissue. The protocol used must be tailored to the materials and tissues being used. Since the procedure is so variable, appropriate controls to confirm sterility of processed tissues is required.

The effects of plant age, variety or sampling location on the endophytes isolated have shown mixed results. While some authors (Gagne et al., 1987; Mason et al., 2015) found these factors to be unimportant; most culture independent works have found significant influences of plant genotype, plant age, season of isolation, and geography on both the endophytes isolated and the inferred community structure (Adams & Kloepper, 2002; Kuklinsky-Sobral et al., 2004, Siciliano et al., 1998; Zimmerman & Vitousek, 2012; Rajala et al., 2014). Soil type has been found to alter the endophytic genera present (Conn & Franco, 2004); unsurprising considering that many putative endophytes successfully colonize the plant from a larger population of soil-borne microbes (Hallmann et al., 1997). It is therefore important to report the environmental conditions present when studying endophytes.

2.5 Culture-Independent Endophyte Methods

Endophytes with fastidious growth requirements, that do not form easily seen colonies, are very slow growing, or are killed during the process of tissue sterilization will be under-represented in culture based assessments (Sun et al., 2008; Sessitsch et al., 2004). In such cases various molecular techniques can be used to attempt to attain a more complete picture of the endophytic community. Ribosomal RNA (rRNA) or DNA (rDNA) targets are common in the identification of microbes. The most commonly chosen targets are the16S rRNA (bacteria), and 23S rDNA (fungi). This is due to their essential function in the organism, rendering the genes ubiquitous and present at high copy numbers. They are also generally more conserved than other regions of the chromosome, and have a length large enough for use in statistical tests and differentiation between species (Janda & Abbott, 2007). Internal Transcribed Spacer (ITS) sequences are found between functional regions of the RNA and are more freely varying than regular rRNA and rDNA sequences; allowing for greater distinction between species or genus (Gardes et al., 1991; Roth, et al. 1998; Schoch et al., 2012). ITSs are found at high copy numbers and

have been used to successfully identify organisms including mammals, plants, bacteria, and fungi (Hadjiolova et al., 1984; Yokota et al., 1989; Roth et al., 1998; Gardes et al., 1991). Among other identifying markers, the large ribosomal subunit (LSU) region has additionally been used in mycological studies, as this region evolves very slowly and can allow for greater support of deep relationships (ex. family level) than the small subunit ITSs alone (Porras-Alfaro et al., 2013). The combined use of ITSs and ribosomal sequencing can allow robust identification of many isolates in fungi, which currently does not have the single gene marker such as the 16 S rDNA used for bacteria.

Insights from sequence data can be used to compare against isolates obtained from culture dependent methods, although this has been done rarely. Arnold et al. (2007) found that the assemblage of fungal endophytes had four times the number of basidiomycete members than previous culturing attempts had suggested. Similarly, U'ren et al. (2014) have found that the endolichenic and fungal endophytic membership showed important differences in comparisons between cultured and non-cultured members. U'ren et al. noted that some cultured isolates were absent from sequence read data, and concluded that pure cultures could still provide important insights. Cultivable microbes can be used for applicative purposes and are more amenable to additional studies than the non-cultivable community. More studies that examine the groups present in culture vs. non-culture works are required to better inform the appropriate methods to use in future studies, so as to gain the most holistically possible picture of a plants interior community.

2.6 Endophyte Characterization

Following isolation of pure cultures of endophytes, identification and biochemical characterization allow for a better understanding of their unique aspects; and is informative for their possible function(s) in the plant host. Researchers must screen endophytes carefully prior to use in agricultural systems, especially considering whether a close relationship with known human pathogen exists. While not pathogenic by default, members of bacterial families such as *Burkholderia*, *Klebsiella, Nocardia,* and *Mycobacterium,* and fungi such as *Alternaria, Colletotrichum, Diaporthe,* and

Lasiodiplodia have been isolated as endophytes from a variety of crop species (Engelhard et al., 2000; Araujo et al., 2002; Conn & Franco, 2004; Delaye et al.; 2013; Guo et al., 2004; Photita et al., 2005; Gomes et al., 2013; Qian et al., 2014).

Even if an endophyte is not inherently dangerous, these species may have an elevated risk of horizontal gene transfer of virulence or viral elements from pathogenic sources (Taghavi et al., 2005). Since endophytes are competitive microbes that must survive plant defenses, it is sensible that many can be opportunistic pathogens of plants and/ or people. Studies showing that many endophytes seem to have evolved from pathogens supports this inference (Spatafora et al., 2007; Delaye et al., 2013), and the plasticity of some genomes has been cited as a mechanism for conversion between these lifestyles (Padhi et al., 2015). Other studies have found them to form their own, separate group from pathogenic strains within the same species, and it could be expected that vertically transmitted endophytes may display this feature more frequently (Photita et al, 2005). Many endophytes do in fact belong to families previously associated with saprophytes and phytopathogens (Rosenblueth & Martinez-Romero, 2006; Rodriguez et al., 2009). As mentioned previously, this reflects which organisms possess the toolkit required for initial colonization i.e., cell-wall degrading enzymes, however it also reflects a gap in collective knowledge about the non-pathogenic roles that these genera might play in the ecosystem.

Changes in environmental conditions (Arnold & Engelbrecht, 2007) and the genotype of either host (Lamit et al., 2014; Siciliano et al., 2001) or microbe may alter the endophytes' end effect on the host. Therefore, researchers must carefully consider if and when the microbes may become harmful to the plant (Sturz & Matheson, 1996; James et al., 1997; Pineda et al., 2013). Judicious selection can lead to great benefits. There is ample evidence that they can be used to improve the environment and plant growth and do so in a much safer and more sustainable method than the previously used, chemical toolbox.

2.7 Crop Improvement through Endophytes

Endophytes have been found to have direct effects on their host through synthesis of plant hormones (Glick et al., 2007; Khan & Doty, 2009), and through improved nutrient uptake, assimilation, or creation through the action of phosphatases, siderophores, nitrogen fixation, etc. (Puente et al., 2009; Boddey et al., 1991). The production of chemical cocktails, including possible anti-microbial metabolites (Mousa & Raizada, 2013; Higginbotham et al., 2013), and volatiles can alter the local microbial environment as well as the host phenotype (Gagne-Bourque et al., 2013; Ryu et al., 2003).

Broadly, endophytes have been found to be active partners with their hosts against microbial and insect pathogens in a huge variety of plant life, including: oak, pine, rice, potato, cotton, and pepper, cactus, and many broad-leafed tropical species (Brooks et al., 1994; Oono et al., 2014; Poon et al., 1977; Sturz & Cristie, 1995; Chen et al., 1995; Vicari et al., 2002, Yi et al., 2013; Puete et al., 2009; Arnold et al., 2007). Plant Growth Promoting (PGP) or Biological Control Agent (BCA) relationships such as these have been found to be ripe areas for natural product mining and have been used to benefit the plant in previous studies (Strobel, 2003; Strobel & Daisy, 2003; Zhang et al., 2015).

Endophytes can alter the plant's interaction with predators or parasites via the production of various metabolites (Rosenblueth & Martinez-Romero, 2006; Pineda et al., 2013). This is especially well known in fungal endophytes of grass species where the protective effect of endophyte harboring in grasses is well documented (Faeth et al., 2006). Generally, herbivory by grazing mammals and insects is toxic and livestock have been found to avoid endophyte infected strands where alternatives exist (Clay, 1988; Faeth et al., 2006; Rodriguez et al., 2009).

Endophytes have successfully been found by many authors to improve host growth under nitrogen or drought stresses (Naveed et al., 2014; Hubbard et al., 2014; Khan et al., 2012; Gagne-Bourque et al., 2015). They are also frequently found to have anti-microbial attributes co-cultured with pathogens, including widespread and economically important fungal (Purdy et al., 1979; Dean et al., 2012) and

bacterial pathogens such as *Sclerotinia sclerotiorum* (Forchetti et al., 2007), *Botrytis cinerea* (Barka et al., 2002), and *Ralstonia solanacearum (*Ji et al., 2008; Chen et al., 2013*).*

Manipulation of the host plant itself can manifest through a hormonal interchange, using well known plant hormones such as Indole-3-Acetic Acid (IAA), 1-Aminocyclopropane-1-Carboxylate (ACC) deaminase, or via cocktails of cocktail organic compounds (VOCs). Endophytes can thereby contribute to the host plants' health (Clay, 1988; Rodriguez et al., 2009)

2.8 Volatiles, IAA and ACC

Endophytes are often screened for plant hormone production during characterization (Ahmad et al., 2008; Spaepen & Vanderleyden, 2011; Mohite, 2013; Nadeem et al., 2010). The emission of VOCs has effects on plant growth ranging from extremes of plant death, to greatly increasing biomass (Blom et al., 2011). Ryu et al. (2003) were one of the first to target VOCs, and demonstrated that the alcohol 2, 3-butanediol synthesized by a plant growth promoting rhizobacteria was responsible for visible increases in leaf surface area and improved general health of the plant. Since their work, a variety of VOCs that improve plant growth include have been identified (Blom et al., 2011; Naznin et al., 2014; Song et al., 2013). VOCs released by microbes are complex mixtures and vary depending upon environment conditions (Morath et al., 2012).

IAA is a well-known stimulatory hormone that is vital for root, leaf, shoot, and floral growth (Spaepen & Vanderleyden et al., 2010; Chen et al., 2007). Auxins such as IAA arise from both the root and stem apical meristem tissues (Zhao et al., 2011, Petersson et al., 2009). As a chief plant hormone, IAA production, transportation and degradation is carefully regulated; disruption of pathway elements is associated with aberrant growth phenotypes (Sato & Yamamoto, 2008; Woodward & Bartel, 2005; Zhao et al., 2011). IAA can be produced by plant-associated microbes and the quantity synthesized has been found to be dependent upon conditions such as pH, culture media, and temperature (Mohite, 2013). Known producers of IAA, both rhizospheric and endophytic, have been used to achieve improved growth in plants; termed phyto-stimulation (Spaepen & Vanderleyden, 2010).

ACC deaminase is a plant hormone that hydrolyzes the immediate precursor of ethylene, ACC into α-ketobutyrate and ammonia (Arshad et al., 2007). This monopolizes ACC and has a net result of decreasing *in planta* ethylene concentrations. Ethylene elevates the division rate of cells (Schaller, 2012), and restricts plant cell size. Increased production of ethylene is associated with environment stresses such as salt or drought (Nadeem et al., 2010), and the initiation of senescence, chlorosis and abscission (Glick et al., 2007). Ethylene acts to reduce plant growth, therefore ACC deaminase can be important in increasing plant growth; it can mitigate plant stress by managing upstream activation. Application of ACC deaminase producing microbial strains have resulted in improved plant growth, most especially for root tissues (Bal et al., 2013; Nadeem et al., 2010) and has improved host resilience in harsh abiotic conditions such as saline soils (Baltruschat et al., 2008). Salinity is an increasingly worrisome problem that arable lands are, and will continue to be, contending with; therefore, a natural, microbial aid for this problem is a positive development.

Some VOCs have additional potential as BCAs. Some BCA identified compounds include: 2-pentane 3-methyl, methanethiol, and 2-undecene (Ting et al., 2011). Hydrogen cyanide (HCN) is a potent volatile that is toxic to aerobic microbes (Pal & Gardener, 2006), however its application should be undertaken with care as HCN can also act to reduce plant growth, especially roots (Bakker & Schippers, 1987). Fungi in particular have been found to be productive volatile producers and endophytic fungi are no different (Morath et al., 2012). The fungal endophyte *Muscodor crispans* was found to possess anti-microbial VOCs (Mitchell et al., 2010), as was a novel actinomycete endophyte from cucumber (El-Tarabily et al., 2009) and the growth of phytopathogens such as *Rhizoctonia solani*, *Phytophthora capsici*. Endophytic microbes could have multiple applications, depending upon their unique characteristics. As several post-harvest pathogens' have been reduced through the production of endophytic VOCs and PGP has also been demonstrated, the flexibility in some endophytes is enticing (Mercier & Manker, 2005; Lee et al., 2009).

2.9 Biological Control Agents (BCAs)

Since endophytic microbes naturally occur in the plant, they are both adapted to the environment and do not pose the same regulatory problems that modified organisms have. Endophytes have thus become prime targets for use as BCAs. Antagonistic action of endophytes can involve antibiosis; however simple competition for nutrients and space can effectively halt disease prior to its manifestation (Bacon et al., 2002; Sessitsh et al., 2004; Alstrom, 2001). The monopolization of limiting factors such as sugars or iron can also reduce the growth of microbes below disease causing populations. BCA endophytes against important pathogens, for example fungi such as *Rhizoctonia* and *Fusarium* species (Senthikumar et al., 2009), bacterial pathogens such as *Curtobacterium flaccumfaciens* pv. *Flaccumfaciens*, and pathogenic nematodes (Hsieh et al., 2005; Moosavi & Zare, 2012) have been documented.

The anti-bacterial properties of endophytes from a variety of plant hosts towards single and multiple pathogen targets are well documented (Rosenblueth & Martinez-Romero, 2006). Endophyte introduction can be specific to conditions that can make determination of their benefits difficult. For example, in addition to the genotypes of both microbe and host plant, abiotic conditions such as drought, change the manifested protection that could otherwise be hidden (Malinowski et al., 2011). Reductions in plant photosynthetic capacity and altered water conductance without causing overt disease symptoms have been recorded as a result of harboring endophytes (Costa Pinto et al., 2000; Arnold et al., 2007). Prior to widespread endophyte introduction, the overall crop yield of a host plant must be considered, as yield penalties can occur (Sturz & Kimpinski, 2004).

Somewhat unrelated to direct agricultural uses is the discovery of endophytes with the goal of discovery of novel metabolites. Many of these chemicals may have uses as BCAs as well and antibiotic discovery continues to be a research goal (Christina et al., 2013). Antibiotics have a history of being found in the natural world, and endophyte populations have been noted as possessing a larger number of novel, biologically active compounds than expected (Strobel, 2003; Schulz et al., 2002). These

compounds are also more likely to be tolerated by living systems since the microbe must co-exist within its host (Strobel, 2003).

2.10 Nutrient mobilization

Essential nutrients are those elements required for cellular life including: carbon, hydrogen, nitrogen, phosphorous, sulfur, and minute quantities of micronutrients such as iron. The deficiency of any of these elements can manifest itself as disease/chlorotic symptoms in plants. Phosphorous is required in both DNA and RNA structures; used centrally in energy molecules and co-enzymes such as adenosine triphosphate and has a major role in many signaling cascades, ex. kinases. It occurs naturally in the soil however it is most often found as phosphate groups (PO_4^-), and is frequently complexed with other elements to form mineral precipitates. These compounds sequester phosphorous from the environment, making it biologically unavailable. Considering its importance in the cellular life of an organism, increasing the availability of phosphorous is a means to bolster growth. Fertilizers containing phosphorous are created using trapped phosphate within rocks in the earth's crust (Gilbert, 2009), a finite resource. Mining provides 160 million tons of phosphorous annually; with a total estimated reserve of 62 billion tons, much of which is difficult to refine or unviable (Gilbert, 2009). Solubilized phosphate, the most biologically available form of phosphate, is present at very small amounts in soil, normally at 1 ppm (1 $\mu mol*L^{-1}$) or less (Goldenstein, 1994; Sashidhar & Podile, 2009). Additionally, in this form phosphate may be lost as mineral runoff causing both waste and environmental damage. Microbes are able to solubilize non-available phosphorous sources to liberate it for biological uses, often using acid based phosphatases (Rodriguez & Fraga, 1999). Strains belonging to the genera: *Rhizobium*, *Pseudomonas*, and *Bacillus* are among the most powerful solubilizing microbes of phosphate (Rodriguez & Fraga, 1999).

Nitrogen is another major nutrient deficiency that can be remedied through microbial amendments. Nitrogen deficiency can cause stunted growth and chlorosis, and as it is the essential mineral needed in highest net quantities (Taiz & Zeiger, 2010); it makes up a large portion of many fertilizers. Nitrogen is vital for protein and nucleic acid synthesis in the cell (Taiz & Zeiger, 2010).

