Université de Montréal

Endophytes of commercial Cranberry cultivars that control fungal pathogens

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Ce mémoire intitulé(e)

Endophytes of commercial Cranberry cultivars that control fungal pathogens

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Résumé

Les endophytes sont des microorganismes (généralement des bactéries et des champignons) qui vivent dans les tissus végétaux mais n'activent pas le système immunitaire/défense des plantes, contrairement aux pathogènes végétaux qui activent généralement les réponses immunitaires des plantes. Des recherches récentes ont montré que pratiquement toutes les plantes cultivées en plein champ contiennent un certain nombre d'endophytes, et que certains endophytes stimulent la croissance des plantes et renforcent la résistance contre les agents pathogènes. Les endophytes sécrètent des composés chimiques (métabolites secondaires) qui suppriment la croissance des agents pathogènes, un processus connu sous le nom de biocontrôle. En raison de ces propriétés de biocontrôle, les endophytes sont une alternative potentielle aux pesticides chimiques pour lutter contre les maladies des plantes. En conséquence, le biocontrôle est devenu un domaine de recherche important.

Mon projet de recherche comportait les objectifs spécifiques suivants : (i) isoler les endophytes des plants de canneberges acquis auprès de deux producteurs commerciaux de canneberges de la variété Stevens situés au Québec, Canada (Bieler Cranberries Inc, et Gillivert Inc.) ; (ii) tester l'activité de biocontrôle des endophytes contre une collection de champignons pathogènes et ensuite inoculer les endophytes les plus actifs dans des plants de canneberges obtenus par germination de la variété Stevens (Bieler Cranberries Inc.) et Scarlet Knight (Daniele Landreville) ; et (iii) identifier des groupes de gènes de métabolites secondaires en séquençant, assemblant et annotant le génome d'un endophyte qui présentait de fortes caractéristiques de biocontrôle.

Dans le cadre de ce projet de recherche, des tests antagonistes in vitro ont été réalisés avec des endophytes de la canneberge et un champignon pathogène, qui ont montré que *Pseudomonas* sp. CSWB3, *Pseudomonas* sp. CLWB12 et la souche fongique *Lachnum* sp. EFK28 étaient les plus actifs et ces souches ont donc été sélectionnées pour des études plus approfondies. Des expériences de germination de semis in vitro et d'inoculation d'endophytes ont montré que les souches bactériennes *Pseudomonas* sp. CSWB3 et *Pseudomonas* sp. CLWB12 amélioraient la croissance des semis de canneberges de la variété Stevens.

Comme les *Pseudomonas* sp. CSWB3 et *Pseudomonas* sp. CLWB12 ont tous deux un effet antagoniste élevé sur les champignons pathogènes, un seul (*Pseudomonas* sp. CSWB3) a été

soumis à une analyse du génome. Le séquençage, l'assemblage, l'annotation et l'analyse du génome de *Pseudomonas* sp. CSWB3 a révélé que cette souche possède cinq groupes de gènes biosynthétiques de métabolites secondaires qui codent pour les protéines responsables de la biosynthèse des composés antifongiques/antimicrobiens : pyrrolnitrine, pyoluteorine, putisolvine, 2,4-diacétylephloroglucinol, bicornutine A1 et bicornutine A2.

Sur la base des résultats de ces travaux, nous concluons que certains endophytes de la canneberge qui possèdent des groupes de gènes codant pour des métabolites secondaires antifongiques peuvent supprimer les pathogènes fongiques et améliorer la croissance des plantes.

Mots-clés: endophyte, bactéries, champignons, pathogène, biocontrôle, activité antifongique, métabolites secondaires, canneberge, *Pseudomonas* sp., analyse du génome, groupes de gènes, génomique comparative.

Abstract

Endophytes are microorganisms (typically bacteria and fungi) that live within plant tissue but do not activate the plant defense/immune system, unlike plant pathogens that typically do activate plant immune responses. Recent research has shown that virtually all plants grown under field conditions contain a number of endophytes, and that certain endophytes stimulate plant growth and enhance resistance against pathogens. Endophytes secrete chemical compounds (secondary metabolites) that suppress pathogen growth, a process known as biocontrol. Because of these biocontrol properties, endophytes are a potential alternative to chemical pesticides for combatting plant disease. Accordingly, biocontrol has become an important field of research.

My research project was comprised of the following specific aims: (i) isolate endophytes from cranberry plants that were acquired from two commercial producers of cranberries of the Stevens variety located in Quebec, Canada (Bieler Cranberries Inc, and Gillivert Inc.); (ii) test the biocontrol activity of endophytes against a collection of fungal pathogens and then inoculate the most active endophytes into cranberry seedlings that were obtained by germinating Stevens (Bieler Cranberries Inc.) and Scarlet Knight (Daniele Landreville) seeds; and (iii) identify secondary metabolite gene clusters by sequencing, assembling, and annotating the genome of one endophyte that exhibited strong biocontrol characteristics.

As part of this research project, *in vitro* antagonistic tests were conducted with cranberry endophytes and fungal pathogen, which showed that *Pseudomonas* sp. CSWB3, *Pseudomonas* sp. CLWB12, and the fungal strain *Lachnum* sp. EFK28 were the most active and therefore these strains were selected for further studies. *In vitro* seedling germination and endophyte inoculation experiments showed that the bacterial strains *Pseudomonas* sp. CSWB3 and *Pseudomonas* sp. CLWB12 enhanced the growth of cranberry seedlings of the Stevens variety.

Since *Pseudomonas* sp. CSWB3 and *Pseudomonas* sp. CLWB12 both had a high antagonistic effect on fungal pathogens, only one (*Pseudomonas* sp. CSWB3) was subjected to genome analysis. Sequencing, assembly, annotation, and analysis of the *Pseudomonas* sp. CSWB3 genome revealed that this strain possesses five secondary metabolite biosynthetic gene clusters that encode proteins responsible for the biosynthesis of the antifungal/antimicrobial compounds pyrrolnitrin, pyoluteorin, putisolvin, 2,4-diacetylephloroglucinol, bicornutin A1, and bicornutin A2.

Based on the results of this work, we conclude that certain cranberry endophytes that possess gene clusters encoding antifungal secondary metabolites can suppress fungal pathogens and enhance plant growth.

Keywords: endophyte, bacteria, fungi, pathogen, biocontrol, antifungal activity, secondary metabolites, cranberry, *Pseudomonas* sp., genome analysis, gene clusters, comparative genomics.

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List of Symbols

μl: microliter

°C: degree Celsius

min: minute

s: second

nm: nanometer

Kb: kilobase

g: gram

mg: milligram

L: Liter

ml: milliliter

%: percentage

μg : microgram

SE : Standard Error

List of Abbreviations

NADH: Nicotinamide adenine dinucleotide

ABA: Abscisic Acid

ACC: S-adenosyl-L-methionine and cyclic non-protein amino acid

AMF: Arbuscular Mycorrhizal Fungi

ANOVA: Analyzed of Variance

BCA: Biocontrol Agent

BGCs: Biosynthetic Gene Clusters

BLAST: Basic Local Alignment Search Tool

bp: base pair

CDS: Coding Sequence

C-endophytes: Clavicipitaceous endophytes

DAPG: 2, 4-diacetylphloroglucinol

DNA: Deoxyribonucleic acid

DNTPs: Deoxyribonucleotide Triphosphate

EDTA: Ethylenediaminetetraacetic acid

ET: Ethylene

ETI: Effector-Triggered Immunity

faa: protein FASTA file

GAs: Gibberellins

gbk format: gene bank format

IAA: Indole-3-Acetic Acid

ITS: Internal Transcribed Spacer

MAMPs: Microbial-Associate Molecular Patterns

miscRNA: miscellaneous Ribonucleic Acid

MTI: MAMPs Triggered Immunity

NC-endophytes: Non-Clavicipitaceous endophytes

NRPS: Nonribosomal peptide Synthetase

OD: Optical Density

PATRIC: Pathosystems Resource Integration Center

PCR: Polymerase Chain Reaction

PDA: Potato Dextrose Agar

PGPR: Plant Growth–Promoting Rhizobacteria

pH: potential hydrogen

PKS: Polyketides Synthetase

PRRs: Pathogen-Recognition Receptors

rDNA: Ribosomal Deoxyribonucleic acid

RN: Root Nodule

rRNA: ribosomal Ribonucleic Acid

SDS-PAGE: Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

SPAdes: St. Petersburg genome assembler

tmRNA: transfer-messenger Ribonucleic Acid

tRNA: transfer Ribonucleic Acid

UV: Ultraviolet

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CHAPTER.1 – LITERATURE REVIEW

1. Literature Review

1.1. Endophytes

Plant endophytes are microorganisms that live within plant tissue, at least for part of their life cycle. Endophytes were first identified in 1809 by the German botanist Heinrich Friedrich (Dey, Datta, Saha, Parida, & Panda1, 2019), who defined endophytes as a specific group of parasitic fungi that live in plants. Later, in 1991, the description of endophytes became more factual and practical, defined by Orlando Petrini as "all organisms inhabiting plant organs that at some time in their life cycle can colonize internal plant tissues without causing apparent harm to their host" (Hardoim *et al*., 2015).

In an agronomical sense (i.e., practical, non-scientific sense), endophytes may have beneficial effects by stimulating plant growth as well as by inhibiting plant pathogens (Gupta *et al*., 2016). However, the qualifications 'beneficial' or 'without causing apparent harm to their host' are problematic and need to be defined for a specific, given context, as some endophytes are pathogenic to certain plants but enhance the growth of other plants. This may be caused by many factors, including the environment, the precise genotype of the endophyte isolate, and other similar types of factors. We therefore use the term 'endophyte' in a more general sense, as 'microbes (bacteria or fungi) that colonize internal plant tissues', without referring to pathogenicity or agronomic benefits.

1.2. Diversity of Endophytes

Endophytes encompass a wide variety of microorganisms, in particular bacteria and fungi. More than 200 plant-associated species of bacteria have been identified, the majority of which belong to the phyla *Actinobacteria, Proteobacteria,* and *Firmicutes* (Golinska et al., 2015). The phylum *Actinobacteria* is comprised of Gram-positive bacteria that includes *Streptomyces*, whereas the phylum *Proteobacteria* is comprised of Gram-negative bacteria, including *Pseudomonas*, *Escherichia*, and *Salmonella*. The majority of the phylum *Firmicutes* is comprised of Gram-positive bacteria, such as *Bacillus* and *Clostridium* (Golinska *et al*., 2015; Hui, Yan, Qing, Renyuan, & Yongqiang, 2013). Many of these bacterial endophytes secrete secondary metabolites

that possess antibiotic, antifungal, and antitumor activity; secondary metabolites produced by *Streptomyces* species have been investigated in great detail (Berdy, 2012).

Fungal endophytes are found within the tissue of a wide variety of plants. The relationship between fungal endophytes and plants may be mutualistic and asymptomatic (i.e., no signs of disease throughout the fungus life cycle), in which plants provide food and energy sources to endophytes while in return endophytes enhance the resistance of plants against pathogens (Saikkonen, Faeth, Helander, & Sullivan, 1998). However, as noted above, fungal endophytes may also exhibit pathogenic traits. Fungal endophytes have been widely studied because of their relationship with plants and their association with plants, which started approximately 400 million years ago (Krings et al., 2007).

Based on taxonomy, host and tissue specificity, and function, endophytic fungi are classified into two main groups (Bamisile, Dash, Akutse, Keppanan, & Wang, 2018), which are clavicipitaceous endophytes (C-endophytes) and non-clavicipitaceous endophytes (NC-endophytes; for instance the wide-spread zygomycete AMF, see below). C-endophytes are associated with different grasses and produce bioactive metabolites that enhance the resistance of plants to insects, nematodes, and fungal pathogens. C-endophytes also produce alkaloids that are toxic to humans and animals but are tolerated by the plant host. C-endophytes include Hypocreales and other Ascomycota. NC-endophytes contribute to several functions in the host plant, including avoidance of abiotic stress, inducing synthesis of plant hormones, and protecting the plant from pathogenic fungi (Rodriguez, White, Arnold, & Redman, 2009).

Endophytic bacteria and fungi have been isolated from the *Vaccinium* genus within the family *Ericaceae,* which includes cranberries, blueberries, and strawberries. Ericoid mycorrhizal fungal endophytes have been isolated in China from blueberries; these fungal endophytes colonize the roots of blueberry plants and help increase the supply of nutrients to the plant under harsh environmental conditions. The ericoid mycorrhizal fungi isolated from blueberry belong to the genera *Clavaria*, *Oidiodendron*, *Lachnum*, *Acephala*, and *Phialocephala* (Yang *et al*., 2018). Additionally, a diverse range of endophytic bacteria has been found to be associated with blueberry plants. These endophytic bacteria produce indole acetic acid and other bioactive compounds involved in biological control activities that stimulate plant growth. In a study conducted by Ortiz-Galeana *et al*., the most common bacterial endophytes found to be associated with blueberry plants

belong to the genera *Pantoea*, *Pseudomonas*, *Burkholderia,* and *Bacillus*. The majority of the species belonging to these genera perform activities that are advantageous to the blueberry plant (Ortiz-Galeana *et al*., 2018).

1.3. Symbiotic Interactions

Plants and endophytes communicate through biological mechanisms that lead to changes at the genetic, signaling, and metabolic levels. Two types of symbiotic communication between plants and either fungi or bacteria have been vigorously studied. The first and most common type are arbuscular mycorrhizal fungi (AMF) (Kawaguchi & Minamisawa, 2010) and *zygomycete* fungi (a paraphyletic taxon) that interact with a large variety of plants. Interactions between these fungi and plants initiate with the entry of fungal hyphae into the root epidermis, followed by an expansion of hyphae to reach the inner cortex where arbuscules are formed. These branched hyphae enhance absorbance of phosphate and other nutrients by the fungus from the soil (Lee, Eo, Ka, & Eom, 2013). The second type of bacterial symbiotic interaction is called root nodule (RN), which consists of a nitrogen-fixing symbiotic interaction that is formed between rhizobacteria and the roots of leguminous plants. With RN, chemicals are produced that mediate highly specific signaling and communication between the host plant and the rhizobacteria (Clua, Roda, Zanetti, & Blanco, 2018). The process of nodulation initiates by the secretion of flavonoids by the plant root, which subsequently stimulate the expression of genes responsible for nodulation. This leads to production of lipochito-oligosaccharide, which is a nodulation factor that stimulates the growth of cells in the root cortex that ultimately leads to the formation of root nodules (Q. Wang, Liu, & Zhu, 2018). Symbiotic interactions between roots and nodules may also be a mechanism that allows bacterial pathogens to enter plant tissue, including certain non-nitrogen-fixing species that harbor antibiotic resistance genes specific to cefoxitin, ampicillin, and cefuroxime-axetil (Muresu, Maddau, Delogu, Cappuccinelli, & Squartini, 2010).

1.4. Plant Pathogens

1.4.1. Overview of Pathogenicity

Plant pathogenicity is defined as the potential of microorganisms to cause damage to crops. The majority of plant pathogens are fungi. Fungal pathogens are classified according to their pattern of nutrition into necrotrophic, hemibiotrophic, and biotrophic. Necrotrophics include bacteria and fungi that secrete lytic enzymes (e.g., cell wall-degrading enzymes) that weaken plant defenses,

which ultimately can lead to destruction of plant tissue (necrosis). The fungus *Botrytis cinerea* that causes grey mold disease is an example of a necrotrophic pathogen. *Botrytis cinerea* forms an infectious structure on the outer surface of the leaf that mediates penetration of plant leaves, which is followed by secretion of lytic enzymes that dissolve cutin (Laluk & Mengiste, 2010).

Unlike necrotrophic pathogens, biotrophic pathogens establish a mutualistic relationship with host plants. Biotrophic pathogens avoid host recognition and detection by secreting lytic enzymes that suppress the immunity of the host plant, which allows these pathogens to live within the plant tissue and benefit from plant-derived nutrients that gradually weakens and damages the host plant (Laluk & Mengiste, 2010). Biotrophic pathogens can also penetrate plant tissue and produce protein-based inhibitors of β-1,3-glucanases, which are enzymes produced by plants that are capable of dissolving fungal cell walls (Gebrie, 2016). An example of a biotrophic pathogen is *Hyaloperonospora arabidosidis,* which causes downy mildew disease in *Arabidopsis*. Sequencing of the *H. arabidosidis* genome revealed 134 *RXLR* genes that encode for RXLR effectors. The function of most of these RXLR effectors is not known, and only 13 of the RXLR effectors were shown to have a small effect on the host immune response, which suggests that inhibition of the host plant immune response by *H. arabidosidis* pathogen is possibly caused by the combined action of all 134 RXLR effectors (Coates & Beynon, 2010; Pel *et al*., 2014).

Nutrient acquisition by hemibiotrophic pathogens requires a living host plant. Hemibiotrophic pathogens go through a biotrophic phase during the primary stages of pathogenicity then switch to the necrotrophic phase. An example of a hemibiotrophic pathogen is the fungus *Zymoseptoria tritici* that is the causal agent of septoria leaf blotch, which affects the leaves of wheat. *Zymoseptoria tritici* infections start with the growth of hyphae on the outer surface of leaves, followed by pathogen penetration. The fungus grows slowly inside plant tissue. The infected plant does not display any pathogenic symptoms for 8–11 days, and then the fungus switches to its necrotic phase, which results in plant death. Following one month of infection, the pathogen starts to form sexual structures (Garcia-Sanchez, Bernales, & Cristobal, 2015).

1.4.2. Plant Defense against Pathogens

Several morphological, biochemical, and molecular mechanisms are involved in the response of plants to pathogenic infections. Following infection, a plant attempts to kill or weaken the

pathogen. Disease symptoms manifest after the plant's defense mechanisms are sufficiently weakened by a pathogen. There are two types of plant defense mechanisms, described below.

1) Constitutive defense mechanisms

Structural defense mechanisms: This type of plant defense mechanism is considered to be the first line of defense against invading pathogens. Structural defense mechanisms include the wax layer and cuticle found on the surface of the plant, which give support and rigidity to plant tissue. Other plant structures act as physical barriers to prevent pathogen penetration, including the epidermal layer, actin cytoskeleton, guard cells, and trichomes (Doughari, 2015).

