Characterization of Bacterial Endophytes Isolated from Brassica Carinata and their Potential Use to Decrease Nutrient Requirements in Crops

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CHARACTERIZATION OF BACTERIAL ENDOPHYTES ISOLATED FROM
BRASSICA CARINATA AND THEIR POTENTIAL USE TO DECREASE NUTRIENT
REQUIREMENTS IN CROPS

BY

ALEX SOUPIR

A dissertation submitted in partial fulfillment of the requirements for the
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This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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ABBREVIATIONS

ANI -- average nucleotide identity

ARS NRRL -- Agricultural Research Service Northern Regional Research Laboratory

ATCC -- American Type Culture Collection

comb. nov. -- Combinatio nova, ‘new combination’

DF minimal media -- Dworkin and Foster minimal media

DNA -- deoxyribonucleic acid

DW -- dual wavelength optical density

-- (Sample OD_{590nm} – Sample OD_{750nm}) –

(Control OD_{590nm} – Control OD_{750nm})

E-value -- Expect value: with database of current size one might expect
to see the same score E times, simply by chance

Fe-HClO -- Salkowski’s reagent

FSA -- Fisheries Stock Analysis

HCl -- hydrochloric acid

HSD -- honest significant difference

k sds/ac -- thousands of seeds per acre

lb/ac -- pounds per acre

LSD -- least significant difference

M -- molar

Mbp -- mega-base pair

mM -- millimolar
NaCl -- sodium chloride
NBRIP -- National Botanical Research Institute Phospahte
NFb -- nitrogen free bromothymol
nMDS -- nonmetric Multi-Dimensional Scaling
nr/nt database -- nr is a protein database in amino acids
-- nt is a nucleotide database
OD -- optical density
PCR -- polymeric chain reaction
PDA -- potato dextrose agar
PGPB -- plant growth promoting bacteria
Q25 -- $Q = -10 \times \log_{10}P$
-- Phred quality score of 25 (~1 incorrect in 316 base call)
rRNA -- ribosomal ribonucleic acid
SDS -- sodium dodecyl sulfate
sds/ac -- seeds per acre
tmRNA -- transfer-messenger ribonucleic acid
tRNA -- transfer ribonucleic acid
TSA -- tryptic soy agar
USDA -- United States Department of Agriculture
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ABSTRACT

CHARACTERIZATION OF BACTERIAL ENDOPHYTES ISOLATED FROM *BRASSICA CARINATA* AND THEIR POTENTIAL USE TO DECREASE NUTRIENT REQUIREMENTS IN CROPS

Alex Soupir

2020

Bacterial endophytes have the capability to enhance plant growth by producing plant growth hormones, solubilizing phosphates, suppressing pathogenic fungi, and reducing plant stress hormones. These capabilities make them desirable limiting the amount of nutrients and pesticides that are applied to crops. Through these assays and isolations, it is possible to identify novel bacterial species. In-vitro testing had shown 9 of the 20 isolates possess the ability to produce indole-3-acetic acid (IAA) with *Pantoea agglomerans* BC09 producing a concentration of 30.2 ng/µl over 4 days. BC09, *Bacillus subtilis* BC10, and *Pantoea sp.* BC12 were able to solubilize calcium phosphate, 7 endophytes exhibited amplification of the 1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase) gene (*acdS*), and the plant pathogen *Fusarium proliferatum* had shown suppression from 17 endophytes. In-planta studies with wheat show differences between spring wheat genotypes, with BC12 and *Bacillus cereus* BC14 increasing Boost’s root volume more than 80% and *Enterobacter sp.* BC05 increasing root volume of Prevail 87%. The corn genotype 5126RR was significantly increased 72% in root volume with *Bacillus pumilis* BC07 applications. Brookings soybean variety increased most across endophyte applications with BC07 increasing seedling mass and root volume more than 71%. Greenhouse trial responses did not always respond to the same endophytes as root
architecture with *Bacillus thuringiensis* BC15 drastically increasing root dry biomass of Boost 102% under low nitrogen conditions and Prevail showing a significant increase in shoot under low or high nitrogen from 9 endophytes. Corn genotype 9714/G root and shoot biomass responded positively to *Bacillus safensis* BC16 inoculation under high nitrogen conditions. Codington soybean genotype significantly increased root biomass 23% after BC15 inoculation. When testing endophytes ability to increase yields under field conditions, all 3 crop species did not show a significant increase. Concentration of nitrogen in plant tissue was similar to controls for both soybean and corn while phosphorus concentrations only differed in Codington at R3 after *Bacillus sp.* BC20 applications. Additionally, isolation of endophytes leads to previously unsequenced and uncharacterized novel bacteria belonging to *Methylophilus* under the proposed species *M. endophytica.*
CHAPTER 1: GENERAL INTRODUCTION

1.1 Wheat, Soybean, and Corn

*Zea maize*, *Glycine max*, and *Triticum aestivum*, more commonly known as corn, soybean, and wheat, are the 3 crops in the United States that take up the most farm land (1). Corn was America’s largest anticipated crop in the 2019 growing season, consuming between 90 and 91.7 million acres of cultivable farmland (1, 2). Of the harvested crop, a third is used for animal feed, a little over a third is used for ethanol production used in E10/E15/E85 gasoline, and the last portion is used for human consumption and other commercial products (3). Production of 2018 to 2019 decreased from 14.4 billion bushels to 13.7 billion bushels (1). Soybean was grown on cultivable farm land covering roughly 89 million acres in 2018 and 76 million acres in 2019 (1). Yield of 2019 for soybeans averaged 46.9 bu/ac, producing 3.5 billion bushels in total (1). *Triticum aestivum* is common wheat and is the 3rd largest row crop in the United states by cultivated land, and was grown on 47.8 million acres or 45.2 million acres planted in 2018 and 2019, respectively (1). The production of wheat, including winter wheat, durum wheat, and spring wheat totaled 1.9 billion bushels with an average of 51.7 bu/ac (1).

Of these three economically important row crops, soybeans do not require the application of nitrogen fertilizers due to the symbiotic relationship with rhizobia bacteria in specialized tissues called root nodules where biological nitrogen fixation takes places (4-6). However, nitrogen is sometimes applied to soybean fields as starter fertilizer which aids in rood development and photosynthesis while marginally increasing yields (7). However, corn and wheat do require nitrogen fertilizer applications to maintain yields since they do not form root nodules for nitrogen fixation. The South Dakota State
University Extension suggests 1.2 lbs of nitrogen and 2.5 lbs of nitrogen per bushel of yield goal per acre for corn and wheat, respectively (8). Of the fertilizer that is applied to the fields, corn potentially takes up less than 40% and the environmental runoff can be greater than 50% of the applied nitrogen and phosphorus fertilizers (9, 10). The leaching of nitrates into marine environments causes an increase in aquatic plant and algae growth, depleting the waters of oxygen which in turn decreases the ability for fish to thrive (11, 12). Additionally, estimated 75% of the greenhouse gas emissions from crop cultivation is associated with inputs from nitrogen fertilizers (13).

Although nitrogen is the largest contributor to runoff and of high concern due to its effects on greenhouse gas emissions, phosphate fertilizers also environmental concerns (9, 13). One problem is that the production of phosphate fertilizers monoammonium phosphate (MAP) and diammonium phosphate (DAP) can cause heavy metal pollutions in nearby environments and areas of application (14, 15). Phosphate fertilizers are produced from rock phosphates which are nonrenewable resources and predictions put the peak of phosphate production between 2070 (16, 17). Since high production goals for corn, soybeans, and wheat require large amounts of fertilizers that are causing environmental damages and are being depleted, other avenues of nutrient acquisition or higher nutrient use effeminacy methods must be explored.

1.2 Bacterial Endophytes and their Plant Growth Promoting Capabilities

Endophytes are microorganisms reside within plant tissues and they do not cause harm to the plant (pathogens) while those that live on the surfaces of plant tissues are referred to as epiphytes (18). The term “endophyte” is derived from Greek ‘endon’ –
inside, and phyton – plant and is generally associated with either bacteria or fungi that are living in these tissues (19). Initially, endophytes also included arbuscular or other mycorrhizal fungi that also grow outside of the roots they colonize, but in 1995 it was suggested in to limit the term endophyte to only those fungi that grow wholly within plant tissues (20-22). Fungal endophytes were more highly researched in the late 1980’s and 1990’s, and even in 2018 the Web of Science database has roughly 2.5 x the number of entries for fungal endophytes than for bacterial endophytes (19). This difference provides an area for further exploration into bacterial endophytes.

Endophytes can be isolated from plant tissue after surface sterilization, often with alcohol, sodium hypochlorite, or chlorine gas, or through the extraction of wash fluids (23-28). Surface sterilized tissues are then minced and placed in a culturing media which influence the community that can be isolated. For example, the culturing using nitrogen free media selects for those bacteria which are able to live in an environment without readily available nitrogen, and are able to fix gaseous nitrogen through biological nitrogen fixation (29, 30). Following isolation of pure cultures, the bacterial endophytes are than tested for their plant growth promoting capabilities, for example for their ability to mobilize recalcitrant phosphorus, suppress fungal pathogens, and produce the auxin indole-3-acetic acid (IAA) and the enzyme 1-amino-1-cyclopropane carboxylate deaminase (31-37). Bacterial endophytes that possess plant growth promoting capabilities are called plant growth promoting bacteria, or PGPB.

One of the many interests in studying the use of bacterial endophytes is their potential for increasing the nutrient availability for plants. Bacterial endophytes have been shown to increase the nitrogen use efficiency (NUE) and nitrogen availability in
sugarcane, rice, corn, and strawberries (32, 38-41). The ability for a bacterial endophyte to fix atmospheric nitrogen to ammonium for plant use through biological nitrogen fixation requires \( \textit{nif} \) genes (42). Endophytic bacteria have shown the ability to solubilize mineral-bound phosphate when inoculated onto crops like soybeans and corn (43, 44). Phosphate solubilization by bacterial endophytes has been attributed to the secretion of low molecular weight organic acids which decrease the pH of the soil (45). Increasing the production of indole-3-acetic acid in plant growth promoting endophytes has also been shown to increase the amount of nitrogen the bacteria are able to fix (46).

Indole-3-acetic acid (IAA) is an auxin class phytohormone that promotes cell growth and differentiation of plant cells (47). \textit{Bacillus} strains that are able to produce high levels of IAA have been for example to increase the development of soybean nodules as well as plant dry weight and root length (48). IAA levels have also been shown to increase when ethylene levels increase (48, 49). Ethylene is a plant stress hormone that has the precursor 1-aminocyclopropane-1-carboxylate (50).

An increase in the amount 1-aminocyclopropane-1-carboxylate has been shown in Arabidopsis to increase the levels of IAA (49). However, an increase in levels within plant tissues is a signal of stress. Some endophytes are able to produce ACC deaminase that is able to break down 1-aminocyclopropane-1-carboxylate to \( \alpha \)-ketobutyrate and ammonia before ACC oxidase can converts it to ethylene (51). Decreasing ethylene levels with the application of ACC deaminase producing endophytes has been shown to increase plant growth under saline and heavy metal contaminated conditions, and promote nodulation in common beans (52-54).
Decreasing stress responses in plants also involve the ability for the bacterial endophytes to suppress the growth of fungal pathogens. Fungal pathogens can cause reduced yields and can produce mycotoxins that make the consumers of grains ill (55). The capability of bacterial endophyte to suppress fungal pathogens can be through the production of antimicrobial compounds or competition for nutrients (56). These antimicrobial compounds include volatile organic compounds, phenazines, and chitinases which are able to break down fungal chitin cell wall (57, 58). The relationship between the in-vitro assays for fungal suppression and the ability of the endophyte to suppress the pathogen in-planta varies for different pathogens but is suggested to be a strong correlation (59).

Bacterial endophytes can colonize the tissue of plants through vertical transmission from a parent plant to the seed or through horizontal transmission and the environment. The endophytes that are taken up from the environment enter plant tissues through have leaf stomata or damage caused by insects feeding on tissue. In the roots, endophytes may enter into the roots at root junctions or they may enter at locations where roots have been damaged from the soil they are growing through. In either situation, there is a requirement for the endophyte to have the ability to move unless the endophyte is injected into the plant with insect saliva (60).
1.3 References

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CHAPTER 2: BENEFICIAL PLANT MICROBE INTERACTIONS AND THEIR EFFECT ON NUTRIENT UPTAKE, YIELD AND STRESS RESISTANCE OF SOYBEANS

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2.1 Abstract

Plants are meta-organisms that are associated with complex microbiomes. Many of the microorganisms that reside on plant surfaces (epiphytes), or within plant tissues (endophytes) do not cause any plant diseases, but often contribute significantly to the nutrient supply of their host plant and can help the plant to overcome a variety of biotic or abiotic stresses. The yield potential of any plant does not only depend on successful plant traits that improve for example the adaptation to low input conditions or other stressful environments, but also on the plant microbiome and its potential to promote plant growth under these conditions. There is a growing interest to unravel the
mechanisms underlying these beneficial plant microbe interactions, because the activities of these microbial communities are of critical importance for plant growth under abiotic and biotic stresses and could lead to the development of novel strategies to improve yields and stress resistances of agronomically important crops. In this review, we summarize our current understanding of the beneficial interactions of soybean plants with arbuscular mycorrhizal fungi, nitrogen fixing rhizobia, and fungal and bacterial endophytes, and identify major knowledge gaps that need to be filled to use beneficial microbes to their full potential.

2.2 Introduction

The plant rhizosphere and phyllosphere is colonized by a wide range of epiphytic and endophytic microorganisms and these microorganisms can establish beneficial, neutral, or detrimental associations of varying intimacy with their host plant. Recent developments in sequencing technologies have enabled us to study the composition and function of plant microbiomes, but plant microbiomes are dynamic, and differ among different plant tissues, and in response to the environment. The plant microbiome can also be seen as “the second plant genome” or pan-genome and can consist of 10 times more genes than typical plant genomes (1). Beneficial microorganisms that are associated with plants hold enormous potential to be developed into microbial fertilizers or microbial pesticides (2) and new biotechnological tools to increase the nutrient efficiency and stress tolerance of crops, and environmental sustainability of agroecosystems. Specific interactions between microbes and plants, such as the *Rhizobium*-legume symbioses, are well understood, but the majority of the plant microbiome, and its contribution to the extended phenotype of the host, is not yet well defined.
Soybeans form interactions with nitrogen-fixing rhizobia and this symbiosis plays a key role for the nitrogen (N) nutrition of the plant, but also for agricultural productivity since soybean root residues provide N for other plants in crop rotations (3, 4). Arbuscular mycorrhizal (AM) fungi colonize the root system of the majority of land plants, including soybeans, and transfer nutrients such as phosphate (P), N, potassium (K), and other nutrients to their host plants, and improve the resistance of their host plant against abiotic (e.g. drought, salinity, heavy metals), and biotic stresses (5). In addition, soybeans are associated with endophytes that live inside their plant host for at least part of their lives, without causing apparent disease symptoms as a result of this colonization. Plant endophytes exhibit a wide range of plant growth promoting capabilities, including the production of phytohormones, an improved nitrogen (N) nutrition through biological nitrogen fixation (diazotrophic endophytes), the biosynthesis of ACC (1-aminocyclopropane-1-carboxylate) deaminase, the capability to solubilize phosphate, and also the biosynthesis and release of antimicrobial metabolites or siderophores to inhibit the growth of pathogenic microorganisms.

The plant microbiome is a largely unexplored resource of beneficial microorganisms with diverse properties and a hidden potential to manipulate plant growth and success in stressful environments. However, while the symbiosis of soybeans with rhizobia and with AM fungi is well characterized, the functional role of endophytes is only known for a limited number of isolates. Our functional understanding of these interactions is mainly based on experiments with individual symbionts, but there is increasing evidence that individual symbionts can also affect the interactions of the plant with other symbionts (6-9). We summarize here the effects of different beneficial microbes on nutrient uptake,
yield and stress resistance of soybeans, and identify knowledge gaps that hinder the application of these interactions to their full potential in soybean production systems.

2.3  Beneficial plant microbe interactions of soybean plants

2.3.1  Arbuscular mycorrhizal symbiosis

The arbuscular mycorrhizal (AM) symbiosis is arguably the most important symbiosis on Earth and is formed by more than 65% of all known land plant species (n > 200,000), including all legumes and many other agronomically important crops, such as wheat, corn, and rice (10). AM fungi are classified into the fungal subphylum Glomeromycota that consists of less than 350 fungal species (11). AM fungi co-exist relatively morphologically unaltered with plants for more than 400 million years, and there is evidence that suggests that the AM symbiosis played a critical role for land plant evolution (12).

It is long known that AM fungi can increase the nutrient uptake of their host plant, and are able to deliver substantial amounts of P, N, K, sulfur (S), and trace elements, such as copper (Cu) and zinc (Zn) to the plant. Many AM fungi also provide non-nutritional benefits for their host that are critical for plant survival or fitness, and improve for example the resistance of plants against abiotic (e.g. drought, heavy metal, salinity) and biotic (pathogens) stresses (5). In return for these benefits, host plants transfer up to 20-25% of their photosynthetically derived carbohydrates to the fungal symbiont (13). It was generally believed that carbon is transferred to the fungus in the form of hexoses (14), but recent evidence suggests that also fatty acids can move across the mycorrhizal interface to the fungal partner (Figure 1) (15-17).
AM fungi are ubiquitous in soils and can account for up to 50% of the microbial biomass in soils (18). AM fungi form extensive hyphal networks in soils, and the extraradical mycelium (ERM) of the fungus acts as an extension of the root system and increases the nutrient absorbing surface of the root. The ERM with its mycorrhizosphere (interface between fungal hyphae and the soil) acts as an important conduit between microbial communities and the host plant (19) and can provide soil microbial communities with plant-derived carbon (C) inputs in large distance from the root. The mycorrhizosphere represents in soils an important ecological niche for diverse microbial communities that are specifically adapted to this mycorrhizosphere. According to estimates, the bacterial density in the mycorrhizosphere is 4 to 5 times higher than in the plant rhizosphere (20). However, the presence of AM fungal mycelia does not only lead to quantitative, but also to qualitative changes in the microbial community composition in soils (21). The presence of AM fungal hyphae plays an important role in the bacterial community assembly during decomposition (21) and affects the access of members of these microbial communities to C sources during decomposition (22).

Within the host root, the fungus can spread intercellularly, but also penetrates the root cortex intracellularly, and forms here highly branched specialized structures, called arbuscules that are separated from the plant symplast by the plant periarbuscular membrane (23). Some AM fungal species also form vesicles, thick-walled, lipid containing storage organs in the roots. Arbuscules are the site of nutrient exchange between the plant and the fungus, and both the fungal cell membrane and the plant periarbuscular membrane are characterized by the presence of specific transport proteins.
that play a critical role for the resource exchange between both partners (Figure 1) (14, 24, 25).

The colonization of host roots by AM fungi is based on a molecular dialog between both partners that facilitates partner recognition and triggers responses in both partners that are critical for the establishment of the symbiosis (26). After fungal spore germination, an extensive hyphal branching in close proximity to host roots can be observed that is triggered by strigolactones and other compounds in root exudates (27). After attachment to the host root surface and the differentiation of a fungal hyphopodium, the fungus penetrates the root, and spreads with the help of a prepenetration apparatus (28), and forms arbuscules in the cells of the root cortex. Initiated is this process by the release of lipochitooligosaccharides, or Myc factors by the fungus, that are perceived by specific receptors on the host root surface and trigger a cascade of molecular responses in the host root. The pathway is called the common symbiotic signaling pathway (CSSP), since similar responses can be observed after the perception of rhizobial Nod factors (26, 29). A key role for the perception of fungal Myc or Nod factors by the rhizodermis plays the membrane-bound receptor-like kinase SYMRK that activates the mevalonate (MVA) biosynthetic enzyme HMGR1 (3-hydroxy-3-methylglutaryl CoA reductase 1). A second set of CSSP proteins is located in the nuclear pore complex and includes the three nucleoporins NUP133, NUP85, and NENA, the ATP-powered Ca²⁺ pump MCA8, and cation channels encoded by CASTOR and POLLUX involved in the strong Ca²⁺ oscillations in the nucleus of rhizodermal cells that can be observed shortly after Myc factor perception. Another set of proteins is located in the nucleoplasm and decodes these Ca²⁺ signals (29, 30). CCaMK phosphorylates with the help of calmodulin CYCLOPS,
which then regulates gene expression either directly, or through GRAS transcription factors such as NSP1, NSP2, and RAM1 (29-31). The elucidation of the CSSP is mainly based on studies in the model legumes *Medicago truncatula* or *Lotus japonicus*, but the fact that the proteins of the CSSP are highly evolutionary conserved, and even present in plants that are unable to form AM interactions, suggest that this pathway is also established in soybeans.