Synthetic nitrogen creation typically uses the energy intensive Haber-Bosch method (Chanway et al., 2014), however it is highly dependent upon fossil fuels, and is thus both finite and highly polluting. The misuse of fertilizers resulting in eutrophication and degradation of aquatic environments from agricultural runoff has long been known (Ma, 1997), as has microbial nitrogen fixation; most famously in nodule forming, legume associated rhizobial bacteria. Unsurprisingly, many endophytes which are often related to soil dwelling microbes like rhizobia have the ability to fix nitrogen as well. The endophyte *Gluconacetobacter* (previously known as *Acetobacter) diazotrophicus* for example, was found to be responsible for much of the nitrogen fixation in sugarcane, and could eliminate the need for external nitrogen fertilizers in this crop (Boddey et al., 1991; Dong et al., 1994). Endophytes can help in less traditionally means of nitrogen sourcing as well. For example, an endophytic fungus, *Metarhizium spp*. which also parasitizes insects, has been found to transfer insect derived nitrogen to its host plant (Behie et al., 2012). The internal growth of endophytes means that they are able to contribute more directly to their hosts' needs. An area of ongoing interest is using nitrogen-fixing endophytes as a means to reduce mineral stress and fertilizer needs in crops unable to establish traditional rhizobial partnerships, such as most cereals (Cocking, 2003; Chanway et al., 2014).

Like phosphorous and nitrogen, iron is also an element with very low natural bioavailability in soil; being found at concentrations 10^{20} fold less than preferred for many microbes (Saha et al., 2013). When absorbed by the plant, iron is rapidly fixed into plant tissues as oxides and enzymes such as phytoferrin (Tais & Zeiger, 2010). This is important because iron-oxygen hydroxyls can cause damage when free in the cell (Halliwell & Gutteridge, 1992). Iron is a vital micronutrient that is pivotal for many enzymes, and for electron transfer (Taiz & Zeiger, 2010). The sequestration of iron from the soil environment can be seen in a biocontrol aspect in addition to a possible PGP mechanism in capable microbes (Berg, 2009). Iron is sequestered by microbes through the action of siderophores, low weight molecules with high iron affinity. There are three chemical families, depending upon the nature of electron donators to the iron molecule: catceholates, hydroxymates or mixed (Miethke et al., 2007).

Siderophore production is thought to be the main mechanism of suppressive soils which contain *Pseudomonas spp*., a genus well known as siderophore producers (Kloepper et al., 1980). Siderophores have been found to help plants deal with heavy metal stresses, and through binding of these elements, allow normal cellular machinery to continue unencumbered (Rajkumar et al., 2010). Therefore, siderophore producing microbes have a well-known, positive impact on plants that are used for phytoremediation of contaminated sites, such as willow (Khan et al., 2014), poplar (Moore et al., 2006) and ryegrass (Sun et al., 2014).

Discovery and use of microbial endophytes from plants is providing new ways to improve the global future of food security through offering an ecologically friendly alternative means to improve crop yield and quality. Characterization of newly discovered species from a basic research perspective, is informing us about how little we truly understand the interplay between a plant, and the internal biome that it supports. Greater exploration is leading to shifts in our understanding of the pathogen-symbiont continuum from a classification into a more fluid and responsive scaling system. Industrial hemp is a crop that can provide materials which are being employed in novel and unique ways and is gaining increasing consumer and producer acceptance. The Canadian farming community stands as a strong source of high quality hemp products and can benefit from this widespread interest. The isolation and characterization of microbes endogenous to the hemp plant was thus undertaken with the twin goals of improving scientific knowledge about this crop, and for potential future uses to improve overall crop yield of industrial hemp.

Chapter 3: Materials and Methods

3.1 Microbial Isolation & Characterization

3.1.1 Farm Site, Cultivars and Tissue Collection

Seeds of *Cannabis sativa* L. cultivars Anka, CRS-1, and Yvonne were obtained from Health Canada. All three are representative multi-use hemp varieties; the former two cultivars were grown locally by Québec farmers, and Yvonne showed promising preliminary data in abiotic stress response studies (Mayer et al., 2015). Plants were grown at the Emile A. Lods Agronomy Research Centre, at the Macdonald Campus of McGill University (20965 Chemin Ste-Marie Sainte-Anne-de-Bellevue; Québec; Canada). Soil pH was 6.22 and mineral composition was as follows (Kg/ ha): phosphate 160, potassium 195, calcium 2622, magnesium 273, and aluminum 2667. Plants were seeded in mid-May and were grown in plots 1.3 m x 5 m, containing 7 rows, spaced 18 cm apart. No herbicide was applied, and manual weeding was done on all plots throughout the growing season. Only naturally occurring precipitation and light exposure were used throughout the plants' growth; plot GPS locations are detailed (Table 1).

The seeds used were collected from the harvests in 2012 and 2013 by M. Aubin, in addition to non-passaged seeds (2013) that were provided directly from Health Canada approved suppliers. Anka and Yvonne seeds were collected from plots containing an additional 110 kg/ ha of nitrogen. CRS-1 seeds were collected from plants grown in fertilizer free plots. Seeds were field harvested and stored at 4 °C in 50 ml falcon™ tubes until use. Prior to sterilization, approximately 50 seeds were taken from storage and imbibed overnight on filter paper in dH_2O, and de-hulled using a sterile scalpel and tweezers to better facilitate tissue surface access and sterilization protocol effectiveness.

Leaves and stems were collected from plants sown in mid-May, 2013; and were harvested bi-weekly beginning mid-June until mid-August. Approximately two dozen leaf brackets, including the vascular petiole tissue of each cultivar were harvested at each time point, using multiple plants within the

plot. Field samples were placed in labeled Ziploc® bags on ice for transportation, stored in a 4 °C walk-in cold room, and processed within 48 h of collection.

3.1.2 Endophyte Isolation

All plant tissues were sterilized following a procedure of stepwise, agitated immersion in ethanol (70 %), sodium hypochlorite (3.5 % available Cl-), and sterile de-ionized water (dH_2O) according to Schulz (1993). To maximize the isolation of cultivable organisms from different tissues following sterilization, leaves were sectioned (0.5 cm) using a sterile scalpel, and placed directly onto the surface of selective media. Petiole sections (a dozen pieces of ~10 mm each), and seed embryos (~approximately 40) were homogenized in 5 ml of sterile water and 150 µl of the petiole or embryo macerates were spread-plated onto selective culture media. The effectiveness of the sterilization procedure was tested using both imprinting, and wash plating methods depending upon the material being used (Schulz et al, 1993; Ji et al., 2008; Kaga et al., 2009).

3.1.3 Microbial Purification & Culturing

Bacteria: To ensure the purity of the emergent microorganisms, bacterial colonies from sterilized tissues were passed through four rounds of single colony isolation via streaking on Luria-Bertani (LB; 1.0% Tryptone, 0.5% Yeast Extract, 1.0% NaCl, 1.5 % agar; Difco, Lawrence, KS, USA) or nutrient agar plates (NA; Difco, Lawrence, KS, USA) amended with filter sterilized, anti-fungal agent benomyl (10 mg/ L, Wilson's, USA). Pure cultures were stored at -80 °C in 25 % glycerol stock cultures.

Fungi: Emergent fungal mycelia were isolated on Potato dextrose agar (PDA; Difco, Lawrence, KS, USA) or malt dextrose agar (MEA; Difco, Lawrence, KS, USA) amended with 100 mg/ L penicillin and 100 mg/ L streptomycin (Sigma-Aldrich Co., ON, Canada). Fungal isolates were purified through 4 rounds of re-isolation by carefully removing a 3 mm section from the edge of the growing colony, transferring to fresh, amended plates and being incubated for an additional 24-48 h before subsequent re-isolation. Pure fungal cultures were stored at -80 °C in 25 % glycerol.

3.2 Molecular Identification and Characterization of Endophytes

Bacteria: Bacteria were grown at room temperature for 16-18 h with agitation (175 RPM) to achieve adequate growth, and cell concentrations between 10^8-10^{10} CFU/ml, depending upon the genus. Cells were collected for Gram reaction staining (Steinbach & Shetty, 2001), and DNA extraction using the Presto™ Mini gDNA Bacteria Kit (FroggaBio., ON, Canada) according to manufacturer instructions. DNA quality was confirmed via a 1 % agarose gel prior to subsequent reactions.

The bacterial universal primer pair 27F and 534R were used according to previously published protocols (Gagne-Bourque et al., 2013; Table 2) to identify over 130 bacterial endophytes. The iProof™ High-Fidelity (HF) PCR kit (Bio-Rad, ON, Canada) and 40 ng of genomic DNA was used per 50 µl reaction. A positive, amplified genomic DNA sample and negative control without DNA were run concurrently with each reaction. An annealing temperature of 63 °C and 35 cycles were used. The amplified PCR product quality was confirmed prior to additional cleaning using a Gel/PCR DNA Fragments Extraction Kit (FroggaBio, ON, Canada) as instructed, and sequenced at Genome Québec (Montreal, QC, Canada). Results were queried between December 2013 and March 2014 using NCBI's BLASTn software program (Altschul et al., 1990). The top, named search results were used to identify isolates to the genus level where possible.

Fungi: Over fifty fungal isolates were grown on malt dextrose agar for one week prior to extraction of total genomic DNA using the Extract-N-Amp Plant DNA extraction kit (Sigma-Aldrich Co., ON, Canada) as directed. The primers ITS1F and LR3, corresponding to the first ITS of the small subunit, and the large subunit sections of ribosomal DNA were used to amplify a fragment 1 to 1.2 kbs in length using previously described cycle conditions (Hoffman & Arnold, 2010). The PCR product was cleaned using Exo-SapIT reagents (Affymetrix) as directed, and Sanger sequenced at the University of Arizona Genetics core (Tucson, AZ, USA).

Sequences were examined and trimmed manually using Sequencher 4.5 (GeneCodes Corp., MI, USA) to obtain a high quality consensus read. In cases of discrepancies in the consensus read (5.7 % of

isolates), the ITS1F and LR3 sequences were assessed separately and search results were compared. A single consensus sequence was also generated in Mesquite version 3.02 (Maddison & Maddison, 2015) from the ITS1F and LR3 sequences. The consensus identity within the top, named hits was used to identify isolates to the family level, and where possible, to the genus level.

Fungal sequences were clustered into 90, 95 and 100 % OTU groups based upon sequence similarity using the phylogenetic tool Mesquite. Isolate identity was conducted using the 95 % similarity group, which has been found to roughly correspond to the species level in other endophytic fungi (U'ren et al., 2009).

3.3 Phenotypic and Chemical Characterization of Endophytes:

Bacteria**:** For enzymatic assays and biochemical tests (Table 3), a single colony of the bacteria was grown in 4 ml of LB broth solution overnight (16-18 h, 175 RPM). Following appropriate dilution in LB broth, 25 µl of 10^5 CFU/ ml were used in all tests unless otherwise stated.

3.3.1 Cellulase and Phosphatase Solubilization

Cellulases were assayed on indicator plates with carboxymethyl cellulose (Sigma-Aldrich Co., ON, Canada) amendment, and stained in Congo-red solution (0.2 %) as outlined (Gupta et al., 2012). The ability of endophytic bacteria to solubilize inorganic phosphate was assayed on agar medium containing inorganic phosphate (g per 1 L: glucose, 10; NH_4Cl, 5; NaCl, 1; MgSO4*7H2O, 1; $Ca_3(HPO_4)_2$, 0.8; agar, 15, pH 7.2) according to Verma et al. (2001). Development of solubilization of the indicator was shown via halo around the growing colonies.

3.3.2 Siderophores

Bacteria were assessed for siderophore activity by growing cultures on Chrome azurol S (CAS) media, a complex mixture of Fe-CAS (Sigma-Aldrich Co., ON, Canada) indicator, piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES) (Sigma-Aldrich Co., ON, Canada), micronutrients, and 10 % sterile casamino acid (Difco; KS, USA) solutions; as outlined in Alexander & Zuberer (1991).

3.3.3 Hydrogen cyanide (HCN)

HCN gas production was assessed based upon a change from bright yellow to orange in picric acid (0.5 %, RICCA Chemicals) soaked Whatman filter, lining the lid of a sealed petri dish, as described in Bakker et al. (1987).

3.3.4 Antibiotic Resistance

Antimicrobial agent resistance was tested individually on agar plates containing antibiotics following the procedure of Gagne-Bourgue et al. (2013) at either 100 µl /ml: kanamycin, rifampin, (Sigma-Aldrich Co., ON, Canada) streptomycin (Bioshop, ON, Canada) and tetracycline (Fisher Chemicals, ON, Canada); or 125 µl /ml: ampicillin, gentamicin, (Sigma-Aldrich Co., ON, Canada) chloramphenicol, (ICN Biomedicals; OH, USA) and hygromycin (Santa Cruz; TX, USA). Bacteria were considered sensitive to an antibiotic at the concentration tested if no visible growth was observed on treatment plates, and there was visible growth on control plates after 48 h of incubation.

3.3.5 Organic acid production (OAP)

OAP was determined using a modified methyl red test (Voges & Proskauer, 1898). Briefly, bacteria were grown at room temperature, 175 rpm in 5 ml of glucose-phosphate broth for 4 days. A small amount (3-5 drops) of methyl red solution was added to the culture and the tubes were mixed gently. Colour change was recorded after 5 minutes. Change to deep red was scored as positive, deep orange was scored as a weak reaction and maintenance of yellow colouration was scored negatively. Pure LB or HCl were used as negative and positive controls.

All of the above tests were conducted in biological triplicate, and incubated at 28 °C for 4-5 days (save OAP) before evaluation. An initial scoring of a single representative strain of endophyte chosen at random from each genus isolated was subjected to cellulose, phosphate solubilization, HCN production, and siderophore activity tests. Results are found in Table A1. The well characterized switchgrass endophyte *Bacillus subtilis* B26 (Gagne-Bourque et al., 2013) was added as a control for select tests. The genera that appeared to be of elevated interest: *Bacillus*, *Cedecea*, *Enterobacter*, *Pantoea, Pseudomonas,*

and *Strenotrophomons* had the number of isolates expanded and re-tested. Four isolates that were found to be of high interest from these repeated screenings were continued for additional testing.

3.3.6 Indole Acetic Acid Production

Indole-Acetic-Acid (IAA) production was assessed as described by Husen et al. (2003) using L-Tryptophan (final concentration: 5 mM, Sigma-Aldrich Co., ON, Canada) amended LB media, shaken for 4 days (23 °C, 175 RPM). The culture supernatant was mixed with $FeCl_3$-$HClO_4$ reagent (1:2 ratio) and permitted to react in the dark for 30 minutes prior to being spectrometric scoring at 530 nm. Biological replicates and the IAA chemical standards were both done in triplicate to provide accurate quantification. Results were compared to a standard curve of known IAA concentrations solubilized in LB broth with other reagents used in the bacterial quantification, to account for background activity.

3.3.7 1-Aminocyclopropane-1-Carboxylate (ACC) Deaminase Activity

ACC deaminase testing followed the protocol of Penrose and Glick (2003). Briefly, 200 µl of toluenized cells were mixed with 20 µl of ACC and incubated for 15 min at 30°C for reactions to occur. Two trials with either 1 or 100 µl of the reaction volume was mixed with HCl (1 ml, 0.56M) and vortexted, added with 800 µl of HCl (0.56 M) along with 300 µl of 2,4-dinitrophenylhydrazine, and returned to incubation for an additional 30 min (30°C). NaOH (2 ml, 2 N) was added and readings were taken at 540 nm.

