Biological control of potato common scab disease by endophytic plant growth promoting bacteria associated with undomesticated plants

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August 15

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

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Abstract

Potato, the most agriculturally significant member of the *Solanaceae* family, suffers the effects of many drastic diseases, among them potato common scab (CS), caused by the pathogenic bacterium *Streptomyces scabies*, which synthesizes the phytotoxin thaxtomin. Beneficial bacteria that establish themselves in the root zone, known as plant growth-promoting rhizobacteria (PGPR), play crucial roles in enhancing plant development and managing plant pathogens.

Hence, multiple series of experiments were carried out to formulate potential bioproducts aimed at promoting more sustainable potato production. To begin, promising bacterial strains with the capacity to enhance plant growth and alleviate potato common scab disease were collected and isolated from a reservoir of plant roots from five undomesticated (dandelion, heal-all, milkweed, wild lettuce, coltsfoot) from the Sainte-Anne-de-Bellevue region of southwestern Quebec. A total of 75 strains were effectively isolated from the plant roots using a range of microbial growth media. The bacterial isolates were subsequently subjected to a rapid screening assay to evaluate their potential in enhancing plant growth, such as the production of beneficial plant hormones (IAA), the capacity for inorganic phosphate solubilization, and the ability to fix nitrogen. This screening aimed to identify these bacterial isolates as potential biofertilizers.

Based on the findings of this *in vitro* study, strains that clearly exhibited plant-growth-promoting effects were carefully selected for further assessment in a subsequent screening phase. The bacterial strains selected through the initial screening assay were identified taxonomically, to the genus level and tentatively to the species level, by 16S rRNA gene sequencing. The research results revealed that ten strains exhibited superior performance in the primary screening assay.

Subsequently, a second round of *in vitro* screening was conducted to assess the potential antagonistic effects of the ten bacterial strains selected for activity against the pathogenic

Streptomyces scabies on petri plates. This evaluation aimed to elucidate any inhibitory effects exhibited by these bacterial strains against the growth and activity of *Streptomyces scabies.* Once again, based on the results of the *in vitro* study, the strains that displayed clear antagonistic activities against *Streptomyces scabies* were selected and used throughout the subsequent phases of the research. Three out of the ten selected endophytic bacterial isolates exhibited antagonistic effects against the pathogen *Streptomyces scabies*. These strains belong to the genera *Bacillus* and *Pseudomonas* and were tentatively identified as *Bacillus velezensis, Pseudomonas chlororaphis,* and *Bacillus amyloliquefaciens,* assigned the strain designations S1, S2, and S3, respectively. These three strains were utilized in the rest of the experiments within this study.

The three selected strains underwent comprehensive testing to reveal the intricate mechanisms by which they control the common scab pathogen. The examined mechanisms encompass the production of hydrogen cyanide, siderophores, and biosurfactants, further enriching our comprehension of their effectiveness as biocontrol agents. The three selected strains all showed the capability to produce hydrogen cyanide (HCN), generate siderophores, and synthesize biosurfactants.

The concluding phase of this research examined the mobility of the selected bacterial strains, their coexistence within a multispecies consortium, growth rate, and resistance to commonly used antibiotics. The results indicated significant antibiotic resistance and rapid growth across all three strains. Their movement on agar plates demonstrated swimming capacity, and in co-culture and cross-streak testing, strains S2 and S3 showed compatibility with one another.

To summarize, this project highlighted the substantial potential of the newly isolated endophytic bacteria, specifically the three chosen strains, to be utilized effectively against common scab disease of potato and to promote plant growth.

Résumé

La pomme de terre, le membre le plus important sur le plan agricole de la famille des solanacées, souffre des effets de nombreuses maladies graves, parmi lesquelles la gale commune de la pomme de terre (CS), causée par la bactérie pathogène *Streptomyces scabies*, qui synthétise la phytotoxine de thaxtomine. Les bactéries bénéfiques qui s'établissent dans la zone racinaire, connues sous le nom de rhizobactéries favorisant la croissance des plantes (PGPR), jouent un rôle crucial dans l'amélioration du développement des plantes et la gestion des agents pathogènes des plantes.