Biochemical defense: Various chemicals are produced by plants in response to invading pathogens. Toxic or lytic reactions mediated by these chemicals may directly affect the pathogen or may indirectly affect the pathogen by stimulating microflora on the plant surface to produce toxic chemicals. These types of biochemical compounds that are secreted by plant tissues include antimicrobial organic substances such as saponins and alkaloids that act as inhibitors of microorganism growth. Another biochemical defense mechanism is mediated by toxic inhibitors, which are metabolites found in plant tissue that degrade toxins secreted by pathogens, which contributes to the plant's resistance against the invading pathogen (Doughari, 2015).

2) Induced defense mechanisms

As previously described, pathogens have the ability to suppress the immune system of host plants and produce inhibitors of cell wall-degrading enzymes produced by the host plant. However, if a pathogen overcomes the plant's pre-existing defenses, additional structural, cellular, or biochemical response mechanisms may be induced in order to kill the pathogen (van Baarlen, van Belkum, Summerbell, Crous, & Thomma, 2007).

Structural defense: Structural defenses include lignification of the cell wall that increases its rigidity, which prevents penetration of fungal pathogen hyphae (Jones & Dangl, 2006). Another structural defense is suberization that includes conversion of the cell wall into cork tissue by suberin formation, which helps to isolate infected cells away from healthy tissue, which ultimately helps to minimize the spread of the infection (van Baarlen *et al.*, 2007). Another important structural defense mechanism is gum deposition within infected cells (Doughari, 2015), and formation of a secondary wall and papillae. Papillae are polysaccharide polymers that are secreted

in response to an infection, especially in cereals, to increase their resistance (Anderson *et al*., 2010; Freeman & Beattie, 2008).

Biochemical defenses: Biochemical defenses are the last line of host defense against penetrating pathogens (Jan, Azam, Ali, & Haq, 2011). Biochemical immunity consists of two main layers: microbial-associated molecular pattern (MAMP)-triggered immunity (MTI) and effector-triggered immunity (ETI). Protection against an invading pathogen initiates with pathogen recognition, which stimulates a defensive response (termed a hypersensitive response) that induces infected cell death, which helps to protect neighboring uninfected cells. A group of proteins called pathogen-recognition receptors (PRRs) are localized to the plant cell membrane and the cytoplasm and recognize MAMPs that subsequently activates MTI. Pathogens secrete effectors that bind PRRs, which inhibits plant MTI (Swarupa, Ravishankar, & Rekha, 2014).

ETI is stimulated by a plant's intracellular resistance gene in response to the detection of pathogenic type III secreted effectors (T3SEs); ETI is linked to the programmed cell death of infected cells (Doughari, 2015). Plants produce various other biochemical molecules in response to infection, including toxic substances such as phenolic compounds and phytoalexins. Additionally, plants produce proteins such as lectins, ricin, protease inhibitors, and hydrolytic enzymes such as chitinases and glucanases that play important roles in inhibiting pathogens (Doughari, 2015).

The innate immune system of plants can discern between endophytes and pathogens, which allows endophytes to enter the host plant without triggering the immune system. This lack of immune response helps to maximize the benefits received from this interaction, which ultimately boosts the immune reaction of plant-endophytes against invading pathogens (Khare, Mishra, & Arora, 2018). As mentioned previously, the first line of plant defense is the recognition of MAMPs by PRRs located on the surface of plant tissue (Swarupa et al., 2014). Fungal endophytes can avoid host immune recognition by producing chitin deacetylases that deacetylate chitosan oligomers that are part of the plant cell wall. Certain endophytic bacteria possess MAMPs that inhibit recognition by the PRRs found on the surface of the plant cell wall (Khare et al., 2018).

In conclusion, the entry of endophytes into host plants is facilitated by the ability of endophytes to avoid the plant immune system. Additionally, endophytes help plants to defend themselves against pathogens by secreting biochemical substances that suppress pathogen growth.

1.5. Mechanisms of Enhancing Plant Growth

1.5.1. Endophytes and Plant Nutrition

Bacterial and fungal endophytes in the soil (termed the rhizosphere) can enhance plant growth by increasing the availability and uptake of nutrients by plants; hence, they are often referred to as biofertilizers. The number of plant-growth-promoting rhizobacteria (PGPR) in the soil depends on the environmental conditions and the soil type. PGPR are found near the roots of a plant and induce plant growth *via* direct and indirect mechanisms. Direct mechanisms include bacterial secretions, nitrogen fixation, and solubilization of phosphorus (Olanrewaju, Glick, & Babalola, 2017). Nitrogen and phosphorus are essential nutrients for plant growth. However, these nutrients are often only available in limited quantities in the soil, due to the loss of nitrogen by leaching and the low bioavailability of phosphorus (due to it predominant insoluble form as aluminum and iron phosphates). Collectively, PGPR are important for plant growth because of their nitrogen fixation and phosphorous solubilization functions (Martinez-Viveros, Jorquera, Crowley, Gajardo, & Mora, 2010). In addition, PGPR can increase the absorbent surface of plant roots by stimulating root growth and branching as well as stimulating other non-pathogenic symbionts (endophytes) of the host (Saia *et al*., 2015; Vessey, 2003). Certain PGPR known as **endophytic** plant growth-promoting bacteria employ unknown mechanisms to enter plant tissue. The various functions performed by endophytic plant growth-promoting bacteria are similar to those performed by non-endophytic PGPR, including facilitating the acquisition of nutrients such as nitrogen, phosphorus, and iron from the rhizosphere and the direct transfer of these nutrients into plant roots (Santoyo, Moreno-Hagelsieb, Orozco-Mosqueda Mdel, & Glick, 2016). With fungal endophytes, nutrients can be transferred *via* mycelia that extend from mycorrhizal fungi living in plant root tissue (White et al., 2019). Endophytes such as *Bacillus* spp. that colonize plant roots have high affinity for organic acid-metal complexes and facilitate the transfer of these complexes from the soil into plants (White et al., 2019).

1.5.2. Phytohormone production and regulation of ethylene levels

Phytohormones are bioactive molecules that act as messengers in signaling pathways, which contributes to plant growth and developmental regulation. The biological, morphological, and physiological processes of a plant can be affected by very low concentrations of phytohormones

(Martinez-Viveros *et al*., 2010). Several endophyte species (both bacteria and fungi) can produce phytohormones (Tsavkelova, Klimova, Cherdyntseva, & Netrusov, 2006), including gibberellins (GAs), indole acetic acid (IAA), abscisic acid (ABA), and jasmonic acid (JA). JA and ABA play crucial roles in the regulatory processes that control the heat-stress response, while GAs and IAA enhance plant growth and development (Waqas *et al*., 2015). Interactions between plants and microorganisms such as endophytes, rhizobacteria, free-living bacteria, and some pathogens results in conversion of tryptophan to IAA, which is one of the auxins produced from tryptophan. IAA may also be produced *via* tryptophan-independent pathways. The main function of IAA is to stimulate the division, expansion, and differentiation of plant cells, which enhances plant growth (Martinez-Viveros *et al*., 2010). (Swain, Naskar, & Ray, 2007) showed that the length of the stem and root of the white yam plant (*Dioscorea rotundata*) increased following inoculation of IAA-producing *Bacillus subtilis* as compared to non-inoculated plants.

GA is another phytohormone that regulates plant growth and plays an important role in seed germination, leaf elongation, and flowering, and works with auxins to increase root length. GA levels are regulated by various factors, such as light, temperature, and auxin concentrations (Stamm & Kumar, 2010). A recent study by (Hamayun et al., 2017) showed that treatment of soybean plants with GA-producing *Porostereum spadiceum* (an endophytic fungus) resulted in enhanced growth as compared to untreated plants.

Ethylene (ET) is a gaseous phytohormone produced from the precursor methionine via S-adenosyl-L-methionine and cyclic non-protein amino acid (ACC). ET is involved in many stages of plant growth, including the ripening of fruit (Wani, Kumar, Shriram, & Sah, 2016). High concentrations of ET may inhibit plant growth by affecting cellular processes and stimulating defoliation. Rhizobacteria have the capacity to regulate ET levels in the soil by producing ACC deaminase, which degrades ACC into alpha-ketobutyrate and ammonium (Martinez-Viveros *et al*., 2010).

1.5.3. Biocontrol of pathogens

Microbial pathogens can cause various diseases in plants that ultimately leads to decreased crop yields. Chemical pesticides have been widely used to reduce or eliminate pathogenic infections, but these compounds negatively impact human health and the environment. In addition, with prolonged use, pathogens may become resistant to chemical pesticides, and consequently new pesticides must be developed. Based on previous research, biological methods may be suitable

alternatives to chemical pesticides, which would reduce the harmful impact of chemicals. These biological methods are based on using soil microorganisms and plant-associated endophytes as bio-pesticides, since they exhibit biocontrol activity against pathogens. Biocontrol is the term used to describe the secretion of bioactive compounds (e.g., antibiotics, hydrogen cyanide, and many other secondary metabolites) that suppress pathogen growth and consequently promote plant growth (Martinez-Viveros *et al*., 2010). The biocontrol properties of endophytes are the core subject of this research and are discussed in more detail in the following section.

1.6. Biocontrol Mechanisms

Biocontrol, an abbreviation for "biological control", describes the use of microorganisms to suppress the growth of pathogens that ultimately reduces or eliminates disease symptoms. An antagonistic microbe with bioactive properties that suppress the growth of a pathogen is known as a biocontrol agent (BCA). BCAs produce compounds that influence the plant host as well as the pathogen. These compounds are secreted in response to specific and non-specific interactions between the plant, endophytes, and pathogens. BCAs have been widely used in the field of plant pathology (Pal & Gardener, 2006).

It is well known that various microorganisms, including bacteria and fungi, secrete bioactive compounds, some of which have been used as therapeutic drugs. Penicillin, obtained from *Penicillium glaucoma* in 1896, was the first such fungal bioactive substance identified, and has been widely used as an antibiotic for treating a wide range of bacterial infections (Suryanarayanana *et al*., 2009).

Various mechanisms of biocontrol have been described that enhance plant growth by suppressing or killing pathogens, including induction of host resistance (which was explained in the section on plant defense) and secretion of antibiotics and lytic enzymes.

1. Antibiotics

The production and secretion of antibiotics is one of the most powerful biocontrol mechanisms. Our research is focused on secondary metabolites secreted by endophytes that function as biocontrol agents, which include antibiotics. Antibiotics are low molecular weight organic substances that are secreted by various microbes. Previous studies showed that the antibiotics produced by PGPR have an impact on the growth of pathogens; i.e., production of sufficient levels

of an antibiotic by endophytes suppresses pathogens in close proximity. A single antibiotic can control one or more pathogens (Pal & Gardener, 2006).

Recent studies have shown that certain bacteria, such as *Bacillus* spp. and *Pseudomonas* spp., function as biocontrol agents by producing bioactive compounds with antimicrobial activity that suppress the growth of fungal pathogens. Certain *Bacillus* spp. and *Pseudomonas* spp. harbor gene clusters that are responsible for the secretion of secondary metabolites such as non-ribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs) (Palazzini, Dunlap, Bowman, & Chulze, 2016; Strano, Bella, Licciardello, Caruso, & Catara, 2017).

NRPSs and PKSs are large enzymes or enzyme complexes involved in the biosynthesis of various natural bioactive compounds known as non-ribosomal peptides and polyketides, which are compounds that possess remarkable bioactivity, including antifungal and antimicrobial activity. Non-ribosomal peptides are synthesized by sequential condensation of amino acids, whereas polyketides are synthesized by repetitive insertion of two carbon ketide units obtained from thioester of acetate (Ansari, Yadav, Gokhale, & Mohanty, 2004). The biosynthetic gene clusters (BGCs) that encode NRPSs and PKSs are commonly found in the phyla *Proteobacteria*, *Actinobacteria*, *Firmicutes,* and *Cyanobacteria* (H. Wang, Fewer, Holm, Rouhiainen, & Sivonen, 2014). NRPS often have three catalytic domains: the adenylation (A) domain that recognizes and activates amino acids, the peptidyl-carrier (thiolation) domain that transfers the activated amino acids, and the condensation (C) domain that is responsible for peptide bond formation and peptide chain elongation. Certain NRPS contain additional domains, such as the epimerization domain (E) that convert L-amino acids into D-amino acids, the dual/epimerization domains (E/C) that are responsible for epimerization and condensation, cyclization domains (Cy) that can functionally replace C domains, the oxidation domain (Ox) that is responsible for the oxidation of the thiazoline ring that leads to the formation of an aromatic thiazol, and thioesterase domains (TE) that are responsible for cyclization or hydrolysis of the final peptide in the NRPS module that releases it (Amoutzias, Chaliotis, & Mossialos, 2016). The resulting NRPS molecules may be further modified by subsequent biochemical reactions, leading to an even broader spectrum of bioactive substances.

Type I PKSs typically have three main domains: the acyl-transferase (AT) domain that incorporates malonyl or methylmalonyl-CoA, the KS domain that is responsible for C-C bond formation, and the acyl-carrier (thiolation) domain, which is similar to the peptidyl-carrier domain of NRPSs. PKSs have additional domains, such as the ketoreduction (KR) domain, the dehydration (DH) domain, and the enoylreductase (ER) domain (Amoutzias et al., 2016).

NRPSs and PKSs are structurally and functionally similar and therefore often form hybrid gene clusters (hybrid NRPS/PKS) (Muller *et al*., 2015). BGCs that encode both an NRPS and a PKS can produce complex NRPS/PKS-derived hybrid bioactive compounds called peptide-polyketide secondary metabolites, which can be produced directly by the NRPS/PKS hybrid enzyme or indirectly as non-ribosomal peptides and polyketides that bind together (Amoutzias et al., 2016).

Pseudomonas spp. harbor gene clusters that encode various NRPs. NRP compounds include pyoverdines, pyochelin, pseudomonine, paerucumarin, pseudoverdine, lipopeptides, safracin, tabtoxin, phaseolotoxin, pyrrolnitrin, and indole-3-acetic acid (IAA) (Gross & Loper, 2009). *Bacillus velezensis* produces NRPs such as fengycin, surfactin, bacilysin, iturin and corynebactin (Palazzini et al., 2016).

PKSs produce several different natural products that possess antibiotic, chemotherapeutic, and phytotoxic activities. Polyketides secreted by *Pseudomonas* spp. include mupirocin (pseudomonic acid A), 2,4-diacetylphloroglucinol (DAPG), and 2,5-dialkylresorcinols (Gross & Loper, 2009), while polyketides secreted by *Bacillus velezensis* include bacillaene, difficidin and macrolactin (Palazzini *et al*., 2016).

A previous study found that the biocontrol isolate *Bacillus velezensis* RC 218 produces non-ribosomal peptides and polyketides that can suppress the fungal pathogen *Fusarium*, which can be used in agricultural applications. This same study also analyzed the genome of *B. velezensis* RC218 and identified the BGCs responsible for the biocontrol activity of this strain (Table 1) (Palazzini *et al*., 2016).

Table 1.- NRPS and PKS gene clusters and bioactive secondary metabolites produced by *B. velezensis* RC218. Adapted from (Palazzini et al., 2016)

Compound	Synthetase type	Genes	Size (kb)	Bioactivity
Fengycin	NRPS	*fen*A, B, C, D, E	37.7	Antifungal
Surfactin	NRPS	*srf*AA, AB, AC, AD	26.2	Surfactant, antibiotic
Bacilysin (Chlorotetaine)	NRPS	*bac*A, B, C, D, E, F, ywfH	6.7	Antibacterial
Iturin	NRPS	*itu*D, A, B, C	37.2	Antifungal
Bacillaene	PKS	*bae*B, C, D, E, acpK, *bae*G, H, I, J, L, M, N, R, S	72.5	Antibacterial
Difficidin	PKS	*dfn*A, Y, X, B, C, D, E, F, G, H, I, J, K, M, L	69.5	Antibacterial
Macrolactin	PKS	*mln*A, B, C, D, E, F, G, H, I	53.2	Antibacterial

2. Lytic enzymes

Endophytes can directly suppress the activity and growth of pathogens by secreting lytic enzymes, which prevent the growth of plant pathogens by destroying their cell walls. Lytic enzymes include β-1,3 glucanases, chitinases, and cellulases. β-1,3 glucanases are involved in the biocontrol activity of *Lysobacter enzymogenes* strain C3 and have been found to suppress fungal pathogens of plants (Pal & Gardener, 2006). In fact, mutations in the β-1,3 glucanase genes of *L. enzymogenes* strain C3 lead to a reduction in the biocontrol effects against *Pythium* and *Bipolaris*, which causes damping-off of sugar beets and leaf spot in fescue, respectively (Gao, Dai, & Liu, 2010).

1.7. Cranberry Plants and Endophytes

American cranberries (*Vaccinium macrocarpon*) are a member of the evergreen species of *Ericaceae*. American cranberries are prostrate and relatively short plants and grow mainly in North

America (i.e., Canada and USA) (Hisano, Bruschini, Nicodemo, & Srougi, 2012; Polashock *et al*., 2014). North America is the main producer of cranberries worldwide (Diaz-Garcia et al., 2019).

1.7.1. Cranberry Endophytes

A large number (several hundreds) of endophytes were previously isolated from cranberry plant tissue in B. F. Lang's laboratory over a period of approximately five years. In this research more endophytes were isolated to increase the chance of isolating strains with stronger biocontrol, suppression of the largest possible spectrum of fungal pathogens, and potentially, the future identification of novel antifungal antibiotics of medical interest.

The most common endophytes that showed biocontrol properties when tested against various cranberry fungal pathogens include the following:

Bacillus velezensis**:** *B. velezensis* is a Gram-positive bacterium that forms endospores. *B. velezensis* produces enzymes, antibiotics, insecticides, and other bioactive compounds that inhibit pathogens and promote plant growth (Ruiz-Garcia, Bejar, Martinez-Checa, Llamas, & Quesada, 2005). The *B. velezensis* genome contains several gene clusters that encode secondary metabolites that function as fungicides; thus, *B. velezensis* may potentially be a powerful biocontrol agent that inhibits pathogens and enhances plant growth (Liu *et al*., 2017).

***Lachnum* sp.:** *Lachnum* is a genus of fungi in the family *Hyaloscyphaceae* that produces abundant amounts of bioactive compounds such as antimicrobial substances that have been used for medicinal and agricultural purposes (Rukachaisirikul, Chantaruk, Pongcharoen, Isaka, & Lapanun, 2006). An *L. abnorme* strain was previously isolated from stems of the *Ardisia cornudentata* Mez plant; additionally, it was shown that this fungus and three other *Lachnum* species can produce at least 35 secondary metabolites, such as benzenoids and coumarins. Subsequent investigation of these metabolites revealed that they possess biocontrol activity (Chang *et al*., 2016).