3.3.8 Antifungal Activity

The ability of selected endophytes to restrict the radial growth of a variety of fungi (Table 5) was performed as previously described (Gagne-Bourgue et al., 2013). The experiment was performed at three separate time points per fungi and in biological triplicate for each bacterium per trial. Results were tabulated using the equation for reduction of growth where C=control radial growth and T= bacterial treatment radius.

$$Inhibition = \left(\frac{(C - T)}{C}\right) x\ 100$$

Fungi**:** Fungi were tested for cellulase and lignase activities using plate based methods as described (Gupta et al., 2012; Sundman & Nase; 1974). Cellulose amended MEA, and indulin-AT (MeadWestvaco, VA, USA) amended water agar were used for testing cellulose and lignase activities, respectively. Fungi were allowed to grow for an additional week prior to lignase testing. For both tests, indicator dye solutions were applied to assess activities at 4- 8 days (cellulases) or 11-15 days (lignases). Assays were performed in biological triplicates and repeated at least once to obtain a consensus. In the case of positive results, a third test was frequently performed to confirm the isolates ability to produce an enzyme.

3.4 Genetic Characterization:

Bacteria**:** Putative nitrogen fixing ability was probed using culture-independent methods via PCR amplification of *nifH* (Gaby & Buckley, 2012). Primers DVV and IGK3, previously identified as being excellent for widespread *nifH* screenings (Ando et al., 2005; Gaby & Buckley, 2012), were used with the genomic DNA of isolates used for their initial identification (Table 2). Previously extracted DNA from the model species *Bradyrhizobium japonicum* was used as a positive control. PCR conditions followed the same conditions as ITS amplification excepting a change in amplification temperature to 54 °C.

Fungi: Whole genomic fungal DNA was cleaned of impurities via ethanol precipitation and subjected to PCR amplification using the bacterial 27F and 1492R 16S rRNA primers. PCR conditions followed previous protocols (Hoffman & Arnold, 2010). Amplified products were cloned using the StrataClone PCR cloning kit (Agilent Technologies Inc., CA, USA) as directed, and sent to the University of Arizona Genetics core for sequencing as described previously. Samples were manually edited in the software Sequencher and identified using BLASTn. Top reads, regardless of whether the result was formally named as a species, were taken as the identity of putative EHB.

3.5 Microbial Phylogenetic Tree Generation

Reverse sequences of 534R amplified sequences from bacterial endophytes were generated using the Reverse Sequence tool from Bioinformatice Organization (MA, USA) and aligned into a single contig in BioEdit v7.2.5 (Ibis Biosciences; CA, USA). Contigs were aligned using the CLUSTAL tool:

MUSCLE (EMBL-EBI, Cambridgeshire, UK) to be used in tree generation. Fungal sequences, previously generated into a single contig, were similarly aligned for generation of a fungal phylogenetic tree using MUSCLE.

Phylogenetic trees for both bacterial and fungal endophytes were constructed separately from aligned sequence files using the freeware program MEGA version 6.0 (Tamura et al., 2013). Trees were generated using Maximum Parsimony methods and used a Subtree-Pruning-Re-grafting algorithm (Nei & Kumar; 2000). Positions with fewer than 95% site coverage were eliminated. The bootstrap consensus tree was inferred from 1000 replicates, and branches under 70% bootstrap replicate support were collapsed. The analysis for bacteria involved 136 nucleotide sequences, and 53 for fungi. All positions containing gaps and missing data were eliminated. In the bacterial tree there were a total of 310 positions in the final dataset and for fungi there were 331 positions.

3.6 Plant Response to Endophyte Colonization

3.6.1 Endophytic Bacterial Growth and Inoculum Preparation

Bacterial strains BTC6-3, BTC8-1, BTG8-5 and BT14-4 were all isolated as members of the endogeneous endophytic community arising from the petiole tissue of field grown industrial hemp (*Cannabis sativa* L.). Isolates were identified via ITS ribotyping as *Pseudomonas sp.*, and putatively identified to the species *P. fulva* (BTC6-3, BTC8-1), or *P. orientalis* (BTG8-5 and BT14-4). Cultures were revived from -80 C storage in 25 % glycerol stocks; grown on LB plates and a single colony was used for liquid culture in overnight growth in 5 ml of LB (23 °C, 175 RPM).

3.6.2 Effect of Bacterial Volatiles on Tomato Seedlings

The ability of the bacteria to effect plant growth was assessed using surface sterilized *Solanum lycopersicum* cv. Beefsteak (Veseys, PE, Canada) grown in polystyrene bi-plates (100 mm x 15 mm; Fisher Chemicals, ON, Canada), that allowed the co-culture of plant and bacteria using a modified method from Ryu et al., (2003). Ten milliliters of MS media (Sigma-Aldrich Co., ON, Canada), amended with filter sterilized sucrose (final concentration of 0.3 %) or 10 ml of LB agar, were used for plant and

bacterial growth respectively. Both culture media were amended with the anti-fungal agent benomyl (10 mg/ L).

Five seeds of tomato (*S. lycopersicum*), sterilized using stepwise immersion in 30 % hydrogen peroxide and sterile dH_2O washes (Miller et al., 2012), were placed with flame sterilized forceps onto the MS media comprising one half of the plate. To the other half, 50 µl of freshly grown bacteria at a concentration of 10^5 CFU/ ml or sterile LB alone were added. A non-volatile producing *E. coli*, DH5α (Qiagen Inc., CA, USA) was used as a control (Gagne-Bourgue et al., 2013). Plates were sealed with parafilm; placed randomly into a single row in a growth chamber (Controlled Environments Ltd., MB, Canada), and incubated under 16/8 h light/dark cycles, light intensity of 260 µmol m^{-2} s^{-1} with a temperature regime of 20/ 17 °C (day/ night), and humidity of 46 % for 14 days. Plates were monitored frequently for contamination until harvest. In total, four bacterial treatments, an *E. coli* bacterized, and a bacteria-free control using sterile LB broth were tested. Five seeds were placed per petri dish. Five plates were used for each treatment, giving a total of 25 plants per treatment per trial. The experiment was repeated to give a total of three biological replicates for all treatment groups. At two weeks of growth tomato seedlings were removed, and their total length, root lengths and fresh weights were recorded. Dry weights were recorded following 2 days of drying at 58 °C. Statistical differences between groups and control plants was assessed via ANOVA and Tukey's Honest Significance Test at p= 0.05 in the program R (R Studio, 2012).

3.6.3 Detection and Colonization of Tomato Seedlings by Endophytic Bacteria

S. lycopersicum cv. Beefsteak seeds (3-4 seeds), surface sterilized as previously described, were planted in 10 g (3 cm deep) of sterilized Agromix® potting soil G12 (Fafard et Frères LTD., QC, Canada) in magenta boxes. The sterilized soil was plated on non-amended PDA, and LB media and monitored for 2 days to confirm sterility prior to seeding. Following seeding, 5 ml of 1 X Hoaglands solution (Hoagland & Arnon; 1950) was added per box. Seedlings were grown for one week at 16/8 h, day/ night cycles and 25/ 20 °C before being thinned to a single, healthy plant per box. Plants were soil drenched with 5 ml of

10^5 CFU/ ml of freshly grown bacteria in LB broth at 14 days post-seeding (DPS). Control plants were drenched in 5 ml of sterile LB broth, giving two treatment groups and a control for each trial. The experiment was repeated once in a temporally separated trial, and approximately 10 seedlings survived to harvest for each treatment.

At 28 DPS seedlings were harvested, cut into root, stem or leaf sections using sterile scalpels. Tissues were sterilized in hydrogen peroxide as previously described (see section 3.1.2). A subset of sterile tissue was retained for PCR probing, while the remainder was ground with 5 ml of sterile water. A dilution series was created; 100 µl was spread on non-amended LB and incubated at 23 °C for 2 days prior to CFU scoring. Isolates were also retained and colony purified for additional testing.

Pseudomonas species specific primers BT14-4F1/BT14-4R1 (Table 2) designed using Biology Workbench (SDSC, University of California, CA, USA) were used in PCR reactions to detect and confirm colonization of the target endophytes in plant tissues. Amplification was conducted using HF Bio-Rad PCR as described previously (see section 3.2), with an annealing temperature of 67.5 ºC.

3.6.4 Plant Growth and Colonization Assessment following Re-introduction of Endophytes into C. sativa L. cv. *Anka Seedlings*

Plant Growth Conditions: Greenhouse trials were conducted at MacDonald Campus of McGill University between May and August, 2015 to assess potential effects of bacterial inoculation on the original plant host *C. sativa* L. One hundred pots (KORD Regal, 5 inch, standard) were cleaned in dilute bleach solution, filled with Agromix® G6 soil (Fafard et Frères LTD., QC, Canada), and dampened thoroughly prior to arrangement in a 10 x 10 grid, using 5 cm row spacing between treatment groups. Approximately three hundred hemp seeds (cv. Anka) were surface sterilized via successive immersion in dilute bleach (3.5 % available Cl-), ethanol and sterile dH_2O as outlined previously. Two to four seeds were placed directly on the soil surface; covered with a thin layer of soil and watered, as needed. Plants were thinned to a single, healthy seedling 9 DPS.

Seedlings received 5 ml of 1 X Hoaglands' solution to the soil 3-4 cm from the base of the plant weekly. The volume was increased to a final volume of 10 ml 1 X Hoaglands' solution at 14 DPS, and maintained until final harvest. At 14 DPS, treatment groups were formed from a randomly chosen pot position and arranged into a rectangular pattern with two rows, each comprised of 10 pots per treatment, with 7-10 cm of space between groups. Plants were bacterized via soil drench directly at the plant base with 10 ml of 10^5 CFU/ ml of each freshly grown bacteria, pelleted from LB growth media and washed twice using sterile water prior to re-suspension in sterile water. Control plants received a soil drench of sterile water (10 ml) alone and were placed 4 m from treatment groups to reduce possibility of contamination. Beginning at 20 DPS multiple chlorophyll readings (SPAD 502, Spectrum Technologies Inc., IL, USA) were recorded from central leaflets in the highest fully unfurled leaf-sets, and averaged / leaf to assess the general health of the plants.

Plants were harvested at 42 DPS, and the following growth parameters were recorded: root length, shoot length, leaf number, stem diameter, and dry weights of stem or root tissues following incubation for 2 days at 56 °C (Table 11). Hemp inoculation trials were replicated in three separate trials (I, II, III) with an average of 15 plants per treatment group across trials. Measurements were assessed using one-way ANOVA followed by Tukey's Honest Significant Difference using the statistical program R (R Studio, 2012).

3.6.5 Assessment of endophytic colonization:

At harvest, 5 plants were selected at random from each treatment for sampling, and either used immediately in pseudomonad population determination or frozen for later use. Tissues were either frozen directly in liquid nitrogen (non-sterile condition, NS; Trials II and III) or surface-sterilized in 3.5 % OCl^- as previously described (sterile condition, S, Trial I), prior to freezing and storage at -80 °C. Tissues used for CFU determination were ground in 5 ml of sterile dH_2O, and 100 µl of a dilution series was spread and incubated at 23 °C for 2 days prior to counts. Each dilution point was done in triplicates for each tissue and each treatment for each trial. Soil samples were also collected for storage at -80 °C and

population abundance determination. Approximately 0.4 g (fresh weight) of rhizospheric or bulk soil was agitated in 10 ml of sterile distilled water for 1 h (175 RPM) to dislodge organisms from the soil matrix. A dilution series was created and 100 µl of multiple points were plated and counted as previously described in triplicate, using un-amended LB agar plates.

3.6.6 Culture Independent Colonization Confirmation:

S and NS tissues, as well as rhizospheric and bulk soil samples were ground to a fine powder in liquid nitrogen prior to DNA extraction. Between 100 and 400 mg of sample was used in a modified CTAB method of DNA extraction (Carrigg et al., 2007). Briefly, the sample was incubated with lysis buffer (100 mM Tris-HCl, pH 8.0; 2 M NaCl; 25mM EDTA, pH 8.0; 5 % polyvinylpyrrolidine; 3 % Cetyl trimethylammonium bromide (CTAB)), 2- mercaptoethanol, and 1 µl RNAse A (Bioshop; ON, Canada) for one hour at 70 ºC. Samples then underwent one phenoly:chloroform:isoamyl alcohol (25:24:1) extraction; 2 rounds of chloroform:isoamyl alcohol (24:1) extraction and overnight precipitation in 2/3 volume isopropanol and 1/10 volume 3 % sodium acetate (pH 5.2) at room temperature (23 ºC). Precipitated DNA was collected, washed in 70 % ethanol and eluted into pure dH_2O (pH 7.0). DNA was visualized on a 1 % agarose gel to confirm quality prior to amplification using the *Pseudomonas* specific primers BT14-4F1/BT14-4R1 (Table 2). Amplification used the HF Bio-Rad PCR amplification as described previously with an annealing temperature of 67.5 ºC.

Chapter 4: Results

4.1 Patterns of Isolation:

Over 1000 tissue segments, collected from hemp cultivars throughout the growing season of 2013, were screened for the presence of cultivable endophytic microorganisms. A total of 137 bacterial strains were isolated and identified and the majority was isolated from petiole (67 %), followed by leaf (19 %) tissues (Fig. 1A). Hemp seeds harbored some endophytes, and the majority of them (90.5 %) were bacterial. The number of isolated bacterial strains was generally similar among the three hemp cultivars (Fig. 1C) and their isolation pattern throughout the collection period showed an early spike followed by gradual increase in bacterial isolates (Fig. 1E). Unsurprisingly, the most common genera to be isolated followed the trend seen, i.e. the most common bacteria were isolated from petiole most often (Table 4).

The majority, 70 % of the identified fungal strains (53), were isolated from leaf tissues, while 26 % arose from petioles, and 4 % originated from seed tissues (Fig. 1B). Unlike bacteria, nearly half (45 %) of isolates originated from various tissues of the cultivar Anka (Fig. 1D). There was no major trend in fungal isolate incidence with the number of fungal endophytes isolated at each time-point generally consistent, with a slight decrease over the season overall (Fig. 1E).

Our data trends suggest the identity of both host and tissue specificity in certain genera. Of those bacterial isolates found in seed tissues, *Pantoea sp.* were the most common, followed by *Staphylococcus sp*. and *Bacillus sp*. (Table 4). The most common bacterial leaf endophytes were *Pseudomonas sp.* and *Bacillus sp*. or *Staphylococcus sp*. and the most common petiole bacteria genera were *Pseudomonas sp.* and *Pantoea sp.* Among bacteria, *Pantoea sp*. were rarely found in Yvonne tissues however *Microbacterium sp*. were specific to Yvonne petioles (Fig. 3). *Bacillus spp.* were not found in Anka tissues, and likewise *Staphylococcus sp.* were never isolated from CRS-1 (Table 4). Genera such as *Acinetobacter* and *Erwinia* were only isolated at early dates, and some isolates, such as *Cedecea* or *Microbacterium* were collected from a single time-point, cultivar, and tissue, showing a transient presence

in the plants rather than the systemic one demonstrated by the *Pseudomonas*, *Staphylococcus* and *Bacillus* found throughout the growing season (Fig. 3, Table 4).

Fungi showed both host and tissue specificity as well. The most common fungal leaf endophytes of hemp were *Cochliobolus sp*. and *Alternaria sp.* and a single member of both genera were also found as the only fungal seed endophytes. The most common genera in petioles were *Aureobasidium sp.* and *Cladosporium sp.*; interestingly neither *Alternaria* or *Cochliobolus* were found from petiole tissues (Table 5). Among fungi, *Aureobasidium sp.* was also never isolated form Yvonne tissues. There was a decided change in the assemblage of the fungi colonizing the plant at different times in the year. Early in collection, i.e. Late June/ early July, most fungal endophytes isolated were members of *Alternaria* and *Aureobasidium*; by the final collection dates i.e. Mid-August, the most common isolates were: *Cladosporium*, *Cochliobolus* and *Drechslera* members (Fig. 3).

4.2 Isolate Identification:

Initial sequencing using ITS primers 27F/534R allowed unambiguous determination of bacteria to their genera and in many cases to the species level. Additional cloning and sequencing was done for isolates of increased interest to confirm species designation using the universal ITS primers 27F and 1509R (Table 2). Eighteen different bacterial genera were identified (Fig. 2A) with *Pseudomonas* (34 %), *Pantoea* (18 %), *Staphylococcus* (15 %), and *Bacillus* (9 %) being the most prevalent genera. A phylogenetic tree constructed for bacterial isolates clearly supports the BLAST sequence identification of large groups of bacteria, however some unexpected mixing emerges in closely related, large member groups such as *Pantoea* and *Pseudomonas* (Fig. 4). The dominant isolate groups remain well-delineated; and illustrate clearly the preference of common endophytic bacterial genera.