Figure 2.1. Overview of the mycorrhizal nutrient uptake pathway in AM roots of soybean plants via the extraradical mycelium of the fungus (a), and the mycorrhizal interface consisting of the fungal arbuscule in root cortical cells surrounded by the periarbuscular membrane of the host (b). Both, fungal cell membrane and plant periarbuscular membrane are characterized by the presence of mycorrhiza specific transporters.
that play a critical role for the nutrient exchange across the mycorrhizal interface of soybean plants (e.g. GmPT7 or GmAMT4.1, see also below).

Mycorrhizal plants have two pathways that are involved in the nutrient uptake from the soil: the ‘plant pathway’ via high- and low-affinity transporters in root epidermis and root hairs or the ‘mycorrhizal pathway’ that first involves the uptake of nutrients via the ERM of the fungus, transport to the arbuscules, and then the uptake by the plant from the interfacial apoplast through specialized transporters in the periarbuscular membrane. In response to the colonization with AM fungi, transporters that are involved in the plant pathway are often down-regulated, while mycorrhiza-specific transporters in the periarbuscular membrane are induced (32), indicating that there is a shift in the nutrient acquisition strategy, and that the mycorrhizal pathway can become the dominant pathway for nutrient uptake (33, 34).

2.3.1.1 Importance of arbuscular mycorrhizal fungi for yield and nutrient uptake of soybeans

Under both greenhouse and field conditions, increases in nutrient content, yield and overall fitness of soybeans in response to an AM colonization have been reported (35, 36), and soybean yields were found to be significantly correlated to the colonization of the roots with AM fungi (37). Many reports clearly demonstrate the positive effects of AM fungi on the nutrient uptake of soybeans, and here particularly on the uptake of phosphorus (P) and of nitrogen (N) (38-40). However, the effects can differ greatly among AM fungi. Our own studies demonstrated for example that while the AM fungus Rhizophagus irregularis can increase the P nutrition of soybeans with low or high P acquisition efficiency, Glomus custos had no effect and Glomus aggregatum even led to slight growth depressions under medium P supply conditions (38).
Some of the observed differences among these AM fungi seem to be related to the impact of the AM fungus on plant P transporter expression. Fourteen genes of the Ph1 family have been identified in soybeans (41), and three of these transporters show high expression levels in mycorrhizal roots (42). While the colonization of the roots with *R. irregularis* led to the down-regulation of *GmPt4*, a high affinity P uptake transporter that is presumably involved in the uptake of P from the soil, was the expression of *GmPt9*, and *GmPt10* up-regulated in AM roots. *GmPt9* and *GmPt10* cluster with the mycorrhiza-inducible P transporters *OsPt11* of *Oryza sativa* (rice) and *MtPt4* of *Medicago truncatula* that play a critical role for the P uptake from the mycorrhizal interface (25, 43). *GmPt9* was up-regulated by *G. aggregatum* and *R. irregularis*, but *GmPt10* was only upregulated by *R. irregularis*, indicating that this transporter is involved in the P uptake from the interface, and that *GmPt10* expression can serve as an indicator for mycorrhizal P benefits in soybean plants. *GmPt7*, another soybean P transporter, shows a high expression in cells with mature and active arbuscules, but is not expressed in cells with collapsed and degenerated arbuscules, suggesting that this transporter may also play a role for the P transport across the AM interface. However, *GmPt7* is not a mycorrhiza specific transporter, and is also expressed in columella cells of root caps and in lateral root primordia of non-mycorrhizal roots (44). Similarly, out of the 16 ammonium (NH$_4^+$) transporters of soybean, five transporters are mycorrhiza-inducible, and one of them, *GmAMT4.1* is specifically expressed in arbusculated cells (Figure 1), indicating that this transporter could be involved in the NH$_4^+$ transport across the AM interface (45).

There is evidence from the model legume *Medicago truncatula*, that AM fungi can also improve the acquisition of other macronutrients such as potassium (K) or sulfur (46,
K deficiency is a common problem in soybeans and can lead to yellowing of the leaves, stunted growth and reduced yields and can become particularly severe under drought stress. Although transcriptional and physiological responses to K deprivation have been studied in other legumes (48), whether AM fungi also play a role in the K acquisition of soybean plants is not yet known.

2.3.1.2 Importance of arbuscular mycorrhizal fungi for the stress resistance of soybeans

AM fungi can also increase the resistance of soybeans against other abiotic stresses such as drought, salinity or soil contaminations. It is known for several decades that the AM colonization can improve the tolerance of soybeans against drought (49). AM fungi can influence leaf water potential, solute accumulation, and oxidative stress of soybeans under drought stress (50), and delay nodule senescence triggered by water deprivation (51). In AM soybeans, plasma membrane aquaporins were down-regulated in response to drought stress, and this could reduce the permeability of membranes for water and contribute to water conservation (52). In addition, both fungal and plant mitogen-activated protein kinases (MAPKs) are up-regulated in AM soybean plants under drought stress. MAPK cascades are known to regulate many cellular processes in response to various stimuli, including abiotic and biotic stresses (53). AM fungi also improve the tolerance of soybeans against salinity. AM plants had a higher biomass, and proline concentrations in roots, but reduced proline and Na concentrations in the shoot under salt stress. When the fungus was pre-treated with NaCl, the alleviating effects were even stronger, indicating that the acclimation of the fungus to salinity may play a role for the stress response (54). AM fungi can also improve the tolerance of soybeans against arsenic (55) and aluminum (56) by reducing the uptake of these toxic metals.
Soybean yield and productivity is also threatened by many fungal or bacterial diseases, and soil inhabiting nematodes. Soybean cyst nematodes (SCN, *Heterodera glycines*), brown spot (*Septoria glycines*), charcoal rot (*Macrophomina phaseolina*), rot and stem rot (*Phytophthora sojae*), and soybean rust (*Phakopsora pachyrhizi* and *P. meibomiae*) are among the most important pathogens of soybeans and cause substantial yield losses in the U.S. (57). SCN are often responsible for hidden yield losses, since soil infestations remain often undetected since they become severe. SCN can spread easily from field to field via soil movements with machinery, wind, or by humans, and can now be detected in 90% of the soybean producing states in the U.S. (58). SCN infestations can lead to yield losses of more than 30% and are responsible for about $1.5 billion in soybean crop damage each year in the U.S. AM fungi can protect soybeans against a wide range of pathogens, including fungi, bacteria, nematodes or insects (59), and reduce the SCN egg population in soils by 70% (60). The positive impact of AM fungi on biotic stresses has been attributed to the overall positive effect on nutrient uptake and a damage compensation effect, the competition for root space and soil nutrients, induced systemic resistance (ISR) and altered rhizosphere interactions. In addition, AM fungi form extensive hyphal networks in soils and can connect plants of the same or of different plant species by common mycelial networks (CMNs). CMNs play an important role in the plant-to-plant communication and can transfer infochemicals and warning signals from infested plants to uninfested plants and stimulate defence reactions in these plants (61).

2.3.2 Nitrogen fixing symbiosis with rhizobia
Most legume plants are able to interact with N-fixing bacteria, called rhizobia that are able to reduce atmospheric dinitrogen (N₂) into ammonia (NH₃) in specialized root nodules. The symbiosis evolved in legumes between 25 and 50 million years ago (62, 63), and plays an important role for plant nitrogen (N) nutrition. Rhizobia can contribute with up to 70% to the total N nutrition, and grain legumes can gain up to 300 Kg N, and legume trees (e.g. *Acacia* sp.) up to 600 Kg N per ha and year from these interactions (4, 64). Free living rhizobia produce Nod factors that are perceived by plant roots and act as triggers for the common symbiotic signaling pathway (CSSP; see above). Nod factors are also lipochitooligosaccharides that are composed of chitin chains with various lipid modifications. Chitin is the main constituent of fungal but not of bacterial cell walls, and the functional and structural similarities between Nod and Myc factors has led to the assumption that rhizobia adopted the evolutionary far more ancient (~ 450 million years) CSSP to establish this endosymbiotic interaction with legumes (65). Nod factors stimulate the curling of root hairs, and entrapped bacteria within these curls are transported within infection threads, to the inner zone of developing root nodules. Inside of cortical cells, the rhizobia divide and multiply, and are released into vesicles, called symbiosomes, in which they differentiate to fully functional bacteroids. One or more differentiated bacteroids are surrounded by the plant symbiosome membrane, that represents a barrier by which the host plant can control the movement of solutes to the bacteroids through specialized transporters or channels (66).

Bacteroids express the nitrogenase complex that consists of six protein subunits (two each of NifH, NifD, and NifK) and two [4Fe–4S] and two (Fe₈S₇) iron–sulfur clusters and two iron–molybdenum cofactors (Fe₇MoS₉N) called FeMoco, which catalyze
the N₂ reduction to NH₃ (67). The nitrogenase metallo-centres are all oxygen-labile and must operate in an environment with a low level of free oxygen, and nodules provide their bacterial symbionts with this oxygen reduced environment for optimum N fixation (68). N fixation by bacteroids is a highly energy consuming process, and rapid respiration in the bacteroids is necessary to produce the 16 ATP required for the conversion of each atmospheric N₂ into two NH₃.

\[
N_2 + 8H^+ + 8e^- + 16ATP = 2NH_3 + H_2 + 16ADP + 16P_i
\]

The product of biological N fixation (BNF) is ammonia, which diffuses out of the bacteroids into the acidic symbiosome space and is here protonated to ammonium. The symbiosome membrane is energized by an H⁺-ATPase, that pumps protons into the symbiosome space and thereby promotes the uptake of NH₃/NH₄⁺ into the plant cytosol, where NH₄⁺ is rapidly assimilated into amino acids, and the ureides allantoin and allantoic acid (68). A candidate for the uptake of NH₄⁺ from the symbiosome space is NOD26, that was first identified in soybeans (69). NOD26 belongs to the major intrinsic protein/aquaporin (MIP/AQP) channel family, and is exclusively localized in the symbiosome membrane (66). The ureides allantoin and allantoic acid serve as the dominant long-distance transport of N from the root nodules to the shoots (70, 71).

Cortex cells and the vascular endodermis of nodules express GmUPS1—1 and GmUPS1-2, which play a role for the transport of allantoin and allantoic acid out of the root nodules to the sink organs. RNAi knockouts of these proteins accumulate ureides in the root nodules, and show a reduced N transport to the shoots (72).

BNF is an energy expensive process, which requires 16 ATP to fuel the reduction of one N₂. Plants allocate up to 30% of their photosynthetically fixed C to rhizobia (73),
which is oxidized in the bacteroids to ATP. The N\textsubscript{2} fixation rate of rhizobia is higher when the nodules receive more C, suggesting that the allocation of C to nodules is a limiting factor for BNF. Transgenic *Medicago sativa* plants that over-express a sucrose phosphate synthase, a key enzyme for sucrose biosynthesis in plants, show higher C contents in nodules, more and larger nodules per plant and an enhanced nitrogenase activity of the root nodules (74). Free living rhizobia can grow on a variety of different sugars, including mono- and disaccharides, but the absence of transporters for these sugars in bacteroids suggests that rhizobia in symbiosis take up dicarboxylates, and here particularly malate from the symbiosome space. The C4-dicarboxylate transport system that is localized in the inner bacteroid membrane is encoded by the *dctA* gene, has a high mobility for malate, and is essential for symbiotic nitrogen fixation (75). Although the mechanisms of N fixation and assimilation are well documented, key steps are still unknown. For example, little is known about the C metabolism inside nodules, the regulatory steps that control the C export to rhizobia, and the proteins involved in the C and N transport between partners. Recent evidence in the model legumes *M. truncatula* and *Lotus japonicus* suggest that sucrose transporters from the Sugar Will Eventually be Exported Transporter (SWEET) family could be involved in the sucrose efflux from the phloem towards nodulated cells.

2.3.2.1 Significance of rhizobia for soybean agriculture

According to estimates, soybeans with their rhizobia populations fix around 20 million tons of N each year, and this has an enormous influence on agricultural productivity, not only on soybeans, but also for other crops in crop rotation systems (3, 4). Soybean residues in the soil enrich the soil with N, improve soil organic matter and
can lead to yield increases in non-legume crops that follow soybeans. Crop rotations or intercropping systems of cereals with legumes can result in higher crop yields without fertilizer additions (76). However, conventional agricultural management practices and other anthropogenic factors can have a negative impact on rhizobial function. In addition, excessive tillage, applications of higher N fertilizer dosages, extended fallow periods can also have detrimental effects on rhizobia populations in soils. As a consequence, integrating this symbiosis more efficiently in modern agricultural practices is crucial to limit the amount of fertilizers used and to make agriculture more environmentally sustainable. Exploring ecologically best fitted ecoregions for soybeans and best adapted soybean cultivars will help farmers to produce more yield with reduced inputs. Rhizobial strains differ in their efficacy in symbiosis with different soybean cultivars, and the input of N into agricultural systems can be increased by the inoculation of legumes with optimized rhizobia for different environments (64). The development of better inoculation strategies, and specifically adapted rhizobia for different soybean cultivars could reduce the dependency of farmers on agrochemicals and enhance food security (64).

2.3.3 Tripartite symbiosis with arbuscular mycorrhizal fungi and rhizobia

In natural environments, legume roots form tripartite interactions, and are simultaneously colonized by both AM fungi and rhizobia (6, 77). Tripartite interactions have been shown to improve plant productivity, seed yield, P and N acquisition, and photosynthetic rates (9, 78, 79). The rhizobial nitrogenase complex requires at least 16 ATP to reduce one N₂ molecule into two NH₃. Consequently, nodules act as strong P sinks in legume root systems to provide sufficient P resources to the bacteroids for
optimum BNF (77, 80). Since AM fungi are able to improve the P nutrition of legume plants, AM fungi can increase the BNF by root nodules by at least 50% (9). Non-mycorrhizal soybean plants have lower nodule numbers and weights, and particularly under low P supply lower N fixation rates (6, 81). AM fungi can also provide their hosts with microelements that are essential for N fixation, including zinc, iron, manganese and molybdenum (82, 83).

AM fungi and rhizobial bacteria can act synergistically and can improve plant productivity, seed yield, and grain quality (6, 9, 79). However, the prior inoculation by either rhizobia or AM fungi can also reduce the subsequent colonization by the other symbiont (84). Plants control the extent of root colonization by both symbionts by an autoregulatory mechanism, possibly to limit the high C costs associated with these interactions (81, 85). Whether AM fungi and rhizobia interact antagonistically or synergistically depends on the environmental context (79), and the compatibility between symbiotic partners (9, 86). For example, the rhizobial strain STM 7183 is more compatible with the AM fungus Rhizophagus clarus, and leads to higher nodulation rates, nitrogenase activities, and plant growth responses than STM 7282 (9). Similarly, plant productivity and seed yields of nodulated soybeans were higher when the plants were co-inoculated with the AM fungus Rhizophagus irregularis, than with Acaulospora tuberculata or Gigaspora gigantea (86). Soybean cultivars also differ in their ability to benefit from their microbial communities (87). Consequently, the symbiotic efficiency should be integrated into soybean breeding programs, and AM fungi and N-fixing bacteria with high compatibility should be identified to improve the productivity and stress resistance of soybeans and other legumes.
Both interactions are costly, and the host plant allocates up to 20% of its photosynthetically fixed C to its fungal (13, 88), and up to 30% to its N-fixing symbionts (Figure 2) (73). C acts as an important trigger for symbiotic functioning, and a reduction in the C fluxes to the symbionts decreases BNF by rhizobia (89), and P and N uptake and transport by AM fungi (90-92). Considering the high C costs of these symbioses for the host, plants are under a selective pressure to strongly regulate the C fluxes to both root symbionts, but these control mechanisms are currently poorly understood. Resource exchange between host and AM fungi are controlled by a reciprocal reward mechanism that is driven by biological market dynamics (93). Our own results recently demonstrated that similar mechanisms may also control the resource to C exchange in tripartite interactions, and that Medicago plants allocate C to the different root symbionts in tripartite interactions in response to nutrient demand conditions, and that the AM fungus becomes a stronger competitor for C resources from the host, when the fungal partner has access to N (77).
AM fungi have stronger effects on plant gene expression than rhizobia (94), but our current understanding of the molecular mechanisms involved in the C allocation to individual root symbionts is limiting. An overexpression of a leaf sucrose phosphate synthase of *M. truncatula* increases starch production, allowing the plant to allocate more photosynthates to root nodules and consequently improved nitrogenase activity and overall plant growth (74). There is evidence that suggests that sucrose transporters (SUT) could be involved in the regulation of beneficial C fluxes towards the fungal symbiont (95), and the expression of *MtSUT2* and *MtSUT4*-1 has been shown to be positively correlated to the C allocation to different symbiotic partners in tripartite interactions (77). *MtSWEET1b* and *MtSWEET6* of the Sugars Will Eventually be Exported Transporter family (SWEET) are highly expressed in AM roots, and preferentially transport hexoses such as glucose, and could be involved in the transport of hexoses or fatty acids across the mycorrhizal interface to the fungal partner (77, 96). *MtSWEET11* is specifically expressed in root nodules, and could be involved in the sugar distribution within root nodules, but loss-of-function mutants indicate that *MtSWEET11* is not essential for BNF (97). A better understanding of these processes is critical, because it may be key to improve the resource exchange between plants and symbionts, and ultimately to enhance productivity of agronomically important legumes.

### 2.3.4 Symbiosis with endophytic bacteria or fungi

Endophytes are defined as organisms that live inside plant hosts for at least part of their lives, without causing apparent disease symptoms in the host as a result of this colonization (98). Fungal and bacterial endophytes are nearly ubiquitous across all groups of vascular plants (99), but there is a large biological diversity among endophytes, and it
is not rare for some plant species to host hundreds of different endophytic species (100). Fungal endophytes have been shown to enhance growth and seed production or protect against environmental stresses such as drought or P deficiency or provide defense against herbivory through the synthesis of various biologically active metabolites, such as alkaloids. In soybeans diverse communities of fungal endophytes can be found, and several of these endophytes have plant growth promoting capabilities, and enhance for example soybean growth in nickel or copper contaminated soils by reducing the levels of stress-related phytohormones such as abscisic acid and jasmonic acid (101), and increase glutathione activities and thereby reduce oxidative stress (102). The inoculation of soybean plants with fungal endophytes can also lead to higher shoot biomasses, chlorophyll contents, and photosynthetic rates compared to non-inoculated soybeans under salt stress and decrease the abundances of SCN in soils (103).

Soybeans host also a diverse group of bacterial endophytes, and many endophytic bacteria have plant growth promoting capabilities (104), such as the ability to produce plant growth hormones, or ACC (1-aminocyclopropane-1-carboxylate) deaminase, to solubilize phosphate, or to release antimicrobial metabolites or siderophores that can inhibit the growth of pathogenic microorganisms. ACC deaminase reduces the levels of ethylene, an important stress hormone in plants. Several endophytic bacteria are also diazotrophs, and have like rhizobia bacteria the ability to fix N. Bacterial endophytes also interact with rhizobia bacteria, and can enhance root nodulation, and activity, and as a consequence the N content of soybean plants (105). The dual inoculation with rhizobia and a salt tolerant bacterial endophyte led to synergistic responses and promoted the fitness of soybean plants under salt stress (106).
2.4 Important research gaps and future challenges

Beneficial plant microbe interactions with AM fungi, rhizobia, or bacterial and fungal endophytes have enormous potential to improve plant growth and nutrient uptake in stressful environments and to increase the environmental sustainability of soybean agriculture. However, while the beneficial effects of AM fungi and rhizobia on soybean productivity are long known, the effect of only a small number of endophytes is currently known. The plant microbiome is a still unexplored resource of microorganisms with a so far hidden potential to promote plant growth, and success under abiotic or biotic stress conditions, and with unknown effects on the plant phenotype.