1.7.2. Cranberry Pathogens

A variety of fungal pathogens are capable of infecting cranberry plants and can cause losses in total fruit production of up to 33% if fungicides are not used (Conti, Cinget, Vivancos, Oudemans, & Belanger, 2019). Fruit rot is the most common disease that affects cranberry plants, which is caused by approximately 12 different fungal pathogens. Identification of the fungus responsible for fruit rot can be accomplished by culturing rotten cranberry fruit on culture medium followed by analysis

of growth characteristics. Different fungal pathogens cause different symptoms in cranberry plants (Wells & McManus, 2013).

Coleophoma empetri: This fungal species causes Ripe Rot disease. During growth on culture medium, the mycelia initially have a white color then turn dark brown or black.

***Colletotrichum acutatum*:** This fast-growing fungal species causes a disease called Bitter Rot. During growth on culture medium, the color of this fungus is initially white then turns pinkish-orange because of the red pigments produced by this fungus.

Colletotrichum gloeosporioides: Similar to *C. acutatum*, *C. gloeosporioides* is fast-growing and causes Bitter Rot. *C. gloeosporioides* is white in color during the early stages of growth on culture medium, then becomes dark gray as the colonies mature.

Phomopsis vaccinii: This fungal species causes Viscid Rot/Upright Dieback disease. During the early stages of growth on culture medium, this fungus is white in color then turns dark gray.

Phyllosticta vaccinii: This fungal pathogen causes Early Rot disease and has a very slow growth rate as compared to other cranberry fungal pathogens. It produces mycelia that are olive to black in color with irregular edges during growth on culture medium.

Phyllosticta elongata: This fungal pathogen causes Berry Speckle/Botryosphaeria fruit rot. The mycelia of this fungus are greenish-gray in color and has a very similar appearance to *P. vaccinii* during the early stages of growth on culture medium, then becomes recognizable by its morphology and fast growth rate.

Physalospora vaccinii: This fungal pathogen causes Blotch Rot. Two different strains were previously isolated from rotten fruit: a dark strain that produces brown-gray mycelia and a light strain that produces white mycelia.

1.8. Hypothesis

Bacterial and fungal endophytes have the potential to enhance cranberry growth and yield because of their ability to inhibit the growth of fungal pathogens *via* secretion of bio-pesticides (secondary metabolites).

1.9. Objectives

The objectives of this research are to:

1- Isolate endophytes from cranberry plants obtained from two commercial sources and then classify the isolated endophytes into three groups based on fruit yield: very good yield, good yield, and weak yield.

2- Identify which isolates are the most potent in terms of suppressing cranberry pathogens.

3- Use young plants obtained by seed germination to evaluate the biocontrol activity of the most potent isolates identified in Aim 2.

4- Classify the most active biocontrol isolates by ribotyping, and then sequence, assemble, and annotate the genome of one isolate, followed by the identification of the genes potentially involved in biocontrol.

CHAPTER 2 – MATERIALS AND METHODS

2. Materials and Methods

2.1. Endophytes and growth medium

Endophytes were isolated from cranberry plants obtained from two commercial sources: Gillivert Inc. and Bieler Cranberries Inc. Endophytes were cultured on potato dextrose agar (PDA), which is a commonly used general purpose medium for culturing fungi. PDA was prepared by adding 12 grams of potato dextrose broth and 7.5 grams of potato agar to 500 ml of distilled water in a one-liter glass bottle. The bottle was placed on a stirrer to mix and dissolve the contents. The pH of the PDA was adjusted to 7.0 by the gradual addition of NaOH. The PDA medium was then sterilized at 121°C in an autoclave, and once sufficiently cooled, the medium was poured into Petri plates.

2.2. Sample collection and handling

Professor Lang's laboratory team collected cranberry plants from two cranberry fields of the Stevens variety: one field owned by Bieler Cranberries Inc. and the other owned by Gillivert Inc., both located in Quebec. Plants were classified into three groups based on fruit yield: very good yield, good yield, and weak yield. Similarly, plants from the Gillivert field were classified into two groups: good yield and weak yield. Plants were labelled and placed in a cold room for long-term preservation.

2.2. Surface sterilization

Cranberry plants were selected randomly and washed for 15 minutes with tap water. Roots were placed in tap water overnight to eliminate soil particles. The surface disinfection procedure was performed under laminar air flow as follows: cranberry plants from very good yield, good yield, and weak yield of Bieler Cranberries and Gillivert fields were cut into three parts: roots, stems, and leaves. Each group was separately sterilized. Plant parts were immersed for 2 minutes in a 2% mild liquid detergent solution (Neutrad, Decon Lab Inc,) and were then immersed for 3 minutes in a solution of 0.79% sodium hypochlorite (bleach) and 0.1% Tween 80. Plant parts were then dipped into 70% ethanol for 30 seconds and were then rinsed three times with sterilized distilled water. Disinfected plant parts were placed in sterilized petri plates to dry.

2.3. Isolation of endophytes

Surface-sterilized plant parts (leaves, stems, and roots) were cut into small segments using a sterilized scalpel blade that were then transferred onto fresh PDA medium petri plates. Inoculated plates were incubated at 25°C. After 4-7 days of incubation, endophyte growth was visually noticeable. Each endophyte was transferred from a small section of growth to a fresh PDA plate (purification step) that was then incubated at 25°C. Several rounds of endophytes re-isolation were performed to obtain pure isolates.

2.4. Biocontrol of fungal pathogens by endophytes

The antagonistic activity of isolated endophytes were tested on 19 fungal pathogens. For this, four endophytes were grown at the edge of a fresh PDA plate for 4 days, and then one of the 19 fungal plant pathogens was placed in the center of the PDA plate. Control PDA plates contained pathogens only in the center. Three replicates of each plate were prepared. The plates were incubated at 25°C. Endophyte and pathogen growth was evaluated every 3 days and measurements of pathogen growth were taken after 3, 6, 15 and 30 days of growth.

The 19 fungal pathogens that were tested included *Rhizopus* sp., *Trichoderma* sp., *Cadophora luteo-olivacea*, *Botrytis cinerea*, *Alternaria alternata*, *Physalospora vaccinii*, *Fusarium graminearum*, *Verticilium dahlia*, *Diaporthe vaccinii*, *Cytospora* sp., *Godronia cassandrae, Peniophora* sp., *Diaporthe* sp., *Penicillium* sp., *Colletotrichum* sp., *Physalospora* sp., and three uncharacterized fungal pathogens.

2.6. Compatibility test

Confrontation tests were performed to determine which combinations of endophytes have the potential to enhance the biocontrol effect when inoculated together in plant tissue. A combination consisting of two endophytes was tested by placing the combination on a fresh PDA plate at short distance 5-10 mm. Controls containing only the tested endophytes were done. The plates were incubated at 25°C and were observed each day. Incompatible endophytes were identified *via* inhibition zone formation. Experiments were repeated three times to generate statistically relevant results.

2.7. Molecular characterization of endophytes

An SDS-based laboratory protocol was used to extract genomic DNA from endophytic fungus. A small piece of the fungus was placed in an Eppendorf tube containing glass beads and then 50 µl of TE (2 ml Tris 100mM + 5 µl EDTA 5mM) were added. The fungal piece was crushed and mixed vigorously with a sterile plastic pestle to lyse the cells. Next, 150 µl of TE, 4 µl of 20% SDS, and 4 µl of proteinase K were added, followed by incubation at 37℃ for 30 min. Next, the tube was centrifuged for 20 min at 11,000 rpm (maximum speed). The supernatant was transferred to a new Eppendorf tube and then a ¼ volume of 5 M NaCl was added. The tube was vortexed, placed on ice for 1 hour, then centrifuged for 10 min. Using a pipette, the supernatant was transferred to a new Eppendorf tube and then EtOH/AMC (95% ethanol/0.5 M ammonium acetate) (2.5-times the volume) mixed with 2.5 of the volume of, the tube was placed on ice for 20 min and centrifuged for 15 min at maximum speed. The supernatant was carefully discarded making sure DNA pellet is still in the tube, 175 µl of 70% ethanol were added, the sample was mixed gently and was then centrifuged for 5 min at 11,000 rpm (maximum speed). Next, the ethanol was carefully discarded using a pipette and then 21 µl of TE were added, followed by gentle mixing. Extracted DNA was stored at 4℃ or -20℃.

The fungus isolate was identified by ribotyping in which the ITS (internal transcribed spacer) regions of the rDNA were PCR amplified using primers BMB-CR-fwd: 5'-GTACACACCGCCCGTCG-3’ (forward primer) and ITS4 5'-TTCCTCCGCTTATTGATATGC-3' (reverse primer) (Ihrmark *et al*., 2012).

Endophytic bacteria were identified by PCR amplifying 16S rDNA using the universal primers 27F 5'-AGAGTTTGATCCTGGCTCAG-3' (forward primer) and LPW58 5′-AGGCCCGGGAACGTATTCAC-3 (reverse primer) (Sabat et al., 2017). Rapid PCR on a single colony was performed.

The final volume of each PCR was 50 µl and contained 5 µl of 10x Taq polymerase buffer, 1 µl of 10 mM dNTPs, 0.8 µl of (50ng/ml) RNase, 5 µl of 20 mM $MgSO_4$, 2 µl of 10 pM forward primer, 2 µl of 10 pM reverse primer, 33.8 µl of PCR-grade water, and a bacterial colony. Fungal ITS amplification reactions contained 5 µl of 10x Taq polymerase buffer, 1 µl of 10 mM dNTPs, 0.8 µl of (50ng/ml) RNase, 5 µl of 20 mM MgSO4, 2 µl 10 pM of forward primer, 2 µl of 10 pM reverse primer, 28.8 µl of PCR-grade water, and 5 µl of fungal genomic DNA. PCR was performed

on a Bio-Rad thermal cycler using the following cycling parameters: for bacteria, 96°C for 5 min, followed by 31 cycles of denaturation at 95°C for 15 s, primer annealing at 56°C for 15 s, and primer extension at 72°C for 50 s. For fungi, 96°C for 5 min, followed by 31 cycles of denaturation at 95°C for 15 s, primer annealing at 58°C for 15 s, and primer extension at 72°C for 1 min.

Agarose gel was prepared by adding 1 g of agarose to 100 ml of TAE (40 mM Tris-acetate, 1mM EDTA) buffer. The solution was heated until agarose was completely dissolved. The 1% agarose gel was placed on the bench to cool down to about 50°C. 30 ml of 1% agarose gel was poured into a plastic beaker, and 2.5 µl of Ethidium bromide was added to the agarose gel and mixed well. Agarose gel with Ethidium bromide was poured into an agarose tray with a comb in the appropriate place to create the wells and then placed on the benchtop to cool down. The gel tray was then placed into the electrophoresis apparatus with TAE (40 mM Tris-acetate, 1mM EDTA) buffer.

Gel electrophoresis was used to visualize the PCR products. A 2 µl aliquot of each sample was mixed with 1 µl of loading dye and 2 µl of nano-pure water. A 1µl aliquot of the 1 kb ladder was loaded into a well as a size reference and 5 µl of each sample mixture were added to each respective well. Agarose gel electrophoresis was conducted at 100 V for 50 minutes.

DNA fragments in the agarose gel were visualized using a UV trans-illuminator with an attached camera. The size of the amplified DNA fragments was estimated based on the 1 kb ladder. To identify the species corresponding to the most active isolates, PCR products were extracted from the gel and were sent to IRIC | Université de Montréal for Sanger sequencing. Sequencing results were analyzed by BLAST searches of nucleotide data collections on the NCBI website, and species were identified based on similarity matches.

2.8. Seed germination

Mature seeds were collected from cranberry fruits obtained from two varieties, the Stevens variety from Bieler Cranberries Inc and the Scarlet Knight variety from Groupe Landreville Nadeau. Seeds were sterilized using the surface sterilization protocol described above. Seeds were dried under laminar air flow on sterilized petri plates.

Minimal medium was used to germinate and grow cranberry seeds. Minimal medium per liter was prepared by combining 800 ml of Nanopure distilled water, 1 g of sucrose, 4 g Gel Gro (Gelzan, 0.4% agar), 10 ml macroelement solution (100x), 10 ml $Ca(NO_3)_2 \cdot 4H_2O$ solution (100x), 5 ml of

NaFe EDTA (200x), 1 ml microelements solution (1000x), and 1 ml of potassium iodide (KI) (1000x) in a sterilized 1L glass bottle . The bottle was placed on a heated stirrer to mix and dissolve the contents and then Nanopure distilled water was added to bring the volume to 1 L. The pH of the solution was adjusted to 5.5 with KOH. The solution was then autoclaved at 121°C. After cooling, 1 ml of vitamin solution (1000x) was added under sterile conditions under laminar air flow. Minimal medium was poured into sterilized plastic boxes (with lids) that were used for seed germination.

Ten cranberry seeds were placed into each box (25 boxes per field) that were then incubated in the dark at 25℃ for one month. Boxes were checked every week and contaminated boxes were eliminated. The study contained three treatments (*Pseudomonas* sp. CSWB3, *Pseudomonas* sp. CLWB12, *Lachnum* sp. EFK28) and two controls (negative and positive). The positive control was *Lachnum* sp. EC5; a biocontrol fungus that was previously isolated in the laboratory by Lila Salhi; this strain was obtained from the Stevens variety cranberries of a Pierre Fortier field (Notre-Dame-de-Lourdes, Quebec). Each treatment was repeated four times (separate boxes).

2.9. Endophyte inoculation

One month after seed germination, the small cranberry plants were inoculated with four endophytes: (i) *Lachnum* sp. EC5; (ii) *Lachnum* sp. EFK28 (Bieler Cranberries); and (iii) and (iv) two *Pseudomonas* spp. (CSWB3 and CLWB12) (Gillivert). Fresh bacterial liquid cultures were prepared in potato dextrose broth one day before inoculation and were incubated at 25℃. The next day, the optical density (OD) of the bacterial culture was measured at 600 nm using a spectrophotometer. The OD of the bacterial culture was adjusted to 0.1 prior to inoculation of plants.

For *Lachnum* sp. EC5, 10-15 days prior inoculation of the plants, fresh potato dextrose broth containing a small piece of the fungus was incubated at 25℃. Two days prior to inoculation, the fungus was removed from the liquid culture and ground in a sterilized blender to break up the septate hyphae. A 20 µl aliquot of the fungal suspension was grown on a PDA plate in order to estimate fungal viability and to exclude potential bacterial contamination that would interfere with the plant growth assay described below.

Twenty boxes containing small seedlings (Steven's cultivar) were inoculated as follows: four boxes were inoculated with 20 µl (in each hole) of sterilized potato dextrose broth as negative controls,

four boxes with inoculated with 20 µl of *Lachnum* sp. EC5 as positive controls, four boxes were inoculated with 20 µl of *Lachnum* sp. EFK28, four boxes were inoculated with 20 µl of *Pseudomonas* sp. CSWB3, and four boxes were inoculated with 20 µl of *Pseudomonas* sp. CLWB12.

The same procedure was repeated using another 20 boxes with small seedlings obtained from Scarlet Knight seeds. The inoculated boxes were incubated at 25℃ for two months and observations were made one a week.

2.10. Seedling measurement

Two months following endophyte inoculation, the seedlings were harvested, washed carefully with tap water to remove remaining agar and endophytes, and measured and weighed. Next, stem lengths were measured, and each small plant was separated into two stems and roots that were transferred into petri plates and dried at 55°C for 24 hours. Dry weights of stems and roots were measured and recorded using a micro balance.

2.11. Isolation of genomic DNA from *Pseudomonas* sp. CSWB3

The bacterial isolate *Pseudomonas* sp. CSWB3 was selected for whole genome sequencing, because it had the strongest biocontrol effect on fungal pathogens. An overnight culture (10 mL) of *Pseudomonas* sp. CSWB3 was grown in potato dextrose broth and the optical density was measured at 600 nm using a spectrophotometer. The ideal OD for bacterial DNA extraction is 0.3 at 600 nm (Genomic-tip 20/G manual from QIAGEN, preparation Gram-negative and some Gram-positive bacterial samples). A 1 mL aliquot of the overnight culture was subjected to genomic DNA purification. Sample preparation and processing followed the protocol supplied with the QIAGEN DNA purification columns (Genomic-tip 20/G manual from QIAGEN, preparation of buffers B1, B2, QBT, QC and QF). The 1 ml aliquot of the bacterial culture was centrifuged at 5000 x *g* for 10 min to pellet the bacterial cells, from which the supernatant was removed and discarded. The bacterial pellet was resuspended in 1 ml of buffer B1 (containing 2 µl of RNase A solution) by vortexing at top speed. Next, 20 µl of lysozyme solution (100 mg/ml) and 45 µl of QIAGEN proteinase K stock solution were added, and the tube was incubated at 37℃ for 30 min. Following incubation, 0.35 ml of buffer B2 were added and the tube was inverted several times to mix, followed by incubation at 50℃ for 30 min. A Genomic-tip 20/G was equilibrated with 1ml of buffer QBT and was allowed to empty by gravity flow. The DNA sample was then transferred to the

equilibrated QIAGEN genomic-tip and entered the resin by gravity flow. The column was then washed with 3 x 1 ml of buffer QC. The genomic DNA was eluted with 2 x 1 ml of buffer QF, precipitated with ethanol, and centrifuged at 5000 x g for 15 min at 4℃. The DNA pellet was washed with 1 ml of cold 70% ethanol, air-dried, resuspended in 50 µl of TE buffer, and dissolved at 55℃ for 2 hours. The quantity and quality of the extracted genomic DNA was estimated using gel electrophoresis and NanoDrop spectrometry.

2.12. Genome sequencing using Illumina MiSeq

The *Pseudomonas* sp. CSWB3 DNA was sequenced by Le Centre d'Expertise et de Services Génome Québec (Montréal, Québec, Canada), using an Illumina MiSeq instrument and the NEBNext Ultra II DNA Library Preparation Kit for Illumina (New England Biolabs). Sequencing was conducted as paired-end reads with a maximum read length of ~300 bp. The resulting FASTQ read files were downloaded from the genome center's website for further processing.