In fungi, sequences amplified by the ITS1F/ LR3 primer pair (Table 2) were used to delineate OTU groupings of 90, 95 and 100 %; corresponding roughly to the genera, species and isolate levels respectively (U'ren et al., 2009) using the program Mesquite version 3.02 (Maddison and Maddison, 2015). Results indicated the presence of 13 genera, being members of 17 species, and having 33 unique

strains (Fig. 2B). All fungal isolates were members of the dikarya, with the majority (96 %) being *Ascomycota*. Only two of the 53 isolates were members of *Basidiomycota*; namely: *Irpex*, and *Cryptococcus* (Fig. 5). The most commonly occurring fungal family was *Pleosporaceae* (Fig. 2B), and made up 47 % of all isolates tested. Examination at a higher taxonomic resolution showed the most common genera to be *Aureobasidium* (24 %), followed by *Alternaria* (19 %), *Cochliobolus* (19 %), and *Cladosporium* (15%). Groups generally show fewer mixed clades in comparison to the bacterial tree, which could be a result of the sequences used or sample size differences; i.e. 136 bacterial (Fig. 4) vs 53 fungal sequences aligned (Fig. 5).

4.3 Bacterial Screening, Selection, and Characterization:

Early testing and selective procedures were performed to maximize resources for performing in-depth characterization assessments (see section 3.3). Initial screening showed the production of cellulases to be the most commonly occurring trait found across genera (10/19 genera), while phosphate solubilization (5/19 genera), and HCN production (3/19 genera) were less common (data not shown). The expanded testing repeated the previous tests, in addition to tests for the presence of the *nifH* gene, organic acid, and siderophore production. In total 36 isolates were considered to determine the best performing isolates for additional testing (Table A1). The most common traits were siderophore production (80.6 %), cellulose (47.2 %), phosphate solubilization (44.4 %), and production of organic acids (38.5 %). HCN production was found in only 25 % of the tested strains. None of the isolates possessed the *nifH* gene (data not shown). The top 4 strains were all members of the *Pseudomonas* genus: BTC6-3, BTC8-1, BTG8-5, and BT14-4. All four showed varying levels of solubilization of inorganic phosphate, production of siderophores, and all except BTC6-3 demonstrated cellulytic activity (Table 6). HCN production was noted for only BTC6-3 and BTC8-1, and organic acid production was positive in BTC6-3 and BTG8-5.

These strains were additionally tested for antibiotic resistance, ACC deaminase activity, and fungal inhibition in direct confrontation assays. Antibiotic resistance was variable across the strains and compounds (data not shown). BTC6-3 and BT14-4 were found to produce the greatest amounts of IAA

(10.96 and 9.46 µg/ ml respectively), although all tested strains showed some level of IAA presence. No stain showed ACC activity, even using 100-fold increased quantities of supernatant (data not shown).

Ten fungi, covering a wide range of lifestyles were chosen for confrontation assessment against BTC6-3, BTC8-1, BTG8-5, and BT14-4. *Sclerotinia sclerotiorum* and *Botrytis cinerea*, both pathogens of hemp (McPartland, 1999) were of particular interest. Of all fungi tested, *S. sclerotiorum* was found to be most significantly affected by co-culture, displaying radical reductions in radial growth of 17 to 54 % against BTC6-3 and BTC8-1, respectively. Growth of *B. cinerea* was significantly reduced by only BTG8-5 (22 % reduction, Tukey's HSD test (THSD) p= 0.0242). *Rhizoctonia solani* was significantly reduced by BT14-4 (28 %, p=0.0141), and the bi-nucleate strain of *R. solani*, was significantly affected by co-culture with strains BTC8-1 and BTG8-5 (23.4 and 31.3 % reductions, p=0.0373 and 0.0065), however no other tested fungi were significantly affected (THSD, p=0.05; Table 7).

4.4 Fungal Characterization:

All 53 identified fungal strains were assessed for the production of cellulases and lignases. Cellulase activity in fungal isolates was found to be widespread. Over 70 % of isolates showed cellulose activity with 5.7 % of the strains exhibiting excellent activity; producing halos extending to over 10 mm from the colony edges. The production of lignases was limited to only 2 strains: FL11-3, a *Pleosporales sp*. that showed moderate strength, and more weakly by FL12-3, a *Stagonosporopsis sp*. (Table 8).

Preliminary assessment for the presence of EHB using the genomic DNA of a representative fungus from each genus identified revealed 6 putative host candidates (Table 8). BLAST search results suggest that putative EHB matched primarily uncultured and often unnamed bacterium. Among named hits were *Acinetobacter*, and *Staphylococcus.* Of those fungi showing putative EHBs, 4 showed *Mycoplasma* infection (Table 9).

4.5 Effect of Endophytic Bacterial Volatiles on Plant Growth

A significant reduction in root and total lengths were observed in all tomato seedlings bacterized with *Pseudomonas* strains as compared to both LB or *E coli* DH5α control plants (THSD, p<0.05).

Reduction in fresh weights was also significant for seedlings exposed to *Pseudomonas* strains BTC6-3, BTC8-1, and BT14-4, with the most significant reductions observed in the BTC6-3 treatment (Table 10). No significant difference between the growth of tomato seedlings exposed to the non-volatile producing *E. coli* DH5α and non-bacterized broth control plants was observed in any physiological metric measured. There was no significant germination difference among any treatment, or in dry weights of the control vs. treated seedlings, however the standard deviation was in some cases greater than average dry weight values.

4.6 Re-inoculation of Plants with *Pseudomonad* Endophytes

4.6.1 Tomato Seedlings:

Tomato seedlings, grown in sterile soil in magenta boxes showed some reductions in root length in treated conditions but otherwise appeared normal at harvest (data not shown). Bacteria that matched the Pseudomonad phenotype in treated plants was detected in colonized plant homogenates while control plants did not show bacterial colonies of any kind. Strain BTC8-1 was detected at similar levels in roots, stems and leaves, with log cfu/ml counts ranging from 1.70 to 4.13 (Fig 6A). BTG8-5 showed a marked decrease in stem tissue relative to leaf or root tissues (between 1.30 and 6.42 log cfu/ml) and appeared to be nearly absent in one trial (0.82 log cfu/ml). Amplification of DNA collected from sterilized tissue samples using designed *Pseudomonas* specific primers BT14-4F1/BT14-4R1 gave the expected band size in all treated samples, and was absent in control tissues (Fig. 6B).

4.6.2 Hemp Seedling Re-Inoculation:

Treatment was not found to have a significant effect on any parameter measured when compared to water treated control plants (THSD, p= 0.05, Table 11). The abundance of Pseudomonad strains expressed as cfu/ml, was most predominant in soil samples (Fig. 7A). Comparable cfu/mls were detected in bulk and rhizosphere soils in treated and untreated soils (Fig. 7A).

Generally, leaf and root tissues that underwent sterilization prior to homogenization failed to yield bacterial colonies (Trial I). In contrast, bacteria were recovered from both leaf and root tissues that did not undergo surface sterilization (Trials II and III). Abundances from these non-sterilized tissues showed consistent trends, with averages of 2.13 and 3.78 log cfu/ml in roots and leaves, respectively. Trial II treatment BTC6-3 showed an unusual inverse trend in which bacterial colonies were found at greater abundance in leaf tissues than roots (Fig. 7A). Bacterized plants did not have higher than expected levels of Pseudomonads based upon results seen in control tissues. Counts showed high variability, with standard deviation often approaching average values.

Pseudomonas specific primers designed for isolates BTG8-5 and BT14-4 amplified bands of the expected size in leaf samples (Fig. 7B). Root sample DNA amplification was poor and soil tissues were reticent to amplification of DNA extraction (data not shown). The presence of the introduced bacteria could not be confirmed via molecular means in these samples and combined with abundance cfu data, suggests possible epiphytic rather than endophytic colonization.

Chapter 5: Discussion

5.1 Effects of Cultivar and Tissue Source on Endophyte Assemblage

Host and microbial genetics are among the factors that have been reported to influence the endophytic microbiome (Sapkota et al., 2015; Sun et al., 2012). In this study the impact of host genotype on endophytic communities was examined in three cultivars of *Cannabis sativa* L.: Anka, CRS-1 and Yvonne. They are multi-purpose cultivars and include strains widely used by the local agricultural community. This study attempted to gain an understanding of the microbial endophytic community present in these strains and probed cultivable isolates for potential PGP and BCA applications.

Cultivar did not appear to have a large impact upon the total number of isolated bacterial endophytes, however there was a larger proportion of isolated fungal endophytes from cultivar Anka than expected (Fig. 1D). Genotype played a role in bacterial and fungal endophyte distribution specificity; shown by the bacterial genera *Microbacterium* (specific to Yvonne), *Xanthomonas* and *Acinetobacter* (Anka), and the fungi genera *Pezizomycetes* (Yvonne specific) and *Irpex* (CRS-1). Host specificity has been found previously in Cannabis and has been postulated to be in part due to the myriad of secondary compounds that this plant manufactures (Winston et al., 2014). Other endophytes were isolated in two but never the third cultivar, such as the bacteria *Staphylococus* (Yvonne and Anka), *Curtobacterium* (Yvonne and CRS-1), and fungi *Alternaria* or *Aureobasidium* (Anka and CRS-1). These results are in agreement with previous studies on both bacterial (Winston et al., 2014; Rasche et al., 2006; Rosenblueth et al., 2006), and fungal endophytes (Wearn et al., 2012; Park et al., 2012; Rodriguez et al., 2009).

Recent research conducted on various medical cultivars of *C. sativa* demonstrated that there was a significant cultivar effect upon microbial community structure within the plants' endorhiza (Winston et al., 2014). The interaction between host cultivar and endophyte has been found to alter host growth patterns (Gao et al., 2010; Lowman et al., 2015), and continued study of the impact of host genetics over multiple study years could yield important insights. The cases of microbial specificity or apparent aversion to a cultivar could be combined with chemical and genetic profiling of the different host plant

strains to inform potential reasons for the results found here. To date, study of hemp microflora over multiple years to control for environmental fluctuation and chemical profiling of different cultivars has not been undertaken in hemp, or is at least not available to the public.

Leaf assemblages of endophytes have become the most examined tissue source in many recent works due to the ease of tissue sampling; giving a correspondent ability to have larger study scope. As seed transmitted microbes are thought to have effects of greater importance than many horizontally transmitted ones, frequently being associated with plant resistance to both biotic (Clay, 1988; Li et al., 2015), and abiotic factors (Baltruschat et al., 2008; Gagne-Bourque et al., 2015; Naveed et al., 2014) they have also been studied heavily. Roots were not examined here, however leaf, petiole and seed assemblages offer insight into the above ground microflora of hemp, as seen through differences found between the colonization behaviors of the bacterial and fungal communities.

Tissue type is an important factor for endophyte colonization (Compant et al., 2011; Arnold et al., 2003) and it is interesting that the most heavily colonized tissue differed between bacterial and fungal isolates in *C. sativa*. The trend of increased bacterial endophytes in petiole as opposed to leaf tissues is corroborated in studies of other plants (Abraham et al., 2013; Trotel-Aziz et al., 2008). A recent study to the contrary by Massimo et al. (2015) showed that more fungal endophytes were found in stems than leaf tissues of desert plants. This is unsurprising as the stem tissue in these plants was the primary photosynthate producing tissue, and in terms of sugars would be more analogous to the leaves in temperate plants.

Many studies involving fungi have focused upon leaf tissues (Arnold et al., 2007) and findings such as *Alternaria* and *Cocliobolus*, both well known as phytopathogens of leaves (Soltani et al., 2014; Schoch et al., 2006); being found exclusively in leaf tissues as endophytes is interesting as it may support the theory that many endophytes are latent saprophytes or pathogens that have infected a non-host plant or lost pathogenicity (Esser, 2007; Delaye et al., 2013). Few have examined the fungal and bacterial

consortium at the same time, and it would be of particular interest to see if the tissue source trend found here is widespread in other plants.

There are over 130 bacterial genera reported as seed endophytes; the majority of which belong to *Proteobacterial* genera such as *Bacillus, Staphylococcus, Pseudomonas* and *Pantoea* (Truyens et al., 2014; Rosenblueth et al., 2012). In this study, seed embryos harboured the lowest numbers of bacterial or fungal isolates, 14 and 4 % of all endophytes isolated respectively, which is consistent with general trends elsewhere (Truyens et al., 2014), and is reasonable considering likely host mechanisms to restrict deleterious infection in sexual propagates. Major seed endophytes found in hemp: *Pantoea sp.*, *Staphylococcus sp*. and *Bacillus sp*., have also been found as seed endophytes in other crop plants (Rosenblueth et al, 2010; Mano and Morisaki, 2008; Truyens et al, 2014). Only 2 fungal endophytes (*Alternaria sp.* and *Cladosporium sp*.) were recovered in seeds, and both were recovered as seed endophytes elsewhere (Hodgon et al., 2014); although typically most fungal seed endophyte research has been on very host specific commensals of grasses such as the *Clavicipitacae* species *Epichloë* and *Neotyphodium* (Clay and Schardl, 2002; Saikkonen et al., 2010).

Previous research has demonstrated very high endophytic fungal diversity in leaves (Arnold et al., 2003), and supports the finding of highest fungal diversity in these tissues. The well-known saprophytes *Alternaria* and *Aureobasidium* are also among the most widely reported endophytes; frequently shown to be BCA or PGP agents (Dugan & Lupien, 2003; Soltani et al., 2014; Polizzotto et al., 2012; Schena et al., 1999, Schena et al., 2003) and have been found to synthesize intriguing metabolic compounds (Polizzotto et al., 2011). *Cladosporium* and *Cochliobolus* are most often associated with disease, but also exist as saprophytic community members (Schoch et al., 2006), and have been found as endophytes elsewhere (Hodgson et al., 2014; Campos et al., 2008). Excellent antifungal activity of endophytic *Cochliobolus sp.* has been shown in competition assays (Qadri et al., 2013); the crude extract thereof was capable of over 90 % reductions in the growth of important tropical disease agents (Campos et al., 2008). *Alternaria sp.* endophytes are well-known producers of novel metabolites (Wang et al., 2015), some of which are likely

responsible for host benefits during periods of stress (Waqas et al., 2015). It would be worthwhile to perform metabolic and biological control studies on strains of *Alternaria* and *Cochliobolus* isolated from hemp to ascertain whether they have similar value against pathogens.

Pseudomonas spp. were the most common endophyte isolated from hemp. They are well-known plant epiphytes (Espinosa-Urgel, 2004), giving members of this genus ample opportunity to integrate into the interior plant community; explaining their prevalence as endophytes in multiple host, and tissue systems (Mercado-Blanco & Bakker, 2007; Khan & Doty, 2009; Rosenblueth & Martinez-Romero, 2006). Pseudomonads often have attributes beneficial in plant defense such as iron-scavenging siderophores (Malla & Pokharel, 2008) or unique antimicrobials (Mavrodi et al., 2001; Ramette et al., 2010). Early studies established *Pseudomonas s*p. to be capable of movement from initial root inoculum, and confirmed colony establishment in leaf tissues in a variety of monocot and dicot plants (Lamb et al., 1996). Various endophytic Pseudomonads have demonstrably benefited the growth of their hosts (Weyens et al., 2010; Ozawa et al., 2007; Mercado-Blanco & Bakker, 2007). In terms of defensive potential, both *Pseudomonas* and *Bacillus sp*. have been found to improve their hosts' defense against *Botrytis cinerea*, an important and widely affecting fungal pathogen (Trotel-Aziz et al., 2008), and much recent research has gone into a promising *Bacillus sp*. strain that has shown plant growth protective effects and abiotic stress mitigation (Gagne-Bourque et al., 2015).