The obligate lifestyle of AM fungi, has made for a long time the production of fungal inoculum in large quantities difficult, but the development of sterile transgenic root organ cultures has led to an increased commercialization of AM fungal inocula for the utilization in agroecosystems (107). Although increases in yield and biomass have been reported in different crops after inoculation with these inocula (35, 108), in other studies inconsistent or neutral effects were observed (109). AM fungi differ in the benefit that they provide for their host plant (110), and mycorrhizal growth responses are highly context-dependent. Several factors can alter the success of AM fungal inoculation in agroecosystems, including plant/fungal compatibility, degree of competition with the native microbial population, or timing of inoculation (111). All these aspects need to be taken into consideration to find the most adapted and specific conditions for an efficient use of AM fungal inocula in a given field, or for a certain crop. Our current understanding of the effect of beneficial plant microbes on soybeans is mainly based on studies with single symbionts, but plant productivity and stress resistance in
agroecosystems depends on diverse microbial communities, and the interactions among the different microorganisms in these communities.

Identifying and characterizing the molecular mechanisms responsible for the functioning of different plant microbe interactions is crucial to harness these symbiotic microorganisms in agroecosystems. Currently, most knowledge is gathered on model legumes, such as *Medicago truncatula*, but the information about soybeans is limited. However, the accumulation of genomic and transcriptomic data, along with the development of molecular tools such as stable transformations (e.g. 112), CRISPR-Cas9 system (113), or mutant populations will provide us with a better understanding of these interactions in soybeans.

2.5 Acknowledgements

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CHAPTER 3: CHARACTERIZATION OF BACTERIAL ENDOPHYTES ISOLATED
FROM THE OILSEED PRODUCING CROP *BRASSICA CARINATA*: PART 1 –
IN-VITRO CHARACTERIZATION

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3.1 Abstract

Bacterial endophytes were isolated from the oil seed crop *Brassica carinata* onto nitrogen free media. *Brassica carinata* is host to many bacterial endophytes with plant growth promoting characteristics that can protect the plant in conditions that are stressful for the host plants. The endophytes were characterized for biotic and biotic stress mitigation and identified through 16S sequencing. Abiotic and biotic stresses include the production of indole-3-acetic acid, solubilization of calcium phosphate, 1-amino-1-cyclopropane (ACC) deaminase gene identification through genome PCR amplification, and the ability to suppress common fungal pathogens of wheat and corn. It was found that 9 endophytes possessed the ability to produce indole-3-acetic acid, 3 endophytes (*Pantoea agglomerans* BC09, *Bacillus subtilis* BC10, and *Pantoea sp.* BC12) were able to solubilize calcium phosphate, 7 endophytes had shown amplification of genomic DNA using primer specific for 1-aminocyclopropane-1-carboxylate, and that the fungal pathogens *Fusarium proliferatum* and *F. graminearum* were more easily suppressed by


the collection of endophyte than was *F. oxysporum* and *F. acuminatum* when challenged in petri dish assays.

### 3.2 Introduction

*Brassica carinata* also known as Ethiopian mustard seed is primarily grown in the Great Plains of the United States and in Canada. However, carinata is also grown in its native Ethiopia, where its green leaves are farmed for sustenance and its seeds for oil (1). It is a feedstock for biofuel industries and is used in the production of both ethanol and biodiesel (2). Other qualities that make carinata to a viable bioenergy and economically important crop are its ability to be planted in heavy metal polluted fields and that the seed meal, left over from processing, can be fed to livestock and cattle (3, 4). While carinata is very tolerant to warmer, arid climates, it is also being considered as a potential winter crop that could be planted in subtropical areas, such as in Florida in the United States (5). Carinata, could in theory, be cultivated year-round for an increased supply of feedstock for biofuels adding to the consideration of this crop as a viable and long-term bio-fuel source.

Interactions between plants and microbes is extremely complex and small changes can cause unintended shifts to the plant system. These interactions can benefit both parties and resulting in strong plant-bacterial bonds which may be passed down to progeny. *Brassica carinata* and other *Brassica* species are not colonized by mycorrhizal fungi and do not form root nodules, that in many other plant species improve the nutrient acquisition from soils (4). It has been hypothesized that carinata instead relies on other organisms from its own microbiome for nutritional needs. The endophytic community in
plants is formed from environmental, host and even its own input from bacterial community members (6). It’s these bacterial endophytes that can help plants to grow and thrive in their environment (7).

The bacterial endophytes can aid in plant growth through several mechanisms such as limiting abiotic stresses (fixing atmospheric nitrogen to forms that are usable for plants and solubilization of soil bound phosphates for plant uptake), biotic stresses within plants or within the soil (caused by fungal pathogens, bacterial pathogens, and nematodes), production of phytohormones (auxins, cytokinins and jasmonates), and modulating plant ethylene levels (ACC deaminase cleaving 1-aminocyclopropane-1-carboxylate to ammonia and α-ketobutyrate) (8, 9). Plant growth-promoting bacteria (PGPB) are those that possess at least one of these growth-promoting characteristics and otherwise do not cause harm to the host plant. Biological nitrogen fixation and the production of phosphatases and organic acids have the potential to decrease the amount of nitrogen and phosphates applied to crops, which in turn can down on ecosystem pollution and nutrient runoff (10). Suppression of pathogens is critical for crops such as small grains like wheat where pathogens like *Puccinia graminis* cause up to 70% losses and *Fusarium sp.* produce mycotoxins which additionally can reduce grain and forage quality (11, 12). Augmenting the plant production of phytohormones, bacterial endophytes can produce auxins to that signal cellular elongation and division which has shown to significantly increase shoot and root growth, and increase trends in root hair counts in rice (13). ACC deaminase is able to break down the precursor to ethylene (plant stress hormone) and the expression of the *acdS* can promote nodulation and growth in
beans, decrease impacts of salinity stress on tomatoes and wheat, and heavy metal stress in *Brassica napus* (14-17).

In this study, endophytic bacteria were isolated from *Brassica carinata* and screened for their plant growth promoting capabilities, such as mobilizing and transferring nutrients, producing plant growth hormones and suppressing pathogen growth. To our knowledge, this is the first study where bacterial endophytes were isolated and characterized from *Brassica carinata*.

### 3.3 Results

#### 3.3.1 Isolation and Identification of Endophytes

All isolates were obtained on nitrogen free agar as a primary screening step to select for bacteria that are able to fix gaseous nitrogen through biological nitrogen fixation. The capability of the bacteria for biological nitrogen fixation was confirmed on nitrogen free agar, and all bacteria were able to change the color of the medium from green to blue due to the production of ammonia, and raised the pH of the Agar from slightly acidic (pH = 6.8) to a more basic pH. Most of the isolated endophytes were isolated from shoot (9 isolates) and head tissues (7 isolates), while from leaves (2 isolates), flowers (1 isolate), and rhizosphere (1 isolate) lower numbers of endophytes were isolated. Identification by 16S rRNA gene sequencing revealed that 11 isolates were from the genus *Bacillus* (*B. pumilis* BC01, *B. paralicheniformis* BC02, *B. pumilis* BC07, *B. pumilis* BC08, *B. subtilis* BC10, *B. pumilis* BC13, *B. cereus* BC14, *B. thuringiensis* BC15, *B. safensis* BC16, *B. safensis* BC18, and *B. sp.* BC20), 4 from the genus *Enterobacter* (BC03, BC04, BC05, and BC06) and 3 were from the genus *Pantoea* (*P.
agglomerans BC09, P. agglomerans BC11, and P. sp. BC12). The genera Lysinibacillus (L. sp. BC17) and Xanthomonas (X. sacchari BC19) had 1 isolate each out of the 20 identified (Error! Reference source not found.).

3.3.2 Phosphate Solubilization

All 20 isolates were screened for their ability to solubilize phosphate by plating them on Pikoviskaya’s agar. Three of the bacteria, BC09, BC10, and BC12, created halos around bacterial colonies, indicating the ability for them to solubilize the calcium phosphate in Pikovskaya’s media.

3.3.3 Indole-3-Acetic Acid (IAA) Production

IAA production was assessed at d 4 and the results are shown in Figure 3.1. All isolates were initially tested for their ability to produce IAA after 4 d and 9 of the 20 endophytes were able to produce some level of the plant growth hormone. Of the isolates that were able to produce IAA were used for further studies and the IAA production was standardized to an OD₆₀₀ of 1.000 to allow the comparisons among the different isolates. From all tested isolates isolate BC09 had the largest production of IAA, producing 30.2 ng/µl, followed by BC12 producing 21.76 ng/µl, BC16 producing 21.55 ng/µl, and BC11 producing 20.24 ng/µl (Figure 3.1). BC05 produced the lowest concentration of IAA with 7.87 ng/µl, while BC03, BC06, BC17 and BC18 produced 14.3 ng/µl, 11.82 ng/µl, 11.12 ng/µl, and 16.76 ng/µl, respectively.
3.3.4 ACC deaminase

Genomic DNA was subjected to PCR with ACC deaminase primer sets. Of the 20 *B. carinata* isolates, seven had shown some level of amplification with these primers. The seven bacterial endophytes that had amplification using the ACC deaminase primers were BC02, BC05, BC07, BC10, BC12, BC13, and BC16 (Figure 3.2).

3.3.5 Fungal Suppression

All *B. carinata* isolates were tested against fungal isolates from the fungal plant pathogens *Fusarium proliferatum*, *F. oxysporum*, *F. graminearum*, and *F. acuminatum* in a plate assay. In the event of suppression, the fungal growth between the 2 streaked lines of the isolate would be oblong rather than circular. All isolates with the exception of BC07, BC16, and BC17 (due to ambiguity) were able to suppress *F. proliferatum* (Error! Reference source not found.). BC01 – BC04, BC06 – BC10, BC14 and BC15, and BC18 had shown the ability to suppress the growth of *F. graminearum*, while *F. oxysporum* and *F. acuminatum* had been inhibited by very few of the isolates (Table 3.1). The only suppression of *F. oxysporum* was from the challenge with BC14, and BC08, BC18, and BC20 were the endophytes that were able to suppress *F. acuminatum*.

3.4 Discussion

In this study we studied 20 bacterial endophytes isolated from surface sterilized *Brassica carinata* tissues for their plant growth-promoting characteristics. These plant growth-promoting characteristics included the ability to grow on nitrogen free media, solubilize calcium phosphate, produce the auxin indole-3-acetic acid, the presence of 1-
amino-1-cyclopropane carboxylase deaminase gene in the genomic DNA, and the ability to suppress the growth of 4 fungal pathogens: *Fusarium proliferatum*, *Fusarium oxysporum*, *Fusarium graminearum*, and *Fusarium acuminatum*. The presented results indicate that several of the bacterial endophytes have the potential to assist plants under various stress conditions, specifically nitrogen stress and in competition with the pathogens *F. proliferatum* and *F. graminearum*.

### 3.4.1 Potential to Alleviate Nutrient Deficiency

All of the endophytes isolated from *Brassica carinata* tissues were extracted after surface sterilization onto nitrogen free media (Nitrogen Free bromothymol), forcing the bacterial endophytes to acquire nitrogen from other sources such as gaseous nitrogen (18). When the bacteria grow on the nitrogen free bromothymol plates, the media color turns from green color to blue, which indicates an increase in pH from 6.8 to more basic pH. Growth on the nitrogen free media isn’t completely indicative of the bacterial endophytes’ ability to fix atmospheric nitrogen; endophytes could be utilizing helper bacteria or other metabolic pathways to grow on nitrogen free media. Bacterial isolates that do fix atmospheric nitrogen can have that ability quantified using the acetylene reduction assay (19-21). The colony size on the NFb plates were extremely small (less than 0.5 mm) after allowing the colonies to grow for a week which could indicate that the bacterial cultures are under nutrient stress.

The ability to solubilize calcium phosphate on Pikovskaya’s agar is shown by a clearing zone within the agar around the bacterial colonies. Often, the growth of the bacterial colonies on the Pikovskayas agar is slowed because of the general screening
purpose of this media and not all nutrients will be available to a wide assortment of bacteria and the bacteria is putting energy into solubilizing phosphate in the media for use. Solubilization of phosphate on plates could indicate the bacterial endophytes’ ability to solubilize calcium phosphate in the rhizosphere that can be used by the host plant. The only isolates from Brassica that were able to solubilize phosphate were of the *Pantoea* genus. *Pantoea*, among other genera, have been found to be the primary contributor to phosphate mobilization in soils and has also been proposed as a bio-fertilizer that could be applied to crops, such as soybean (22, 23).

3.4.2 Bacterial Endophyte Identification

Bacterial identification through 16S rRNA gene sequencing revealed a large number of the bacterial endophytes were from the genus *Bacillus*. *Bacillus* species have been isolated from a variety of crops, such as apples and maize (24, 25). Species of *Bacillus* have also been isolated from members of the *Brassica* genus, and in *B. juncea* (mustard) and *B. napus* (canola) they made up the primarily the cultureable isolates that also promoted the growth of Brassica in in-vitro and in greenhouse studies (24, 26, 27). *Bacillus* species are also dominant endophyte species such as *Camellia sinensis* (tea) or of a closely related genus such as *Paenibacillus* and *Lysinibacillus* (28). We also considered the microbial community similar in *Brassica* species, *Brassica napus* (canola), and found that one of the most abundant genera was *Bacillus*, to look for potential targets to compare to when studying isolates from *B. carinata*. This genus is also present in high numbers in the seeds of *B. napus* and then colonizes the plant as it grows and is then passed on to its progeny (29). *Bacillus* has also been extensively studied and research has
shown the potential for *Bacillus* species to increase plant growth, for example in corn, and has also been shown to be useful as a bio-control agent (30, 31).

### 3.4.3 Indole-3-Acetic Acid Production

Indole-3-acetic acid (IAA) is a plant auxin that signals plant cells to elongate and divide. Nine of the bacterial endophytes (BC03, 05, 06, 09, 10, 11, 12, 15, and 18) showed the ability to produce this auxin in an environment with tryptophan. The production of IAA by bacterial endophytes is measured in-vitro by a colorimetric assay, where after growing in spiked LB solution with tryptophan, Salkowski’s reagent is added a pink color change is then observed if IAA has been produced and be calculated colorimetrically. Three of the isolated genera: *Bacillus*, *Pantoea*, *Enterobacter* and *Lysinibacillus* all produced IAA in varying amounts but the *Pantoea* isolates showed the highest biosynthesis of IAA. *Lysinibacillus* only produced 11.1 ng/µL IAA over a 4 day period and was also found to be a IAA producer when it was isolated from corn tissue (32). However, this strain was isolated from the carinata rhizosphere and not from the carinata tissue, which could potentially explain the lower biosynthesis of IAA. For bacteria to have optimal IAA production, it should form a symbiosis with a plant or within plant tissue. This would be more advantageous to the bacteria because the plant could offload carbon and other nutrients to the bacteria as well as offer itself as a habitat. This would also aid in confirming that these bacteria are using a tryptophan dependent pathway, since tryptophan is usually plant exuded or supplied (33). The other bacterial isolates that tested positive for IAA production were from carinata tissue and showed higher values of IAA production over a 4-day period.
3.4.4 Fungal Suppression

Fungal pathogen suppression or biocontrol is usually performed by artificial agents such as pesticides, fungicides, and herbicides. Fungal pathogens sourced from the surrounding area and from soybean and Ethiopian mustard crops was tested against bacterial isolates. Isolates and fungal pathogens were plated on PDA plates and observed after incubation if there was any pathogen suppression that would be indicative of fungal isolate growing away from the bacterial isolate or stunted growth when compared to a control plate with no bacteria added. Almost all isolates had some form of suppression ability of *Fusarium proliferatum*, except for BC07 and BC16. *Fusarium graminearum*, also known *Gibberella zeae*, was suppressed by 12 of the 20 isolates (BC01-04, BC06-10, BC14 and BC15 and BC18). When looking at the suppression of *Fusarium oxysporum*, only one isolate, BC14 had the ability to suppress this fungus. This fungus is known to infect a wide variety of plant crops when looking at just the species level of the fungus. It can cause severe damage in loss in vegetable and field crops as well as flowering crops. What makes this pathogen dangerous is that there is very few, if any fungicides that can control it and only resistant varieties of plants can overcome infection and help to control it (34). This could be why only a select few endophytes can suppress its growth. A similar theme can be seen when isolates are paired against *Fusarium acuminatum*, only BC08, BC18 and BC20 suppressed growth. *F. acuminatum* is known for its ability to cause indistinguishable disease symptoms and that it also creates a disease complex with other species of *Fusarium* (35). The pathogenicity of these two fungal species could and their ability to co-exist in disease complexes with other similar species could be one
reason why they seem to not allow other beneficial microbes to inhabit the same environment.

3.4.5 ACC deaminase Screening and Quantification

The ability for bacterial strains to aid in stress relief of plants can be seen in a variety of abilities including the production of ACC deaminase. This enzyme cannibalizes ACC, the precursor to ethylene, which is known to halt root and shoot growth in plants that are under stressful conditions. This in-turn allows the growth of roots and shoots to continue. Endophytes were first screen for the ACC deaminase gene, \textit{acdS} (36). Of the 20 endophytes seven (BC02, BC05, BC07, BC10, BC12, BC13 and BC16) had a putative gene that resembled \textit{acdS} when screening with PCR. After PCR, isolates were grown in media that contained only ACC as the carbon source and were then processed to look for $\alpha$-ketobutyrate after breaking down ACC (37). When testing the resulting solution colorimetrically, there was no detectable $\alpha$-ketobutyrate present. This could be because the expression levels of the \textit{acdS} gene are low or not actively being produced. It could also be that the in-vitro conditions were not suitable for the production of ACC deaminase and expression was lowered. Future work should include looking at gene expression of the \textit{acdS} gene and possible alternative routes for quantifying the amount of $\alpha$-ketobutyrate produced.
3.5 Materials and Methods

3.5.1 Isolation of Nitrogen Fixing Endophytes from *Brassica carinata*

*Brassica carinata* tissues were surface sterilized as follows: 1 min in 70% ethanol, 30s in sterile water, 5 min in 5% bleach, 1 min in 70% ethanol, followed by 4 successive 30s washes in sterile water. Semi-solid Nitrogen Free bromothymol (NFb), described in Kirchhof et al (1997) was used as a selection media, with 5 replicates per plant tissue (leaf, shoot, flower, head) (18). After pellicle formation, the bacteria were streaked on NFb plates with the same components as the NFb described above but using 15gL⁻¹ agar and incubated at 30°C. Streaking for isolation was performed until single colonies were formed and were plated in pure culture. Pure cultures were used to create cryogenic stock cultures and stored at -80°C.

3.5.2 Identification based on 16S rRNA gene

Isolates were grown in nutrient broth for 24h. The bacterial suspension was combined with 35% glycerol solution 1:1 in a volume of 200 µL. Suspensions were sent to GENEWIZ for sequencing with the primers 27F, 1492R, and a proprietary 16S rRNA gene primer. The resulting sequences were analyzed with BLAST [Basic Local Alignment Search Tool] to determine the genus of all isolates (38).

3.5.3 Phosphate Solubilization

The ability to solubilize phosphate was first determined by the ability to produce halos on Pikovskaya’s agar (39). *Pseudomonas aeruginosa ATCC 27853* was obtained from the American Type Culture Collection as positive control (Himedia’s Technical
Data Sheet) on Pikovskaya’s agar. Bacteria were poked into the media, 5 replications per plate, and allowed to grow for 7 d. Following growth, the halo zones were assessed; bacteria that produced halos were given a plus (+) while those that weren’t able to produce halos were given a (-).

3.5.4 Indole-3-acetic Acid Production (IAA)

IAA production was screened with a method by Ahmad et al. (40) by growing bacteria in LB broth with 500 µg/mL of L-tryptophan sterilized with a 0.22 µm filter. The cultures were harvested by centrifugation (10,000 x g for 5 min) and 2 mL of the supernatant was combined with 4 mL of Fe-HClO (1:2 ratio) for 25 min and the absorbance was measured at 530 nm (41). All isolates yielding an A530 greater than the control was subjected to a further quantitative analysis.

The isolates were grown in LB for 24 h at 30°C and 200 rpm. Following, the isolates were washed with phosphate buffered saline (source) and normalized to an OD of 0.1 at 600 nm by diluting with phosphate buffered saline. LB with 500µg/mL of L-tryptophan (5ml) was supplemented with 100 µL of the bacterial dilution and incubated for 4 d at 30°C and 200 rpm. Each assay was performed 3 times. The IAA production was assessed as described above.

3.5.5 1-Aminocyclopropane-1-Carboxylate Deaminase PCR Amplification

Isolate genomic DNA were subjected to PCR using 1-aminocyclopropane-1-carboxylate deaminase (acdS) primers: reverse 5’- TTD CCH KYR TAN ACB GGR TC -3’; forward 5’- GGB GGV AAY AAR MYV MGS AAG CTY GA -3’. PCR reactions
were performed in 50 µL reactions that contained 20 ng genomic DNA, 2 µM of each primer, 5x Green GoTaq, and nuclease free water. PCR cycles were conducted as described in Nikolic et al. (42).