2.13. Genome assembly and annotation of *Pseudomonas* sp. CSWB3

2.13.1. Genome assembly

Paired-end FASTQ files encompassing both forward and reverse reads were cleaned and corrected using Trimmomatic and Rcorrector (using the clean-MISEQ script developed by our research group). Trimmomatic performs quality control of the paired-end reads (i.e., suppression of reads below a given read call probability and removal of primer sequences). Read errors are corrected by Rcorrector, which uses an approach based on k-mer counts.

Corrected reads were assembled using SPAdes (St. Petersburg genome assembler), a *de novo* genome assembler based on Paths P and Q algorithms that can assemble paired and unpaired reads in either FASTA or FASTQ formats (Bankevich *et al*., 2012). SPAdes assembles genomes based on four principal steps: (i) construction of a de Bruijn assembly graph based on k-mers of a given length followed by graph simplification, (ii) k-bimer adjustment, (iii) construction of a paired assembly graph, and (iv) inference of large contigs (Bankevich *et al*., 2012).

2.13.2. Genome annotation

Genome annotation was performed using Prokka (Seemann, 2014), which is a tool used for rapid bacterial genome annotation. The assembled contigs in FASTA format served as the input data for Prokka. The following external prediction tools are used by Prokka: Prodigal (for prediction of

coding sequences), RNAmmer (for rRNA genes), Aragorn (for tRNA genes) and Infernal (for non-coding RNAs). Annotation of the protein coding genes was performed in two steps: (1) identification of precise gene locations and (2) functional annotation using BLAST to comparisons with known sequences in large databases (Seemann, 2014). Preliminary identification of genes involved in secondary metabolite biosynthesis was done by Prokka. antiSMASH was used to make more precise gene identifications, as described below.

2.14. Genome analysis of *Pseudomonas* sp. CSWB3 and comparative genomics

2.14.1. Core genome of *Pseudomonas* sp. CSWB3

The core genome of *Pseudomonas* sp. CSWB3 was analyzed using Prokka 1.13 and a genome analysis tool provided by Pathosystems Resource Integration Center (PATRIC) version 3.6.2, which is a bacterial bioinformatic database and analysis resource (Wattam *et al*., 2017) that can be found at (https://www.patricbrc.org/).

2.14.2. Taxonomy and phylogenetic analysis

PATRIC version 3.6.2 was used to generate a classification and phylogenetic tree of *Pseudomonas* sp. CSWB3. PARTIC uses Codon Tree to infer a phylogenetic tree, based on all shared proteins of the bacterial genomes included in the analysis. Bacterial data available at PATRIC are linked to the NCBI taxonomy database.

2.14.3. Secondary metabolite analysis

The secondary metabolite gene clusters of *Pseudomonas* sp. CSWB3 were identified and analyzed using antiSMASH version 5.1.0 (Blin *et al*., 2019) (https://antismash.secondarymetabolites.org/) and the gene bank file (gbk) obtained from Prokka annotation. AntiSMASH-characterized gene clusters with 100% match to the reference in the AntiSMASH database were selected for further analysis. For each gene cluster along with the potential compound produced by it, the number of genes involved in each secondary metabolite biosynthesis was identified by referring to previous studies listed on the antiSMASH website. Additional information on potential secondary metabolites were obtained from the antiSMASH-MIBiG database (Kautsar *et al*., 2020) (https://mibig.secondarymetabolites.org/).

2.14.4. Comparative analysis of the *Pseudomonas* sp. CSWB3 genome with the genomes of *Pseudomonas* sp. EB42, *Bacillus velezensis* EB37, *Bacillus velezensis* EBFV, and *Pseudomonas protegens* Pf-5

The *Pseudomonas* sp. CSWB3 genome was compared to the genomes of *Pseudomonas* sp. EB42, *Bacillus velezensis* EB37, *Bacillus velezensis* EBFV (these biocontrol strains were previously isolated in Lang's Laboratory by a PhD student, Lila Salhi), and *Pseudomonas protegens* Pf-5, a biocontrol bacteria used in previous studies (Jing et al., 2020).

Pairwise alignments between the *Pseudomonas* sp. CSWB3 genome in gbk format and the four bacterial isolates were performed using a genomic visualization tool called progressiveMauve (Darling, Mau, & Perna, 2010) (http://darlinglab.org/mauve/). Homologous sequences of aligned genomes were analyzed. Mauve results were visualized *via* an extended multi-Fasta (xmfa) file and (backbone) file that contains conserved regions within the aligned genomes.

The web server Orthovenn2 (Xu *et al*., 2019) (https://orthovenn2.bioinfotoolkits.net/home) was used to compare orthologous gene clusters between the five bacterial strains. The input files for Orthovenn2 were the protein sequence (.faa) format obtained from the Prokka annotation.

Secondary metabolite gene clusters of *Pseudomonas* sp. CSWB3 were individually compared to the four bacterial isolates (*Pseudomonas* sp. EB42, *Bacillus velezensis* EB37, *Bacillus velezensis* EBFV and *Pseudomonas protegens* Pf-5) to determine if there is similarity between their secondary metabolite biosynthesis gene clusters. Sequence alignments were performed with the progressiveMauve software by searching each secondary metabolite gene or protein ID. The BLAST tool in Orthovenn2 was used to compare the amino acid sequences corresponding to the genes of each gene cluster in the five bacterial strains.

NCBI BLAST was also used to compare *Pseudomonas* sp. CSWB3 secondary metabolite gene clusters to the entire NCBI database.

2.15. Statistical analysis

Seed germination data variance were analyzed by ANOVA using RStudio (version 1.2.1335© 2009-2019 RStudio, Inc); statistical significance was established as $p \leq 0.05$. One-way ANOVA was used to establish the significance between the mean values of stem length, stem dry weight, and root dry weight per treatment. Two-way ANOVA was used to evaluate the statistical

significance of each treatment on two cranberry varieties: Stevens from the Bieler Cranberries field and Scarlet Knight from Landreville Nadeau. Bar charts were created using GraphPad Prism 8 software (version 8.4.3).

CHAPTER 3 – RESULTS

3. Results

3.1. Previous results

Results from Lang's laboratory at the University of Montreal generated by the PhD student Lila Salhi showed that six *Bacillus velezensis* isolates (EB4, EB5, EB33, EB36, EB37 and EB55) can suppress the growth of the fungal pathogen *Colletotrichum gloeosporioides,* a common cranberry fungal pathogen (Figure 1). *Bacillus velezensis* EB5 inhibited the growth of *Colletotrichum gloeosporioides* more significantly as compared to the other isolates, but could not suppress the growth of the fungal pathogens *Phomopsis vaccinii, Cytospora chrysosperma,* and *Godronia cassandrae*. *Bacillus velezensis* EB37 was the only isolate that inhibited all of the pathogens that were tested. Therefore, *Bacillus velezensis* EB37 has become an important isolate for further analysis to reveal genes responsible for biocontrol activity.

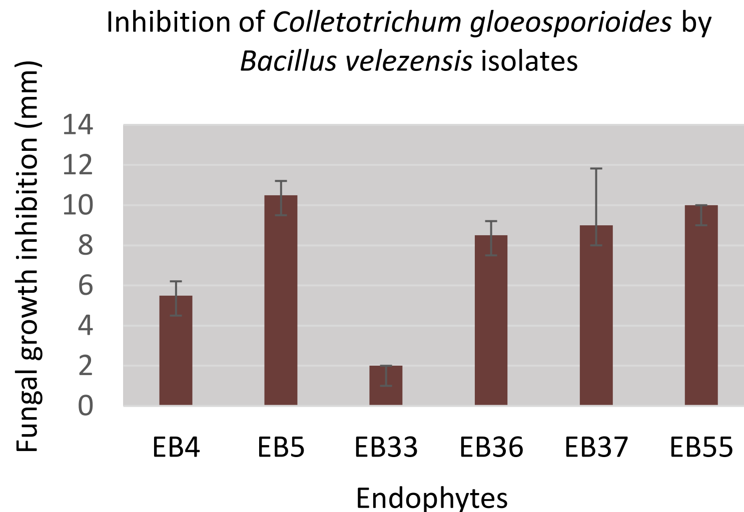

Figure 1. – Inhibition of *Colletotrichum gloeosporioides* by six *Bacillus velezensis* isolates. *Bacillus velezensis* EB5, EB55, and EB37 generated the largest inhibition zone against *Colletotrichum gloeosporioides* (10.5 mm, 10 mm, and 9 mm, respectively)

3.2. Results

3.2.1. Endophytes isolated from cranberry plants

Morphologically different endophytes were isolated from the tissue of cranberry plants obtained from two Stevens cranberry producers: Bieler Cranberries and Gillivert. A total of 124 endophytes were isolated, of which 34 were bacteria and 90 were fungi. The majority of endophytes were isolated from leaves of low yield cranberry plants from the Gillivert field (Table 2).

Table 2.- Total number of endophytes isolated from cranberry plants. Bacteria and fungi were isolated from surface-sterilized cranberry plant tissue corresponding to very good yield, good yield, and weak yield plants from two Stevens variety producers: Bieler Cranberries and Gillivert. The majority of endophytes were isolated from low yield plants.

Field	Plant Group	Plant Part	Bacterial Endophytes	Fungal Endophytes	Total Number of Endophytes
Bieler Cranberries (Stevens variety)	Very Good-Yield	Leaves	-	1	1
		Stems	-	5	5
		Roots	-	-	-
	Good-Yield	Leaves	-	-	-
		Stems	-	3	3
		Roots	1	11	12
	Weak-Yield	Leaves	-	1	1
		Stems	-	10	10
		Roots	1	-	1
Gillivert (Stevens variety)	Good-Yield	Leaves	2	7	9
		Stems	2	12	14
		Roots	2	4	6
	Weak-Yield	Leaves	**14**	**14**	**28**
		Stems	5	**16**	21
		Roots	7	6	13
Total			**34**	**90**	**124**

3.2.2. Biocontrol activity of cranberry endophytes on pathogens

Confrontation tests showed that 25 of 34 bacterial isolates displayed biocontrol effects on at least one of the 19 fungal pathogens, whereas only 22 of 90 fungi showed a biocontrol effect. Among the tested isolates, the bacterial isolates CSWB3 and CLWB12 and the fungal isolate EFK28 showed antagonistic activity against most of the fungal pathogens. Based on bacterial 16S rDNA and fungal ITS rDNA ribotyping, the bacterial isolates CSWB3 and CLWB12 are 99.69% identical to *Pseudomonas* sp. and the fungus isolate EFK28 is 95% identical to *Lachnum* sp. (Table 3). ITS rDNA of *Lachnum* sp. EFK28 is 100% identical to *Lachnum* sp. EC5, which is a biocontrol agent that was previously isolated from the roots of the Stevens variety field "Pierre Fortier" by the PhD student Lila Salhi. It is possible that these two *Lachnum* isolates are identical, transferred by planting material (stolons obtained by mowing of a field) that was supplied by the Pierre Fortier farm, and implying that root endophytes may be transferred this way.

Table 3.- Origin and the closest match of CSWB3, CLWB12, and EFK28 isolates based on BLAST analysis of 16S and ITS sequences

Isolate code	**Isolate origin**			**Closest match in NCBI database (Accession number)**	**Identity (%)**
	Producer	Yield	Plant part		
CSWB3	Gillivert (Stevens variety)	Weak yield	Stems	*Pseudomonas* sp. (KC920926.1)	99.69%
CLWB12	Gillivert (Stevens variety)	Weak yield	Leaves	*Pseudomonas* sp. (KC920926.1)	99.69%
EFK28	Bieler Cranberries (Stevens variety)	Good yield	Stems	*Lachnum* sp. (EU794910.1)	95.70% 100% identical to *Lachnum* sp. EC5

Performance of *Pseudomonas* sp. CSWB3 and *Pseudomonas* sp. CLWB12 was similar and these two isolates suppressed the same fungal pathogens (Table 4). Both *Pseudomonas* sp. CSWB3 and *Pseudomonas* sp. CLWB12 inhibited the growth of the fungal pathogens *Colletotrichum* sp. and *Diaporthe* sp., which are common causal agents of cranberry fruit rot (Figure 2).

Table 4.- Inhibition of fungal pathogens by the most active endophytes (*Pseudomonas* sp. CSWB3, *Pseudomonas* sp. CLWB12, and *Lachnum* sp. EFK28). Bacterial isolates CSWB3 and CLWB12 suppressed the same pathogens and showed similar inhibition zone values.

Pathogens	**Inhibition zone (mm) of pathogens induced by *Pseudomonas* sp. CSWB3 and CLWB12 and *Lachnum* sp. EFK28**		
	Pseudomonas sp. CSWB3	*Pseudomonas* sp. CLWB12	*Lachnum* sp. EFK28
***Peniophora* sp**	29	34	12.5
***Diaporthe* sp**	40.5	42.5	-
***Penicillium* sp**	-	-	-
***Colletotrichum* sp**	33.5	30.5	-
***Physalospora* sp**	11.5	7.5	33.75
***Rhizopus* sp**	-	-	-
***Trichoderma* sp**	-	-	33
Cadophora luteo-olivacea	-	-	-
Botrytis cinerea	-	-	-
Alternaria alternate	25	27.5	30
Physalospora vaccinia	4	10	-
Fusarium graminearum	-	-	-
Verticillium dahlia	12.5	12	12
Diaporthe vaccinia	24	24.5	-

***Cytospora* sp**	27	27	26
Godronia cassandrae	7	6	-
Uncharacterized Strain PatT3	-	-	-
Uncharacterized Strain PatB2	-	-	-
Uncharacterized Strain PatB3	-	-	-

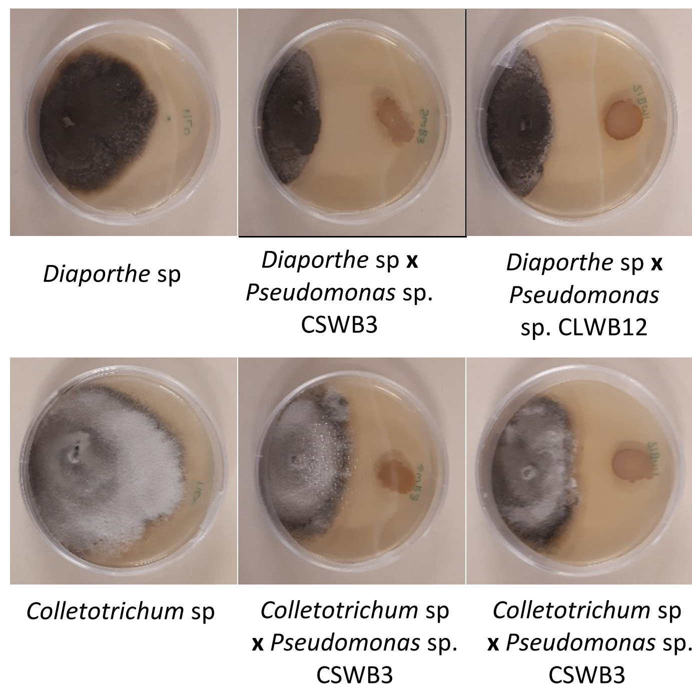

Figure 2. – Growth inhibition of the fungal pathogens *Diaporthe* sp. and *Colletotrichum* sp. by cranberry endophytic bacteria *Pseudomonas* sp. CSWB3 and *Pseudomonas* sp. CLWB12 at day 30

3.2.3. Compatibility between endophytes

The mutual compatibility of the three most promising endophytes (*Pseudomonas* sp. CSWB3, *Pseudomonas* sp. CLWB12, and *Lachnum* sp. EFK28) was tested *in vitro*. Mutual compatibility is an interesting feature because it enhances the synergistic effect that compatible endophytes have on fungal pathogens and hence improves biocontrol of plant diseases. An antagonistic assay that

evaluated combinations of *Pseudomonas* sp. CSWB3, *Pseudomonas* sp. CLWB12, and *Lachnum* sp. EFK28 showed that these isolates are not compatible (Figure 3). Confirmatory antagonistic tests with these same bacterial species were conducted that showed a narrow inhibition zone, which potentially indicates that the two bacterial species may be from different species. According to (Riley & Gordon, 1999), *Escherichia coli* produces bacteriocin called colicins. This compound can mediate population dynamics within species under specific conditions, such as stress and lack of nutrients. Therefore, in the same bacterial population, there are bacteriocin sensitive and bacteriocin resistant bacteria. Whole genome sequencing of both isolates would be needed to confirm this.