Par conséquent, les plusieurs séries d'expériences ont été menées pour formuler des bioproduits potentiels visant à promouvoir une production de pommes de terre plus durable. Pour commencer, des souches bactériennes prometteuses ayant la capacité d'améliorer la croissance des plantes et d'atténuer la gale commune de la pomme de terre ont été recueillies et isolées à partir d'un réservoir de racines de plantes provenant de cinq plantes non domestiquées (pissenlit, guérisseuse, asclépiade, laitue sauvage, tussilage) de la région de Sainte-Anne-de-Bellevue au sud-ouest du Québec. Au total, 75 souches ont été efficacement isolées des racines des plantes à l'aide d'une gamme de milieux de croissance microbienne. Les isolats bactériens ont ensuite été soumis à un test du dépistage rapide pour évaluer leur potentiel d'amélioration de la croissance des plantes, comme la production d'hormones végétales bénéfiques (IAA), la capacité de solubilisation du phosphate inorganique et la capacité de fixer l'azote. Ce criblage visait à identifier ces isolats bactériens comme les biofertilisants potentiels.

Sur la base des résultats de cette étude *in vitro*, les souches qui présentaient clairement des effets favorisant la croissance des plantes ont été soigneusement sélectionnées pour une évaluation plus approfondie lors d'une phase de dépistage ultérieure. Les souches bactériennes sélectionnées

par le test de dépistage initial ont été taxonomiquement identifiées, au niveau du genre et provisoirement au niveau de l'espèce, par séquençage du gène de l'ARNr 16S. Les résultats de la recherche ont révélé que dix souches présentaient des performances supérieures dans le test de dépistage primaire.

Par la suite, une deuxième série de criblages *in vitro* a été menée pour évaluer les effets antagonistes potentiels des dix souches bactériennes sélectionnées pour leur activité contre la bactérie pathogénique de *Streptomyces scabies* sur des boîtes de Pétri. Cette évaluation visait à élucider tout effet inhibiteur présenté par ces souches bactériennes contre la croissance et l'activité de *Streptomyces scabies*. Encore une fois, sur la base des résultats de l'étude *in vitro*, les souches qui présentaient des activités antagonistes claires contre *Streptomyces scabies* ont été sélectionnées et utilisées tout au long des phases ultérieures de la recherche. Trois des dix isolats bactériens endophytes sélectionnés ont montré des effets antagonistes contre le pathogène *Streptomyces scabies*. Ces souches appartiennent aux genres *Bacillus* et *Pseudomonas* et ont été provisoirement identifiées comme *Bacillus velezensis*, *Pseudomonas chlororaphis* et *Bacillus amyloliquefaciens*, auxquelles ont été attribuées les désignations de souche S1, S2 et S3, respectivement. Ces trois souches ont été utilisées dans le reste des expériences de cette étude.

Les trois souches sélectionnées ont subi des tests complets pour révéler les mécanismes complexes par lesquels elles contrôlent l'agent pathogène commun de la gale. Les mécanismes examinés englobent la production de cyanure d'hydrogène, de sidérophores et de biosurfactants, enrichissant davantage notre compréhension de leur efficacité en tant qu'agents de lutte biologique. Les trois souches sélectionnées ont toutes montré la capacité de produire du cyanure d'hydrogène (HCN), de générer des sidérophores et de synthétiser des biosurfactants.

La phase finale de cette recherche a examiné la mobilité des souches bactériennes sélectionnées, leur coexistence au sein d'un consortium multi-espèces, le taux de croissance et la résistance aux antibiotiques couramment utilisés. Les résultats ont indiqué une résistance importante aux antibiotiques et une croissance rapide pour les trois souches. Leur mouvement sur des plaques de gélose a démontré une capacité de nage ; et dans les tests de co-culture et de stries croisées, les souches S2 et S3 ont montré une compatibilité les unes avec les autres.

En résumé, ce projet a mis en évidence le potentiel substantiel des bactéries endophytes nouvellement isolées, en particulier les trois souches choisies, pour être utilisées efficacement contre la gale commune de la pomme de terre et pour favoriser la croissance des plantes.