3.5.6 Fungal Suppression

Fungal isolates that were used to test the capability for *B. carinata* isolates to suppress fungal growth were *Fusarium proliferatum*, *F. oxysporum*, *F. graminearum*, and *F. acuminatum*. Suppression ability was determined using an agar plate assay described by Ji et al. with some modifications: the mycelia plug was placed in the center of the PDA plate and the bacteria were streaked 2 cm on either side (43). Antagonistic effects were determined by inhibition zones between the fungal and bacterial isolates. Isolates were scored based on whether fungal growth was inhibited (+) or not inhibited (-). An endophytes ability to cause the fungal colony to grow in an oval rather than circle as the control was considered positive.

3.6 Acknowledgements

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Table 3.1: The list of *Brassica carinata* isolates with their respective genus from 16S rRNA sequencing and the ability of the isolates to suppress the growth of pathogenic fungi (*F. p.*: Fusarium proliferatum, *F. o.*: Fusarium oxysporum, *F. g.*: Fusarium graminearum, and *F. a.*: Fusarium acuminatum). The amount of phosphate solubilized from calcium phosphate as insoluble-phosphate source after 7 days of incubation indicated by +/−, as well as the amount of IAA produced after 4 days, and the ability of the isolate to breakdown ACC to α-ketobutyate. Also, the plant tissue from which the endophyte was isolated from.

<table>
<thead>
<tr>
<th>Bacterial Isolate</th>
<th>Species</th>
<th>F. p.</th>
<th>F. o.</th>
<th>F. g.</th>
<th>F. a.</th>
<th>Solubilized Phosphate</th>
<th>IAA Production</th>
<th>acdS Activity</th>
<th>Plant Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC01</td>
<td><em>Bacillus pumilis</em></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Flower</td>
</tr>
<tr>
<td>BC02</td>
<td><em>Bacillus paralicheniformis</em></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>0 mM</td>
<td>-</td>
<td>Leaf</td>
</tr>
<tr>
<td>BC03</td>
<td><em>Enterobacter sp.</em></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>14.3 ng/µL</td>
<td>-</td>
<td>Head</td>
</tr>
<tr>
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<td><em>Enterobacter sp.</em></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>7.9 ng/µL</td>
<td>0 mM</td>
<td>Head</td>
</tr>
<tr>
<td>BC05</td>
<td><em>Enterobacter sp.</em></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>11.8 ng/µL</td>
<td>-</td>
<td>Rhizosphere</td>
</tr>
<tr>
<td>BC07</td>
<td><em>Bacillus pumilis</em></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0 mM</td>
<td>Head</td>
</tr>
<tr>
<td>BC08</td>
<td><em>Bacillus pumilis</em></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Head</td>
</tr>
<tr>
<td>BC09</td>
<td><em>Pantoea agglomerans</em></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>30.2 ng/µL</td>
<td>-</td>
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</tr>
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<td>BC10</td>
<td><em>Bacillus subtilis</em></td>
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<td>-</td>
<td>NA</td>
<td>-</td>
<td>-</td>
<td>20.2 ng/µL</td>
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<td>21.8 ng/µL</td>
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<td>BC12</td>
<td><em>Pantoea sp.</em></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Shoot</td>
</tr>
<tr>
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<td><em>Bacillus pumilis</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0 mM</td>
<td>Head</td>
</tr>
<tr>
<td>BC14</td>
<td><em>Bacillus cereus</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Head</td>
</tr>
<tr>
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<td><em>Bacillus thuringiensis</em></td>
<td>+</td>
<td>NA</td>
<td>+</td>
<td>NA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Shoot</td>
</tr>
<tr>
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<td><em>Bacillus safensis</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td><em>Lysinibacillus sp.</em></td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>11.1 ng/µL</td>
<td>-</td>
<td>Shoot</td>
</tr>
<tr>
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<td><em>Bacillus safensis</em></td>
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<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>16.8 ng/µL</td>
<td>-</td>
<td>Shoot</td>
</tr>
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<td>BC19</td>
<td><em>Xanthomonas sacchari</em></td>
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<td>-</td>
<td>NA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Shoot</td>
</tr>
<tr>
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<td><em>Bacillus sp.</em></td>
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<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Leaf</td>
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</table>
3.8 Figures

Figure 3.1: IAA production after 4 days of incubation. IAA was standardized to the optical density of the bacteria. Error bars are standard errors of the mean.

Figure 3.2: Electrophoresis gel of the PCR amplification of the ACC deaminase gene within the genomic DNA of 7 bacterial endophytes isolated from *Brassica carinata*.
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CHAPTER 4: CHARACTERIZATION OF BACTERIAL ENDOPHYTES ISOLATED FROM THE OILSEED PRODUCING CROP \textit{BRASSICA CARINATA}: PART 2 – APPLICATION OF ENDOPHYTES ON WHEAT

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4.1 Abstract

Endophytic bacteria that were isolated from \textit{Brassica carinata} demonstrated in in vitro assays different plant growth promoting characteristics and for example grew on nitrogen free medium, produced indole-3-acetic acid, solubilized calcium-phosphate, and suppressed the growth of different fungal pathogens. To determine the practical application of these bacterial endophytes and their plant growth promoting capabilities, \textit{in planta} assays were conducted to assess the impacts on seedling root development, greenhouse biomass, and yield, test weight, and protein content under field conditions. In short term root architecture experiments bacterial endophyte \textit{Bacillus paralicheniformis} BC02 has shown to significantly increase seedling root mass by up to 42.7\%, root length by 57.9\%, root surface area by 41.6\%, and root volume by 26.2\% in the winter wheat variety Redfield with other endophyte isolates having varying levels of positive and negative impacts. Differences also presented between spring wheat genotypes Boost, Prevail, and Surpass, with inoculations of Surpass eliciting a more significant negative response. Spring wheat grown under low and high nitrogen greenhouse conditions
furthered distinctions between genotypes, with endophytes often performing differently under different nutrient conditions. Prevail had shown the most positive benefit from the different endophyte applications under low and high nitrogen conditions, and Boost benefitted more under high nitrogen conditions than low. The largest increase was in Boost roots under low nitrogen conditions with BC16 application (102.4%) while Prevail had a non-significant increase in root mass from BC11 (one extremely high performing plant). Redfield winter wheat grown under field conditions with natural nitrogen levels did not respond in a significant way to the *B. carinata* endophyte applications. This study outlines the potential in bacterial endophytes isolated from *Brassica carinata* in increasing wheat yields while decreasing the application of nitrogen.

### 4.2 Introduction

Wheat is one of the most cultivated crops in the world, and is the third largest row crop in the U.S. by land usage and yield behind corn and soybean (1). In 2019, wheat was produced on 47.8 million acres and generated roughly 1.9 billion bushels of grain at an average of 51.7 bushels per acre (1). To achieve these yields in wheat, 2.5 pounds of nitrogen per bushel must be applied, i.e. 50 bu/ac yield goal would require 125 lb N/ac (2). Nitrogen fertilization is responsible for 75% of the carbon foot print in farming (3). To offset these high fertilizer needs and the environmental impacts that are associated with nitrogen fertilizers, endophytic bacteria have the potential to decrease the amount of applied nitrogen needed (4).

Fungal or bacterial endophytes are organisms that grow within plant tissues (not on the surface) and do not cause harm to the plant. Many bacterial endophytes possess
plant growth promoting capabilities and are part of a group referred to as plant growth promoting bacteria, or PGPB. Since bacterial endophytes are located within plants, they need ways to get inside of those plant tissues. It is hypothesized that endophytes are selected for their ability to benefit their hosts, with the passage of endophytes from parent plant to seedling being referred to as vertical transmission (5). However, endophytes can enter plants also from the environment e.g. from the rhizosphere, and this transmission is referred to as horizontal transmission (6, 7). This horizontal endophyte transfer can be used to inoculate plants with target endophytes.

The capabilities that bacterial endophyte may possess can range from alleviation of abiotic stresses, like nitrogen fixation in the scope of nitrogen deficient soils, to biotic stresses like nematode and pathogenic fungi infections (6, 8, 9). Here, we tested bacterial endophytes isolated from *Brassica carinata* for their impact on seedling root architecture and ability to decrease nitrogen stress in wheat under greenhouse and field conditions (10).

### 4.3 Results

Sequencing of 16S rRNA gene previously revealed that 11 isolates were from the genus *Bacillus* (*B. pumilis* BC01, *B. paralicheniformis* BC02, *B. pumilis* BC07, *B. pumilis* BC08, *B. subtilis* BC10, *B. pumilis* BC13, *B. cereus* BC14, *B. thuringiensis* BC15, *B. safensis* BC16, *B. safensis* BC18, and *B. sp.* BC20), 4 from the genus *Enterobacter* (BC03, BC04, BC05, and BC06) and 3 were from the genus *Pantoea* (*P. agglomerans* BC09, *P. agglomerans* BC11, and *P. sp.* BC12). The genera *Lysinibacillus*
(L. sp. BC17) and Xanthomonas (X. sacchari BC19) had 1 isolate each out of the 20 identified (10).

4.3.1 Root Architecture

The application of the Brassica isolates to Boost seeds often led to large and significant responses (Figure 4.1). For example the application of BC01, BC11, BC12, BC14, BC15, and BC16 resulted in significant increases in seedling mass, root length, surface area, and volume. The application of BC17 caused a decrease in seedling mass, root length, surface area, and volume compared to the non-treatment control. BC09, BC17, and BC20 led to a significant decrease in root volume.

The response of the wheat genotype Prevail was similar to that of Boost, but more of the endophytes led to growth depressions (Figure 4.2). BC05 inoculated plants significantly increased root length and surface area, while root volume trended positively but not significant due to the spread of the root volume data. Mass and root characteristics were all negative for BC02, BC03, BC04, BC07, BC08, BC10, BC11, BC13, BC14, BC18, and BC19, while only BC05 and B12 increased all four metrics compared to the control.

The spring wheat variety Surpass also showed an overall negative impact from the application of endophytic bacteria to the seeds; BC19 and BC03 were the only 2 isolates that increased the seedling mass, and root length, surface area, and volume compared to the control, while BC01, BC02, BC11, BC12, BC13, BC14, BC15, and BC16 led to negative responses (Figure 4.3). Isolate BC10, BC19, and BC20 were the only isolates that increased the seedling mass significantly to the control.
Winter wheat variety Redfield had a greater response than Surpass but was overall not as responsive as Boost and Prevail (Figure 4.4). The greatest change compared to the control came from the application of BC02 where root length increased 58% while the greatest negative change was from the application of BC12 causing a 27% decrease in root volume. *Brassica carinata* isolates BC01, BC02, BC03, BC05, BC06, BC11, BC12, BC13, BC14, and BC15 all significantly increased the root length of Redfield.

4.3.2 Greenhouse Trials

The *B. carinata* endophyte application to spring wheat variety Boost decreased shoot biomass under both high and low nitrogen fertilization in contrast to the benefits seen by endophyte applications in short root architecture trials. Isolate BC01 and BC19 led to significant decreases in shoot biomass under low nitrogen conditions compared to the control while BC20 was the only isolate that was able to significantly increase the shoot biomass under high nitrogen conditions (Figure 4.5). Boost had a significant increase in root biomass under high nitrogen conditions from the inoculation of BC15, and a significant increase in root biomass under low nitrogen conditions from the inoculation BC11 and BC16 (Figure 4.6). Only under low nitrogen conditions were statistically significant decreases in root biomass observed. These significant decreases were caused by isolates BC02, BC07, BC10, BC13, BC15, BC20.

Prevails shoot response was largely a positive trend, with a greater number of significant increases being among the high nitrogen applications (Figure 4.7). Isolates BC03, BC04, BC13, BC14, BC18, and BC19 increased Prevail’s shoot mass under the high nitrogen conditions, while BC06, BC09, and BC11 significantly increased shoot
biomass under low nitrogen conditions. Prevail’s root biomass contained more variation within treatments but were still largely positive trending in growth compared to the control, with only BC05 having significantly decreased the root biomass under low nitrogen conditions (Figure 4.8). BC01, BC02, BC10, BC18 and BC19 significantly increased the root biomass under high nitrogen conditions. Isolate BC19 was also able to increase the root biomass under low nitrogen conditions. Isolate BC11 shows a surprisingly high percent change in root biomass to the control under low nitrogen conditions, but not significant. This is due to a single replicate having a biomass 6x higher than the next greatest biomass, so the data is highly skewed and percent change is calculated using the samples’ mean.

The shoot biomass response for spring wheat Surpass was similar to that of Boost, with many having a percent change between -20% and 20% (Figure 4.9). Isolates BC01 and BC08 were able to significantly increase the shoot biomass when plants were grown under high nitrogen conditions. None of the isolates were able to significantly increase Surpass’s shoot mass under low nitrogen conditions, with BC09 and BC11 significantly decreasing the shoot biomass under low nitrogen conditions. Isolate applications under high nitrogen conditions did not show any significant decreases in biomass. Isolate BC01 was also able to increase root biomass compared to the control under high nitrogen conditions, along with BC02, BC08 and BC19 (Figure 4.10). Isolates BC08, BC13, BC19, and BC20 had shown significant increases in root biomass, with BC09, BC11, and BC12 significantly decreasing root biomass under the low nitrogen. The isolates BC05, BC06, BC09, BC12, BC16, and BC17 caused significant decreases in root biomass under high nitrogen conditions.
4.3.3 Field Trial

Metrics measured for Redfield under field conditions was yield (corrected to a moisture level of 13%), test weight, and protein content (corrected to a moisture level of 13%) (Figure 4.11). The non-treatment control achieved an average yield of 64 bushels/acre. None of the bacterial endophytes created a difference in the yields from the control, but BC09 had the highest average yield with 65.4 bu/ac and BC02 had the lowest yield of 60.4 bu/ac. Test weight across all treatments was very consistent ranging from 60.1 lbs/bu and 61.5 lbs/bu (Figure 4.12). All isolates were statistically similar in test weight with only statistical differences between BC12 and BC13 and BC15. The protein content was also consistent across all isolates, maintaining between 13.3% and 13.5%. All treatments were statistically similar (Figure 4.13).

4.4 Discussion

Bacterial endophytes from *Brassica carinata* have numerous growth-promoting capabilities when applied to wheat. When comparing *in vitro* results to *in planta* results, as in the root architecture after endophyte application, it can be seen that certain endophytes that produce indole-3-acetic acid, l-amino-cyclopropane carboxylic acid and phosphate solubilization capabilities do have an ability to increase plant growth in root and shoot growth.

Genotypic differences can be observed when applying different endophytes to different host plants. This in turn can lead to host specificity depending on the species of plant and the microbe in question (11). When strictly looking at the different wheat genotypes, Boost, had a more overall positive trend with interactions between isolates.
Six strains (BC01, BC11, BC12, BC14, BC15, and BC16) were found to have positive growth effects in the four metrics observed (root weight, length, surface area, and volume). When comparing to the other wheat genotypes, growth promotion did not always shift to other genotypes when the same endophytes where applied. In Prevail, only BC05, BC06, BC15 and BC16, in Surpass, only BC03 and BC19 and in Redfield only BC02, had shown any significant increases in root architecture. This shows that even when endophytes are applied to the same genus of a specific hostplant, specie or genotype variation can lead to detrimental response and underperforming strains. Plant defense systems and tissue specificity can also compound the difficulty of endophyte establishment and growth promotion in non-native host plants (12). It should also be thought of that certain strains of endophytes could have coevolved with a plant over time (13) and have developed a rapport with their plant-hosts that lead to non-discriminatory actions against a non-host plant.

However, some strains did not show any indications of growth promotion and some have even shown to reduce plant growth. This seems to reaffirm work done by Majeed et al and Maggini et al (14, 15). It is not clear what inhibitory effects could be the cause of the of detrimental impacts on certain plant species. One idea could be postulated that the microbe-host relationship and plant-endophyte specificity could cause endophytes turn pathogenic (16). It should be noted that even if strains do have growth promoting capabilities, this does not always translate into growth promotion that can be visually seen. Plant robustness and root architecture also play a role in plant fitness, which could translate into a plant possibly not having a larger increase in biomass but being able to withstand more environmental stressors.
Greenhouse trials also indicate that certain strains of endophytes do deliver growth promoting attributes and have shown that biomass increase can be observed. Being in an environment that allows plants additional room to grow can aid in the growth promotion characteristics seen in the *in vitro* testing. However, this can cause variability in that this system is not a closed or axenic system and additional input from outside sources can now be seen. Plant genotype again seems to play a role here as well, however, different parts of the plant also respond differently when interacting with the newly applied endophytes. Boost showed very little significant plant growth promotion when considering just the shoot portion, where only BC20 showed a significant response under high nitrogen nutrient application. Only two endophytes, BC11 and BC16 showed any significant response under low nitrogen, where other endophytes showed a negative effect. Prevail had only six isolates (BC03, BC04, BC13, BC14, BC18, and BC19) show shoot growth under low nitrogen conditions whereas under high nitrogen applications isolates BC01, BC02, BC10, BC18 and BC19 had shown to significantly increase shoot growth. This increase in growth under high nitrogen conditions could indicate that the isolates are dependent on extra nitrogen applied to better augment plant growth. This could also be due to the coevolution of this organisms grow in soil with a high nutrient availability (17, 18). Prevail root biomass only showed significant growth promotion when isolates BC01, BC02, BC10, BC18 and BC19 were applied under high nitrogen nutrition. This again reaffirms that these endophytes may very well be dependent on higher N availability. The genotype Surpass, showed little to no significant shoot growth promotion under low N conditions, however, isolates BC01 and BC08 were able to increase shoot growth under high N. Surpass root showed increase in biomass after
application of isolates BC08, BC13, BC19 and BC 20 under low N while under high N, isolates BC01, BC02, BC08, and BC18 aided in root growth promotion. All of these inputs can make trying to find statistically significant treatments more difficult than in *in vitro* settings. In work that was performed by Lally *et. al* (19), variable results were seen in the outcomes from greenhouse testing showing that not all strains will respond the same throughout different areas of testing. However, this is still an important step in the down selection of strains to field purposes. This can assist in selecting bacteria when going to field trials, in a sense, if bacterial strains cannot perform in a greenhouse conditions where there is relatively little outside input then the strain will more than likely have a harder time out in a field setting. The wheat genotype, Redfield, was planted in the field and no endophytes showed any significant growth in yield, with the highest yield being from the application of BC09, which only displayed a small increase in yield. Redfield final test weight and protein content values were all relatively consistent, regardless of endophyte application. Endophyte application could be one cause of the of the stable field metrics; our application dosage concentration was relatively low. This and the additional of rhizospheric microbial competitors could have lowered the numbers of endophytes applied (20). Again, N dependency could also be a factor in how these endophytes interact with hostplants could indicate why, under low N nutrient applications, very little or sporadic growth promotion is observed.
4.5 Materials and Methods

4.5.1 Surface Sterilization of Seeds

Seeds were sterilized using a chlorine gas method described by Lindsey et. al. (21). Instead of using “100 mL” of bleach, 96 mL of bleach was used to react with 4 mL of HCl. Containers were left for 12 h in a fume hood. Once, the seeds had been sterilized, they were stored in beakers that had been autoclaved to maintain surface sterility.

4.5.2 Root Architecture

The impact of each isolate on the root architecture was determined for 3 different regional crops: 4 wheat varieties (3 South Dakota State University spring wheat and 1 South Dakota State University winter wheat), 2 corn varieties (5126RR and 9714-G), and 3 soybean varieties (South Dakota State University Brookings, Codington, and Davison varieties). Isolates were grown in LB for 24 h at 30°C and 200 rpm. Cultures were standardized to an OD of 0.05 at 600 nm with PBS. Each isolate, along with a control, was measured in 45 replications per variety.

Seed inoculation of wheat was completed by placing 45 surface sterilized seeds into a sterile 15 mL tube and adding 2 µL/seed (90 µL) of the standardized bacterial suspension. Tubes were then shaken to coat all seeds evenly. Four sheets of heavy weight germination paper measuring 21.84 cm x 16.5 cm were wet with 50 mL of polyethylene glycol 6000. Fifteen seeds were placed between the 2 center sheets 2.54 cm down from the long edge, maintaining equal spacing between seeds and 0.635 cm from the short edge of the paper. All 4 sheets were then transferred to a square Petri dish. This was repeated for 3 Petri dishes per isolate totaling 45 seeds. Completed Petri dishes were
placed within a growth chamber (16 h: 25°C, 60 % relative humidity, light; 8 h: 20°C, 60 % relative humidity, dark) horizontally for 24 h before rotating upright for 5d.