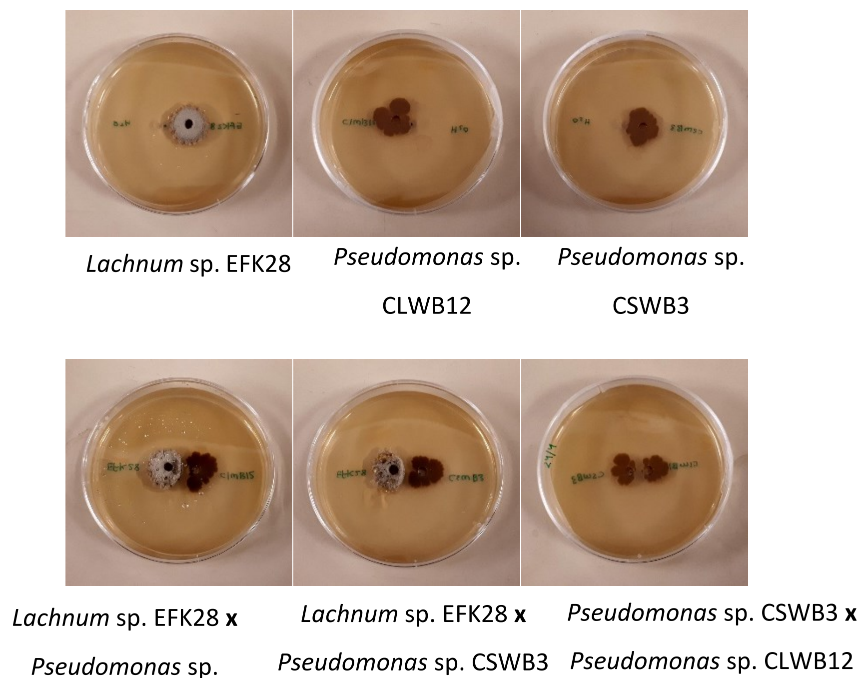

Figure 3. – Compatibility tests between *Pseudomonas* sp. CSWB3, *Pseudomonas* sp. CLWB12, and *Lachnum* sp. EFK28

3.2.4. Effect of *Pseudomonas* sp. CSWB3, *Pseudomonas* sp. CLWB12 and *Lachnum* sp. EFK28 on the growth of Scarlet Knight and Stevens cranberry seedlings

3.2.4.1. Stem length

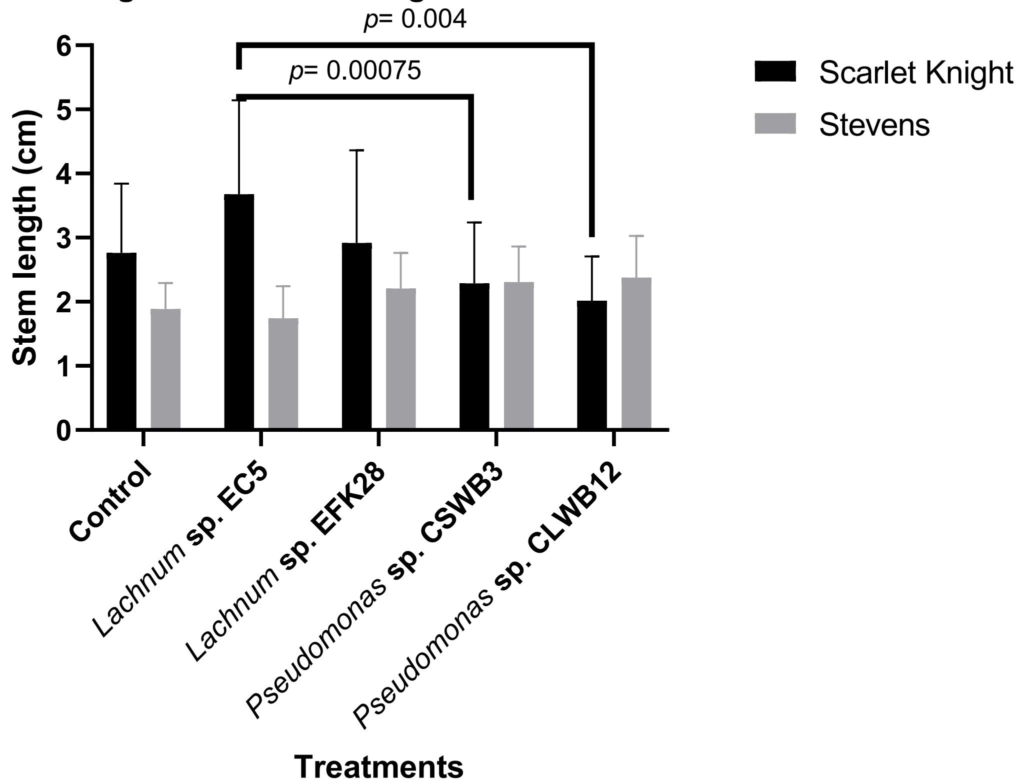

Figure 4. – The effect of endophyte inoculation on the stem length (cm) of cranberry seedlings obtained from two varieties (Stevens and Scarlet Knight). Young plants were inoculated with the negative control, *Lachnum* sp. EC5 fungus as a positive control, *Lachnum* sp EFK28, and *Pseudomonas* sp. CSWB3 and CLWB12. Standard error was calculated from three independent replicates.

The average stem length of Scarlet Knight seedlings inoculated with *Lachnum* sp. EC5 (3.7 cm) was significantly higher as compared to seedlings inoculated with *Pseudomonas* sp. CSWB3 and

Pseudomonas sp. CLWB12 (~ 2 cm) (*p*-values of 0.00075 and 0.004, respectively) (Figure 4). In contrast, the mean stem length of Stevens seedlings inoculated with *Pseudomonas* sp. CSWB3 (2.3 cm) was significantly higher as compared to seedlings inoculated with the negative control (1.9 cm) and *Lachnum* sp. EC5 (1.7 cm) (*p*-values of 0.04 and 0.01, respectively) (Figure 4). Additionally, seedlings inoculated with *Pseudomonas* sp. CLWB12 had taller stems (2.4 cm) as compared to *Lachnum* sp. EC5 (1.7 cm) (*p*-value of 0.02). The mean stem length of seedlings treated with *Lachnum* sp. EFK28 (2.1 cm) was significantly higher as compared to seedlings treated with *Lachnum* sp. EC5 (1.7 cm) (*p*-value 0.02) (Figure 4).

Two-way analysis of variance showed that the same treatments (negative control, *Lachnum* sp. EC5, *Lachnum* sp. EFK28, *Pseudomonas* sp. CSWB3, and *Pseudomonas* sp. CLWB12) applied to both cranberry varieties (Scarlet Knight (Landreville Nadeau) and Stevens (Bieler Cranberries)) were statistically not different ($p \geq 0.05$).

3.2.4.2. Stem and leaf dry weight

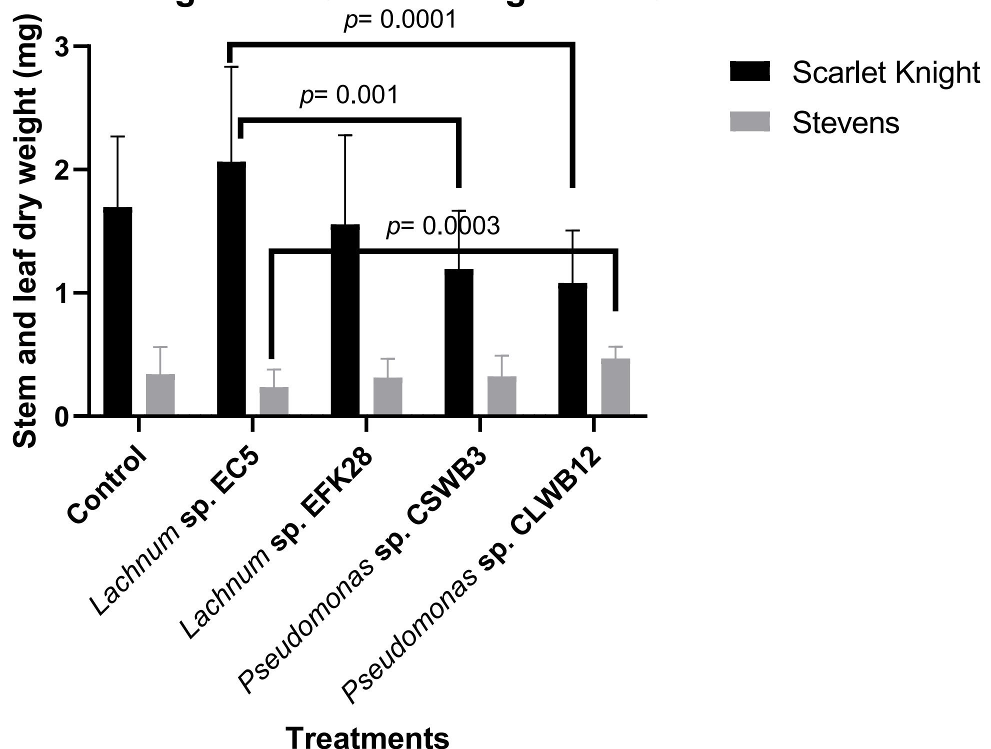

Figure 5. – The effect of endophytes on cranberry seedling stem and leaf dry biomass (mg). Cranberry seedlings of two cranberry varieties (Stevens and Scarlet Knight) were inoculated with the negative control, *Lachnum* sp. EC5 fungus as a positive control, *Lachnum* sp. EFK28, and *Pseudomonas* sp. CSWB3 and CLWB12. Standard error was calculated from four independent replicates.

The stem and leaf dry biomass (mg) of Scarlet Knight seedlings inoculated with *Lachnum* sp. EC5 (2 mg) was significantly higher as compared to seedlings inoculated with *Pseudomonas* sp. CLWB12 (1 mg) and *Pseudomonas* sp. CSWB3 (1.2 mg) (p-values of 0.0001 and 0.001, respectively) (Figure 5). The above-ground biomass of negative control seedlings (1.7 mg) was significantly higher as compared to *Pseudomonas* sp. CLWB12 (p value of 0.005) and *Pseudomonas* sp. CSWB3 (p value of 0.02) (Figure 5).

Seedling stem and leaf biomass of Stevens was significantly higher in those inoculated with *Pseudomonas* sp. CLWB12 (0.47 mg) as compared to *Lachnum* sp. EC5 (0.2 mg), control (0.3 mg), *Pseudomonas* sp. CSWB3 (0.3 mg), and *Lachnum* sp. EFK28 (0.3 mg) (p-values of 0.0003, 0.02, 0.02, and 0.03, respectively) (Figure 5).

Based on two-way ANOVA analysis, the same treatments (negative control, *Lachnum* sp. EC5, *Lachnum* sp. EFK28, *Pseudomonas* sp. CSWB3, and *Pseudomonas* sp. CLWB12) that were applied to both varieties (Scarlet Knight and Stevens) were not significantly different ($p \geq 0.05$).

3.2.4.3. Root dry weight

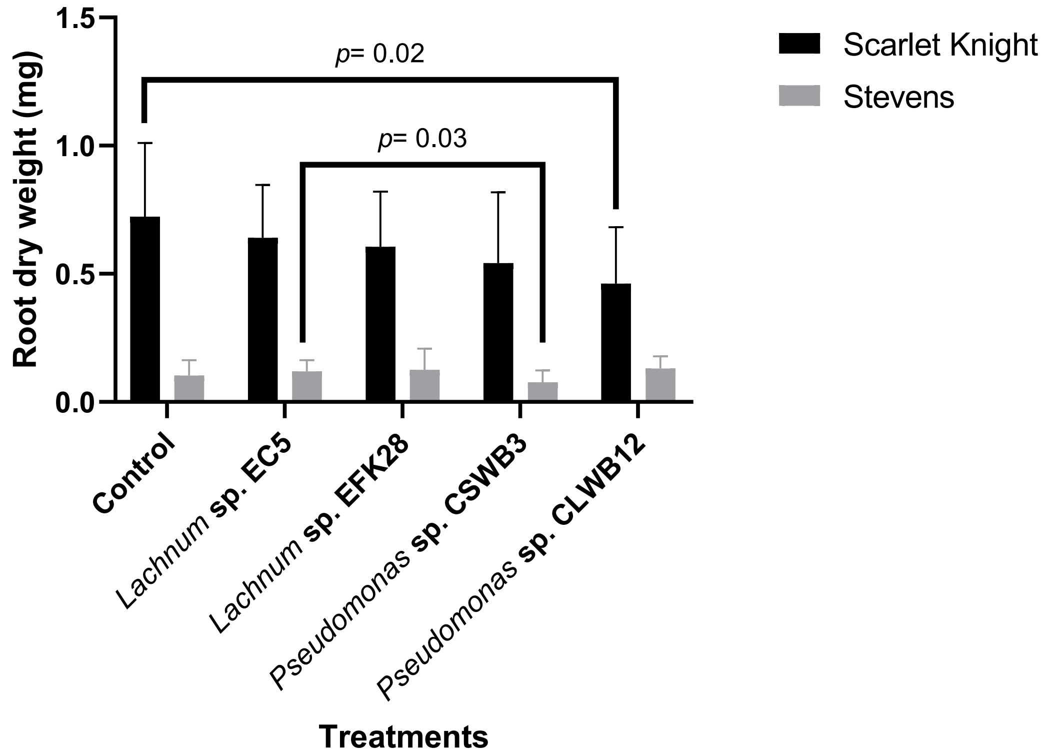

Figure 6. – The average dry root biomass (mg) of cranberry seedlings of two varieties (Stevens and Scarlet Knight) were inoculated with the negative control, *Lachnum* sp. EC5 fungus as a positive control, *Lachnum* sp. EFK28, and *Pseudomonas* sp. CSWB3 and CLWB12. Standard error was calculated from four independent replicates.

The root dry weight (mg) of Scarlet Knight seedlings was larger than the root dry weight of Stevens seedlings. The root dry weight of Scarlet Knight seedlings was significantly higher for the control seedlings (0.72 mg) as compared to the seedlings inoculated with *Pseudomonas* sp. CLWB12 (0.46 mg) (*p*-value of 0.02), whereas for the Stevens variety, the root dry biomass was larger for seedlings inoculated with *Lachnum* sp. EC5 (0.1 mg) as compared to seedlings inoculated with *Pseudomonas* sp. CSWB3 (0.07 mg) (*p*-value 0.03) (Figure 6).

Based on two-way ANOVA analysis, the same treatments (negative control, EC5, EFK28, CSWB3, and CLWB12) applied to both varieties (Scarlet Knight and Stevens) were not significantly different ($p \geq 0.05$).

3.2.4.4. Root: Stem dry weight ratio

Statistical analysis showed that endophyte inoculation did not significantly influence the ratio of root dry biomass to stem dry biomass of Scarlet Knight seedlings, whereas this ratio was significantly higher for Stevens seedlings inoculated with *Lachnum* sp. EC5 (0.64) as compared to seedlings inoculated with *Pseudomonas* sp. CSWB3 (0.33) or *Pseudomonas* sp. CLWB12 (0.28) (*p*-values of 0.01 and 0.03, respectively) (Table 5).

Two-way ANOVA showed that there was an interaction between treatments (C, EC5, EFK28, CSWB3 and CLWB12) for both fields (Scarlet Knight and Stevens); however, these same treatments were not significantly different in both fields and showed a consistent effect on root:stem ratio.

Table 5.- The average root:stem dry weight ratio ± standard error of cranberry seedlings of two varieties (Stevens and Scarlet Knight) that were inoculated with the negative control, *Lachnum* sp. EC5 fungus as a positive control, *Lachnum* sp. EFK28 fungus, and *Pseudomonas* sp. CSWB3 and CLWB12

Seedlings	Control	*Lachnum* sp. EC5	*Lachnum* sp. EFK28	*Pseudomonas* sp. CSWB3	*Pseudomonas* sp. CLWB12
Scarlet Knight	0.47 ± 0.04	0.34 ± 0.03	0.46 ± 0.07	0.47 ± 0.05	0.67 ± 0.22
Stevens	0.43 ± 0.09	0.64 ± 0.07*	0.51 ± 0.10	0.34 ± 0.06*	0.28 ± 0.03*

3.2.5. Genome analysis of *Pseudomonas* sp. CSWB3

3.2.5.1. General features of the *Pseudomonas* sp. CSWB3 genome

The *Pseudomonas* sp. CSWB3 genome was sequenced using Illumina MiSeq. A total of 7,239,103 sequencing reads were obtained that were assembled into 25 contigs using SPAdes. The assembled genome size of *Pseudomonas* sp. CSWB3 is 6,902,922 bp, with a G+C content of 63.4%. A total of 6,328 genes were predicted by Prokka version 1.13 (Seemann, 2014), including 6,180 coding sequences (CDS) (Table 6).

Table 6.- General features of the *Pseudomonas* sp. CSWB3 genome generated by Prokka version 1.13

Feature	***Pseudomonas* sp. CSWB3**
Contigs	25
Genomic size (bp)	6,902,922
GC%	63.4%
Genes	6,328
Plasmids	0
Chromosomes	0
CDS	6,180
rRNA	6
tRNA	68
tmRNA	1
misc_RNA	73

The distribution of the annotated *Pseudomonas* sp. CSWB3 genome including CDS and RNA genes on 25 contigs obtained from the PATRIC genome analysis tools is shown in Figure 7.

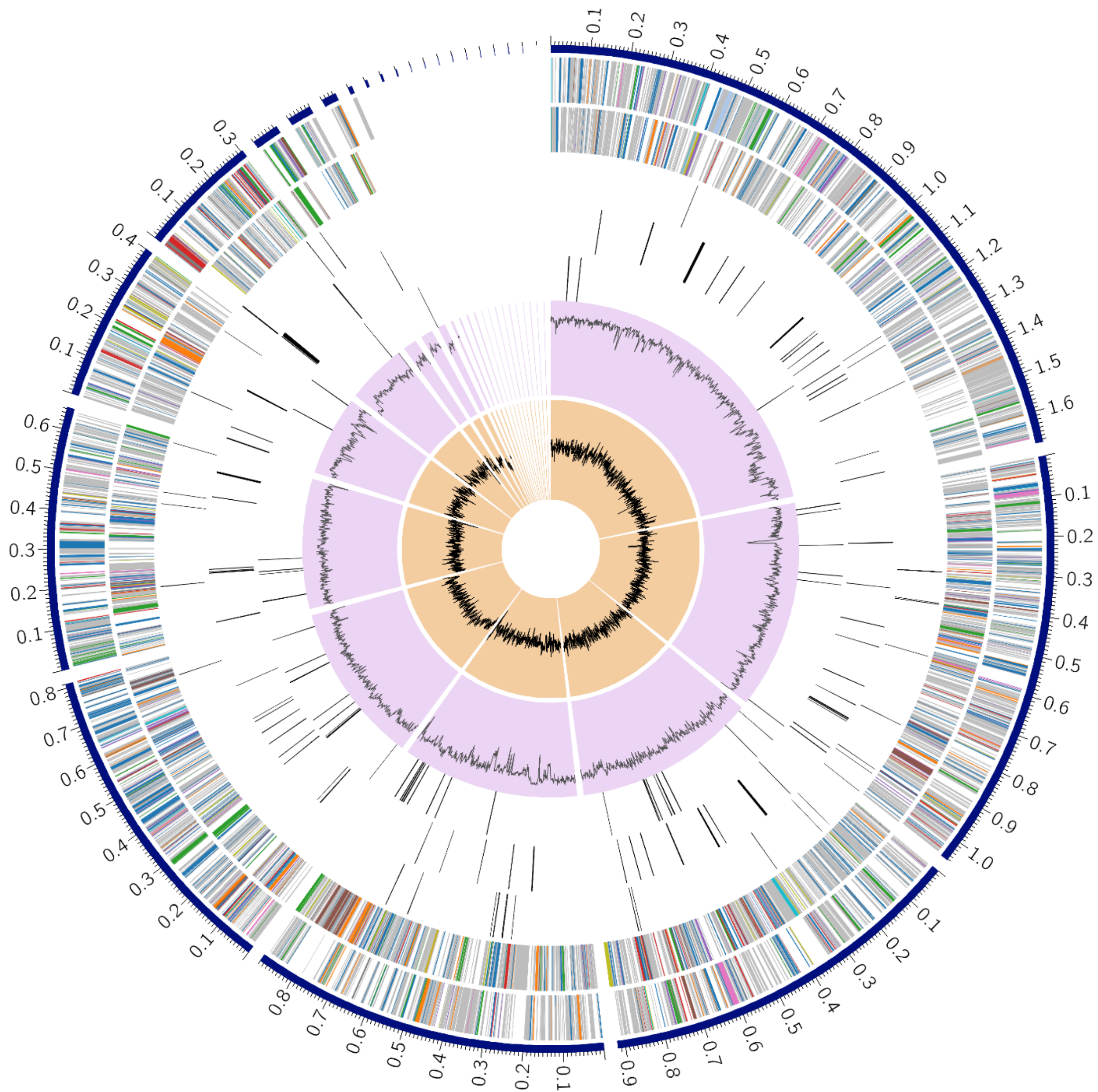

Figure 7. – Circular distribution display of the annotated genes of *Pseudomonas* sp. CSWB3. Starting with the outer ring, the display is organized as follows: contigs, forward strand CDS, reverse strand CDS, RNA genes, CDS associated with antimicrobial resistance, CDS with homology to known virulence factors, GC content, and GC skew. The color of each CDS on the forward and reverse strands indicate the subsystem that these genes belong to (see the subsystems graph below). PATRIC 3.6.2 (Wattam *et al*., 2017)

3.2.5.2. *Pseudomonas* sp. CSWB3 taxonomy and phylogenetic analysis

Based on molecular phylogenetic analysis, the *Pseudomonas* sp. CSWB3 genome is highly similar to the genome of *Pseudomonas protegens*, with 100% sequence coverage (Table 7 and Figure 8).