It is sensible that petiole tissue, which is chiefly vasculature in nature may harbour endophytes that are flagellated or otherwise motile, such as *Pseudomonas*. The most common petiole colonizers of petiole mirrored the leaf community, with *Pseudmmonas sp*., attaining the majority of isolates and *Pantoea sp*. the second most common genus. *Pantoea sp.* are also well known bacterial endophytes; discovered in tissues of *Oryza sativa*, and in citrus tree branches for example (Mano et al., 2006; Feng et al., 2006; Araujo et al, 2002). *Aureobasidium sp*. and *Dothideomycetes* members (including *Cladosporium*), are among the most common petiole fungal endophytes. *Dothideomycetes* are well known saprophytes and endophytes and often have cellulytic potential (Schoch et al., 2006) and

Aureobasidium sp. are among the most widely reported fungal endophytes (Dugan & Lupien, 2003; Gonzalez & Tello, 2011; Pugh & Buckley, 1971).

5.2 Temporal Effects:

The temporal effects on endophyte colonization are known in tree systems (Suryanarayanan & Thennarasan, 2004; Thongsandee et al., 2012); in tree-associated lichen, and in bark tissues (Beck et al. 2014). Generally positive trends in the total number of isolates over time (Suryanarayanan & Thennarasan, 2004) and/ or genus specific patterns of colonization have been found (Thongsandee et al., 2012; Shen & Fulthorpe, 2015). While similar studies in herbaceous plants are rarer, Wearn et al. (2012), recently found both tissue and seasonal effects on the isolate assemblages of root and leaf fungal endophytes in grassland forbes.

The pattern of bacterial isolation is consistent with previous studies, as there was a general increase in the number of isolates recovered over the growing season (Mahaffee & Kloepper, 1997), and inconsistencies in abundance as seen in the Early-July peak, have been noted elsewhere (Shen & Fulthorpe, 2015). This early abnormality in the bacterial trend could have resulted from short term favourable environmental conditions for a particular group of bacteria (Shen & Fulthorpe, 2015), the plant reaching an amiable stage for colonization (Keller et al., 2000; Sugiyama et al., 2014), or an aberrant result of under sampling, since this study only examined cultivable isolates from a single year. Members specific to later growth included the *Enterobacter*, *Xanthomonas*, and *Ochrobactrum* genera; genera well known as plant colonizers (Hauben et al., 1998; Studholme et al., 2011; Trujillo et al., 2005).

It is not uncommon to be unable to determine endophytes to the species level (Qadri et al., 2013; Yuan et al., 2011), however most endophytes isolated in this study could be identified to genus. The number of bacterial genera isolated (18) is consistent with recent studies in Chinese cabbage (23) (Haque et al., 2015), and rice (20) (Hameed et al., 2015). The number of fungal genera, described as groups at the 95 % OTU similarity level (13), is somewhat smaller than is often found elsewhere; 19 OTU were

identified in bamboo seed (Shen et al., 2014), and 27 were found in various plants native to the Himalayas, including *C. sativa* (Qadri et al., 2013). The ratio of *Ascomycota* to *Basidiomycota* is consistent with previous studies (Shen et al., 2015, Qadri et al., 2013), as *Ascomycota* make up the grand majority of most previous studies' fungal isolates (Gazis & Chaverri, 2010; Jin et al., 2013). Both fungal and bacterial strains seem to show a greater number of genera traditionally classified as pathogens or saprophytes at later dates than early ones, ex. *Xanthomonas, Cochliobolus*, *Enterobacter* appearing at later dates. This may be the result of senescing tissue allowing previously inactive endophytes to become active, or due to epiphytes having invaded the plant tissue more readily than earlier in the plants' growth.

5.3 Characterization:

In preliminary testing, cellulose activity, and siderophore production was fairly widespread among the isolates tested, while phosphate and HCN production occurred more rarely. There was a range of IAA production, from 0.88 to 10.96 µg/ ml, which, while less than some previously studied endophytic productions (Shi et al., 2011; Hoffman et al., 2013) are not insignificant amounts of IAA in planta (Wojcikowska & Gaj; 2015; Pencik et al., 2015), and fall within the range (0.35 and 10 µg/ ml) of other IAA producing bacteria (Khan & Doty, 2009).

Four endophytic *Pseudomonas sp.* strains: BTC6-3, BTC8-1, BTG8-5 and BT14-4 demonstrated various levels of cellulose, organic acid, hydrogen cyanide (HCN), phosphatase, IAA and siderophore production. Siderophore and IAA production are traits that can be considered both PGP and BCA applications (Verma et al., 2011). Phosphatase, cellulose, HCN production are among the tests used elsewhere for characterization of endophytes for screening purposes to good effect (Ahmad et al; 2008; Husen, 2003; Gagne-Bourque et al., 2013). HCN, siderophore, and IAA production has previously been shown to be common in *Pseudomonas* (Ahmad et al., 2008). Dinitrogen fixation potential was screened as has been performed with a variety of environmental isolates elsewhere (Ando et al., 2005; Gaby & Buckley, 2012) but isolates were found to be negative. This was surprising as other endophytic *Pseudomonas* have been found to possess nitrogen fixation genetics (Desnoues et al., 2003), and one

isolate from hemp was putatively identified as *P. nitroreducens* and was expected to test positively. ACC deaminase activity has been reported in fluorescent Pseudomonads (Saravanakumar & Samiyappan, 2006) and was also tested (Penrose & Glick, 2003), however isolates were not found to possess ACC deaminase activities, even when methods were modified to use 100-fold sampling (Penrose & Glick, 2003).

Competitive growth inhibition assay variation as documented in this study, is expected as a result of the various combinations of fungi to antagonist (Qadri et al., 2013; Soltani et al., 2014). In direct confrontation challenge against 10 different fungal strains, BTC6-3 did not show any significant inhibition, in some cases actually marginally (although in-significantly) promoting fungal growth. Kai et al. (2008) previously reported that the majority of bacteria producing volatiles caused an inhibitory response rather than contributing to growth in the tested fungus, although promotion was also possible. *S. sclerotinium* has previously been found to be among the most sensitive fungi tested to inhibitory effects of bacterial volatiles (Kai et al. 2008).

Volatiles are a strong means by which microbes and plants communicate. Young *Solanum lycopersicum* L. cv. Beefsteak tomato seedlings showed reduced growth when co-cultured with the endophytes BTC6-3, BTC8-1, BTG8-5 and BT14-4. A stunted root phenotype was the most striking development among these plants. The production of HCN, a possible BCA mechanism, has been described elsewhere as the mode of action of weed control (Heydari et al., 2008), and could be the cause of reduced root growth. Bacterial volatiles are also known for activation of plant immune responses (Ryu et al., 2004); in the absence of stress, this response is non-beneficial and can result in reductions in plant fitness (Heil & Baldwin, 2002; Cipollini et al., 2002). Metabolic analysis of the volatiles produced by the endophytes and subsequent tests with isolated compounds would be necessary to confirm the source of the effect, and could be a valuable avenue for future research.

The majority of fungal endophytes were good producers of cellulases with 73.6 % of tested strains showing some degree of activity; however, a minority of fungal isolates were found to have lignase activity (3.8 %). This is likely due to the tissue source used for initial isolation. Most fungal isolates were found from leaf tissues or petioles, which are typically not as heavily lignified as stems. Additionally, hemp in particular has a cellulose based profile when compared to other fibrous plants (Dorez et al., 2014), and would naturally select for cellulytic, as opposed to lignase producing cohabiters.

Endohyphael bacteria (EHB) are predominantly members of the proteo-bacteria that reside within various lineages of endophytic fungi (Hoffman & Arnold, 2010). Studies have found that they can confer PGP traits when present within their fungal host (Hoffman et al., 2013), however the relationship is facultative and is easily lost though sub-culturing or fungal culture on antibiotic treated media (Hoffman & Arnold, 2010). As our initial isolation used PDA or MEA containing a mixture of penicillin and streptomycin, previously determined to have wide anti-bacterial use, it is unsurprising that of the 17 strains tested, only 6 showed putative EHBs, despite the fact that EHB are believed to be widespread (Hoffman & Arnold, 2010; Sharma et al., 2008). The methods used in this study were employed to reduce contamination, giving the greatest certainty of isolates' endophytic nature, and to retain the most representative sample of cultivable organisms possible. A side-effect of this was the likely loss of many EHB. Some bacterial-fungal relationships have shown to be resilient against antibiotic curing, including *Acinetobacter* (Sharma et al., 2008); one of the EHBs discovered here.

Many of the EHB sequences could only be identified as currently uncultured bacteria, members of the proteo-bacteria or mycoplasmas: well-known commensal or parasitic organisms in both animal and plant systems. Mycoplasma often escape antibiotic treatment due to their cell-free nature and are found as a common contaminant in cell-culture lines (Miles & Nicholas, 1998). There is little research into their infection of fungal lines or putative role in the biology of a fungal host. It is possible that, similar to the newly discovered EHB, they play a role in host attributes. Pending more in-depth study, we have thus presented them as possible EHBs. These preliminary EHB results are encouraging, and could be benefited

from more in depth probing, through either microscopic or chemical means (Hoffman & Arnold, 2010), to confirm the presence of these EHB within the hemp endophytic fungi and test their possible function(s) in the host fungus.

5.4 Re-Introduction of Bacterial Endophytes to Plant Hosts:

Cellulase activity is believed to be of importance in microbial competence in re-infection of a plant host, especially for root and seed penetration (Hallmann et al., 1997; Rosenblueth et al., 2006; Truyens et al., 2015). Therefore, finding it to be widespread among isolates, and in particular among isolates selected for further work, was important. Isolates BTC8-1 and BTG8-5 both possessed this attribute, were representatives from the *Pseudomonas fulva* and *Pseudomonas orientalis* species and were selected for initial proof of concept re-colonization trials, using the model dicot plant tomato. Tomato is a model dicot in horticulture, and re-infection in the absence of a competing microbial flora was deemed promising for further greenhouse studies. Hemp seeds could not be rendered completely sterile without significant loss of viability. The presence of introduced *Pseudomonas sp.* was confirmed via culture dependent and in-dependent methods with some minimal signs of root growth inhibition in otherwise healthy plants.

Our greenhouse studies with *Pseudomonas* strains BTC6-3, BTC8-1, BTG8-5 and BT14-4 using the original host plant grown under a non-sterile greenhouse environment suggest that the *Pseudomonas* endophytes remained epiphytic rather than systematically re-colonizing the host plant as was expected from previous testing. Other studies have identified *Pseudomonas* as among the most persistent and promising for re-colonization of plants (Ryan et al., 2008; Zinniel et al., 2002), and it is widely acknowledged that they are abundant throughout the environment (Mercado-Blanco & Bakker, 2007). This study's population abundance numbers yielded much lower titers than has been found elsewhere (Elvira-Recuenco & van Vuurde, 2000; Germaine et al., 2004), and could be due to a plethora of causes.

While it is generally well accepted that some endophytes are capable of improving their hosts' growth in the face of biotic and abiotic stresses (Naveed et al., 2014; Larriba et al., 2015; Gagne-Bourque et al., 2015) this is not always found (Tian et al., 2015). Some studies have suggested that decreased primary metabolism, such as photosynthesis, as a result of endophytic infection can decrease host growth in the absence of stress (DuPont et al., 2015). Indeed, previous studies have found re-infection of otherwise beneficial microbes are facilitated by stressful conditions, and typically do not occur under ideal conditions, such as when well fertilized (Wahab & Abd-Alla, 1996). Hemp plants in this study were not nutrient, water or light deficient; all plants were maintained under optimal greenhouse conditions, fertilized and watered on a regular basis. It is possible that plants did not respond to bacterial re-infection because their needs were being met through other means.

Another explanation is the length of time between seeding and microbial re-introduction via soil drench. The soil microbiome is diverse and challenging for niche creation. Rhizoplane colonization is a vital step to any biological delivered via the soil, and one that has been a problem in the past (Compant et al., 2010). Bent and Chanway (1998) have suggested that the reduced efficiencies found in colonization and PGP benefits in the presence of other bacteria could be a result of mismatch of a strains adaptation to controlled environments and more challenging ones. Many microbes selected in laboratory studies are only employed in re-colonization trials after extensive study and culture passages; possibly altering the fitness of the strain. *Pseudomonas sp.* has elsewhere been found above $5.5\log_{10}$ within three days of introduction of the bacteria (Berggren et al., 2004), and considering its cosmopolitan presence in the environment, it was not expected to experience difficulty in growing to high titres as was seen here. It is possible that introducing the bacteria at two weeks of growth via soil drench, in contrast to a drench at potting, or as a seed coating may have made it challenging for the bacteria to create a niche in the environment from which to re-infect the host. While this genus is versatile and can attain very high densities, sometimes being one of the dominant genera in the rhizosphere (Zinniel et al., 2002; Mahaffee & Kloepper; 1997), it is possible that these particular strains were not able to withstand the extended

competition in a novel growing environment used here (Compant et al., 2010), especially if native *Pseudomonas spp*. were monopolizing the environment.

Should the above reasoning be sound, then the particular strains used here would have difficulty to adapt under greenhouse conditions. It is uncertain how the highly variable and stressful field environment could affect the relationship between the plant and putative endophyte. Testing of these concerns could be done by replication of the trial with inoculation at planting via drench at various cell densities, or experimentation with seed coating, a technique that has been used elsewhere to good effect (Colla et al., 2015). Additional testing under stressful conditions such as a cold shock (frost), fertilizer or water with-holding (nutrient deficiencies or drought), or challenging mid-grown plants with pathogens are designable, would more closely approach field conditions and may prompt colonization behaviour.

Chapter 6: Conclusions

Knowledge institutions are not the only beneficiaries of research focused on endophytic study. Endophytic research is believed to provide an underexplored and valuable niche for bioprospecting of novel compounds (Strobel & Daisy, 2003), important for antibiotic discovery among other applications. In agriculture specifically; novel compounds, especially secondary metabolites, have result in increased disease resistance in endophyte hosts (Larriba et al., 2015; Straub et al., 2013), and research looking to capitalize on natural, plant, and microbe derived compounds could benefit. A recent study found that one third of host genes examined could be altered by endophytic infection and that secondary metabolism gene expression was upregulated to the detriment of primary metabolism (Dupont et al., 2015). At a minimum, researchers should at least consider the endophytic status of the plant under study at the design stage.

This study identified and characterized the endopytic microflora of hemp with the goal of better understanding attributes of this native flora, and for downstream application using the isolated microbes. Many fungi are members of genera known to produce useful metabolites, and six of these fungi gave early indication of some EHB infection; both would warrant more serious follow up study. Four bacterial endophytes, selected based upon good PGP and BCA enzymatic activities, produced interesting but currently unidentified volatiles that effected the growth of tomato seedlings. Competent endophytic recolonization in sterile conditions did not preclude endophytic colonization under greenhouse conditions for these selected strains. Epiphytic colonization was confirmed, and experimental suggestions to address the discrepancy between sterile and non-sterile colonization trends are proposed. This study has enhanced the general knowledge of endophytes in industrial hemp grown in NE North America. It has added valuable avenues for further study, and putative applications for isolated strains through the careful testing of bacterial and fungal endophytes for PGP and BCA possibilities.

Chapter 7: Recommendations for Future Studies

This study offers the opportunity to study many aspects related to the strains isolated from *C. sativa* L.