Acknowledgments

I begin this thesis by acknowledging the blessings and guidance of Allah, the Most Merciful and the Most Compassionate. In every step of my academic journey, I have felt His presence and His support, which have been a source of strength and inspiration. I am deeply grateful for the knowledge and wisdom He has bestowed upon me, enabling me to pursue this study.

This project was made possible through the collective efforts of numerous individuals and partners, each of whom contributed significantly. Without their support, the successful execution of this project would not have been achievable. I would like to extend my foremost appreciation to Dr. Donald Smith, my supervisor, who played a pivotal role in guiding me through the experiment's preparation, execution, and analysis. His invaluable insights into agricultural research methodologies and his unwavering encouragement provided me with numerous academic opportunities.

I also wish to express my gratitude to the faculty members of the Plant Science department, whose continuous assistance played a crucial role in my growth as a graduate student. Special acknowledgment goes to those who actively engaged with my proposal and final seminars.

Gratitude is also owed to the to the members of the Smith Lab. Your warmth and camaraderie, coupled with your profound scientific insights, have left a lasting impact. Special acknowledgments are due to Alfred, whose guidance and expertise steered my project on its correct trajectory, even on multiple occasions.

I am deeply thankful to the Islamic Development Bank (IsDB) for their vital financial backing, which played a pivotal role in making my study a reality. Their generous support not only facilitated the successful completion of my project but also underscored their commitment to advancing education and research. This funding was instrumental in covering various aspects of

my study, including resources, materials, and necessary expenses. The IsDB's dedication to promoting academic pursuits has had a profound impact on my ability to contribute to my field of study, and I am truly grateful for their assistance.

I also extend my heartfelt appreciation to my companions and Colleagues who played a crucial role in the completion of my thesis. Mohamed Abubaker, Levini, Mahamed Antar, Jolvis and Mina, your consistent support, brainstorming sessions, and constructive feedback were instrumental in refining my research. Your contributions will forever be cherished as integral parts of this achievement.

Lastly, but certainly not least, I want to convey my profound appreciation to my family. Their unwavering encouragement, invaluable insights, and steadfast support have been the cornerstone of my journey. Through their guidance and wise counsel, they have not only provided me with the strength to overcome challenges but have also been a source of inspiration. Their belief in my endeavors has been a constant driving force, and I am truly fortunate to have such a strong and nurturing foundation.

Contribution of Authors

Following the McGill Guidelines for a traditional-based thesis, Mahamoud Rabileh served as the primary author for this project under the guidance of Prof. Donald L. Smith, who is affiliated with the Department of Plant Science at McGill University in Sainte-Anne-de-Bellevue, Quebec, Canada. All experiments in this research project were planned, designed, and implemented by Mahamoud Rabileh (primary researcher) with input, recommendations, and guidance from my thesis supervisor Prof. Donald L. Smith. I conducted laboratory experiments, analyzed, wrote the thesis, and finally put it together under the supervision of Dr. Smith. This thesis is written in a traditional monograph format comprising the following chapters:

Chapter 1 Introduction– Mahamoud Rabileh structured and wrote the first draft, which was reviewed by Dr. Smith, Department of Plant Science, McGill University**.**

Chapter 2 Review of Literature – Mahamoud Rabileh searched the literature and wrote the first draft, which was reviewed by Dr. Smith, Department of Plant Science, McGill University.

Chapter 3 Materials and Methods – Mahamoud Rabileh set up the experiments and conducted the research with the assistance and guidance of Dr. Smith. *Streptomyces scabies* strains were provided by Carole Beaulieu, Professor at University of Sherbrooke. Mahamoud Rabileh wrote the first draft, which was reviewed by Dr. Smith, Department of Plant Science, McGill University.

Chapter 4 & 5 Results and Discussion– In the Results and Discussion section, the author presented and interpreted the research findings, connecting them to the thesis objectives, the author provides valuable insights and interpretations of the research findings, offering a unique perspective that advances the field of study. Dr. Smith contributed by supplying foundational scientific knowledge, offering essential intellectual context, and providing extensive editorial

guidance. Mahamoud Rabileh wrote the first draft, which was reviewed by Dr. Smith, Department of Plant Science, McGill University.