Table 7.- Taxonomy of *Pseudomonas* sp. CSWB3 generated by PATRIC 3.6.2 (Wattam *et al*., 2017)

Percent Coverage	Rank	NCBI Taxon ID	Scientific Name
100	Root	1	
100	Root 1	131567	cellular organisms
100	Domain	2	Bacteria
100	Phylum	1224	*Proteobacteria*
100	Class	1236	*Gammaproteobacteria*
100	Order	72274	*Pseudomonadales*
100	Family	135621	*Pseudomonadaceae*
100	Genus	286	*Pseudomonas*
100	Genus 1	136843	*Pseudomonas fluorescens group*
100	Species	380021	*Pseudomonas protegens*
64	Species 1	1124983	*Pseudomonas protegens* CHA0
16	Species 1	1420599	*Pseudomonas protegens* Cab57

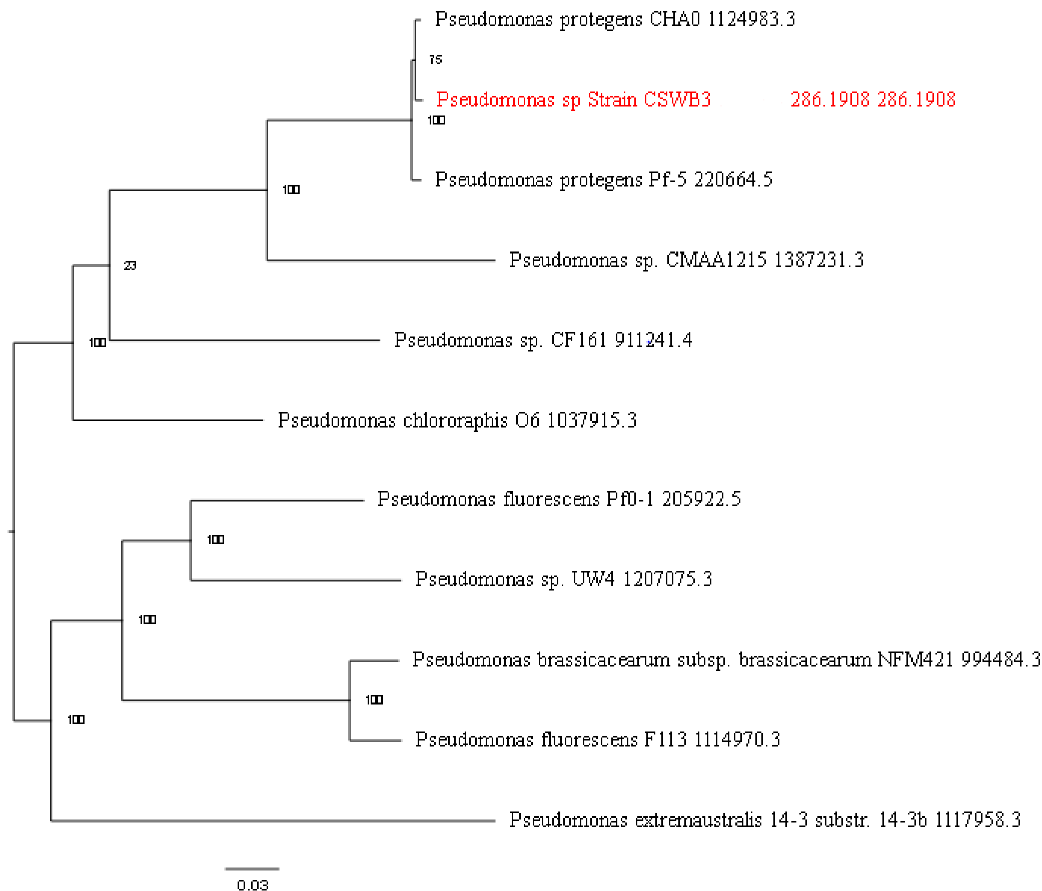

Figure 8. – Phylogenetic tree of *Pseudomonas* sp. CSWB3. Using PATRIC tools (Wattam *et al.*, 2017) and based on the whole genome sequence, *Pseudomonas* sp. CSWB3 shows high affinity to the *Pseudomonas protegens* genome

3.2.5.3. Secondary metabolite clusters within the *Pseudomonas* sp. CSWB3 genome

A total of 17 characterized and unknown secondary metabolites gene clusters were predicted by an analysis of the *Pseudomonas* sp. CSWB3 genome using gene bank file format (gbk) in antiSMASH version 5.1.0. We focused only on BGCs that perfectly matched the reference in antiSMASH (i.e., 100% match), because these BGCs encode proteins that have been previously studied and are known to synthesize specific secondary metabolites. Five BGCs predicted by antiSMASH were identified in the *Pseudomonas* sp. CSWB3 genome, including Type I Polyketide Synthase (T1PKS), Type III Polyketide Synthase (T3PKS), NRPS, Others, and NRPS-like clusters. These BGCs encode proteins that are involved in the synthesis of the following antimicrobial secondary metabolites: pyoluteorin, 2,4-diacetylphloronglucinol, putisolvin, pyrrolnitrin, bicornutin A1, and

bicornutin A2, respectively (Table 8).

Table 8.- BGCs identified in the *Pseudomonas* sp. CSWB3 by AntiSMASH with 100% similarity to the reference and secondary metabolites produced

Compound	Synthetase type	Genes	Size (kb)	Bioactivity	Reference
Pyoluteorin	T1PKS (polyketide)	*bltB, bltC*	12.7	Antibiotic, antifungal	*Pseudomonas protegens* Pf-5
Pyrrolnitrin	Others	*PmA, B, C, D*	5.8	Strong antifungal	*Pseudomonas chlororaphis*
Putisolvin	NRPS	*psoA, B, C; macA, B*	43.7	Antimicrobial, cytotoxicity, and surfactant	*Pseudomonas putida*
2,4-diacetylphloroglucinol	T3PKS (polyketide)	*phlA, C, B, D, E, F*	7.2	Antifungal	*Pseudomonas fluorescens*
Bicornutin A1/Bicornutin A2	NRPS-like	*bicA*	0.855	Antimicrobial	*Xenorhabdus budapestensis*

3.2.6. Comparative analysis of *Pseudomonas* sp. CSWB3 with four biocontrol bacterial strains

Major findings of the comparative analysis of the *Pseudomonas* sp. CSWB3 genome with the genomes of *Pseudomonas* sp. EB42, *Pseudomonas protegens* Pf-5, *Bacillus velezensis* EB37, and *Bacillus velezensis* EBFV are described below.

3.2.6.1. Orthologous average nucleotide identity between the genomes of *Pseudomonas* sp. CSWB3, *Pseudomonas* sp. EB42, *Pseudomonas protegens* PF-5, *Bacillus velezensis* EB37, and *Bacillus velezensis* EBFV

Percent similarity between the genomes of the isolates listed above was determined using the orthologous average nucleotide identity (OrthoANI) tool. This analysis showed that the

Pseudomonas sp. CSWB3 genome is most similar to the genome of *Pseudomonas protegens* Pf-5 (98.64% identity) (Figure 9).

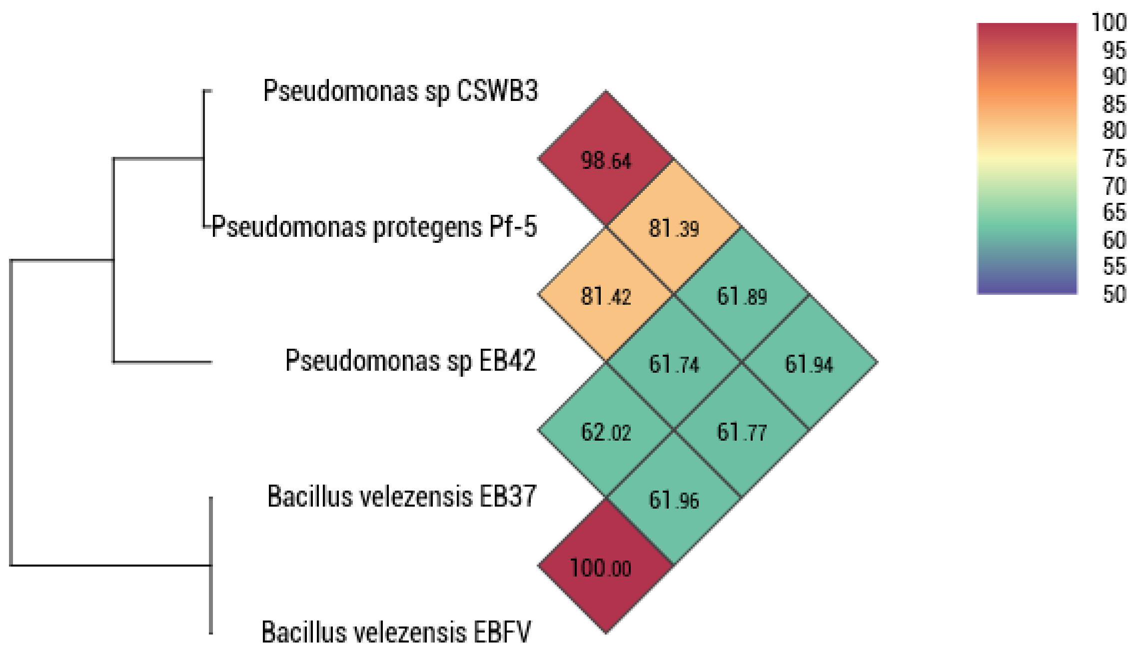

Figure 9. – Comparison of OrthoANI values between the genomes of *Pseudomonas sp*. CSWB3, *Pseudomonas sp*. EB42, *Pseudomonas protegens* Pf-5, *Bacillus velezensis* EB37, and *Bacillus velezensis* EBFV

3.2.6.2. Core genomes of *Pseudomonas* sp. CSWB3, *Pseudomonas* sp. EB42, *Pseudomonas protegens* PF-5, *Bacillus velezensis* EB37, and *Bacillus velezensis* EBFV

Orthovenn2 analysis showed that the genome of *Pseudomonas* sp. CSWB3 contains 5,886 gene clusters that encode 6,180 proteins. Five of these clusters are unique to *Pseudomonas* sp. CSWB3 (i.e., they are not found in the other four strains). These five unique clusters encode seven proteins of unknown function and four proteins of known function. Of these known proteins, two are involved in bacteriocin immunity that makes the bacterial cells resistant to bacteriocin produced by other bacteria. Another two of the known proteins are involved in capsule production that protects 2 proteins responsible for encapsulation and external protection of this strain.

Orthovenn2 analysis showed that 817 orthologous clusters are common in the five strains, 16% of the common genome is dedicated to the biological process, and among these clusters, 16% are

involved in metabolic processes that include secondary metabolite biosynthesis. No specific orthologous clusters were identified in the genomes of *Bacillus velezensis* EB37 and *Bacillus velezensis* EBFV, whereas *Pseudomonas* sp. EB42 contains 90 unique clusters and shares 78 clusters with *Pseudomonas* sp. CSWB3. Among these shared clusters, 8% are involved in metabolic processes. *Pseudomonas* sp. CSWB3 shares 1328 common clusters with *Pseudomonas protegens* Pf-5, of which 18% are involved in metabolic processes. The three *Pseudomonas* sp. strains (CSWB3, EB42, and *protegens* pf5) share 3,587 common gene clusters; among these clusters, 17% are involved in metabolic processes (Figure 10).

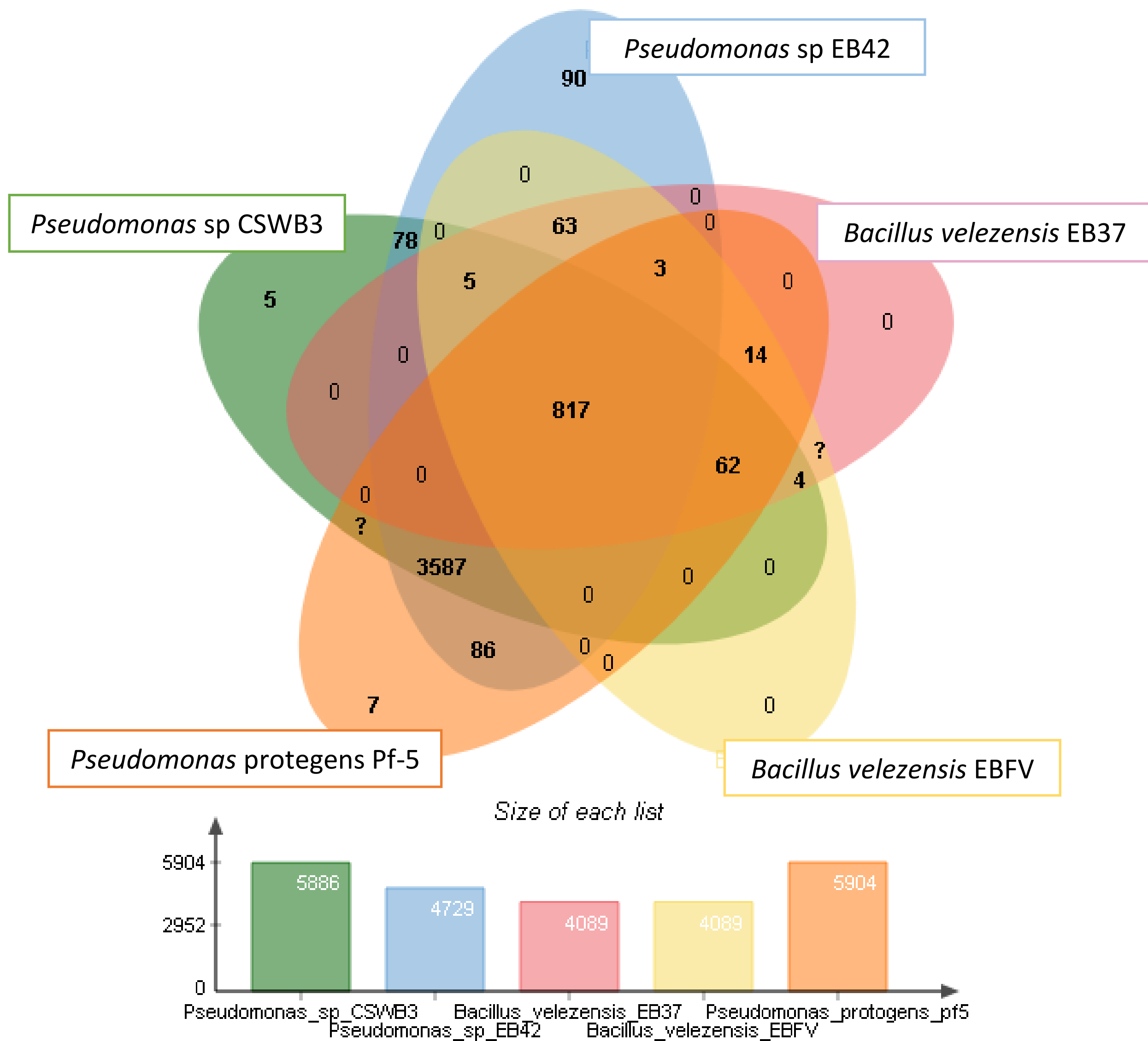

Figure 10. – Venn diagram generated by Orthovenn2 showing orthologous gene clusters for *Pseudomonas* sp. CSWB3, *Pseudomonas* sp. EB42, *Pseudomonas protegens* Pf-5, *Bacillus velezensis* EB37, and *Bacillus velezensis* EBFV

3.2.6.3. Comparative genomic insight into secondary metabolite biosynthesis

The *Pseudomonas* sp. CSWB3 genome contains five BGCs identified by antiSMASH with 100% similarity to the reference (Table 9). These BGCs consist of genes that encode large PKS or NRPS proteins that may produce the antimicrobial secondary metabolites pyoluteorin, pyrrolnitrin, putisolvin, 2,4-diacetylphloronglucinol, bicornutin A1, and bicornutin A2.

Orthovenn2 BLAST was used to compare the amino acids sequence of each secondary metabolite secreted by *Pseudomonas* sp. CSWB3 to the secondary metabolites secreted by the other four bacteria (*Pseudomonas* sp. EB42, *Pseudomonas protegens* Pf-5, *Bacillus velezensis* EB37, and *Bacillus velezensis* EBFV). Only *Pseudomonas protegens* Pf-5 has 100% sequence coverage and 99% identity for pyoluteorin, pyrrolnitrin, putisolvin, and 2,4-diacetylphloronglucinol secondary metabolites gene clusters with an *E-value* of 0.0, whereas the gene cluster responsible for the biosynthesis of bicornutin A1 and bicornutin A2 secondary metabolites was unique to *Pseudomonas* sp. CSWB3. Other strains showed low sequence coverage and identity for these gene clusters. Orthologous secondary metabolite gene clusters were also visualized using progressiveMauve in which each gene code or protein ID was searched for individually and the same results were obtained.