- Replication of the study over multiple growth years would add valuable data to support or refute trends found. It could also offer the opportunity to perform metagenomic study to capture an idea of non-cultivable colonizers and compare to cultivable isolates as has been done elsewhere (Arnold et al., 2007).
- Modification of greenhouse trials following the development and testing of GFP tagged isolates would visually confirm colonization of host plants with the putative endophyte (Verma et al., 2004; Torres et al., 2013; Compant et al., 2005). When coupled with alternative visualization techniques such as TEM (Gagne-Bourque et al., 2015) or FISH (Compant et al., 2011), visual identification of bacteria to the genus level can be possible and could allow temporal stages of colonization to be better studied
- Modification of greenhouse growing conditions such as adding (a)biotic stress to the plant and assessing colonization and/ or changes in physiology. Results from field trials would also be the most directly predict endophyte effects for the agricultural community of primary producers
- Strengthening of screening procedures. Simplifying attributes most likely to lead to selection of isolates with application. This can benefit future works as seen in recent work developed a screening method that resulted in isolation of metabolites with a high success rate against pathogenic agents such as malaria, Leshmaniasis (Higginbotham et al., 2013), pathogenic *E. coli* and *Stapholococcus aureus* (Qadri et al., 2013)
- Further *in planta* studies would continue to move these strains to more practical application and value

Chapter 8: Literature Citations

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Chapter 9: Tables

Table 1. GPS Co-ordinates of Hemp plants whose tissues where used for endophyte discovery

Global Positioning System Co-ordinates				
Cultivar (Year)	**Tissue Tested**	**Plot Number**	**Longitude**	**Latitude**
Anka and Yvonne (2012)	Seeds*	1	N45°26´02.4´´	W073°55´55.6´´
			N45°26´03.2´´	W073°55´52.9´´
		2	N45°26´02.3´´	W073°55´55.5´´
			N45°26´03.1´´	W073°55´52.8´´
CRS-1 (2012)	Seeds* (Control)	1	N45°26´02.0´´	W073°55´55.6´´
			N45°26´02.3´´	W073°55´54.4´´
		2	N45°26´01.5´´	W073°55´55.0´´
			N45°26´01.8´´	W073°55´54.0´´
	Seeds*(Nitrogen)	1	N45°26´02.4´´	W073°55´54.2´´
			N45°26´02.7´´	W073°55´53.2´´
		2	N45°26´01.8´´	W073°55´53.8´´
			N45°26´02.2´´	W073°55´52.9´´
Yvonne (2012)	Leaves/Petioles	1	N45°26´02.4´´	W073°55´55.6´´
			N45°26´03.2´´	W073°55´52.9´´
Anka and CRS-1 (2013)	Leaves/ Petioles	1	N 45° 26' 4.5"	W 73° 55' 52.9"

*Seeds were provided from Health Canada certified sources prior to field use. Both seeds collected from harvest and control seeds direct from providers were assessed.

Table 2. PCR primers used in this Study

Name	Target	Sequence (5' – 3')	Annealing Temperature (ºC)	Size of Amplified Product (bp)	Source*
Universal Primers 27F/534R	Bacterial 16S rRNA	AGAGTTTGATCMTGGCTCAG/ GGTTACCTTGTTACGACT	60	450	1
Universal Primers ITS1F/ LR3	Fungal Sm/ Lg rDNA subunits	CTTGGTCATTTAGAGGAAGTAA/ GGTCCGTGTTTCAAGAC	54	1000-1200	2, 3
DVV/ IGK3	NifH gene	ATM GCR AAM CCM CCR CAM ACM ACR TC/ GCM WTH TAY GGM AAR GGM GGM ATH GGM AA	54	310	4
M13F/ M13R	Standard M13 cloning vector	GTA AAA CGA CGG CCA GT/ GGA AAC AGC TAT GAC CAT G	53	various	5
14-4F1/ B14-4R1[$]	*Pseudomonas sp*. specific	TTAATCGGAATTACTGGGCG/ CGCACCTCAGTGTCAGTGTT	67.5	370	This study
rbcL 1F 724R	Plant Rubisco	ATG TCA CCA CAA ACA GAA AC/ TCG CAT GTA CCT GCA GTA GC	59	700	6

[$] Specific to *Pseudomonas* strain BT14-4 and cross hybridizes with *Pseudomonas* strains BTC6-3, BTC8-1 and BTG8-5

*References: 1- Watanabe, 2001; 2- Gardes & Bruns, 1993; 3- Vilgalys and Hester, 1990; 4- Ando, 2005; 5- TOPO® TA-cloning Kit (Invitrogen, CA, USA); 6- Bafeel et al., 2012

Table 3. Biochemical tests used for the characterization of endophytes isolated from Hemp tissues

Target Organism	Chemical Assay	Method of Assessment	Active Ingredients	Reference*
Bacteria	Phosphate-Solubilization	Lysis zone and Halo Formation	$Ca_3(HPO_4)_2$	1
	Siderophore	Lysis zone and Halo Formation	Chrome azurol S (CAS) $FeCl_3$*6H20 piperazine-N,N′-bis(2- ethanesulfonic acid) (PIPES) hexadecyltrimethylammonium bromide (HDTMA) Casamino acids (10 % w:v)	2
	Antibiotic Resistance	Presence/ Absence of Growth	Kanamycin Rifampin Streptomycin Tetracycline Ampicillin Chloramphenicol Gentamicin Hygromycin	3
	HCN	Colormetric	Picric acid (0.05 %)	4
	IAA	Colormetric	L-Tryptophan	5
	ACC deaminase	Colormetric	α- ketobuterate 2,4-dinitrophenyl-hydrazine	6
	Organic Acids	Colormetric	Glucose-phosphate broth Methyl Red dye	7
Bacteria & Fungi	Cellulose Degradation	Lysis Zone and Halo Formation	Cellulose (0.2 %) Cogo Red Stain	8
Fungi	Liginase	Lysis Zone and Halo Formation	Indulin (Lignin) (0.05 %)	9

*References: 1-Verma et al., 2001; 2- Alexander & Zuberer, 1991; 3- Gagne-Bourque et al., 2013; 4-Bakker et al., 1987; 5-Husen et al., 2003; 6-Penrose & Glick, 2003; 7-Voges and Proskauer, 1898; 8-Gupta et al., 2012; 9-Sundman and Nase, 1974

Table 4. Cultivar and tissue origin of bacterial endophytes

Genus[#]	Tissue[&]												Total # of strains Belonging to Genus	% of Bacterial Isolates[*]
	Leaf				Petiole				Seed					
	A	C	Y	**T**	A	C	Y	**T**	A	C	Y	**T**		
Acinetobacter	0	0	0	**0**	3	0	0	**3**	0	0	0	**0**	3	**2.2**
Agrobacterium	0	0	0	**0**	1	0	0	**1**	0	0	0	**0**	1	**0.7**
Bacillus	0	2	1	**3**	0	4	3	**7**	0	2	1	**3**	13	**9.4**
Brevibacterium	0	0	0	**0**	0	1	0	**1**	1	0	0	**1**	2	**1.5**
Cedecea	0	0	0	**0**	0	1	1	**2**	0	0	0	**0**	2	**1.5**
Curtobacterium	0	2	0	**2**	0	0	2	**2**	0	0	0	**0**	4	**2.9**
Enterobacter	0	0	0	**0**	2	2	1	**5**	2	0	1	**3**	8	**5.8**
Enterococcus	0	0	0	**0**	1	0	0	**1**	0	0	0	**0**	1	**0.7**
Erwinia	0	0	0	**0**	0	1	0	**1**	0	0	0	**0**	1	**0.7**
Microbacterium	0	0	0	**0**	0	0	3	**3**	0	0	0	**0**	3	**2.2**
Ochrobactrum	0	0	0	**0**	1	1	0	**2**	0	0	0	**0**	2	**1.5**
Paenibacillus	0	1	0	**1**	0	0	0	**0**	0	0	0	**0**	1	**0.7**
Pantoea	1	1	0	**2**	6	8	1	**15**	4	3	0	**7**	24	**17.5**
Pseudomonas	4	2	5	**11**	4	15	16	**35**	0	0	1	**1**	47	**34.3**
Rhizobium	0	0	0	**0**	0	1	0	**1**	0	0	0	**0**	1	**0.7**
Staphylococcus	2	0	1	**3**	5	0	9	**14**	0	0	4	**4**	21	**15.3**
Strenotrophomonas	0	1	0	**0**	1	0	0	**1**	0	0	0	**0**	2	**1.5**
Xanthomonas	1	0	0	**0**	0	0	0	**0**	0	0	0	**0**	1	**0.7**

[#] Genus determined via BLASTn 16S of sequences

[&] Cultivars are listed below the tissue source. A= Anka, C= CRS-1, Y= Yvonne, T=Total

[*] 137 bacterial isolates total

Table 5. Cultivar and tissue origin of fungal endophytes

Genus[#]	**Tissue**[&]												**Total # of strains Belonging to Genus**	**% of Fungal Isolates**[*]
	Leaf				**Petiole**				**Seed**					
	A	C	Y	**T**	A	C	Y	**T**	A	C	Y	**T**		
Aureobasidium	4	1	0	**5**	1	7	0	**8**	0	0	0	**0**	13	**24.5**
Alternaria	6	3	0	**9**	0	0	0	**0**	0	1	0	**1**	10	**18.9**
Cochliobolus	4	0	6	**10**	0	0	0	**0**	0	0	0	**0**	10	**18.9**
Cladosporium	2	0	0	**2**	1	1	0	**2**	0	0	1	**1**	5	**9.4**
Dothideomycetes	0	0	0	**0**	0	3	1	**4**	0	0	0	**0**	4	**7.5**
Drechslera	2	0	1	**3**	0	0	0	**0**	0	0	0	**0**	3	**5.7**
Sordariomycetes	2	0	0	**2**	0	0	0	**0**	0	0	0	**0**	2	**3.8**
Cryptococcus	0	1	0	**1**	0	0	0	**0**	0	0	0	**0**	1	**1.9**
Eutypella	1	0	0	**1**	0	0	0	**0**	0	0	0	**0**	1	**1.9**
Irpex	0	0	0	**0**	0	1	0	**1**	0	0	0	**0**	1	**1.9**
Pezizomycetes	0	0	1	**1**	0	0	0	**0**	0	0	0	**0**	1	**1.9**
Pleosporales	1	0	0	**1**	0	0	0	**0**	0	0	0	**0**	1	**1.9**
Stagonosporopsis	0	1	0	**1**	0	0	0	**0**	0	0	0	**0**	1	**1.9**

[#] Genus determined via BLASTn 16S of sequences

[&] Cultivars are listed below the tissue source. A= Anka, C= CRS-1, Y= Yvonne, T=Total

[*] 53 fungal isolates total

Table 6. Attributes of four endophytic *Pseudomonas* strains of interest

Strain[$]	Species	Cellulase Activity[γ]	Phosphatase[γ]	Organic Acid[γ]	IAA produced (µg/ ml)	HCN[γ]	Siderophore Production[γ]	% Inhibition of Fungi[*]
BTC6-3	*Pseudomonas fulva*	-	+	+	10.96±0.31	+	+	None
BTC8-1	*Pseudomonas fulva*	+	+	-	3.39±0.59	+	+++	53.8 *S. sclerotiorum* 23.4 *Binucleate R. solani*
BTG8-5	*Pseudomonas orientalis*	+	++	+	3.36±0.05	-	++	47.8 *S. sclerotiorum* 22.0 *B. cinerea* 31.3 *Binucleate R. solani*
BT14-4	*P.seudomonas orientalis*	+	++	-	9.46±0.10	-	+++	50.7 *S. sclerotiorum* 27.6 *R. solani*

$ All strains are *Pseudomonas sp*. All tests were replicated three times and the averages are presented.

γ Halo size was rated as follows: + positive (halo <5 mm), ++ strongly positive (5 mm< and <10 mm), +++ very strongly positive (halo >10 mm)

* Only reductions in hyphal growth that were deemed statistically significant (Tukey's HSD, p=0.05) are indicated. The percent of growth reduction, compared to unchallenged growth is given in parenthesis (see Methods for more information)

Table 7. Inhibition of radial growth of test fungi in confrontation assays

Fungus	Characteristic	Treatment$	Average Radius (cm)#	% Inhibition	Significance* (p value)
Sclerotinia sclerotorum	Broad Pathogen	Control	3.44	-	-
		BTC6-3	2.84	17.4	0.1712
		BTC8-1	1.59	53.8	<0.0001
		BTG8-5	1.79	47.8	<0.0001
		BT14-4	1.69	50.7	<0.0001
Botrytis cinerea	Broad Pathogen	Control	2.96	-	-
		BTC6-3	2.79	5.8	0.9369
		BTC8-1	2.53	14.6	0.2485
		BTG8-5	2.31	22.0	0.0242
		BT14-4	2.56	13.4	0.3321
Rhizoctonia solani	Broad Pathogen	Control	3.29	-	-
		BTC6-3	3.30	-0.3	1.0000
		BTC8-1	2.56	22.3	0.0717
		BTG8-5	2.45	25.6	0.1279
		BT14-4	2.38	27.6	0.0141
Binucleate Rhizoctonia solani	Biocontrol	Control	3.00	-	-
		BTC6-3	2.89	3.8	0.9908
		BTC8-1	2.30	23.4	0.0373
		BTG8-5	2.06	31.3	0.0065
		BT14-4	2.39	20.6	0.0881
Trichoderma virens	Plant Growth Promoting Fungi	Control	2.88	-	-
		BTC6-3	2.89	-0.2	1.0000
		BTC8-1	2.66	7.6	0.8168
		BTG8-5	2.40	16.8	0.1468
		BT14-4	2.61	9.3	0.6763
Colletotrichum gloeosporioides	Broad Pathogen	Control	2.61	-	-
		BTC6-3	2.74	-5.1	0.9937
		BTC8-1	2.45	6.4	0.9734
		BTG8-5	1.90	27.2	0.1740
		BT14-4	2.27	13.2	0.7484
Stachybotrys elegans	Biocontrol	Control	2.64	-	-
		BTC6-3	2.87	-8.7	0.8651
		BTC8-1	2.16	18.3	0.2165
		BTG8-5	2.03	23.2	0.2216
		BT14-4	2.24	15.3	0.3814
Helminthosporium solani	Limited Pathogen	Control	1.87	-	-
		BTC6-3	2.04	-9.4	0.3993
		BTC8-1	2.11	-13.2	0.3805
		BTG8-5	2.10	-12.5	0.0951
		BT14-4	1.85	1.2	0.9999
Fusarium solani	Broad Pathogen	Control	2.52	-	-
		BTC6-3	2.31	8.5	0.9931
		BTC8-1	2.27	9.8	0.8605
		BTG8-5	2.10	16.6	0.6985
		BT14-4	2.30	8.7	0.9848
F. graminarium	Broad Pathogen	Control	1.98	-	-
		BTC6-3	2.04	-3.1	0.8926
		BTC8-1	2.11	-6.7	0.8014
		BTG8-5	1.81	8.8	0.4330
		BT14-4	2.05	-3.5	0.8605

$ All strains are endophyte strains belonging to the genus *Pseudomonas*

Mean of three replicates of the radial growth of fungi measured in cardinal points surrounding the colony.

*Values shown are the results of a Tukey's Honest Significant Difference test of means using p=0.05 comparison between treatment and control levels. Values are rounded to the fourth decimal and are indicated when less than 0.0001 as <0.0001.

Table 8. Fungal isolate characterization [@]

Cellulase		Lignase	
Halo Size	**% of Isolates**	**Halo Presence**	**% of Isolates**
No activity	26.4	Positive	4.5
<5 mm	11.3		
>5 mm & <10 mm	56.6	Negative	95.5
>10 mm	5.7		

@ All 53 identified fungal isolates were tested for the production of cellulose and lignase lytic enzymes. Values are presented as the percentage of fungi that demonstrated the trait in question. Halo measurements are from duplicate and triplicate replications and used the four cardinal points surrounding fungal growth for measurements.

Table 9. List of fungal endophytes of Hemp that possess positive sequences for Endophyael bacteria

Fungal Host	Genus of Fungus (95 % OTU Group Level) *	Putative Endohyphael Bacteria (Top BLAST hits from multiple clones)	Support Value[#] (%)
FLC6-1	*Pezizomycetes*	*Novosphingobium*	99
		Uncultured alpha proteobacterium	99
		Acinetobacter	99
FLC7-7	*Sordariomycetes*	Uncultured rumen bacterium	95
		Uncultured bacterium partial 16S rRNA	99
		Mycoplasma	98
FTG8-1	*Aureobasidium*	Uncultured bacterium partial 16S rRNA gene	99
		Acinetobacter	99
		Staphylococcus	99
FS9-1	*Cladosporium*	*Acinetobacter*	99
		Uncultured bacterium clone	99
		Mycoplasma	99
		Staphylococcus	99
FL11-8	*Cryptococcus*	*Mycoplasma*	98
		Uncultured *Propionibacterium* clone	99
FT13-2	*Dothideomycetes*	*Mycoplasma*	98

*A Single representative fungi was chosen from 17 different OTUs at the 95 % similarity level. An average of 4 separate clones were sequenced per queried OTU. BLASTn searches were conducted April 2015; results of *E. coli*, or fungal species were dismissed as cloning artifacts.