Seed inoculation for corn and soybean was competed using 3 µL of the standardized bacterial suspension per seed. Tubes were shaken to coat all seeds evenly. Two sheets of heavy weight germination paper measuring 60.96 cm x 15.24 cm were wet with 50 mL of autoclaved distilled water. Fifteen seeds were placed between the sheets across the long edge of the germination paper, maintaining 3.8 cm from the long edge and 1 in from the short edge. The 2 sheets were then rolled and placed into glass jars with the seeds upright. Jars with soybeans were placed within the growth chamber (16 h: 25°C, 60 % relative humidity, light; 8 h: 20°C, 60 % relative humidity, dark) for 7d, and after 5 d the jar caps were removed. Jars with corn were left uncapped and placed within the growth chamber for 4 days.

Following seed germination and growth, the germination paper was removed from the containers and each seedling was weighed for fresh weight. After the mass was collected for each seedling, they were placed on a desktop scanner and scanned at 600 dpi resolution. Images were opened with WinRhizo and root length, surface area, and volume were calculated.

4.5.3 Greenhouse Trials – Wheat under Nitrogen Stress:

Preparation for greenhouse trials consisted of standardizing cultures used for seed inoculation. Isolates were grown in LB for 24 h at 30°C and 200 rpm. Cultures were standardized to an OD of 0.05 at 600 nm with PBS. All substrates were steam pasteurized for 30 min before the substrate were added to the pots. All pots were watered using an
automatic sprinkler system that turned on for 16 s every 64 min, keeping media from drying out.

Wheat was grown for 21 d in 165 mL pots containing 70% by volume of sand and 30% by volume of perlite. South Dakota State University spring wheat varieties Boost, Prevail, and Surpass were inoculated with 2 µL standardized culture per seed and grown under high (100% nitrogen based on Hoagland’s solution) and low (10% nitrogen based on Hoagland’s solution) nitrogen conditions. All 20 isolates were used at both nutrient levels, along with an uninoculated control, in 15 replicates for the 3 varieties. After 14 d of growth, 2 mL of the appropriate nutrient solution was added to each pot. The plant tissue was harvested after 21 d, divided into root and shoot for each plant, dried at 70°C, and weighed after 3 d.

4.5.4 Field Trial under Natural Nitrogen Levels

To check the ability of the different endophytes to enhance wheat growth under natural conditions, 10 endophytes were selected based on their effect on root architecture and their plant growth promoting capabilities (BC01, BC02, BC03, BC05, BC09, BC11, BC12, BC13, BC15 and BC16). South Dakota State University’s winter wheat variety Redfield was used and planted at a rate of 1.2 mil seeds per acre (89 g seeds per plot inoculated with 1392 µL of cell suspension) on 21 September 2017. An uninoculated control was used to calculate the effect of the BC endophytes on wheat growth. The plots were 5 ft x 20 ft and extra fertilizer was not applied (phosphate ~ 6 mg/L, nitrate ~ 14 mg/L, and ammonium ~ 2 mg/L). Replications were laid out in a randomized complete block design of 4 replications per treatment. On 18 July 2018 (42 weeks and 6
days after planting), the plots were harvested and yield, protein content, and test weight were measured, correcting yield and protein content to a moisture level of 13%.

4.5.5 Statistical Analyses

Root architecture experiments (n = 45) and greenhouse trials (n = 15) were analyzed using the Dunn’s non-parametric test from the FSA package (v0.8.27) in R (v3.6.2) at p < 0.1 without correcting for multiple comparisons (22). Field harvest data (n = 4) was analyzed with the LSD test from the agricolae (v1.3-2) package in R (23).

4.6 Acknowledgements

We would like to thank the South Dakota State University Experiment Station and Jonathan Kleinjan for their continued support and aiding with field procurement. We would also like to thank Indigo Ag for their financial support and their assistance and encouragement throughout this project.
4.7 Figures

Boost Root Architecture

Figure 4.1: Root architecture percent changes from the control for spring wheat variety Boost using 20 bacterial endophytes isolated from *Brassica carinata*. Asterisk (*) denotes significance at $p < 0.1$ using Dunn's nonparametric analysis without correcting for family-wise error.

Prevail Root Architecture

Figure 4.2: Root architecture percent changes from the control for spring wheat variety Prevail using 20 bacterial endophytes isolated from *Brassica carinata*. Asterisk (*) denotes significance at $p < 0.1$ using Dunn's nonparametric analysis without correcting for family-wise error.
Figure 4.3: Root architecture percent changes from the control for spring wheat variety Surpass using 20 bacterial endophytes isolated from Brassica carinata. Asterisk (*) denotes significance at $p < 0.1$ using Dunn's nonparametric analysis without correcting for family-wise error.

Figure 4.4: Root architecture percent changes from the control for winter wheat variety Redfield using 20 bacterial endophytes isolated from Brassica carinata. Asterisk (*) denotes significance at $p < 0.1$ using Dunn's nonparametric analysis without correcting for family-wise error.
Figure 4.5: Shoot biomass percent changes for spring wheat variety Boost using 20 bacterial endophytes under greenhouse conditions. Asterisk (*) denotes significance at p < 0.1 using Dunn's nonparametric analysis without correcting for family-wise error.

Figure 4.6: Root biomass percent changes for spring wheat variety Boost using 20 bacterial endophytes under greenhouse conditions. Asterisk (*) denotes significance at p < 0.1 using Dunn's nonparametric analysis without correcting for family-wise error.
Figure 4.7: Shoot biomass percent changes for spring wheat variety Prevail using 20 bacterial endophytes under greenhouse conditions. Asterisk (*) denotes significance at p < 0.1 using Dunn's nonparametric analysis without correcting for family-wise error.

Figure 4.8: Root biomass percent changes for spring wheat variety Prevail using 20 bacterial endophytes under greenhouse conditions. Asterisk (*) denotes significance at p < 0.1 using Dunn's nonparametric analysis without correcting for family-wise error.
Figure 4.9: Shoot biomass percent changes for spring wheat variety Surpass using 20 bacterial endophytes under greenhouse conditions. Asterisk (*) denotes significance at p < 0.1 using Dunn's nonparametric analysis without correcting for family-wise error.

Figure 4.10: Root biomass percent changes for spring wheat variety Surpass using 20 bacterial endophytes under greenhouse conditions. Asterisk (*) denotes significance at p < 0.1 using Dunn's nonparametric analysis without correcting for family-wise error.
Figure 4.11: Yield corrected to 13% moisture for winter wheat variety Redfield inoculated with 10 bacterial endophytes and a non-treatment control under field conditions. Letters signify LSD significance at $p < 0.1$ without correcting for family-wise error. Bars with the same letter are statistically similar. Error bars are standard error of the mean.

Figure 4.12: Test weight for winter wheat variety Redfield inoculated with 10 bacterial endophytes and a non-treatment control under field conditions. Letters signify LSD significance at $p < 0.1$ without correcting for family-wise error. Bars with the same letter are statistically similar. Error bars are standard error of the mean.
Figure 4.13: Protein content corrected for 13% moisture for winter wheat variety Redfield inoculated with 10 bacterial endophytes and a non-treatment control under field conditions. Letters signify LSD significance at p < 0.1 without correcting for family-wise error. Bars with the same letter are statistically similar. Error bars are standard error of the mean.
4.8 References


CHAPTER 5: CHARACTERIZATION OF BACTERIAL ENDOPHYTES ISOLATED FROM THE OILSEED PRODUCING CROP *BRASSICA CARINATA*: PART 3 – POTENTIAL OF ENDOPHYTES TO INCREASE SOYBEAN YIELDS

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5.1 Abstract

Previous characterization and testing of plant growth promoting bacterial endophytes isolated from *Brassica carinata* showed that while no significant increases were observed from field trials in winter wheat, greenhouse and short-term root architecture assays suggested that the application of these endophytes can increase plant growth. To assess this in soybean, short term root architecture assays and a greenhouse trial was conducted under low nitrogen conditions, and a final field trial was conducted under natural phosphate levels. The South Dakota State University soybean variety Brookings showed high and significant responses after an inoculation with the endophyte isolates *Enterobacter sp.* BC06, *Bacillus pumilis* BC07 and *Bacillus pumilis* BC08. BC07 increased seedling root biomass by 71.9% and increased the root surface area by 55.8%. The response of the soybean variety Codington was less pronounced, but BC09 increased seedling biomass and root characteristics. The SDSU soybean variety Davison responded mostly negative after the inoculation with the endophytes. While Brookings showed significant increases in root traits in the short-term trial, this could not be confirmed
under greenhouse conditions, and *Bacillus pumilis* BC01 significantly decreased shoot and root biomass. Codington under greenhouse conditions was only significantly increased in shoot biomass by *Bacillus pumilis* BC13 and root biomass by *Bacillus thuringiensis* BC15. Nitrogen and phosphate levels in the plant tissues were not significantly impacted after the endophyte inoculation in the Brookings field trials at both sampling times, however, *Bacillus sp.* BC20 significantly increase tissue phosphorus concentrations in Codington at R3 growth stage. Yields were not significantly increased after the inoculation with endophytic bacteria in Brookings and BC20 significantly decreased yield. Soybean variety Codington yield was significantly decreased in *Enterobacter sp.* BC04, *Enterobacter sp.* BC05, BC07, *Pantoea sp.* BC12, *Bacillus pumilis* BC13, *Bacillus cereus* BC14, and BC20. While potential plant growth promoting endophytes isolated from *Brassica carinata* were not able to significantly increase yields under natural soil phosphate levels, they show promise for increasing soybean biomass under low nitrogen levels.

### 5.2 Introduction

Soybean was the second highest land use crop in the United States in 2019 by the number of acres harvested (75.6 million acres), only second to corn for the use of grain (81.8 mil acres). On these 75.6 mil acres, soybean produced an average of 46.9 bushels/acre totaling just over 3.5 billion bushels (1). Bacterial endophytes have the potential to decrease growth of both common fungal and common bacterial pathogens and possess other plant growth promoting characteristics which can increase these yields and land use efficiency (2, 3).
Bacterial endophytes are bacteria that live within plant tissues and do not cause negative impacts on the plants. In soybeans, they often possess some level of plant growth promoting capabilities. These abilities range from the ability to fix atmospheric nitrogen, to solubilize recalcitrant soil phosphate resources, to the production of siderophores and plant growth hormones, and the suppression of the pathogens, and minimizing stresses on the host plant (4-7). For example, endophytes that were able to suppress the growth of the fungal pathogen *Phytophthera sojae* also showed the ability to produce siderophores and the auxin indole-3-acetic acid (IAA) and the ability to fix atmospheric nitrogen (3). There are correlations between the production of a siderophore that was produced by the endophyte and the suppression activity against *P. sojae* (3).

Others have also found that the addition of endophytic fungi that are capable of producing plant hormones are able to increase the nitrogen content within soybean tissues (8).

The inoculation with halotolerant bacterial endophytes that are able to grow in environments with higher levels of NaCl, can decrease the negative growth impact of saline soils in soybean plants (9). This effect has been attributed to the effects of endophytes on soybean root structure through the production of (IAA) and increased nodulation under relatively high saline environments (4, 9). Cadmium stress has been alleviated in soybeans by inoculation with bacterial endophytes that produce indole-3-acetic acid (10). Bacteria within soybeans are also able to lower the amount of cadmium that is transported into plant tissues while decreasing the hormones of salicylic and abscisic acid (11). While IAA can directly contribute to the increase in root growth, 1-aminocyclopropane-1-carboxylase deaminase (ACCd) produced by bacterial endophytes...
also provides benefits to soybeans by increasing nodulation and growth through the breakdown of a precursor to ethylene, a stress hormone. This decrease in ethylene levels increases root length, shoot height, and biomass produced by the plants (12, 13).

The endophytic community of soybeans that are glyphosate-tolerant differ from those that are non-glyphosate-tolerant, indicating there is an impact to these communities with the application of glyphosate (5). The differences in the glyphosate- and non-glyphosate-tolerant plants is in part due to the affects that individual cultivars have on what are beneficial endophytes, termed the genotype effect. The application of glyphosate-tolerate soybean cultivar N698 positively influenced the amount \textit{Bradyrhizobium} and \textit{Nitrospora} (14). The increase in rhizobium, and co-inoculating with rhizobium results in higher nodule numbers as well as a greater nitrogen use efficiency of the soybean plants (15). Non-rhizobia strains such as \textit{Bacillus subtilis} were able to increase yield of soybeans after they were inoculated on the seeds, and strain OTF-Bs10 able to increase biomass and nodulation under field conditions (16, 17).

In these studies, we assessed the beneficial impact of bacterial endophytes isolated from \textit{Brassica carinata} when applied to soybean seeds. This was done in 3 steps: first, the endophytes were applied to sterile seeds and we determined whether there were impacts on root architecture. From this experiment endophytes were downselected for the next step; a greenhouse trial, and then the endophytes were selected for field trials.
5.3 Results

Sequencing of 16S rRNA gene previously revealed that 11 endophytes isolated from *B. carinata* were from the genus *Bacillus* (*B. pumilis* BC01, *B. paralicheniformis* BC02, *B. pumilis* BC07, *B. pumilis* BC08, *B. subtilis* BC10, *B. pumilis* BC13, *B. cereus* BC14, *B. thuringiensis* BC15, *B. safensis* BC16, *B. safensis* BC18, and *B. sp.* BC20), 4 from the genus *Enterobacter* (BC03, BC04, BC05, and BC06) and 3 from the genus *Pantoea* (*P. agglomerans* BC09, *P. agglomerans* BC11, and *P. sp.* BC12). The genera *Lysinibacillus* (*L. sp.* BC17) and *Xanthomonas* (*X. sacchari* BC19) both each had 1 isolate of the 20 identified (18).

5.3.1 Root Architecture

The soybean variety Brookings showed overall the most positive response after an inoculation with the bacterial endophytes isolated from *Brassica carinata*. The isolates BC01, BC04, BC06, BC07, BC08, and BC10 all caused increases in seedling weight and root growth compared to the control (Figure 5.1). BC04 and BC07 caused a significant increase in seedling mass and root architectural traits. For example, BC07 caused a 72% increase in both seedling mass and root volume. BC13 significantly decreased the seedling mass by 40%, while also causing significant decreases in root metrics, along with BC15 and BC19.

Codington was relatively balanced between positive and negative changes in seedling mass, and root length, surface area, and volume. Isolate BC02, BC03, BC04, and BC05 led to negative percent changes for all 4 metrics, and BC16 and BC18 also decreased seedling biomass (Figure 5.2). The application of BC04 to Codington had a
significant negative impact on seedling mass, and root metrics. BC09 was able to significantly increase seedling mass, and root length, surface area and volume over the respective control.

The SDSU soybean Davison was largely negatively affected by the inoculation with the *Brassica carinata* isolates, with only a few exceptions. Isolates BC01, BC02, BC03, BC04, BC08, and BC20 all had at least one metric with a positive percent change (Figure 5.3). BC02, BC04, and BC08 significantly increased root length, and BC04 significantly increased seedling mass and root surface area. Isolates that had significant negative percent changes for all 4 metrics are BC05, BC06, BC07, and BC09, BC10, BC12, BC14, BC15, BC16, BC17, BC18, and BC19.

5.3.2 Greenhouse Trial

The use of the soybean variety Brookings, BC01 was the only isolate that was able to elicit a significant response in shoot and root biomass, albeit negative in comparison to the control (Figure 5.4). BC01 decreased shoot biomass by about 10.5% and root biomass by 28.4%. More isolates caused significant changes with the soybean variety Codington than with Brookings. The shoot biomass of the variety Codington was significantly decreased after the inoculation with BC01, BC04, BC05, BC06, and BC07, but significantly increased after the inoculation with BC13 (16%) (Figure 5.5). BC01, BC04, BC05, BC06, and BC07 significantly decreased shoot biomass and significantly decreased root biomass. BC15 was the only isolate that significantly increased the root biomass, but root biomass showed positive trends when compared to the control with the application of BC09, BC10, BC12 – BC15, and BC20.
5.3.3 Field Trial

The nitrogen concentrations at V5 of the soybean variety Brookings differed between the low and high phosphate controls, and we found tissue nitrogen concentration levels of 5.62% or 5.88%, respectively (Figure 5.6). Isolates BC07, BC13, and BC20 had tissue nitrogen concentrations that were statistically similar to both the high and low phosphate controls, while the rest of the isolates did not differ statistically significant from the low phosphate control. At R3, the difference in the nitrogen concentrations between the high and low phosphate controls was still established with tissue nitrogen concentrations of 4.84% and 5.4%. The inoculated plants did not differ in their nitrogen concentrations from the low phosphate control. Interestingly, the levels of nitrogen in the plant tissue decreased in all treatments between V5 and R3.

The phosphorus levels of the soybean variety Brookings followed a similar trend than the nitrogen concentrations within the tissues. The difference between the high and low phosphate controls was statistically different at both V5 and R3 (Figure 5.7). At V5, the plants that were inoculated with the isolates BC04, BC07, BC09, and BC15 were similar to the low phosphate control. The other isolates were statistically similar to both the low and high phosphate controls. The phosphorus concentrations in the tissues between V5 and R3 showed a similar decline as the nitrogen tissue concentrations. BC10 was statistically lower than the low phosphate control plants, but the other isolates were similar to the low phosphate controls. While the higher phosphate application increased the yield of the soybean variety Brookings control, none of the inoculations with different
isolates led to yield increases in yields and BC20 significantly decreased the yield by 4.2% (Figure 5.8).

Tissue nitrogen concentrations at V5 of Codington resulted in a statistical similarity between low phosphate control and high phosphate control, but the low phosphate control plot did have a lower average nitrogen concentration; 5.59% versus 5.73% (Figure 5.9). Isolates BC04 and BC07 were statistically similar to the low nitrogen control but not the high nitrogen control. Nitrogen concentrations at R3 also show no statistical difference between the low phosphate control (5.06%) and high phosphate control (5.28%). R3 tissue sampling shows BC05 and BC09 had significantly lower nitrogen concentrations than the high phosphate control, but not different than the low phosphate control. Other isolates were not statistically different than either the high or low controls.

Phosphorus concentrations of Codington at V5 were consistent between all treatments (Figure 5.10). The high phosphate control contained the highest tissue concentration of phosphorus with 0.46% and BC12 contained the lowest phosphorus concentrations at 0.41%. Sampling time R3 showed greater, significant differences between the controls. Low control concentration decreased to 0.39% resulting in significance to the high phosphate control at 0.47%. BC20 was significantly higher than the low control while other isolates were similar to only the low control. The other isolates were statistically similar to only the low control. There is a decreasing trend of phosphorus levels from V5 to R3 except for the high phosphate control actually increasing slightly in phosphorus concentration.
Codington yield of the high and low controls also had differences from established stress (Figure 5.11). BC04, BC05, BC07, BC12, BC13, BC14, and BC20 all decreased the yield of Codington significantly when comparing to the low control. The greatest decrease in yield was caused by BC13 with a 5.8% decrease compared to the low control. The high phosphate control increased yield of Codington by 5.1% over the low phosphate control.

5.4 Discussion

To test for bacterial compatibility and to help down select potential candidates for greenhouse and field, root architectural studies were performed with twenty bacterial endophytes from *Brassica carinata*. Three different genotypes of soybean were used to determine if there are differences among the isolates.

The genotype, Brookings, showed a variety of effects after application of endophytes. Only 8 (BC01, BC04, BC06-10, BC20) led to a significant growth of the four metrics recorded (weight, surface area, length, volume) compared to the control. BC06 and BC07 had the highest overall tissue growth promotion, and BC07 showed an increase of 75% over the control in seedling mass and root volume. Codington showed less increase in growth after endophyte application in only five the isolates (BC09, BC11, BC12, BC14, BC15) resulted in any significant growth promotion when looking at fresh weight, root length, root volume and surface area. Likewise, only a very few the isolates (BC02, BC04, BC08) significantly promoted plant root growth in the genotype Davison. This falls in line with work that were performed by Zhao *et al* and Bai *et al* (19, 20) and the variability they saw with specific endophytes during in-vitro applications to soybean,
even if endophytes showed growth benefits in-vitro, there wouldn’t always be
distinguishable traits after application to plants. Endophytes from non-host species also
could play a role in the variability in the growth promotion of soybean plants.
Endophytes isolated from different varieties of soybean had different effects when
applied to non-host plants and genotypic variation was observed (21). Since these isolates
originated from *Brassica carinata*, applying them to a non-legume plant could have
adverse effects on soybean plants. Host specificity could be playing a role here, and
possibly, since carinata does not have legumes, this could be a factor.