To find the difference in secondary metabolite identity between *Pseudomonas* sp. CSWB3 and *Pseudomonas protegens* Pf-5, another antiSMASH search was performed for the *Pseudomonas protegens* Pf-5 genome. The *Pseudomonas protegens* Pf-5 genome contains 15 BGCs of which four are 100% antiSMASH-characterized and are similar to BGCs found in the *Pseudomonas* sp. CSWB3 genome and synthesize the same secondary metabolites: pyoluteorin, pyrrolnitrin, putisolvin, and 2,4-diacetylphloronglucinol. To identify the differences in each secondary metabolite-synthesizing enzyme in both strains, BLASTp at NCBI sequence alignment was performed. This analysis showed that the T1PKS that produces pyoluteorin differed in 23 amino acids between the two strains. This analysis also found a three amino acids difference in the T3PKS that produces 2,4-diacetylphloronglucinol, a four amino acid difference in the protein that produces pyrrolnitrin, and a 22 amino acid difference in the protein that produces putisolvin between the two

strains (*Pseudomonas* sp. CSWB3 and *Pseudomonas protegens* Pf-5). All of the amino acid changes and substitutions in each protein did not affect the production of the same secondary metabolite, per antiSMASH predictions.

Bicornutin A1 and bicornutin A2 secondary metabolites are produced only by *Pseudomonas* sp. CSWB3 because the NRPS-like gene cluster responsible for their biosynthesis exists only in *Pseudomonas* sp. CSWB3 and not in *Pseudomonas* sp. EB42, *Pseudomonas protegens* PF-5, *Bacillus velezensis* EB37 or *Bacillus velezensis* EBFV.

3.2.6.3.1. Comparison of secondary metabolite gene clusters of *Pseudomonas* sp. CSWB3 with the NCBI database

The five BGCs of *Pseudomonas* sp. CSWB3 were compared to entries in the NCBI database using BLAST. We found that T1PKS that potentially synthesizes pyoluteorin matched the *Pseudomonas protegens* T1PKS, with 74% coverage and 100% identity. The gene cluster classified as (Others) that is responsible for pyrrolnitrin biosynthesis matched the DUF1864 family protein of *Pseudomonas protegens,* with 100% coverage and 99% identity. The NRPS gene cluster responsible for putisolvin biosynthesis matched orfamide A non-ribosomal peptide synthetase (OfaC) of *Pseudomonas protegens*. The T3PKS gene cluster that synthesizes 2, 4-diacetylphloronglucinol matched T3PKS of *Pseudomonas protegens,* with 100% coverage and identity. The NRPS-like gene cluster responsible for bicornutin A1 and bicornutin A2 biosynthesis matched NRPS of *Pseudomonas protegens,* with 100% coverage and 99.65% identity.

CHAPTER 4 – DISCUSSION, CONCLUSIONS, AND PROSPECTIVE FUTURE WORK

4.1. Discussion

It has been reported that certain endophytes secrete compounds that control the growth of plant pathogens and enhance plant growth and yield *via* a process known as a biocontrol reaction; thus, these endophytes are known as biocontrol agents (Gupta et al., 2016). The present study focused on cranberry endophytes that may be capable of controlling fungal pathogens of cranberries and enhancing cranberry production. Cranberry plants are of particular interest because North America is the leading producer of cranberries around the world.

4.1.1. Isolated endophytes

In this study, we investigated the endophytes of commercial cranberry cultivars from two fields of the Stevens variety: Bieler Cranberries and Gillivert. Among the 124 isolated endophytes, 34 were bacteria and 90 were fungi and most of these were isolated from the leaves of Gillivert weak yielding plants. This result correlates with previous studies that showed that endosymbionts are found in almost all plants, irrespective of high or weak yield (Bragina *et al*., 2012). In addition, a wide diversity of endophytic bacteria and fungi have been previously studied. More than 200 species of bacteria have been isolated from plant tissue, with the majority corresponding to the phyla *Actinobacteria, Proteobacteria,* and *Firmicutes* (Golinska et al., 2015). Fungi have been associated with plants for millions of years (Krings et al., 2007). It is notable that endophytes are widely spread in all plant parts with weak and good yield. Furthermore, crop yield depends on multiple interactions and factors such as the environment and soil. In other words, the outcome of plant yield is a cumulative and integrated effect.

4.1.2. Biocontrol activity

Our results demonstrate that *Pseudomonas* sp. CSWB3 and CLWB12 and the fungus *Lachnum* sp. EFK28 are the most active of the isolates, showing antagonistic functions against the majority of fungal pathogens. *Pseudomonas* sp. CSWB3 and CLWB12 suppressed *Diaporthe vaccinii, Cytospora* sp.*, Physalospora vaccinii, Alternaria alternata, Verticillium dahliae, peniophora* sp., *Diaporthe* sp., *Colletotrichum* sp., *Physalospora* sp., and *Godronia cassandrae* fungal pathogens.

Most importantly, *Pseudomonas* sp. CSWB3 and CLWB12 inhibited the growth of *Diaporthe* sp. and *Colletotrichum* sp. (large inhibition zones > 30 mm), which are common culprits of cranberry fruit rot. The fungal isolate *Lachnum* sp. EFK28 showed antagonistic activity against *Peniophora* sp., *Cytospora* sp*., Verticillium dahliae, Trichoderma* sp., *Alternaria alternate,* and *Physalospora* sp. Consistent with previous studies conducted by our laboratory and other studies identified *Pseudomonas* spp. as biocontrol agents because they produce secondary metabolites that act as antimicrobial compounds (Gross & Loper, 2009). The fungus *Lachnum* sp. EFK28 has been previously used in medicine and agriculture because it produces various bioactive compounds with antimicrobial activity (Rukachaisirikul et al., 2006). This suggests that *Pseudomonas* sp. CSWB3 and CLWB12 and *Lachnum* sp. EFK28 are associated with the biocontrol of fungal pathogens, and most importantly with the control of pathogens that cause damage to cranberry plants. We suggest that this biocontrol activity may be due to antifungal secondary metabolites produced by these strains, which would explain why they may inhibit the growth of fungal pathogens.

4.1.3. Seedling inoculation with endophytes

The results of seedling inoculation experiments showed that *Pseudomonas* sp. CSWB3, *Pseudomonas* sp. CLWB12, and *Lachnum* sp. EFK28 enhanced the stem length and weight of Stevens seedlings but not Scarlet Knight seedlings. However, these same strains did not affect the root biomass of both cranberry varieties. These observations are consistent with previous studies that showed that various endophytes produce phytohormones that enhance plant growth including gibberellins (GAs), indole acetic acid (IAA) (Tsavkelova et al., 2006), and IAA that stimulate cell division stimulation, which enhances plant growth (Martinez-Viveros *et al*., 2010). The stem length of yam plants (*Dioscorea rotundata*) was increased following inoculation with IAA-producing bacteria (Swain et al., 2007). GA plays important roles in seed germination, leaf elongation, and flowering (Stamm & Kumar, 2010). The enhancement of plant growth of Stevens variety seedlings mediated by *Pseudomonas* sp. CSWB3 and CLWB12 and *Lachnum* sp. EFK28 may have resulted from the production of phytohormones by these bacteria. However, it is unclear why these isolates did not enhance the growth of Scarlet Knight seedlings.

4.1.4. *Pseudomonas* sp. CSWB3: secondary metabolites and their mechanism of action

The *Pseudomonas* sp. CSWB3 genome contains five characterized BGCs that matched 100% with references in antiSMASH. These BGCs encode large proteins such as NRPSs, PKSs and hybrid

NRPSs-PKSs that synthesize antimicrobial secondary metabolites, namely pyoluteorin, pyrrolnitrin, putisolvin, 2, 4-diacetylphloroglucinol, bicornutin A1 and biocomutin A2. Among these, pyoluteorin, pyrrolnitrin, and 2, 4-diacetylphloroglucinol have strong antifungal activity. Our findings are consistent with previous findings that showed that *Pseudomonas* spp. such as *P. fluorescens* express NRPSs, PKSs, and hybrid NRPSs-PKSs that are involved in the synthesis of secondary metabolites such as pseudomonine, safracin, mupirocin, 2,4-diacetylphloroglucinol, and 2,5-dialkylresorcinols. These metabolites are well known for their antimicrobial effects (Gross & Loper, 2009). It is notable that *Pseudomonas* sp. CSWB3 may control fungal pathogens because it has the potential to produce antifungal compounds that target and inhibit fungal pathogen growth. The antifungal secondary metabolites produced by *Pseudomonas* sp. CSWB3 (pyoluteorin, pyrrolnitrin, and 2, 4-diacetylphloroglucinol) target the main cellular processes of pathogenic fungi. The main target of the antifungal pyrrolnitrin is the cellular respiration of the pathogenic fungi (Tripathi & Gottlieb, 1969). The antifungal compound 2, 4-diacetylphloroglucinol exhibits antifungal activity by causing wrinkling and severe damage to hyphae (Gong *et al*., 2016). The secondary metabolite pyoluteorin is toxic to plant fungal pathogens such as *Pythium ultimum* and can suppress symptoms caused by this fungus (Nowak-Thompson, Chaney, Wing, Gould, & Loper, 1999). Not all *Pseudomonas* spp. secrete both 2, 4-diacetylphloroglucinol and pyoluteorin; to date, only a few *Pseudomonas protegens* strains have been shown to produce both compounds (Yan, Philmus, Chang, & Loper, 2017). It is interesting to note that *Pseudomonas* sp. CSWB3 has the potential to secrete both 2, 4-diacetylphloroglucinol and pyoluteorin, which suggests that this strain is a very effective antifungal agent.

4.1.5. Comparison of the *Pseudomonas* sp. CSWB3 genome with the genomes of *Pseudomonas* sp. EB42, *Pseudomonas protegens* Pf-5, *Bacillus velezensis* EB37, and *Bacillus velezensis* EBFV

Of the strains tested in this work, the genome of *Pseudomonas* sp. CSWB3 is most closely related to *Pseudomonas protegens* Pf-5, with 98.64% sequence identity. These two strains have 3,587 gene clusters in common, of which 17% are involved in metabolic processes that include secondary metabolite production.

Comparison of the amino acid sequences of each secondary metabolite produced by *Pseudomonas* sp. CSWB3 with those produced by the other four bacteria revealed that they have 100% sequence

coverage with *Pseudomonas protegens* Pf-5 and 99% identities for secondary metabolites, namely pyoluteorin, pyrrolnitrin, 2,4-diacetylphloroglucinol, and putisolvin. This suggests that secretion of bicornutin A1 and A2 is unique to *Pseudomonas* sp. CSWB3. These metabolites are produced only by *Pseudomonas* sp. CSWB3, as NRPS-like gene clusters exist only in this strain. Bicornutin is a strong antimicrobial compound that was previously found to be produced by *Xenorhabdus budapestenis*. Bicornutin is active against zoospores by suppressing mycelial growth of *Phytophthora nicotianae* (Boszormenyi *et al*., 2009). Hence, *Pseudomonas* sp. CSWB3 produces antimicrobial compound that targets mycelial growth, which makes this strain more effective at inhibiting fungal pathogens as compared to *Pseudomonas protegens* Pf-5.

4.1.6. Limitations of this study

The cranberry endophytes and cranberry seedlings used in the current study were grown in growth medium rather than in soil, which is a limitation of the current research. Although the use of a growth medium has advantages regarding the use of space, nutrients, temperature, better control, and optimal pH, it also has limitations. Plants growing in soil are influenced by the combined conditions of the soil and its contents, the temperature, and numerous external factors, which are not precisely simulated by growth media. This limits the generalizability of the results obtained in the current study, and therefore, the experiments need to be verified in the field or in a greenhouse.

Despite the many advantages of endophytic organisms, they also produce secondary chemicals that cause irreversible damage to host plants, which may ultimately leads to losses of livestock. Thus, we think that the selection of endophytes for agricultural applications should be made carefully in order to reduce the impact of secondary metabolites produced by endophytes on all organisms across a broad spectrum. The exploitation of plant-endophyte interactions results in enhanced plant growth as well as sustainable agricultural practices for all crops. Genome sequencing of endophytes sheds light on the genes responsible for endophytic properties. The obtained information can be used for transcriptome as well as proteome analysis in studies of other plant-endophyte interactions.

4.2. Conclusions and prospective future work

In conclusion, the results of our study show that certain endophytes have the potential to control fungal pathogens and enhance plant growth. The bacterial strain *Pseudomonas* sp. CSWB3 suppressed the growth of the majority of fungal pathogens that were tested and was able to enhance the growth of cranberry seedlings (Stevens variety). The genomic structure of endophytes influences their behavior and mechanisms of pathogen growth inhibition. Analysis of the *Pseudomonas* sp. CSWB3 genome showed that it possesses five gene clusters that encode large enzymes that are responsible for the biosynthesis of six antimicrobial secondary metabolites. This may be the reason why this strain has the potential to enhance cranberry plant growth and suppress fungal pathogens. Development of this endophyte will greatly enhance cranberry production and protect the environment from the effects of using chemical fungicides. Our findings support our hypothesis that certain endophytes that possess secondary metabolite biosynthetic gene clusters, such as *Pseudomonas* sp. CSWB3, have the potential to enhance the growth of cranberry plants as well as to inhibit cranberry fungal pathogens.

Endophytes are a crucial biological resource that require additional studies to gain a greater understanding of their roles in protecting plants from pathogenic microorganisms. Future work could investigate the antagonistic ability of *Pseudomonas* sp. CSWB3 in greenhouse and field experiments to confirm the findings of the current research. Genetic engineering of the *Pseudomonas* sp. CSWB3 genome could transform this strain into a more effective biocontrol agent by the introduction of genes that encode strong antifungal effects or enhance secondary metabolite production via gene mutations (insertion/duplication) or a gain-of-function mutation that could make the mutant *Pseudomonas* sp. CSWB3 more effective than the wild-type strain at controlling fungal pathogens. *B.subtilis* strain ATCC 6633 is a biocontrol bacteria that produce the lipopeptide mycosubtilin, which is effective in suppressing *Pythium* infection. A recombinant strain was designated by the replacement of the promoter of mycosubtilin operon in the wild-type with promoter originated from the replication gene of *Staphylococcus aureus* plasmid pUB110. Results showed that the recombinant strain can produce 15-fold more mycosubtilin compared to the wild-type, and showed dramatically effective inhibition of *Pythium* infection in tomato seedlings (Wu, Wu, Qiao, Gao, & Borriss, 2015). Antimicrobial secondary metabolites produced by endophytes could also be extracted and used as fungicides.

Bibliography

Amoutzias, G. D., Chaliotis, A., & Mossialos, D. (2016). Discovery Strategies of Bioactive Compounds Synthesized by Nonribosomal Peptide Synthetases and Type-I Polyketide Synthases Derived from Marine Microbiomes. *Mar Drugs, 14*(4). doi:10.3390/md14040080

Anderson, J. P., Gleason, C. A., Foley, R. C., Thrall, P. H., Burdon, J. B., & Singh, K. B. (2010). Plants versus pathogens: an evolutionary arms race. *Funct Plant Biol, 37*(6), 499-512. doi:10.1071/FP09304

Ansari, M. Z., Yadav, G., Gokhale, R. S., & Mohanty, D. (2004). NRPS-PKS: a knowledge-based resource for analysis of NRPS/PKS megasynthases. *Nucleic Acids Res, 32*(Web Server issue), W405-413. doi:10.1093/nar/gkh359

Bamisile, B. S., Dash, C. K., Akutse, K. S., Keppanan, R., & Wang, L. (2018). Fungal Endophytes: Beyond Herbivore Management. *Front Microbiol, 9*, 544. doi:10.3389/fmicb.2018.00544

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., . . . Pevzner, P. A. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol, 19*(5), 455-477. doi:10.1089/cmb.2012.0021

Berdy, J. (2012). Thoughts and facts about antibiotics: where we are now and where we are heading. *J Antibiot (Tokyo), 65*(8), 385-395. doi:10.1038/ja.2012.27

Blin, K., Shaw, S., Steinke, K., Villebro, R., Ziemert, N., Lee, S. Y., . . . Weber, T. (2019). antiSMASH 5.0: updates to the secondary metabolite genome mining pipeline. *Nucleic Acids Res, 47*(W1), W81-W87. doi:10.1093/nar/gkz310

Boszormenyi, E., Ersek, T., Fodor, A., Fodor, A. M., Foldes, L. S., Hevesi, M., . . . Taylor, R. A. (2009). Isolation and activity of Xenorhabdus antimicrobial compounds against the plant pathogens Erwinia amylovora and Phytophthora nicotianae. *J Appl Microbiol, 107*(3), 746-759. doi:10.1111/j.1365-2672.2009.04249.x

Bragina, A., Berg, C., Cardinale, M., Shcherbakov, A., Chebotar, V., & Berg, G. (2012). Sphagnum mosses harbour highly specific bacterial diversity during their whole lifecycle. *Isme Journal, 6*(4), 802-813. doi:10.1038/ismej.2011.151

Chang, H. S., Lin, C. H., Chen, Y. S., Wang, H. C., Chan, H. Y., Hsieh, S. Y., . . . Chen, I. S. (2016). Secondary Metabolites of the Endophytic Fungus Lachnum abnorme from Ardisia cornudentata. *Int J Mol Sci, 17*(9). doi:10.3390/ijms17091512

Clua, J., Roda, C., Zanetti, M. E., & Blanco, F. A. (2018). Compatibility between Legumes and Rhizobia for the Establishment of a Successful Nitrogen-Fixing Symbiosis. *Genes, 9*(3). doi:ARTN 125

10.3390/genes9030125

Coates, M. E., & Beynon, J. L. (2010). Hyaloperonospora arabidopsidis as a Pathogen Model. *Annual Review of Phytopathology, Vol 48, 48*, 329-345. doi:10.1146/annurev-phyto-080508-094422

Conti, M., Cinget, B., Vivancos, J., Oudemans, P., & Belanger, R. R. (2019). A Molecular Assay Allows the Simultaneous Detection of 12 Fungi Causing Fruit Rot in Cranberry. *Plant Disease, 103*(11), 2843-2850. doi:10.1094/Pdis-03-19-0531-Re

Darling, A. E., Mau, B., & Perna, N. T. (2010). progressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. *PLoS One, 5*(6), e11147. doi:10.1371/journal.pone.0011147