BLASTn support for the identified putative Endophyphael bacteria

Table 10. Effect of bacterial volatile compounds on the growth of Tomato seedlings

Treatment	Root Length[#] (cm)	Total Length[#] (cm)	Fresh weight[#] (mg)	Dry weight[@] (mg)	Germination (%)
Control	5.9±1.8	9.9±2.8	183.9±91.5	30.2±71.5	92.0±8.0
E. coli DH5α	6.4±1.2	10.4±2.0	159.5±70.9	26.1±43.3	90.0±2.8
BTC6-3	0.6±0.7*	2.2±1.5*	58.7±21.8*	12.2±2.5	86.0±2.8
BTC8-1	1.2±1.3*	2.6±1.9*	57.7±21.1*	11.3±1.2	82.0±2.8
BTG8-5	1.2±1.5*	3.4±2.8*	122.6±105.3	40.2±98.2	77.3±6.1
BT14-4	1.2±1.3*	2.6±1.9*	57.5±14.0*	10.9±15.1	86.0±14.1

[#] Average values of trials repeated in triplicate with 5 seedlings per plate and 5 plates per trial. Standard deviation is given following ±

[@] Dry weight measurements were taken as the weight of 5 plants within each plate per replication. Five plates per replication was weighed and the experiment was done in triplicate.

[*] Values were significantly different from the control using Tukey's Honest Significant Difference test of means $p<0.05$

Table 11. Growth parameters of re-inoculated Hemp seedlings grown under greenhouse conditions

Trial	Treatment	Leaf Number*	Shoot Height (cm)*	Stem Diameter (mm)*	Root Length (cm)*	Chlorophyll Reading*	Dry Root Weight (mg)*	Dry Shoot Weight (mg)*
I	Control	12.14±1.66	46.41±5.63	3.64±0.93	33.10±6.91	38.17±3.57	0.674±0.144	2.523±0.375
	BTC6-3	12.13±2.00	45.71±5.80	3.25±0.68	32.71±6.01	38.00±4.23	0.659±0.163	2.349±0.361
	BTG8-5	12.75±1.61	47.34±5.46	3.38±0.72	32.59±4.57	38.03±3.42	0.660±0.166	2.412±0.377
	BT14-4	12.53±1.77	42.62±8.05	3.87±0.90	33.77±6.93	37.29±3.78	0.586±0.096	2.367±0.491
II	Control	12.67±1.45	49.97±9.20	3.33±0.90	33.90±4.50	34.39±5.08	0.443±0.150	2.134±0.637
	BTC6-3	13.07±1.49	44.69±2.78	3.73±0.80	32.92±6.01	34.64±4.58	0.488±0.133	2.209±0.482
	BTG8-5	12.53±1.47	43.25±6.90	3.63±096	34.54±8.19	32.19±4.53	0.570±0.143	2.332±0.585
	BT14-4	12.94±1.25	46.57±7.55	3.65±0.79	30.10±4.93	31.89±6.40	0.522±0.157	2.410±0.545
III	Control	12.50±1.93	53.72±12.28	3.92±0.67	34.30±4.84	34.09±2.90	0.562±0.180	2.586±0.797
	BTC6-3	10.60±1.47	59.03±21.35	3.50±0.76	33.42±3.78	40.47±33.43	0.477±0.169	2.503±0.887
	BTG8-5	11.13±1.63	45.99±10.59	3.63±0.72	34.06±5.46	34.22±3.51	0.470±0.147	1.982±0.583
	BT14-4	10.20±2.74	46.42±14.17	3.40±1.17	33.38±6.48	36.23±3.61	0.410±0.241	2.083±1.059

*Values are average results of 15.25, 16.25, and 14.5 plants per treatment across trials I, II and III respectively. Values following the average denote standard deviation.

Chapter 10: Figures

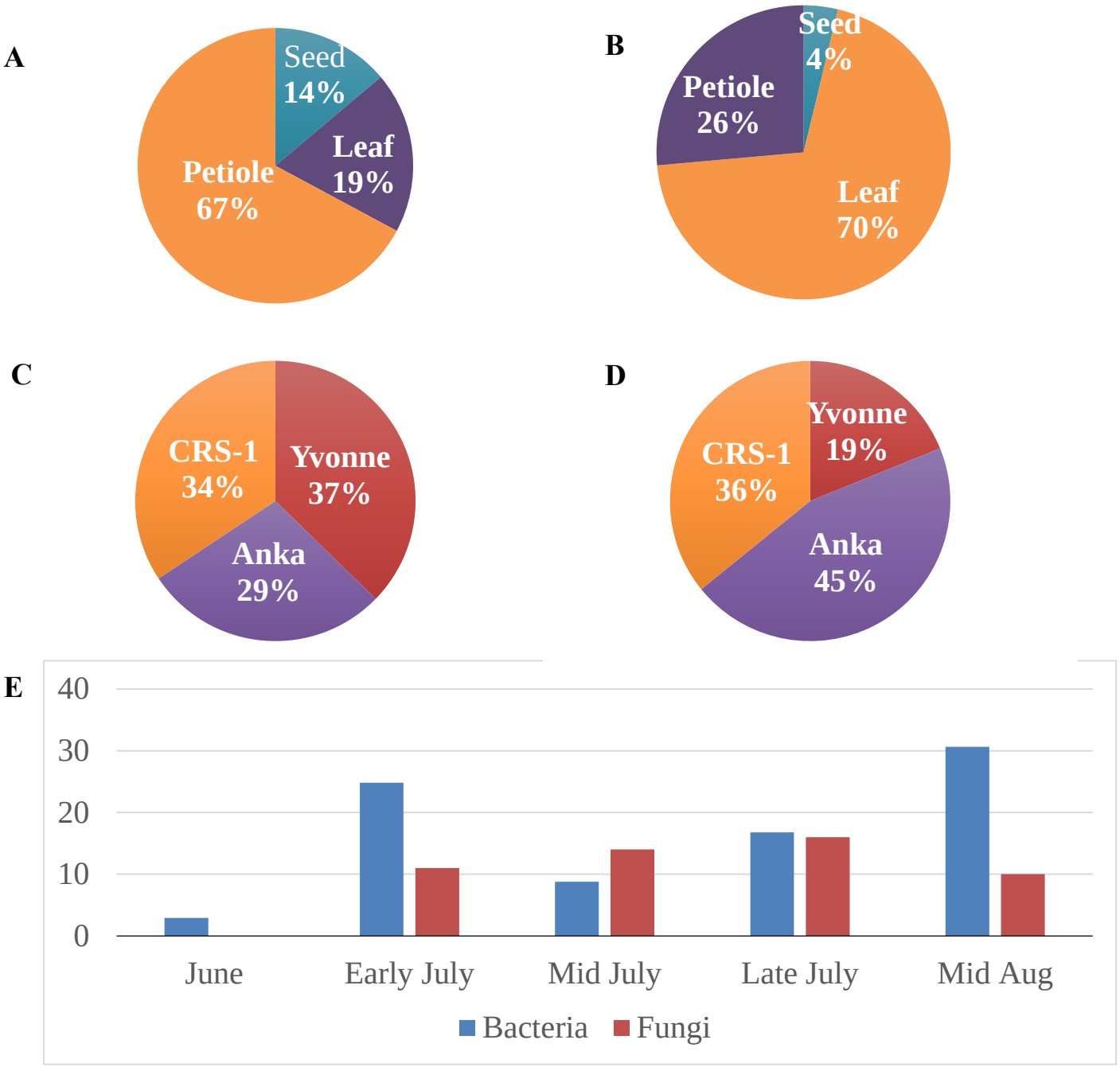

Fig. 1 Distribution of bacterial (A, C) and fungal (B, D) endophytes by tissue (A, B) and by cultivar (C, D) over the growing season of summer 2013 (E).

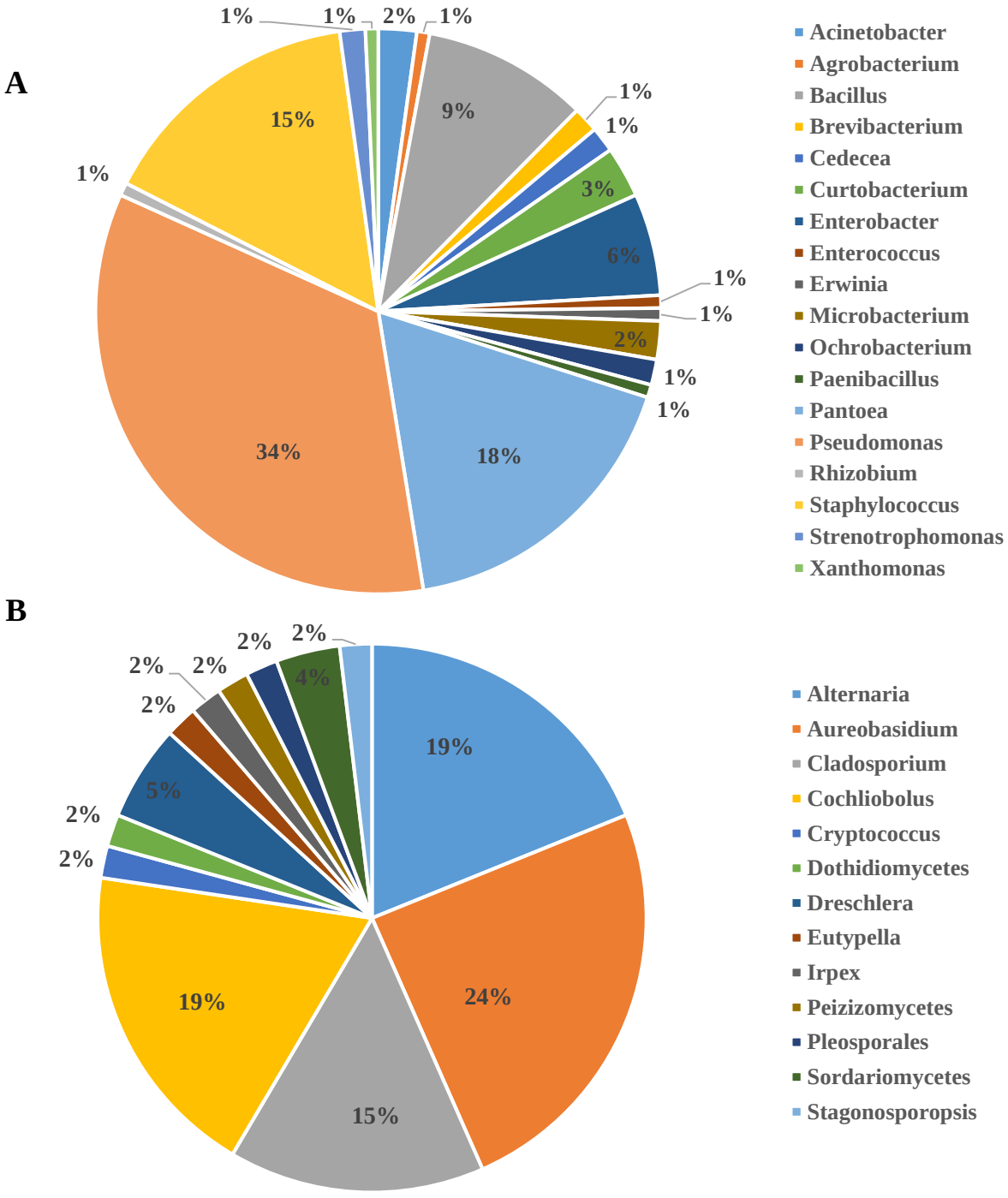

Fig. 2 Genera of microbes isolated from *C. sativa* L. A total of 18 different bacterial genera (A) and 13 genera* (B) were identified from 137 and 53 bacterial and fungal endophytes respectively. *Some fungal groups could not be identified to genus and are presented at the most specific identity supported by available sequence data.

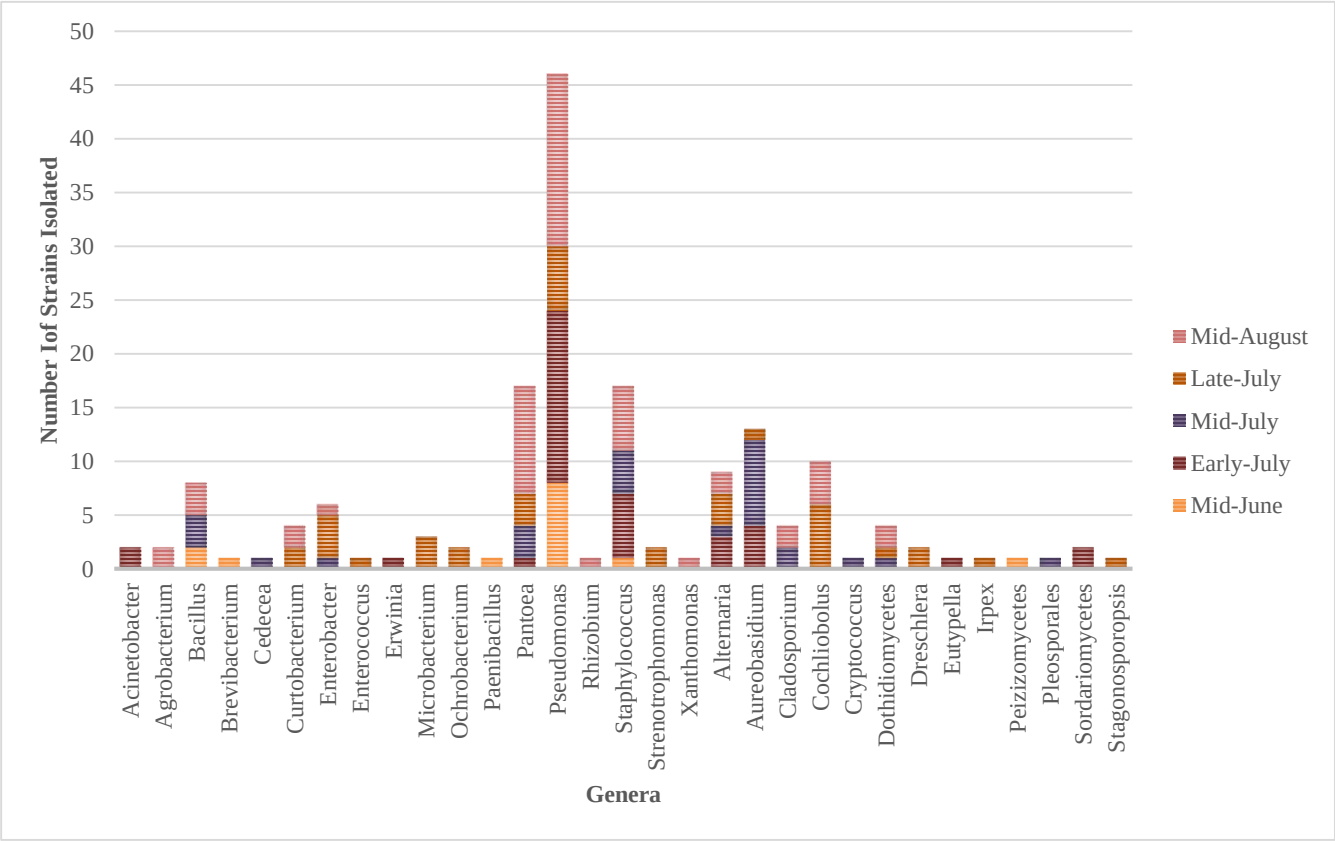

Fig. 3 Temporal distribution of endophytic isolation, Summer 2013. Petioles and Leaves were collected from field grown *C. sativa* L. Isolated genera are distributed based upon the date of tissue harvest.