We conducted soybean greenhouse trials with two soybean genotypes, Brookings
and Codington, under low nitrogen conditions. The genotype Brookings did not show any
significant growth promotion in shoot growth after the inoculation with any of the
endophytes. Significant decreases in growth were observed in the roots when plants were
inoculated with isolates BC01 and BC02. In the genotype Codington, two isolates, BC13
and BC15 led to a significant growth promotion in the shoot and the root, respectively.
Several isolates showed a significant decrease in both root and shoot growth, BC01,
BC02, BC04-08. This level of variability was also observed by Soe *et al* (22), when
applying endophytes to different varieties in a greenhouse setting.

Tissue sampling was performed to determine the nitrogen and phosphorus
concentration of both Brookings and Codington in the field. This was performed at the
growth stages V5 and R3; when plants achieved the maximum of nodes and after
flowering when pods were starting to form. None of the endophytes led to significant
increases in the genotype Brookings compared to the low and high nitrogen controls.
However, when looking at low and high nutrient regimes, at V5, there is a decrease of
5.62% and 5.88% of nitrogen and between low and high phosphate, respectively. At R3, the difference between nitrogen concentrations between low and high phosphate were 4.84% and 5.4%. The level of nitrogen in plant tissues, decreases in all treatments between V5 and R3.

The concentration of phosphorus showed a similar trend as the nitrogen application testing and had a statistically difference between high and low phosphate controls at growth stages V5 and R3. At V5 and R3 no endophyte application had any significant effect and alleviated phosphate stress. This then translates into highly variable results in the field, that could be due to several factors. The only isolate that showed any difference in aiding in phosphate stress was BC15, which trended higher than the low control but was not statistically different than the high control. It is thought that the native rhizobacteria population could outcompete inoculations that are applied to plant tissue and could make it more difficult or an inoculant to establish itself in a non-native host plant (23, 24).

Tissue sampling was also performed on the genotype Codington, to determine the nitrogen and phosphate concentration in the plant tissue after endophyte application. Also, in the genotype Brookings, none of the endophytes led to a significant effect in the nitrogen concentration at growth stage V5. Average nitrogen concentrations for low and high controls, were 5.59% and 5.83%, respectively. BC05, BC09, BC10, BC12, BC13, BC14, BC15, and BC20 did not differ statistically significant from both the low and phosphate controls. At growth stage R3 the nitrogen concentration decreased as it did in the Brookings genotype and showed no statistical difference between the low phosphate and high phosphate controls, 5.06% and 5.28%, respectively. Isolate BC04 and BC07 are
statistically different than the high phosphate control whereas BC05 and BC09 are lower than the high phosphate control. This

Phosphorus concentrations of Codington plant tissue at V5, showed consistent results throughout the high and low controls and bacterial treatments. When, tissue was sampled at R3, the extended time gave the plants more time to take in phosphate and allowed the high control to reach a concentration of 0.47% and the low control was 0.39%, which was a small decrease. When looking at the isolate performances, BC20 was significantly higher than the low control and similar to the high control, whereas, all other isolates performed close to the low control.

Tissue sampling was variable and was more dependent on treatment type with specific endophytes performing better or worse than others. This type of variability and trend was also seen in work done by Knoth et al. (25) in a field setting. Nitrogen concentration in poplar also showed similar treatment effects when grown in field settings after endophyte and microbial treatment in that there were high amounts of variability when tissue nitrogen concentration was measured. The nitrogen could have been redistributed throughout the plant such as the roots and stem and may not be as pronounced in leaf tissue. Work performed by Buckley et al. (26) showed the phosphorus applied in a foliar response, similar to what was performed in this experiment, showed no difference between different varieties of northern oat grass. Single strain inoculations do not yield as much growth promotion as do multi-strain mixtures (25), this could also affect our results and is something that we will have to look at in the future. Many bacterial strains have specific niches and tissue compartments that they inhabit inside of plants. These niches could account for variability of the nutrient concentrations in plant
tissue and could be another factor to study to see how nutrients are taken up and driven throughout the plant lifecycle (27).

Yield data from both genotypes showed the low control plants were under stress and showed differences to the high controls. For the Brookings variety, no isolate produced any significant yield increase compared to the low control. Highest yield obtained was after application of BC15, which had a yield of 57.9 bu/ac, an increase over the low control of 2%. However, some of the isolate inoculations showed a decrease in yield, on average, when compared the low control.

The second variety, Codington, again showed no significant yield increases over the low control. Only BC09, BC10 and BC15 had similar performance compared to the low phosphate control. All other isolates had negative effects on yield. Field locations and weather also can affect endophyte performance with soil chemistry and rainfall as well as humidity having positive or negative effects on endophyte growth promotion potential (28). For certain endophyte treatments it was found that lower rainfall amount would yield a larger growth increase (28). For our data, the month of July, rainfall near Brookings, SD was 9.54 inches, 6.29 inches higher than normal (29, 30). Further research is needed to ascertain how much of an impact weather and climate has on biological applications there is and to what extent the long-term effects are. Competition between other microbes in the soil as well as in planta and establishment and native microbiome competition could have an adverse effect on the inoculant when it is applied in a field setting (31). Arbuscular mycorrhizae and rhizobia bacteria also could have unforeseen interactions after endophytes are applied. All these factors can create a challenging
environment for inoculants to form successful establishments inside of plants and need to be studied in greater detail when developing a bioinoculant.

5.5 Materials and Methods

5.5.1 Surface Sterilization of Seeds:

Seeds were sterilized using a chlorine gas method described by Lindsey et. al. (32) using 96 mL of bleach and 4 mL of HCl. The containers were left for 12 h in a fume hood. Once, the seeds had been sterilized, they were stored in beakers that had been autoclaved to maintain surface sterility.

5.5.2 Root Architecture

The impact of the bacterial endophytes from Brassica carinata on early root growth of soybeans was measured for South Dakota State University varieties: Brookings, Codington, and Davison. Bacterial endophytes were grown for 24 h at 30°C and 200 rpm before being standardized to an OD₆₀₀ of 0.05 with PBS. Surface sterilized soybean seeds were inoculated with 3 µL of standardized bacterial suspension per seed, with 45 replications per endophyte. Two sheets of heavy weight germination paper measuring 60.96 cm x 15.24 cm were wet with 50 ml of distilled water. Fifteen inoculated seeds were planted 1.5 in from the long edge on one germination paper, maintaining equal spacing between seeds and then the other sheet was placed on top. The germination paper and seeds were carefully rolled, placed within a glass jar, and capped. The jars were placed within a growth chamber with 16 h light at 25°C and 8 h dark at 20°C for 7 d. Jar caps were removed after 5 d and the soybeans were allowed to freely
grow for 2 more days. After 7 days, seedling mass was measured, and roots were scanned into the computer for analysis of root architectural traits such as root length, surface area, and volume with the software WinRhizo.

5.5.3 Greenhouse Trial

Based on their in-vitro plant growth promoting capabilities (BC01, BC04-BC10, BC12-BC15, and BC20) and their impact on root architectural traits, the following endophytes (BC01, BC04-BC10, BC12-BC15, and BC20) were down-selected and tested with the soybean varieties Brookings and Codington. Bacterial endophytes were grown for 24 h at 30°C and 200 rpm before standardizing to an OD<sub>600</sub> of 0.05. The seeds were inoculated as described above with 3 µL per seed and allowed to grow 7 d before transplanting them in pots with about 1 inch of the hypocotyl below the surface. Fifteen replicates of each treatment were planted in a complete randomized experimental design. The growth substrate consisted of 10% soil, 20% perlite, and 70% sand by weight and was steam pasteurized and transferred to 3 L pots. In addition to the endophyte inoculated plants, a high and low nitrogen control (high nitrogen was 100% Hoagland’s solution and low nitrogen was 10% Hoagland’s solution) was used, all endophyte treatments were grown under low nitrogen conditions. Pots were watered 3 times a week by hand. Every 14 d, 250 ml of the respective nutrient concentration was applied to the media. After 49 d in pots (56 d old plants), roots and shoot tissues were harvest and dried at 70°C for 3 d before the biomass measurements were taken.
5.5.4 Field Trial

The soybean varieties Brookings and Codington were tested in the field under low phosphate supply conditions, with a natural concentration in the soil of 28 ppm (Olsen phosphate). Bacterial endophytes and a low phosphate control were grown under this low P supply conditions, and in addition a high phosphate control was used to which 104 g monoammonium phosphate per plot added for a theoretical phosphate concentration of 40 ppm. The endophytes for these field trials were down-selected based on the \textit{in-vitro} assays, and the impact on root architecture, and greenhouse trials. The following 10 isolates (BC04, BC05, BC07, BC09, BC10, BC12, BC13, BC14, BC15, and BC20 were used. The soybean plots were prepared in spring of 2018 with 1100 seeds per plot for a population density of 165,000 seeds per acre and inoculated with an OD$_{600}$ of 0.05 at 3 µL per seed. Treatments were arranged in a randomized complete block experimental design. Plant tissue sampling was taken at V5 (5 weeks after planting) and R3 (9 weeks after planting), sampling the newest fully developed trifoliate. Seeds were harvested in the fall of 2018 and yield metrics were collected.

5.5.5 Statistical Analysis

Root architecture experiments ($n = 45$) and greenhouse trials ($n = 15$) were analyzed using the Dunn’s non-parametric test from the FSA package (v0.8.27) in R (v3.6.2) at p < 0.1 without correcting for multiple comparisons (33). Tissue sample data was compared using Student’s t-test (5 samples per plot for 4 plots). Field harvest data ($n = 4$) was analyzed with the LSD test from the agricolae (v1.3-2) package in R (34).
5.6 Acknowledgements

We would like to thank the South Dakota State University Experiment Station for their continued support and aiding with field procurement. We would also like to thank Indigo Ag for their financial support and their assistance and encouragement throughout this project.
5.7 Figures

Figure 5.1: Root architecture percent changes from the control for soybean variety Brookings using 20 bacterial endophytes isolated from Brassica carinata. Asterisk (*) denotes significance at p < 0.1 using Dunn's nonparametric analysis without correcting for family-wise error.

Figure 5.2: Root architecture percent changes from the control for soybean variety Codington using 20 bacterial endophytes isolated from Brassica carinata. Asterisk (*) denotes significance at p < 0.1 using Dunn's nonparametric analysis without correcting for family-wise error.
Figure 5.3: Root architecture percent changes from the control for soybean variety Davison using 20 bacterial endophytes isolated from Brassica carinata. Asterisk (*) denotes significance at $p < 0.1$ using Dunn's nonparametric analysis without correcting for family-wise error.
Figure 5.4: Shoot and root biomass percent changes for soybean variety Brookings using 20 bacterial endophytes under greenhouse conditions. Asterisk (*) denotes significance at $p < 0.1$ using Dunn's nonparametric analysis without correcting for family-wise error.

Figure 5.5: Shoot and root biomass percent changes for soybean variety Codington using 20 bacterial endophytes under greenhouse conditions. Asterisk (*) denotes significance at $p < 0.1$ using Dunn's nonparametric analysis without correcting for family-wise error.
Figure 5.6: Nitrogen concentration in the newest fully developed trifoliate of Brookings soybean variety of 10 bacterial endophytes under low phosphate conditions (28ppm) within the field with a low phosphate control and a high phosphate control (40ppm theoretical). Letters signify significance of Least Significant Difference at $p < 0.1$.

Figure 5.7: Phosphorus concentration in the newest fully developed trifoliate of Brookings soybean variety of 10 bacterial endophytes under low phosphate conditions (28ppm) within the field with a low phosphate control and a high phosphate control (40ppm theoretical).
control and a high phosphate control (40ppm theoretical). Letters signify significance of Least Significant Difference at p < 0.1.

Figure 5.8: Brookings soybean variety yield of 10 bacterial endophytes grown under low phosphate (28ppm) conditions with a high (40ppm theoretical) and low phosphate control. Letter signify significance of Least Significant Difference at p < 0.1.
Figure 5.9: Nitrogen concentration in the newest fully developed trifoliate of Brookings soybean variety of 10 bacterial endophytes under low phosphate conditions (28ppm) within the field with a low phosphate control and a high phosphate control (40ppm theoretical). Letters signify significance of Least Significant Difference at p < 0.1.

Figure 5.10: Phosphorus concentration in the newest fully developed trifoliate of Codington soybean variety of 10 bacterial endophytes under low phosphate conditions (28ppm) within the field with a low phosphate control and a high phosphate control (40ppm theoretical). Letters signify significance of Least Significant Difference at p < 0.1.
Figure 5.11: Codington soybean variety yield of 10 bacterial endophytes grown under low phosphate (28 ppm) conditions with a high (40 ppm theoretical) and low phosphate control. Letter signify significance of Least Significant Difference at p < 0.1.
5.8 References


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CHAPTER 6: CHARACTERIZATION OF BACTERIAL ENDOPHYTES ISOLATED FROM THE OILSEED PRODUCING CROP BRASSICA CARINATA: PART 4 – APPLICATION OF ENDOPHYTES ON CORN

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6.1 Abstract

Endophytic bacteria isolated from the oilseed crop Brassica carinata have shown promise as plant growth promoting bacteria with characteristics of indole-3-acetic acid production, ability to grow on nitrogen free media and having gene amplification of 1-aminocyclopropane-1-carboxylate deaminase. Application to wheat and soybean had previously showed promise for nitrogen stress alleviation under greenhouse growing conditions. Genotypes 5126RR and 9714/G were used for short term root architecture and greenhouse assays. Genotype 5126RR showed significant increases in root length, surface area and volume by BC02 and BC12 by at least 23.0\%, while BC15 - BC17 significantly increased the seedling root mass, length, surface area and volume in 9714/G. Both genotypes were grown under both low and high nitrogen in the greenhouse. Genotype 5126RR was not significantly different from the control except for BC02 significantly decreasing shoot biomass under high nitrogen conditions (-19.17\%). Shoot biomass of 9714/G significantly increased by BC16 under high nitrogen and BC17 and BC18 under low nitrogen conditions and benefitted significantly in root biomass from the
inoculation of BC16, BC18, and BC20 under high nitrogen. Nitrogen and phosphorus concentration at V5 and tasseling in field trials did not deviate from the respective control significantly. Field trial yield was not significantly affected by the endophyte application, but under high nitrogen conditions BC06, BC13, and BC19 did increase yield by 4.33%, 5.75%, and 6.24% from the controls’ 237.85 bu/ac. These experiments indicate a positive trend in some *B. carinata* endophyte inoculations when applied to corn.

### 6.2 Introduction

The largest crop in the United States for 2019 was *Zea mays*, or corn. Corn was planted on 81.8 million acres which yielded 13.7 billion bushels (1). To maintain high yields, the South Dakota State University Extension office recommends applying 1.2 pounds of nitrogen for every bushel of corn yield goal (bushels per acre at harvest time) (2). This rule-of-thumb shows the high nitrogen requirements of corn, and these nitrogen requirements contribute largely to the costs of production (3). Of the nutrients that are applied to fields to maintain crop health, the intake of corn has been estimated as 40% while nitrogen runoff can be 50% of the total nitrogen applied (4-6). Run-off from nitrogen fertilizer applications can increase the number of algae in water, leading to oxygen deprivation for marine life (7, 8).

One option to decrease the needed fertilizer applied to fields is to transform corn with the genes needed for biological nitrogen fixation, however, this has not yet been successful and will take a longer time to develop. Another is the addition of cover crop rotations that prevent the run-off and leaching of nitrogen in to the surrounding environments (6). Also, the use of legume cover crops are a potential for nitrogen sources
of corn, but the benefit the cover crop contributes to corn yield is dependent on the type of legume being used (9). Cover crops can also have the potential to negatively impact the production of corn by decreasing the corn population through competition (10). In addition to these methods, bacterial endophytes pose a potential avenue of plant growth promotion.

Bacterial endophytes are bacteria that reside within plant tissue and do not cause harm to their host plant. Sometimes these bacteria are able to produce compounds that induce host defense systems, preventing pathogens or decreasing the levels of pathogens (11, 12). Plants such as sugarcane may derive up to 80% of their nitrogen from nitrogen fixing bacteria within roots, stems and leaves (3). Other crops such as wheat have shown benefits from the application of nitrogen fixing bacteria when the plants are grown under low nitrogen conditions (13). Nitrogen fixing bacteria also may produce plant hormones that promote cell elongation and cellular division, and the overproduction of indole-3-acetic acid can consequently increase nitrogen fixation (14). Throughout this study we determined the effectiveness of bacterial endophytes with plant growth promoting characteristics to alleviate nitrogen stress in corn under short in-vitro assays, in greenhouse, and in field trials.

6.3 Results

Using BLAST on the sequenced of 16S rRNA gene previously showed that 11 endophytes isolated from B. carinata were from the genus Bacillus (B. pumilis BC01, B. paralicheniformis BC02, B. pumilis BC07, B. pumilis BC08, B. subtilis BC10, B. pumilis BC13, B. cereus BC14, B. thuringiensis BC15, B. safensis BC16, B. safensis BC18, and
B. sp. BC20), 4 from the genus Enterobacter (BC03, BC04, BC05, and BC06) and 3 from the genus Pantoea (P. agglomerans BC09, P. agglomerans BC11, and P. sp. BC12). The genera Lysinibacillus (L. sp. BC17) and Xanthomonas (X. sacchari BC19) both each had 1 isolate of the 20 identified (15).

6.3.1 Root Architecture

Corn variety 5126RR showed an overall positive root growth after the inoculation with isolates that were isolated from Brassica carinata. Only the inoculation with BC03, BC04, BC05, and BC10 led to negative root responses when compared to the respective control (Figure 6.1). BC10 did, however, increase the overall seedling mass. Isolates BC02, BC07, and BC12 significantly increased the root length, surface area, and volume. BC15 through BC20 significantly decreased the root length but had only a minor negative or positive impact on other root characteristics.

Variety 9714/G had a very different response to isolate application than 5126RR. BC01, BC02 – BC12, BC14, BC19, and BC20 decreased seedling mass, root length, surface area, and volume compared to the control while the isolate BC13 and BC15, BC16, BC17, and BC18 increased all metrics (Figure 6.2). BC02 and BC03 decreased seedling mass but increased the volume of the roots, but BC02 decreased both root length and surface area. BC03 decreased root length but increased the root surface area. Max root length was observed after the inoculation with BC15. This isolate increased root length by 61% compared to the control.
6.3.2 Greenhouse Trial

Corn variety 5126RR showed overall larger responses under high nitrogen than under low nitrogen conditions in the shoot tissue (Figure 6.3). BC06 elicited a significant decrease in the shoot biomass under high nitrogen conditions and led to a 19% decrease in biomass. BC12 showed an increase of 21% over the control but this increase was not significant. Under low nitrogen conditions there weren’t any significant changes in biomass, however BC01 and BC14 led to a 17% and 14% increase, respectively. There were no significant changes in 5126RR root biomass from the application of the *Brassica carinata* isolates, but there was a negative trend by many of the isolates (Figure 6.4).

Variety 9714/G showed more positive responses after endophyte inoculation. In shoot biomass, BC03, BC06, and BC12 led to a negative response under high nitrogen conditions compared to the control, while BC16 caused a negative response under low nitrogen conditions (Figure 6.5). All other isolate/nutrient combinations resulted in an increased biomass compared to the control. BC16 significantly increased the shoot biomass under high nitrogen conditions, and BC17 and BC18 significantly increased the shoot biomass under low nitrogen conditions. BC16, BC18, and BC20 were also able to significantly increase root biomass under high nitrogen conditions (Figure 6.6). Eight of the 11 isolates had shown different responses under high and low conditions.

6.3.3 Field Trial

We examined the nitrogen and phosphorus concentrations in the corn tissues in the field trial. At V5, the nitrogen concentration in the low nitrogen treated plants did not differ (Figure 6.7). The average nitrogen concentration was about 4.2% for the low
nitrogen treated plants. High nitrogen treated plants showed a greater variation in the nitrogen concentration among the different isolate treatments, but none of the isolates differed from the controls. The highest concentration of nitrogen of 4.28% was observed after an inoculation BC19, and the concentration was statistically higher than after an inoculation with BC03, with a nitrogen concentration of only 3.92%. Phosphate concentrations within the corn tissue of the low nitrogen treatment ranged from 0.34% with BC14 to 0.38% in BC18 (Figure 6.8). BC14 and BC18 were statistically different using the LSD test at p < 0.1, but the other isolates are statistically similar to each other. Under the high nitrogen application treatment, BC13, BC18, and BC19 did not differ, but differed from BC15, which had a concentration was lower at 0.34%.