Dey, P., Datta, D., Saha, D., Parida, S., & Panda1, D. (2019). Plant-Endophyte Interaction and Its Application to Abiotic Stress Management of Crop Plants. *International Journal of Current Microbiology and Applied Sciences, 8*(7), 2708-2716. doi:doi.org/10.20546/ijcmas.2019.807.332

Diaz-Garcia, L., Rodriguez-Bonilla, L., Phillips, M., Lopez-Hernandez, A., Grygleski, E., Atucha, A., & Zalapa, J. (2019). Comprehensive analysis of the internal structure and firmness in American cranberry (Vaccinium macrocarpon Ait.) fruit. *PLoS One, 14*(9), e0222451. doi:10.1371/journal.pone.0222451

Doughari, J. (2015). An Overview of Plant Immunity. *Journal of Plant Pathology & Microbiology, 6*(11). doi:10.4172/2157-7471.1000322

Freeman, B. C., & Beattie, G. A. (2008). An Overview of Plant Defenses against Pathogens and Herbivores. *Plant Pathology and Microbiology, 94*. doi:10.1094/PHI-I-2008-0226-01

Gao, F. K., Dai, C. C., & Liu, X. Z. (2010). Mechanisms of fungal endophytes in plant protection against pathogens. *African Journal of Microbiology Research, 4*(13), 1346-1351. Retrieved from <Go to ISI>://WOS:000280352100001

Garcia-Sanchez, S., Bernales, I., & Cristobal, S. (2015). Early response to nanoparticles in the Arabidopsis transcriptome compromises plant defence and root-hair development through salicylic acid signalling. *BMC Genomics, 16*, 341. doi:10.1186/s12864-015-1530-4

Gebrie, S. A. (2016). Biotrophic Fungi Infection and Plant Defense Mechanism. *Journal of Plant Pathology & Microbiology, 7*(9). doi:10.4172/2157-7471.1000378

Golinska, P., Wypij, M., Agarkar, G., Rathod, D., Dahm, H., & Rai, M. (2015). Endophytic actinobacteria of medicinal plants: diversity and bioactivity. *Antonie Van Leeuwenhoek, 108*(2), 267-289. doi:10.1007/s10482-015-0502-7

Gong, L., Tan, H., Chen, F., Li, T., Zhu, J., Jian, Q., . . . Duan, X. (2016). Novel synthesized 2, 4-DAPG analogues: antifungal activity, mechanism and toxicology. *Sci Rep, 6*, 32266. doi:10.1038/srep32266

Gross, H., & Loper, J. E. (2009). Genomics of secondary metabolite production by Pseudomonas spp. *Nat Prod Rep, 26*(11), 1408-1446. doi:10.1039/b817075b

Gupta, H., Saini, R. V., Pagadala, V., Kumar, N., Sharma, D. K., & Saini, A. K. (2016). Analysis of plant growth promoting potential of endophytes isolated from Echinacea purpurea and Lonicera japonica. *Journal of Soil Science and Plant Nutrition, 16*(3), 558-577. Retrieved from <Go to ISI>://WOS:000395278500001

Hamayun, M., Hussain, A., Khan, S. A., Kim, H. Y., Khan, A. L., Waqas, M., . . . Lee, I. J. (2017). Gibberellins Producing Endophytic Fungus Porostereum spadiceum AGH786 Rescues Growth of Salt Affected Soybean. *Front Microbiol, 8*, 686. doi:10.3389/fmicb.2017.00686

Hardoim, P. R., van Overbeek, L. S., Berg, G., Pirttila, A. M., Compant, S., Campisano, A., . . . Sessitsch, A. (2015). The Hidden World within Plants: Ecological and Evolutionary Considerations for Defining Functioning of Microbial Endophytes. *Microbiology and Molecular Biology Reviews, 79*(3), 293-320. doi:10.1128/Mmbr.00050-14

Hisano, M., Bruschini, H., Nicodemo, A. C., & Srougi, M. (2012). Cranberries and lower urinary tract infection prevention. *Clinics, 67*(6), 661-667. doi:10.6061/clinics/2012(06)18

Hui, S., Yan, H., Qing, X., Renyuan, Y., & Yongqiang, T. (2013). Isolation, characterization, and antimicrobial activity of endophytic bacteria from Polygonum cuspidatum *African Journal of Microbiology Research, 7*(16), 1496-1504. doi:10.5897/ajmr12.899

Ihrmark, K., Bodeker, I. T., Cruz-Martinez, K., Friberg, H., Kubartova, A., Schenck, J., . . . Lindahl, B. D. (2012). New primers to amplify the fungal ITS2 region--evaluation by 454-sequencing of artificial and natural communities. *FEMS Microbiol Ecol, 82*(3), 666-677. doi:10.1111/j.1574-6941.2012.01437.x

Jan, A. T., Azam, M., Ali, A., & Haq, Q. M. R. (2011). Novel approaches of beneficial Pseudomonas in mitigation of plant diseases - an appraisal. *Journal of Plant Interactions, 6*(4), 195-205. doi:10.1080/17429145.2010.541944

Jing, X., Cui, Q., Li, X., Yin, J., Ravichandran, V., Pan, D., . . . Zhang, Y. (2020). Engineering Pseudomonas protegens Pf-5 to improve its antifungal activity and nitrogen fixation. *Microb Biotechnol, 13*(1), 118-133. doi:10.1111/1751-7915.13335

Jones, J. D., & Dangl, J. L. (2006). The plant immune system. *Nature, 444*(7117), 323-329. doi:10.1038/nature05286

Kautsar, S. A., Blin, K., Shaw, S., Navarro-Munoz, J. C., Terlouw, B. R., van der Hooft, J. J. J., . . . Medema, M. H. (2020). MIBiG 2.0: a repository for biosynthetic gene clusters of known function. *Nucleic Acids Res, 48*(D1), D454-D458. doi:10.1093/nar/gkz882

Kawaguchi, M., & Minamisawa, K. (2010). Plant-microbe communications for symbiosis. *Plant Cell Physiol, 51*(9), 1377-1380. doi:10.1093/pcp/pcq125

Khare, E., Mishra, J., & Arora, N. K. (2018). Multifaceted Interactions Between Endophytes and Plant: Developments and Prospects. *Front Microbiol, 9*, 2732. doi:10.3389/fmicb.2018.02732

Krings, M., Taylor, T. N., Hass, H., Kerp, H., Dotzler, N., & Hermsen, E. J. (2007). Fungal endophytes in a 400-million-yr-old land plant: infection pathways, spatial distribution, and host responses. *New Phytol, 174*(3), 648-657. doi:10.1111/j.1469-8137.2007.02008.x

Laluk, K., & Mengiste, T. (2010). Necrotroph attacks on plants: wanton destruction or covert extortion? *Arabidopsis Book, 8*, e0136. doi:10.1199/tab.0136

Lee, E. H., Eo, J. K., Ka, K. H., & Eom, A. H. (2013). Diversity of Arbuscular Mycorrhizal Fungi and Their Roles in Ecosystems. *Mycobiology, 41*(3), 121-125. doi:10.5941/Myco.2013.41.3.121

Liu, G., Kong, Y., Fan, Y., Geng, C., Peng, D., & Sun, M. (2017). Data on genome analysis of Bacillus velezensis LS69. *Data Brief, 13*, 1-5. doi:10.1016/j.dib.2017.04.053

Martinez-Viveros, O., Jorquera, M. A., Crowley, D. E., Gajardo, G., & Mora, M. L. (2010). Mechanisms and Practical Considerations Involved in Plant Growth Promotion by Rhizobacteria. *Journal of Soil Science and Plant Nutrition, 10*(3), 293-319. doi:10.4067/S0718-95162010000100006

Muller, C. A., Oberauner-Wappis, L., Peyman, A., Amos, G. C., Wellington, E. M., & Berg, G. (2015). Mining for Nonribosomal Peptide Synthetase and Polyketide Synthase Genes Revealed a

High Level of Diversity in the Sphagnum Bog Metagenome. *Appl Environ Microbiol, 81*(15), 5064-5072. doi:10.1128/AEM.00631-15

Muresu, R., Maddau, G., Delogu, G., Cappuccinelli, P., & Squartini, A. (2010). Bacteria colonizing root nodules of wild legumes exhibit virulence-associated properties of mammalian pathogens. *Antonie Van Leeuwenhoek, 97*(2), 143-153. doi:10.1007/s10482-009-9396-6

Nowak-Thompson, B., Chaney, N., Wing, J. S., Gould, S. J., & Loper, J. E. (1999). Characterization of the pyoluteorin biosynthetic gene cluster of Pseudomonas fluorescens Pf-5. *J Bacteriol, 181*(7), 2166-2174. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/10094695

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

Ortiz-Galeana, M. A., Hernández-Salmerón, J. E., Valenzuela-Aragón, B., Santos-Villalobos, S. d. l., Rocha-Granados, M. d. C., & Santoyo, G. (2018). Diversity of cultivable endophytic bacteria associated with blueberry plants (Vaccinium corymbosum L.) cv. Biloxi with plant growth-promoting traits. *Chilean Journal of Agricultural & Animal Sciences, 34*(2), 140-151.

Pal, K. K., & Gardener, B. M. (2006). Biological Control of Plant Pathogens. *The Plant Health Instructor*. doi:10.1094/PHI-A-2006-1117-02

Palazzini, J. M., Dunlap, C. A., Bowman, M. J., & Chulze, S. N. (2016). Bacillus velezensis RC 218 as a biocontrol agent to reduce Fusarium head blight and deoxynivalenol accumulation: Genome sequencing and secondary metabolite cluster profiles. *Microbiol Res, 192*, 30-36. doi:10.1016/j.micres.2016.06.002

Pel, M. J., Wintermans, P. C., Cabral, A., Robroek, B. J., Seidl, M. F., Bautor, J., . . . Pieterse, C. M. (2014). Functional analysis of Hyaloperonospora arabidopsidis RXLR effectors. *PLoS One, 9*(11), e110624. doi:10.1371/journal.pone.0110624

Polashock, J., Zelzion, E., Fajardo, D., Zalapa, J., Georgi, L., Bhattacharya, D., & Vorsa, N. (2014). The American cranberry: first insights into the whole genome of a species adapted to bog habitat. *Bmc Plant Biology, 14*. doi:Artn 165

10.1186/1471-2229-14-165

Riley, M. A., & Gordon, D. M. (1999). The ecological role of bacteriocins in bacterial competition. *Trends Microbiol, 7*(3), 129-133. doi:10.1016/s0966-842x(99)01459-6

Rodriguez, R. J., White, J. F., Jr., Arnold, A. E., & Redman, R. S. (2009). Fungal endophytes: diversity and functional roles. *New Phytol, 182*(2), 314-330. doi:10.1111/j.1469-8137.2009.02773.x

Ruiz-Garcia, C., Bejar, V., Martinez-Checa, F., Llamas, I., & Quesada, E. (2005). Bacillus velezensis sp. nov., a surfactant-producing bacterium isolated from the river Velez in Malaga, southern Spain. *Int J Syst Evol Microbiol, 55*(Pt 1), 191-195. doi:10.1099/ijs.0.63310-0

Rukachaisirikul, V., Chantaruk, S., Pongcharoen, W., Isaka, M., & Lapanun, S. (2006). Chromone derivatives from the filamentous fungus Lachnum sp. BCC 2424. *J Nat Prod, 69*(6), 980-982. doi:10.1021/np060164e

Sabat, A. J., van Zanten, E., Akkerboom, V., Wisselink, G., van Slochteren, K., de Boer, R. F., . . . Kooistra-Smid, A. (2017). Targeted next-generation sequencing of the 16S-23S rRNA

region for culture-independent bacterial identification - increased discrimination of closely related species. *Sci Rep, 7*(1), 3434. doi:10.1038/s41598-017-03458-6

Saia, S., Rappa, V., Ruisi, P., Abenavoli, M. R., Sunseri, F., Giambalvo, D., . . . Martinelli, F. (2015). Soil inoculation with symbiotic microorganisms promotes plant growth and nutrient transporter genes expression in durum wheat. *Front Plant Sci, 6*, 815. doi:10.3389/fpls.2015.00815

Saikkonen, K., Faeth, S. H., Helander, M., & Sullivan, T. J. (1998). Fungal endophytes: A continuum of interactions with host plants. *Annual Review of Ecology and Systematics, 29*, 319-343. doi:DOI 10.1146/annurev.ecolsys.29.1.319

Santoyo, G., Moreno-Hagelsieb, G., Orozco-Mosqueda Mdel, C., & Glick, B. R. (2016). Plant growth-promoting bacterial endophytes. *Microbiol Res, 183*, 92-99. doi:10.1016/j.micres.2015.11.008

Seemann, T. (2014). Prokka: rapid prokaryotic genome annotation. *Bioinformatics, 30*(14), 2068-2069. doi:10.1093/bioinformatics/btu153

Stamm, P., & Kumar, P. P. (2010). The phytohormone signal network regulating elongation growth during shade avoidance. *J Exp Bot, 61*(11), 2889-2903. doi:10.1093/jxb/erq147

Strano, C. P., Bella, P., Licciardello, G., Caruso, A., & Catara, V. (2017). Role of secondary metabolites in the biocontrol activity of Pseudomonas corrugata and Pseudomonas mediterranea. *European Journal of Plant Pathology, 149*(1), 103-115. doi:10.1007/s10658-017-1169-x

Suryanarayanana, T. S., Thirunavukkarasub, N., Govindarajulub, M. B., Sassec, F., Jansend, R., & S.Muralia, T. (2009). Fungal endophytes and bioprospecting. *Fungal Biology Reviews, 23*(1-2), 9-19. doi:doi.org/10.1016/j.fbr.2009.07.001

Swain, M. R., Naskar, S. K., & Ray, R. C. (2007). Indole-3-acetic acid production and effect on sprouting of yam (Dioscorea rotundata L.) minisetts by Bacillus subtilis isolated from culturable cowdung microflora. *Pol J Microbiol, 56*(2), 103-110. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/17650680

Swarupa, V., Ravishankar, K. V., & Rekha, A. (2014). Plant defense response against Fusarium oxysporum and strategies to develop tolerant genotypes in banana. *Planta, 239*(4), 735-751. doi:10.1007/s00425-013-2024-8

Tripathi, R. K., & Gottlieb, D. (1969). Mechanism of action of the antifungal antibiotic pyrrolnitrin. *J Bacteriol, 100*(1), 310-318. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/4310080

Tsavkelova, E. A., Klimova, S. Y., Cherdyntseva, T. A., & Netrusov, A. I. (2006). Microbial producers of plant growth Stimulators and their practical use: A review. *Applied Biochemistry and Microbiology, 42*(2), 117-126. doi:10.1134/S0003683806020013

van Baarlen, P., van Belkum, A., Summerbell, R. C., Crous, P. W., & Thomma, B. P. H. J. (2007). Molecular mechanisms of pathogenicity: how do pathogenic microorganisms develop cross-kingdom host jumps? *Fems Microbiology Reviews, 31*(3), 239-277. doi:10.1111/j.1574-6976.2007.00065.x

Vessey, J. K. (2003). Plant growth promoting rhizobacteria as biofertilizers. *Plant and Soil, 255*(2), 571-586. doi:Doi 10.1023/A:1026037216893

Wang, H., Fewer, D. P., Holm, L., Rouhiainen, L., & Sivonen, K. (2014). Atlas of nonribosomal peptide and polyketide biosynthetic pathways reveals common occurrence of

nonmodular enzymes. *Proc Natl Acad Sci U S A, 111*(25), 9259-9264. doi:10.1073/pnas.1401734111

Wang, Q., Liu, J., & Zhu, H. (2018). Genetic and Molecular Mechanisms Underlying Symbiotic Specificity in Legume-Rhizobium Interactions. *Front Plant Sci, 9*, 313. doi:10.3389/fpls.2018.00313

Wani, S. H., Kumar, V., Shriram, V., & Sah, S. K. (2016). Phytohormones and their metabolic engineering for abiotic stress tolerance in crop plants. *Crop Journal, 4*(3), 162-176. doi:10.1016/j.cj.2016.01.010

Waqas, M., Khan, A. L., Shahzad, R., Ullah, I., Khan, A. R., & Lee, I. J. (2015). Mutualistic fungal endophytes produce phytohormones and organic acids that promote japonica rice plant growth under prolonged heat stress. *Journal of Zhejiang University-Science B, 16*(12), 1011-1018. doi:10.1631/jzus.B1500081

Wattam, A. R., Davis, J. J., Assaf, R., Boisvert, S., Brettin, T., Bun, C., . . . Stevens, R. L. (2017). Improvements to PATRIC, the all-bacterial Bioinformatics Database and Analysis Resource Center. *Nucleic Acids Res, 45*(D1), D535-D542. doi:10.1093/nar/gkw1017

Wells, L. D., & McManus, P. S. (2013). A Photographic Diagnostic Guide for Identification of the Principal Cranberry Fruit Rot Pathogens. *Plant Health Progress*. doi:doi:10.1094/PHP-2013-0729-01-DG

White, J. F., Kingsley, K. L., Zhang, Q., Verma, R., Obi, N., Dvinskikh, S., . . . Kowalski, K. P. (2019). Review: Endophytic microbes and their potential applications in crop management. *Pest Manag Sci, 75*(10), 2558-2565. doi:10.1002/ps.5527

Wu, L., Wu, H. J., Qiao, J., Gao, X., & Borriss, R. (2015). Novel Routes for Improving Biocontrol Activity of Bacillus Based Bioinoculants. *Front Microbiol, 6*, 1395. doi:10.3389/fmicb.2015.01395

Xu, L., Dong, Z., Fang, L., Luo, Y., Wei, Z., Guo, H., . . . Wang, Y. (2019). OrthoVenn2: a web server for whole-genome comparison and annotation of orthologous clusters across multiple species. *Nucleic Acids Res, 47*(W1), W52-W58. doi:10.1093/nar/gkz333

Yan, Q., Philmus, B., Chang, J. H., & Loper, J. E. (2017). Novel mechanism of metabolic co-regulation coordinates the biosynthesis of secondary metabolites in Pseudomonas protegens. *Elife, 6*. doi:10.7554/eLife.22835

Yang, H., Zhao, X., Liu, C., Bai, L., Zhao, M., & Li, L. (2018). Diversity and characteristics of colonization of root-associated fungi of Vaccinium uliginosum. *Sci Rep, 8*(1), 15283. doi:10.1038/s41598-018-33634-1