Fig. 4

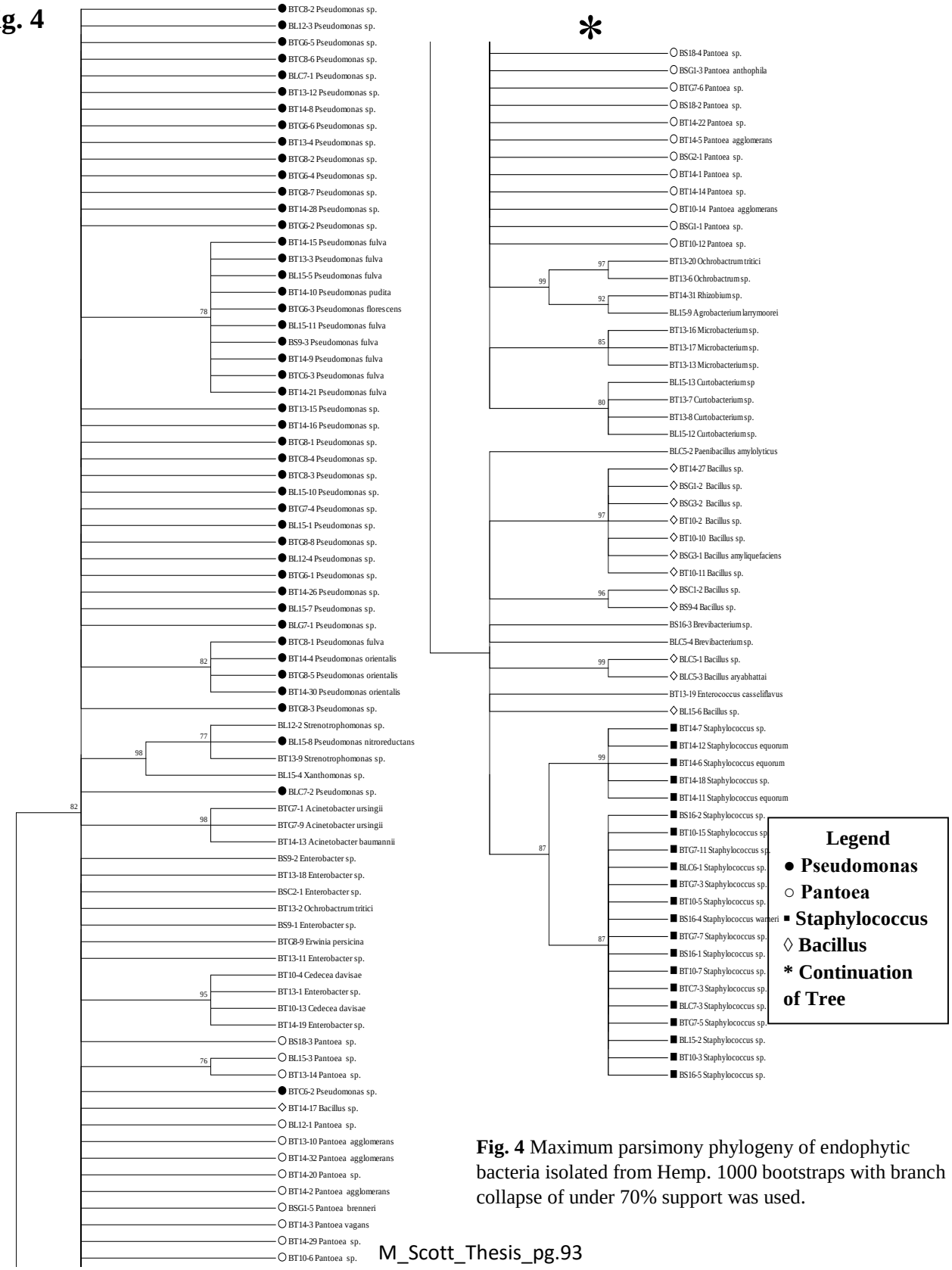

Fig. 4 Maximum parsimony phylogeny of endophytic bacteria isolated from Hemp. 1000 bootstraps with branch collapse of under 70% support was used.

Fig. 5

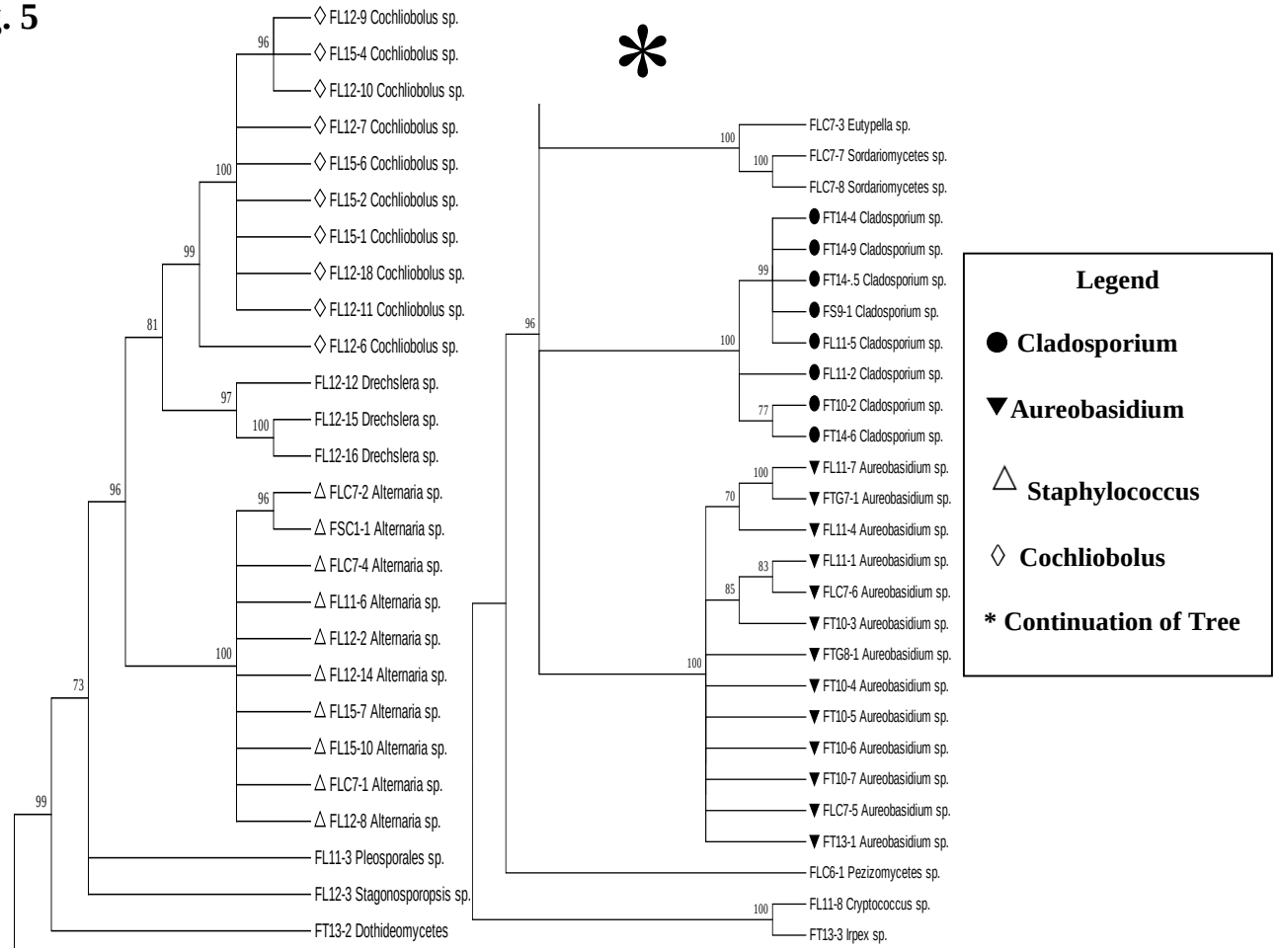

Fig. 5 Maximum parsimony phylogeny of endophytic fungi isolated from Hemp. 1000 bootstraps with branch collapse of under 70% support was used.

A. Dynamics of *Pseudomonas* strains in a dicot model host plant

Treatment	Trial	Tissue	*Log cfu/ml±stdev
BTC8-1	1	Leaf	2.80± 0.14
		Stem	3.20±0.19
		Root	2.83±0.08
	2	Leaf	2.02±0.41
		Stem	1.70±0.00
		Root	4.13±0.19
BTG8-5	1	Leaf	2.99±0.07
		Stem	0.82± 0.34
		Root	1.30±0.49
	2	Leaf	3.24±0.18
		Stem	2.18±0.23
		Root	6.42±0.14

B

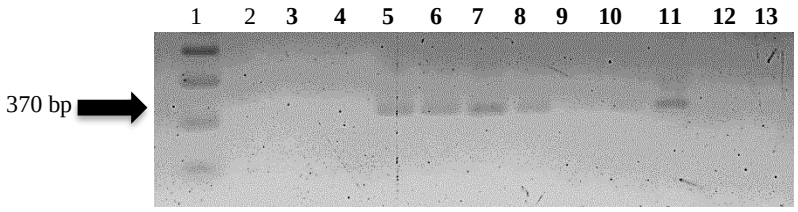

Fig. 6 (A) Colony abundance (CFU/ml) was determined using sterilized tissues of bacterized Tomato seedlings grown under sterile conditions. CFU values were found to be significantly different between trials and are presented separately as averaged values. *Values are a replicate of multiple dilution points done in duplicate and standard deviation is given. Sterilized tissue of non-inoculated control plants did not show colony growth. **(B)** Showing the amplification of size 370 bp using the *Pseudomonas* BT14-4F1/BT14-4R1 primer set designed in this study to demonstrate plant infection. The NEB® 100bp Ladder (1) was used for amplicon size determination. corresponding Sterile tissues bacterized with BTC8-1 (5 – Leaf, 6- Shoot, 7- Root), or BTG8-5 (8- Leaf, 9- Shoot, 10- Root), and genomic DNA from *Pseudomonas* BT14-4 (11) all showed amplification of the expected size (37-bp). Non-Bacterized tissues (2- Leaf, 3- Shoot, 4-Root), loading (12) and PCR (13) negative controls without addition of DNA, showed no amplification.

A Re-colonization of Hemp seedlings by selected endophytes under greenhouse conditions

Trial[#]	Treatment	Tissue CFU per ml Values[*]			
		Bulk Soil	**Rhizosphere Soil**	**Leaf**	**Root**
I	Control	4.70±2.18	4.14±3.27	-	-
	BTC6-3	3.35±2.55	4.37±4.15	-	3.92±3.86
	BTG8-5	3.90±3.60	3.79±3.73	-	-
	BT14-4	4.25±4.07	4.28±3.35	-	-
II	Control	3.10±2.88	1.49±0.00	1.75±1.04	4.34±3.68
	BTC6-3	4.13±3.64	4.21±3.55	4.21±3.62	1.78±1.74
	BTG8-5	5.19±4.44	4.63±3.73	1.45±1.32	-
	BT14-4	4.23±3.36	4.18±3.81	1.79±0.55	3.43±3.66
III	Control	3.50±2.85	3.72±2.84	-	4.36±3.03
	BTC6-3	2.78±2.63	2.85±2.00	2.19±1.95	4.20±3.71
	BTG8-5	3.41±2.95	3.53±1.76	-	4.29±4.19
	BT14-4	3.54±2.24	3.43±3.01	1.39±0.32	4.05±2.75

B

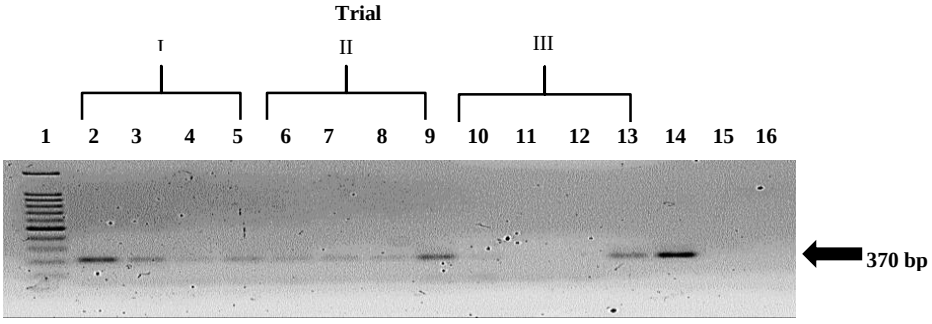

Fig. 7 (A) [#] Three separate trials, each with 80 plants total and 20 plants per treatment group was grown. Five plants were randomly chosen from each treatment group and sampled for tissues. Plant tissues were pooled, homogenized and diluted for determination of colony forming units (CFUs) of *Pseudomonad* bacteria on LB agar.
[*] Values the average of CFU/ml from multiple dilution points with at least 2 plates per point. All values are presented as the Log_{10} value followed by standard deviation.
[-] Indicates that the plate did not have any colony growth or insufficient replicates for averaged log values.
(**B)** PCR results from Hemp Leaf DNA extracts using the *Pseudomonas* specific primer set BT14-4F1/BT14-4R1. The NEB® 100bp Ladder (1) was used for amplicon size determination. Leaf DNA from non-bacterized plants (2, 6, 10), BTC6-3 (3, 7), BTG8-5 (4, 8), and BT14-4 (5, 9, 13), as well as a positive control using genomic DNA template from *Pseudomonas* BT14-4 (14) gave amplification of the expected size (370bp). Some samples in trial III (11, 12), as well as negative controls for both loading (15) and PCR (16) did not show amplification.

Chapter 11: Appendix

Table A1. Characteristics of 36 isolates belonging to genera of interest

Isolate[$]	Putative Genus	Cellulase[λ]	Phosphate solubilisation[γ]	HCN production[λ]	Siderophore	Organic Acid*[γ]	IAA Produced (µg/ ml)
BLC5-1	*Bacillus*	A	+	A	-	-	
BLC5-3		P	++	A	-	+	
BT10-4	*Cedecea*	A	+	A	+	-	
BT10-13		A	+	A	+	-	
BS9-1	*Enterobacter*	A	+	A	++	+	
BT13-11		P	-	A	++	-	
BT14-14	*Pantoea*	A	++	A	-	++	
BTC6-3	*Pseudomonas*	A	+	A	+	++	10.96±0.31
BTG6-1		A	+	A	++	-	
BTG6-3		P	-	P	++	-	
BLG7-1		P	-	A	++	-	
BTG7-4		A	+	A	++	-	
BTC8-1		P	+	P	+++	-	3.39±0.59
BTC8-3		P	-	A	++	-	
BTC8-4		P	-	A	++	+	0.88±.019

BTC8-6	A	++	A	+	-	
BTG8-1	P	-	A	++	-	
BTG8-5	P	++	A	++	+	3.36±0.045
BTG8-8	A	++	A	+	-	
BS9-3	A	-	P	+	++	6.47±0.013
BL12-4	A	+	A	++	-	
BT13-3	A	-	P	+	++	3.52±0.034
BT13-12	P	-	P	-	++	3.97±0.063
BT14-4	P	++	A	++	-	9.46±0.101
BT14-8	A	-	A	-	-	
BT14-9	A	-	A	+	++	
BT14-15	A	-	P	+	++	
BT14-21	A	-	P	+	++	
BT14-26	A	-	A	++	-	
BT14-30	P	++	A	+++	-	
BL15-1	P	-	A	+	-	
BL15-5	A	-	A	+	++	
BL15-7	P	+	A	++	-	
BL15-10	P	-	A	++	-	

BL15-11		A	-	P	+	++	3.81±0.034
BL12-2	*Strenotrophomonas*	P	-	A	+	-	

$- All tests were replicated three times and the average is presented. λ P= present, A= absent, – = negative
γ + positive (halo <5 mm), ++ strongly positive (5 mm< and <10 mm), +++ very strongly positive (halo >10 mm)
*Organic acid was a single test that was then replicated in isolates of interest only