Tissue sampling at the tasseling stage of corn was the final sampling time for nutrient concentrations within plant tissue. Under the low and high nitrogen application treatments, the nitrogen concentration in the tissue was similar between all isolate treatments (Figure 6.9). The average concentration under low nitrogen fertilization was 3.25% and under high nitrogen applications was 3.29%. Phosphorus levels were also similar within the nitrogen applications, with all isolates grown under low nitrogen being similar to one another and all isolates grown under high nitrogen being similar to one another (Figure 6.10). Low nitrogen application resulted in an average phosphorus concentration of 0.29% at tasseling, and the high nitrogen application resulted in an average phosphorus concentration of 0.29% at tasseling. Low and high nitrogen applications were not compared, only within the nitrogen applications.

Yields of SP375 under low and high nitrogen conditions displayed differing levels of significance. Under low nitrogen conditions, all of the isolate applications were
statistically similar according to an LSD test at $p < 0.1$ (Figure 6.11). Even without significance, isolates BC13 and BC17 had shown about 6.5% increase in the average yield of the 4 plots, while BC03 slightly decreased the yield of SP375. For the high nitrogen treatment, all BC isolates were statistically similar to the control (Figure 6.12). However, BC19 had an average yield increase of 6.2% when compared to the control. BC03 also decreased the yield under high nitrogen conditions, decreasing 3.4% compared to the control.

6.4 Discussion

Bacterial isolates were applied to corn seeds and grown in-vitro to determine bacterial compatibility with two corn varieties were used, variety 5126RR and 9174G. These corn varieties are field corn varieties and are typically grown for cattle feed and animal consumption. The majority of endophytes had a positive effect on overall plant growth in the 5162RR variety. Only four caused negative growth responses in the roots. Several metrics were measured to determine the overall compatibility. Root mass was increased after BC10 was applied. Root length, surface area and volume improving when BC02, BC07, and BC12 were added. Isolates BC15-BC20 had little to no effect or a negative effect on root metrics. The variety of 9714G interacted differently with the applied endophytes. Several isolates had negative effects on the seedling mass, root length, surface area and volume when compared to the control. Isolates BC13 and BC15-BC18 showed increases in all metrics that were measured. This diverse effect could be caused from applying endophytes to different plant genotypes and those host systems not being primed for a specific bacteria strain being applied (16). Host specificity could also
play a role, in that the endophytes from carinata were selected and co-evolved with carinata and were primed for the specific plant species. After application to the corn varieties, these endophytes could have outcompeted other beneficial microbes and caused a decreased in plant growth or other negative impacts. Results from O’Brien et al (17) showed that while endophytes do show growth promotion in corn varieties, there is a large variable effect in and between bacterial strain application, not unlike what is shown in our research. Strain specific interactions or selection based on the plant-host could be involved in plant growth response. Our strains such as those of the *Bacillus* genus, often show similar effects on different corn genotypes which could mean that those genotypes could be favoring those specific strains and not others (17). BC15 showed a 61% increase in root length over the control. The decrease in seedling mass by BC02 and BC03 but increase in volume by these two isolates also suggests that non-native bacterial strains while at one point can have negative impacts on certain parts of the plant, they can also have positive impacts on growth of plants as well. Plants that have co-evolved with certain strains of endophytes, may have a better time with handing the influx of transient bacteria after application has occurred. This could be a reason why certain endophytes have both negative and positive effects in different plant parts when applied to non-host crops (18, 19).

During our greenhouse testing, the corn genotype 5126RR, responded in a larger capacity under high nitrogen conditions than low nitrogen, which was to be expected. With an increase in nitrogen fertilizer, the plant would show increased growth and the bacterial endophytes could have also utilized the additional nitrogen and could put more energy into other growth promotion abilities. BC06 led to a decrease in in shoot mass
when under a high nitrogen regime and decreased shoot growth 19% when compared to the control, whereas BC12 had an increase of 21% over the control when in a high nitrogen regime, however, this was not a significant. Low nitrogen regimes similarly showed no significant microbial effects on corn but BC01 and BC14 displayed a 17% and 14% increase over the control, respectively. A negative trend was seen in the genotype 5126RR after an application of endophytes under a low nitrogen regime, showing that these endophytes may require a certain amount of nitrogen to function and provide benefits to plants (20). On the other hand, variety 9714G responded more positively after endophyte application, with shoot biomass increases after an application of all endophytes, except BC03, BC06 and BC12, with BC16 showing negative responses under low nitrogen and not high nitrogen. BC17 and BC18 led to significant increases in shoot biomass under low nitrogen conditions whereas BC16 was indicative of root and shoot growth improvement when applied under low nitrogen and BC20 was able to increase root biomass under high nitrogen regimes.

Using the corn variety SP375, endophytes were applied in a field setting to determine how they would interact with naturally occurring microbes and under low nutrient supply conditions with low phosphate and low nitrogen. Nitrogen concentration in plants under low nitrogen was on average, 4.2% whereas for high nitrogen treated plants had a greater overall variation. BC19 had the highest concentration of nitrogen at 4.28% under high nitrogen conditions. This value was statistically different than the lowest value recorded of 3.92% when BC03 was applied. Phosphate concentrations with corn tissue had a range between 0.34% and 0.38% with BC14 and BC18, respectively. BC14 and BC18 were different after statistical analysis but all other isolates showed no
significant differences after application. When high nitrogen was applied, several isolates had statistically different effects than the control, which were BC13, BC 18 and BC19, which were different than BC15, with a lower concentration of 0.34%. Nitrogen accumulation in corn plants from early in the season could be one reason why the tissue concentration values are very similar to each other and no significant effects occur (21).

When sampling at the tasseling stage, the low and high nitrogen application were observed to be similar between treatments with an average of 3.25% and 3.29% nitrogen concentration under low and high, respectively. These similar amounts could be because of the plant starting to decrease its input of nitrogen into reproduction and that the plant could have stored nitrogen in the early season, and it does not need to take anymore nitrogen in for plant processes (21, 22). This same theme also happens when looking at phosphate levels in plant tissue, with an average of 0.29% at both low and high nitrogen application levels.

Corn yield data of SP375 showed varying levels under low and high nitrogen regimes. Under low nitrogen conditions, all isolates were similar in yield, however, isolates BC13 and BC17 showed a 6.5% increase over the average yield with BC03 showing a decrease in yield. High nitrogen treatments were again statistically similar to each other, however, BC19 had a 6.2% increase over the control and BC03 had a decrease in yield of 3.4% when compared to the control.

Microbial competition as well as plant-microbe compatibility could have been reasons why these isolates had varying performances when applied in a field setting (23). This also can be traced back to in-vitro testing and greenhouse assays. Endophyte origins could also affect how well the inoculum performs, for instance, if an endophyte is from a
nutrient rich area and is then applied to a nutrient poor area, it may not perform as expected with it having evolved in a nutrient rich area (20, 24). This may not show up in in-vitro testing but later down pipelines such as greenhouse and field testing. More research into how non-native host endophytes effect new-host plants, where endophytes originated from and how endophytes co-evolved with plant-hosts will be useful to aid in the search and production of bio-inoculants.

6.5 Materials and Methods

6.5.1 Surface Sterilization of Seeds

Seeds were sterilized using a chlorine gas method described by Lindsey et. al. (25) using 96 mL of bleach and its reaction with 4 mL of HCl. Containers were left for 12 h in a fume hood. Once, the seeds had been sterilized, they were stored in beakers that had been autoclaved to maintain surface sterility.

6.5.2 Root Architecture

BC endophyte (15) influence on the root architecture of corn cultivars 5126RR and 9714-G was measured using the software WinRhizo after 600 dpi root scans of seedlings. The seedlings were inoculated with 24 h old endophyte cultures that were standardized to an optical density at 600nm of 0.05 with PBS, with 3 μL used per surface sterilized seed. For each endophyte, 45 replications were performed per genotype. Inoculated seeds were placed between 2 sheets of heavy weight germination paper measuring 60.69 cm x 15.24 cm, 3.81 cm from the long edge and even spacing between 15 seeds. This was repeated 3 times for a total of 45 seeds per endophyte that was tested.
The germination paper was rolled and secured lightly with a rubber band to prevent the germination paper from unrolling. The 3 sets of 15 seeds rolled in germination paper were placed within a 6 cm section of PVC 12 cm long with a capped bottom, the top was left open. PVC sections were placed inside a growth chamber that maintained 25°C for the 16 h light cycle and 20°C for the 8 h dark cycle. Seedling mass, root length, surface area, and volume were measured after 4 d of growth.

6.5.3 Greenhouse Trial

Endophytes selected based on root architecture and *in-vitro* plant growth promoting capabilities were grown for 24 h at 30°C and 200 rpm before being standardized to OD$_{600}$ of 0.05. For corn variety 5126RR, isolates BC01, BC02, BC03, BC04, BC06 and BC07, BC09 and BC10, BC12, BC15, and BC18 were chosen for greenhouse trials alongside corn variety 9714-G with BC03, BC06, and BC12-BC20 selected based on combination of root architecture results and in-vitro capabilities. The growth substrate was prepared with 70% sand and 30% perlite by volume and pasteurized to decrease the microbial population and then filled in 1 L volume cone pots. Surface sterilized seeds were inoculated with 3 µL of standardized bacterial suspension. Fifteen replications were planted for each endophyte with 3 seeds under low and high nitrogen conditions (low based on 10% Hoagland’s solution and high based on 100% Hoagland’s solution) in a complete randomized experimental design. Control plants were coated with sterile PBS solution. Plants were thinned after 3 weeks to one per pot and 2 mL of nutrients were provided once a week for 5 weeks then 20 mL was provided each week for
4 wks. After 91 d plants were harvested and dried for 3 d at 70°C the biomass was measured.

6.5.4 Field Trial

Bacterial endophytes were grown for 24 h at 30°C and 200 rpm and then standardized to an OD$_{600}$ of 0.05 with PBS solution. Plots measured 3.048 m x 6.096 m and prepped with 250 sds/plot (for a population density of 32,000 sds/ac) and inoculated with 3 µL of standardized cell suspension per seed using PBS as the control. The corn variety used was SP375 and was subjected to low and high nitrogen conditions with 75 lb N/ac or 150 lb N/ac, respectively. A randomized complete block design with 4 replications per treatment was planted in spring 2018. Tissue samples were taken at V5 (6 weeks after planting) and tasseling (10 weeks after planting) of newest fully developed leaf for elemental composition and plants were harvested in the fall of 2018 measuring yield and test weight. Buctril (Bayer Crop Science) was applied in early June and further weed management was handled with gardening hoes.

6.5.5 Statistical Analysis

Root architecture experiments (n = 45) and greenhouse trials (n = 15) were analyzed using the Dunn’s non-parametric test from the FSA package (v0.8.27) in R (v3.6.2) at p < 0.1 without correcting for multiple comparisons (26). Tissue sample data was compared using Student’s t-test (5 samples per plot for 4 plots). Field harvest data (n = 4) was analyzed with the LSD test from the agricolae (v1.3-2) package in R (27).
6.6 Acknowledgements

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6.7 Figures

5126RR Root Architecture

Figure 6.1: Root architecture percent changes from the control for corn variety 5126RR using 20 bacterial endophytes isolated from Brassica carinata. Asterisk (*) denotes significance at p < 0.1 using Dunn's nonparametric analysis without correcting for family-wise error.

9714/G Root Architecture

Figure 6.2: Root architecture percent changes from the control for corn variety 9714/G using 20 bacterial endophytes isolated from Brassica carinata. Asterisk (*) denotes significance at p < 0.1 using Dunn's nonparametric analysis without correcting for family-wise error.
Figure 6.3: Shoot biomass percent changes for corn variety 5126RR using 11 bacterial endophytes under greenhouse conditions. Asterisk (*) denotes significance at p < 0.1 using Dunn's nonparametric analysis without correcting for family-wise error.

Figure 6.4: Root biomass percent changes for corn variety 5126RR using 11 bacterial endophytes under greenhouse conditions. Asterisk (*) denotes significance at p < 0.1 using Dunn's nonparametric analysis without correcting for family-wise error.
Figure 6.5: Shoot biomass percent changes for corn variety 9714/G using 11 bacterial endophytes under greenhouse conditions. Asterisk (*) denotes significance at $p < 0.1$ using Dunn's nonparametric analysis without correcting for family-wise error.

9714/G Greenhouse Shoot

Figure 6.6: Root biomass percent changes for corn variety 9714/G using 11 bacterial endophytes under greenhouse conditions. Asterisk (*) denotes significance at $p < 0.1$ using Dunn's nonparametric analysis without correcting for family-wise error.

9714/G Greenhouse Root
Figure 6.7: Nitrogen concentration in the newest fully developed leaf at V5 of corn variety SP375 of 10 bacterial endophytes under low (75lbs nitrogen/acre) and high (150lbs nitrogen/acre) under field conditions with a control. Letters signify significance of Least Significant Difference at p < 0.1.

Figure 6.8: Phosphorus concentration in the newest fully developed leaf at V5 of corn variety SP375 of 10 bacterial endophytes under low (75lbs nitrogen/acre) and high (150lbs nitrogen/acre) under field conditions with a control. Letters signify significance of Least Significant Difference at p < 0.1.
Figure 6.9: Nitrogen concentration in the newest fully developed leaf at tasseling of corn variety SP375 of 10 bacterial endophytes under low (75lbs nitrogen) and high (150lbs nitrogen/acre) under field conditions with a control. Letters signify significance of Least Significant Difference at p < 0.1.

Figure 6.10: Phosphorus concentration in the newest fully developed leaf at tasseling of corn variety SP375 of 10 bacterial endophytes under low (75lbs nitrogen) and high (150lbs nitrogen/acre) under field conditions with a control. Letters signify significance of Least Significant Difference at p < 0.1.
Figure 6.11: SP375 yield for 10 bacterial endophytes grown under low nitrogen conditions (75lbs nitrogen/acre). Letters signify significance of Least Significant Difference at p < 0.1.

Figure 6.12: SP375 yield for 10 bacterial endophytes grown under high nitrogen conditions (150lbs nitrogen/acre). Letters signify significance of Least Significant Difference at p < 0.1.
6.8 References


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CHAPTER 7: METHYLORUBRUM ENDOPHYTICA SP. NOV., A NOVEL
ENDOPHYTE ISOLATED FROM SOYBEAN SEEDS

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7.1 Abstract

A novel bacterial endophyte, strain Q1 was isolated from soybean seeds. The strain is a Gram-variable facultative aerobe, rod-shaped motile bacterium with polar flagella. Genome sequencing produced 123 contigs that were used to identify Methylobacterium sp. AMS5 as the closest known relative with an 98.91% average nucleotide identity (ANI). However, aligning the trimmed reads of Q1 to the AMS5 genome, demonstrated several large-scale deletions of up to 25 kbp in the Q1 genome in relation to AMS5. With respect the whole genome, the 123 contigs of Q1 and Methylobacterium sp. AMS5 share only 93.60% sequence identity. Consistently, a phylogenetic tree based on 16S rRNA gene sequences showed that Q1 is most closely related to AMS5. Several species of the Methylobacterium genus have recently been reclassified as Methylorubrum, and the chemotaxonomic profile of Q1 was compared to 4 Methylorubrum species that were selected based on the similarity in their 16S sequence. The chemotaxonomic phenotype of Q1 is most closely related to Methylorubrum thiocyanatum ATCC 700647, but shows distinct differences in carbon utilization as well as chemical sensitivity assays. Based on
the results of the genomic sequence analysis, the 16S phylogeny, and the chemotaxonomic profile, we propose *Methylorubrum endophytica* sp. nov. Q1 as a novel species within the *Methylorubrum* genus, and reclassify and rename *Methylobacterium* sp. AMS5 as *Methylorubrum* sp. AMS5.

### 7.2 Introduction

Bacterial endophytes are bacteria that reside within plant tissues and do not cause harm to their host. Many endophytes have plant-growth promoting characteristics, and for example improve the nutrient uptake of their host through their ability to fix gaseous nitrogen or solubilize phosphate, suppress the growth of plant pathogens and increase the resistance of plants against environmental stresses such as drought or high salinity (1-3). Endophytic bacteria have been isolated from many different plant tissues, including the roots, stems, leaves, and seeds, and from a variety of different plant species, including many agronomically important species such as tomato, wheat, and soybean (1, 2, 4, 5). For example, from soybean different bacterial species of the *Enterobacter*, *Pantoea*, *Staphylococcus*, and *Methylobacterium* genus have been isolated (6, 7).

The genus *Methylobacterium* was first described in 1976 (8, 9) by Patt and coworkers and was proposed to encompass Gram-negative bacteria that form pink, circular colonies and can use C1 compounds such as methanol as sole carbon and energy source, although the ability to grow on methane has not been confirmed for all species (8, 9). The genus *Methylobacterium* is classified in the family Methylobacteriaceae and the order Rhizobiales, and some species can reside within root nodules and to fix gaseous nitrogen in legumes (10). However, Methylobacteria have also been isolated from other plants such as poplar trees and *Arabidopsis* (11, 12). Since *Methylobacterium*’s original
In 1976, many species were added to the genus with an increasing variation in the 16S rRNA gene sequence. Due to the growing variation of the 16S rRNA gene sequences within the genus, 11 species that had been originally classified under the genus *Methylobacterium*, were recently reclassified and combined in the new genus *Methylorubrum* (9). The reclassified species include *Methylorubrum aminovorans* comb. nov., *Methylorubrum extorquens* comb. nov., *Methylorubrum podarium* comb. nov., *Methylorubrum populi* comb. nov., *Methylorubrum pseudosasae* comb. nov., *Methylorubrum rhodesianum* comb. nov., *Methylorubrum rhodinum* comb. nov., *Methylorubrum salsuginis* comb. nov., *Methylorubrum suomiense* comb. nov., *Methylorubrum thiocyanatum* comb. nov., and *Methylorubrum zatmanii* comb. nov. (9).

In this study, we used genomic and phylogenetic tools and biochemical tests to compare the proposed novel new species *Methylorubrum endophytica* sp. nov. to six other different bacterial strains, four strains from the *Methylorubrum* genus, *Methylorubrum extorquens* ATCC 43645 (13, 14), *Methylorubrum thiocyanatum* ATCC 700647 (15), *Methylorubrum aminovorans* ATCC 51358 (16), *Methylorubrum rhodesianum* ATCC 43882 (17), and two other strains from the order Rhizobiales, *Sinorhizobium meliloti* Rm1021, and *Bradyrhizobium japonicum* USDA110.

### 7.3 Isolation and Ecology

*Methylorubrum endophytica* Q1 was isolated from field grown soybean seeds at the end of the growing season 2016 close to White, South Dakota, United States (44.384672, -96.571701). The seeds were surface sterilized as follows: 1 min in 70% ethanol, 30 s in sterile water, 5 min in 5% bleach, 1 min in 70% ethanol, followed by 4 successive 30 s wash steps with sterile water. After surface sterilization, the seeds were
crushed and isolated on nitrogen-free semi-solid malate (NFb) medium as previously described (18) in 5 replicates. After a pellicle had formed, the bacteria were streaked onto NFb plates with 15 g L⁻¹ noble agar and incubated at 30°C for 5 days. Isolated colonies were sub-cultured to form pure cultures that were then stored in 65% glycerol at -80°C for preservation.

7.4 16S RNA Phylogeny

For initial 16S rRNA sequencing, a bacterial culture was incubated in Lysogeny broth (LB) for 5 d at 200 rpm. The culture was then prepared in a 17.5% glycerol stock (100 µL bacterial suspension: 100 µL 35% glycerol) and the 16S rRNA was sequenced by GeneWiz (South Plainfield, NJ, USA) using their proprietary primers and the primer 375F. BLASTn (19) was used to identify the original sequence and the search resulted in several hits with high query coverage and identity (Table 7.1). The BLASTn search revealed 3 sequences with a 99% query cover (Methylorubrum extorquens strain CM4 and two sequences of Methylorubrum populi BJ001), and 10 other strains of six different Methylorubrum species, including M. thiocyanatum, M. zatmani, M. rhodesianum, M. aminovorans, M. suomiense, and M. podarium that all had a 99% sequence identity to the 16S rRNA sequence of M. endophytica Q1. Four of these species, M. extorquens (ATCC 43645), M. thiocyanatum (ATCC 700647), M. aminovorans (ATCC 51358), and M. rhodesianum (ATTC 43882) were available through the American Type Culture Collection (ATCC, Manassas, VA, USA) and were later used as reference strains for the Biolog analysis (see below). Additionally, two other representatives of the order Rhizobiales were used as outgroups, Sinorhizobium meliloti Rm1021 (Family Rhizobiaceae), and Bradyrhizobium japonicum USDA110 (Family Bradyrhizobiaceae).
7.5 Genome Features

We isolated genomic DNA following the genomic DNA protocol (20) for bacteria and amended the protocol by adding 0.5 µL of 100 mg mL⁻¹ RNase with 10% SDS and proteinase K and 3 DNA wash steps with 70% ethanol. All centrifugation steps were conducted at 4°C. We determined the quality of the extracted DNA through 1% agarose gel electrophoresis. The Genomic DNA library was made using the Nextera DNA Flex Library Prep kit (Illumina, Inc., San Diego, CA, USA). Sequencing was completed using Illumina’s MiSeq platform and 2 x 300 bp paired end reads. Raw sequences were downloaded from Illumina’s BaseSpace and uploaded to Galaxy (www.usegalaxy.org) (21) for analysis using the Next Generation Sequencing modules FastQC v0.72 for quality control (22), Trimmomatic v0.36.5 for adapter trimming and to eliminate reads below Q25 (paired end reads) (23), and Unicycler v0.4.1.1 for genome assembly (24). The final assembly of Q1 was submitted to GenBank under the accession number SRHQ00000000.

In total, 4,191,170 reads were generated. Since the first 20 nt of the raw sequences contained poor per base sequence content, we removed the adapter sequences, performed a headcrop of 15 nt, and then used a sliding window of 4 bp to trim sequences below a quality of 25 bp by Trimmomatic. The trimmed reads totaled 3,949,548 with an average quality between 22 and 38 bp. An assembly was created that excluded contigs shorter than 100 bp in length by using Unicycler with paired end data.

The quality of the assembly was assessed by QUAST v4.6.3 (25), and it consisted of 123 contigs, 22 of the contigs were greater than 100 kbp and the longest contig was 439,294 bp in length (Table 7.2). The GC content of the Q1 genome is 68.39%. We
performed the annotation of the assembled contigs using the Prokka v1.12.0 pipeline (26, 27) set to a minimum contig length of 200 bp (Table 7.2). In total, 117 of the 123 contigs with a total length of 5,525,230 bp were used for the assembly. This genome length was estimated with Jellyfish v2.2.3 (28), and suggested a full length between 5,688,789 bp and 5,693,451 bp with between 306,244 bp and 306,495 bp of repeats. In contrast, *Methylobacterium sp. AMS5* (7) (top result using BLASTn of the nucleotide database) has a fully assembled genome of 5,599,206 bp, which is about 89,000 bp shorter than the minimum genome haploid length predicted by Jellyfish for the Q1 genome and about 74,000 bp longer than the genome assembly generated by the Prokka pipeline. The length of *Methylorubrum extorquins* AM1 (29) is 5.51 Mbp (68.0% GC content) and *Methylorubrum populi* BJ001 has a length of 5.85 Mbp (69.4% GC content), indicating that the predicted complete genome length of 5.69 Mbp for Q1 is within the reported genome length for other *Methylorubrum* species.

The Prokka assembly revealed 50 tRNA, 3 rRNA, 1 tmRNA, and 4,985 gene coding sequences (CDS). Following assembly, the contigs were uploaded to gVolante for Basic Universal Single-Copy Ortholog (BUSCO) analysis using BUSCO v1 for bacteria (30). All 40 single-copy ortholog genes were detected within Q1’s sequenced genome. According to the Prokaryota subcategory on the SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST) server (31), the genome of Q1 has 53 unique genes that encode proteins involved in flagellar motility.

We conducted a phylogenetic analysis by using the top 20 BLASTn results of the full 16S rRNA gene of Q1, along with *Methylobacterium sp. AMS5* from the Nucleotide collection (nr/nt) database, and *Escherichia coli* strain NW_A26 as an outgroup. Using
the nucleotide database, the 16S rRNA gene from Q1 is 99% identical with *Methylobacterium sp. AMS5*, but this similarity is not confirmed when the 16S ribosomal RNA database is used, since AMS5 does not have a 16S rRNA gene accession. The phylogenetic tree shows that Q1 and *Methylobacterium sp. AMS5* cluster together and form a distinct branch that differs from the 16S rRNA sequences of other bacterial species such as *Methylorubrum zatmanii DSM 5688* and those that cluster with *Methylorubrum extorquens IAM 12631* (Figure 7.1).

Since the whole genome of *Methylobacterium sp. AMS5* has a 100% query coverage to the 16S rRNA sequence of Q1 with 99% identity, we aligned the contigs of *Methylorubrum endophytica Q1* with the *Methylobacterium sp. AMS5* genome. The alignment was completed using NUCmer within the MUMmer pipeline (32) with *Methylobacterium sp. AMS5* as the reference genome and the Q1 contigs as query. There was a high similarity between *Methylobacterium sp. AMS5* and Q1, but the genome of Q1 showed distinct deleted regions (Figure 7.2).

When the Q1 contigs are blasted against the whole genome of *Methylobacterium sp. AMS5*, Q1’s genome covers 93.60% of the *Methylobacterium sp. AMS5* genome using 105 contigs with E-values less than 1E-50. The genome was also uploaded to Microbial Genomes Atlas (MiGA 0.3.6.2 - tinge) webserver for genome relatedness, and the analysis revealed an average nucleotide identity (ANI) of 98.78% and average amino acid identity (AAI) of 97.58% with 88.78% of proteins shared between Q1 and AMS5 (33). According to the NCBI Prok project, the taxonomic species classification of Q1 is *Methylobacterium sp. AMS5* (p-value equal to 0.0016), with the subspecies p-value equal to 0.0519, indicating that the genome sequence of Q1 is similar to AMS5. The average
nucleotide identity (ANI) for Q1 and AMS5 is 98.78%, however, the fraction of the genome shared with AMS5 is only 88.3%.

To identify large sequence differences between the genomes of Q1 and *Methylobacterium sp.* AMS5, the raw reads from Q1 were aligned to *Methylobacterium sp.* AMS5 as the reference sequence allowing large gaps under Transcript Discovery in CLC Genomics Workbench 11 (Qiagen Redwood City, Redwood City, California, USA). Of the 4,191,170 total reads for the *Methylorubrum endophytica* Q1 genome, only 2,730,534 of the paired reads and 85,841 of the single reads mapped to the reference genome. The large gap in mapping to *Methylobacterium sp.* AMS5 shows that several regions as large as 25,000 bp are deleted from the Q1 genome (Fig. 2). In addition to these large deletions, the fact that not all contigs of Q1 can be mapped to the genome of *Methylobacterium sp.* AMS5 suggests that Q1 and *Methylobacterium sp.* AMS5 do not belong to the same species.

7.6 **Physiology and Chemotaxonomy**

The colonies of Q1 are small, circular, raised red colonies with entire margins, smooth, and approximately 0.5 mm to 1.0 mm in diameter after 6 d of growth on tryptic soy agar (TSA) or nutrient agar (NA) at 30°C (Figure 7.3). Growth on TSA or NA at 30°C is better than on NFb, with growth slowing as the temperature decreases. The cells of *Methylorubrum endophytica* sp. nov. Q1 are Gram-variable rods in singles and doubles with most stain as Gram-negative, but some stain as Gram-positive. Also, other species of the *Methylobacterium* genus do not stain well and can appear gram-variable (34). The bacterial cells are able to tolerate a pH of 6, but do not grow at a pH of 5 or lower. *Methylorobrum endophytica* sp. nov. is able to tolerate NaCl concentrations greater than
0.5% (e.g. in TSA), but shows no growth at 1% (see Biolog Gen III results below). The bacterial cells are motile, most likely through flagella as indicated by microscopic observations and the presence of flagellar motility genes in the genome of Q1.

A panel of 94 biochemical tests were used to chemotaxonomically profile 7 different bacterial strains: the proposed novel species *Methylorubrum endophytica* Q1, *M. extorquens* (ATCC 43645, 13), *M. thiocyanatum* (ATCC 700647, 15), *M. aminovorans* (ATCC 51358, 16), *M. rhodesianum* (ATCC 43882, 17), *Sinorhizobium meliloti* Rm1021 (35), and *Bradyrhizobium japonicum* USDA110 (10, 36, 37). We selected these *Methylorubrum* species for the Biolog analysis since they were deposited at culture collections, and are close relatives based on the phylogenetic analysis (Figure 7.1), while *Sinorhizobium meliloti* and *Bradyrhizobium japonicum* were used as other representatives of the Rhizobiales and as outgroups. The Biolog Gen III Microplate (Biolog, Inc., Hayward, California, United States) contains 71 carbon source utilization assays and 23 chemical sensitivity assays.

The plates for all bacteria were grown in Inoculation Fluid C according to the Biolog recommendations up to a 65% turbidity for the *Methylorubrum* species and a 95% turbidity for *Bradyrhizobium japonicum* USDA110 and *Sinorhizobium meliloti* Rm1021 (due to the differences in generation times). All plates were incubated at 30°C for 5 d. Every 24 h, the plates were read with a plate reader at a wavelength of 590 nm and 750 nm. To reduce optical interference, we calculated a dual wavelength O.D. (DW) by subtracting the 750 nm reading from the 590 nm reading. Following, the negative control was subtracted from each well value (setting the negative control well to a value of zero). All wells with an OD of ≥ 30% of the positive control were considered as positive, and <
30 \% as negative (Table 7.4). Q1 shows similar chemotaxonomic characteristics than the other *Methylorubrum* species but differs substantially from the two rhizobia species *Sinorhizobium meliloti* Rm1021 and *Bradyrhizobium japonicum USDA110*.

The results of the Biolog Gen III Microplate of all 7 bacteria were imported into the statistical program R and a nonmetric multi-dimensional scaling was plotted to visualize the data with the MASS package (38) (Figure 7.4). Nonmetric multi-dimensional scaling converts many parameters, in this case biolog results, into distances of X and Y coordinates that can be plotted. Q1 clusters near the other *Methylorubrum* species, between *Methylorubrum thiocyanatum* ATCC 700647 and *Methylorubrum aminovorans* ATCC 51358, but differs clearly from both rhizobia species (Figure 7.4a). When both rhizobia species are removed, it becomes obvious that there are also distinct chemotaxonomic differences between Q1 and the other *Methylorubrum* species (Figure 7.4b). For example, while *M. thiocyanatum* ATCC 700647 can use D-fructose and L-aspartic acid as a carbon source and is able to grow in the presence of 1\% NaCl, Q1 is unable to grow. By contrast, Q1 is able to use alpha-D-glucose, glycerol, L-lactic acid, and D-serine as carbon sources, while *Methylorubrum thiocyanatum* ATCC 700647 is unable to use these carbon or nitrogen sources. After removing the rhizobia strains and running the analysis again, Q1 is most closely related to *Methylorubrum aminovorans* ATCC 51358. In contrast to Q1, *Methylorubrum aminovorans* is able to use D-melibiose, D-fructose, L-aspartic acid, and D-malic acid as carbon sources, and grow in 1\% NaCl, but is unable to use a-D-glucose as carbon source. This illustrates the distinct differences in the chemotaxonomic profiles between Q1 and *Methylorubrum thiocyanatum* ATCC 70064 and *Methylorubrum aminovorans* ATCC 51358.
7.7 Proposal of Methylorubrum endophytica sp. nov.

Similar to *Methylobacterium sp.* AMS5, Q1 was isolated from a soybean seed, and when the 16S rRNA of Q1 is blasted in the nucleotide collection (nr/nt) database, *Methylobacterium sp.* AMS5 is identified as the closest relative. However, when the raw reads of the Q1 genome are aligned to the AMS5 genome, the Q1 genome shows distinct deletions of 6.4% from the AMS5 genome. These deletions are clearly not the results of a missing coverage in these areas, but rather regions where reads must be interrupted for up to 25 kbp to be mapped to the AMS5 reads. We also used chemotaxonomic profiling with the Biolog Gen III system to determine the similarity of Q1 to reference strains that are closely related based on their 16S rRNA sequence. The biochemical results show similarities of the chemotaxonomic profile of Q1 with other *Methylorubrum* species, but also shows distinct differences. Due to the similarities and differences in the chemotaxonomic and genomic results to other *Methylorubrum* spp. and *Methylobacterium sp.* AMS5, we propose to name the new isolate *Methylorubrum endophytica* and to reclassify *Methylobacterium sp.* AMS5 to *Methylorubrum endophytica* AMS5.

7.8 Description of Methylorubrum endophytica sp. nov.

*Methylorubrum endophytica* sp. nov. (*en.do.phy’ti.ca* Gr. pref. *endo* within; *Gr. n. phuton* plant; *L. fem. suff. -ica* adjectival suffix used with the sense of belonging to; *N.L. fem. adj. endophytica* within plant, endophytic)

*Methylorubrum endophytica* is a Gram-variable rod-shaped bacterium in the order Rhizobiales that was isolated from a seed of *Glycine max* harvested near White, South Dakota, USA. It grows well on tryptic soy agar and nutrient agar between 23°C and 30°C
under aerobic conditions, and forms pink colonies from 0.5 mm to 1.0 mm in diameter after 6 d of growth. The bacterium is motile when viewing on wet-mount slides through flagella and as indicated by the presence of flagella motility genes in the genome. *Methylorubrum endophytica* shows resistance against the protein biosynthesis inhibitors troleandomycin, minocycline, and lincomycin, against the bacterial DNA-dependent RNA synthesis inhibitor Rifamycin SV, and against the cell wall biosynthesis inhibitors vancomycin and azetreonam. Although there is a high tolerance to antibiotics, cells do not grow well in more acidic environments below pH 6 or in the presence of NaCl at 1% or higher. Compared to the chemotaxonomic phenotype of other rhizobia, *M. endophytica* is unable to use a broad range of different carbon sources. However, Q1 is able to use alpha-D-glucose, glycerol, methyl pyruvate, L-lactic acid, a-keto-glutaric acid, L-malic acid, bromo-succinic acid, b-hydroxy-D,L-butyric acid, propionic acid, acetic acid, and formic acid as primary carbon sources. The assembled genome has an estimated length of 5.69 Mb with 50 tRNAs, 3 rRNA, 1 tmRNA, and 4,985 protein coding sequences, and a GC content of 68.39%.

### 7.9 Protologue

**Repositories:** The genome sequence of *Methylorubrum endophytica* Q1 has been deposited to DDBJ/EMBL/GenBank and is publicly available under the accession number SRHQ00000000, and the BioSample number: SAMN11281792. The strain has been deposited to the U.S. Department of Agriculture Agricultural Research Service (ARS) Culture Collection under the number NRRL B-65550.
7.10 Author Statements

All authors approved the submission of this manuscript to the International Journal of Systematic and Evolutionary Microbiology. The authors contributed as follows: AS, YQ, TVDT, JP, and BD conducted the experiments, AS, YQ, HB, and JGH conceptualized the project, analyzed the experiments, validated the results, and wrote, reviewed and edited the manuscript, HB and JGH supervised the experiments, and were responsible for the acquisition of the financial support.

7.11 Acknowledgements

The authors would like to acknowledge Katelyn Graber, Lok Joshi, Delayna Paulson, Laura Jackson, Lance Merrick, Jessica Gomes Noll, Gitanjali Nanda Kafle for technical assistance.
7.12 References


7.13 Figures

Figure 7.1: Maximum-likelihood phylogenetic tree created using the maximum-likelihood method with the top 20 results in the 16S ribosomal RNA sequences database of BLASTn, the top hit (Methyllobacterium sp. AMSS5) in the nucleotide collection database, and Escherichia coli strain NW_A26 as the outgroup. Values displayed are bootstrap percentages calculated from 1000 bootstrap resamples of the data set. Only bootstrap values greater than 50% are displayed. Asterisks indicate the type strains that were used for the Biolog trials (Methylorubrum extorquens TK 0001 = ATCC 43645, Methylorubrum thiocyanatum DSM 11490 = ATCC 700647, and Methylorubrum aminovorans JCM 8240 = ATCC 51358).
Figure 7.2: Raw read mapping of the Q1 genome in comparison to the whole genome sequence of *Methylobacterium sp. AMS5*. The *Q1 genome* displays distinct regions that are deleted with respect to the reference strain.
Figure 7.3: Size of Q1 colonies after 6 d on tryptic soy agar, incubating at 30°C.
Figure 7.4: Nonmetric multi-dimensional scaling of the Biolog Gen III results for (a) Q1 strain, *Methyloburum extorquens* ATCC 43645 (M.ext.), *Methyloburum thiocyanatum* ATCC 700647 (M.thi.), *Methyloburum aminovorans* ATCC 51358 (M.ami.), *Methyloburum rhodesianum* ATCC 43882 (M.rho.), *Sinorhizobium meliloti* Rm1021 (S.mel.), and *Bradyrhizobium japonicum* USDA110 (B.jap.), and (b) Q1 strain and the 4 *Methyloburum* strains. The X and Y coordinates were calculated with the MASS package in R and plotted in Excel.
### 7.14 Tables

Table 7.1: Results for the BLASTn query for the 16S rRNA gene sequence of the novel bacterial species *Methylorubrum endophytica* Q1 against the 16S ribosomal RNA sequence database.

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<th>Description</th>
<th>Max Score</th>
<th>Total Score</th>
<th>Query Cover</th>
<th>E value</th>
<th>Ident</th>
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<td><em>Methylorubrum extorquens</em> strain CM4</td>
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Table 7.2: Assembly results of *Methylorubrum endophytica* sp. nov. Q1 using the Unicycler platform.

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Table 7.3: Annotation results of *Methylorubrum endophytica* sp. nov. Q1 using the Prokka pipeline.

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Table 7.4: Biolog Gen III Microplate results for the Q1 strain, *Methylorubrum extorquens* ATCC 43645 (M.ext.), *Methylorubrum thiocyanatum* ATCC 700647 (M.thi.), *Methylorubrum aminovorans* ATCC 51358 (M.ami.), *Methylorubrum rhodesianum* ATCC 43882 (M.rho.), *Sinorhizobium meliloti* Rm1021 (S.mel.), and *Bradyrhizobium japonicum* USDA110 (B.jap.). Positive results are shown as a plus (+) and negative results are shown as (-).

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**Notes:**
- '+' indicates sensitivity to the chemical.
- '-' indicates no sensitivity to the chemical.
Bacteria located within plant tissues away from the external environment (bacterial endophytes) pose a new avenue of plant growth promotion exploration that deviates from the typical increase in nutrient applications and pesticides to fields. Their potential to decrease these nutrients or pesticides lies in the wide range of capabilities that endophytes possess, even different strains of the same bacterial specie. Major focuses of endophytes lie in the ability of some to fix atmospheric nitrogen and solubilize rock phosphates in the soil since nutrient inputs into crops is growing in a time where many are becoming greenhouse gas conscious and phosphate supplies are predicted to diminish within the next century. Crops like *Brassica carinata* do not form associations with rhizobacteria or mycorrhizal fungi but may be benefitting from growth promotion capabilities by endophytic bacteria in plant tissues.

Through the previously mentioned assays and plant growth trials, endophytes from *Brassica carinata* have shown potential in increasing growth under highly controlled environments to causing shifts in plant response under greenhouse and field environments. The plant responses to an endophyte are particular to species, genotype, as well as nutrient applications where high and low nitrogen may elicit very different responses. There is some relationship between in-vitro plant growth promoting characteristics and short-term growth response (root architecture experiments), but with added complexity to plant systems in the greenhouse and field environments those relationships do not persist. As experiments are brought closer to ‘real-world’ conditions, endophyte benefits become more muted possibly due to greater competition of microbes or other environmental factors such as weather.
In this study, we explored the potential use of 20 bacterial endophytes isolated from *Brassica carinata* for their ability to increase plant growth of 3 different crop species, multiple genotypes of each, through different assays. While significant increases were not observed in yields for wheat, soybean, or corn, expansion of endophytes tested from *Brassica carinata* and other crops under field conditions may lead to the discovery of beneficial endophytes as well as novel bacteria. The exploration of endophytes from different sources may show higher levels of compatibility with host species over non-host species.