Chapter 6 General Conclusions and Future directions – In the General Conclusions and Future Directions section, the author's contribution involves summarizing key findings and proposing insightful recommendations for future research, offering a valuable roadmap for further advancement in the field. Mahamoud Rabileh wrote the first draft, which was reviewed by Dr. Smith, Department of Plant Science, McGill University.

Chapter 1: General introduction

1.1 Introduction

Rhizospheric microbes are found on the root surface and in the rhizosphere soil layer around roots, and there are endophytic bacteria that live inside plant tissues, including roots, forming often mutualistic relationships with plants. There are numerous species of beneficial bacteria, many of them plant growth-promoting rhizobacteria (PGPR), these species often include strains within the genera *Pseudomanas, Azospirillum. Azotobacter, Klebsiella, Enterobacter, Alcaligen*, *Arthobacter*, *Burkholderia*, *Bacillus* and *Serratia.* The main roles of the various species of root colonizing bacteria are to assist/promote plant growth and to control against plant pathogens (Rosenblueth and Martínez-Romero, 2006).

Further, rhizobacteria contribute to soil fertility through the solubilization of phosphorus (Vargas et al., 2017), increased nitrogen fixation (Goswami et al., 2016) and helping plants in coping with abiotic stressors and thereby enhancing root growth (Vurukonda et al., 2016). Other benefits of bacteria to plants are the production of phytohormones and improved ability to deal with phytopathogenic microorganisms (Rashid and Chung, 2017). Therefore, there is a need to conduct research to understand the relationships between plants and microbes to add information regarding additional mechanisms of plant growth promotion, bio-control of diseases, and overall enhancement of crop yields.

Besides maize, rice, and wheat, potato, a member of the Solanaceae, is a favourite food crop, which is cultivated and consumed in many regions of the world. Potatoes are a good source of minerals, carbohydrates and caloric energy and contain almost no fat. As such, across the many regions of the world, millions of people consume potatoes daily; a significant number use it as

their staple food (Majeed and Muhammad, 2020). The output of potato can be limited by various biological and nonbiological constraints. For instance, Solanaceae family crops suffer from devastating attacks by bacterial pathogens. Among these pathogens, common scab, which is caused by the bacterial species *Streptomyces scabies*, is one of the most significant diseases of potato production worldwide. There are a range of effects of this pathogen on potato plants. These include swollen, superficial, raised, or perforated scab lesions on the skin of tubers (Majeed and Muhammad, 2020). *Streptomyces scabies* is considered the most problematic species because it can produce thaxtomin A – a phytotoxin (Sarwar et al., 2018). The occurrence of scab infection does not constitute a direct danger to humans. However, its effects threaten humans by reducing potato yields and quality, where severe cases reduce market prices and lead to economic losses. For instance, in Canada, a firm engaged in producing potatoes suffered a $1.2 million reduction in potential earnings over just one year because of scab (Al-Mughrabi et al., 2016).

There has not been adequate development of effective pesticides, specific solutions from conventional practices such as crop rotation or potato cultivar development, to produce potato plants that can endure stress and diseases without significant yield reductions (Vessey, 2003). Thus, it is essential to find a solution for potato production that ensures current and future generations have access to food and relief from the threat of hunger. These factors motivated the launching of an investigation to develop biological control methods that can serve as alternative approaches to suppressing common scab disease, leading to greater potato quality and output. The success of research conducted on indigenous PGPR has facilitated the development of commercial inoculants used to increase crop productivity and fight against plant diseases (Tabassum et al., 2017). However, there is still a need to continue the screening of rhizobacterial diversity for further studies

and to produce more effective and beneficial microbes for enhanced disease resistance and plant growth.

Thus, to develop potential bioproducts for sustainable agriculture, research was conducted to isolate rhizobacteria in Southwestern Québec, Canada, and investigate their impact in alleviating common scab disease and potential for potato growth promotion.

1.2 Objectives and Hypotheses

Overall Objective:

The overall objective of this research is to investigate the cultivable endophytic rhizobacterial community associated with undomesticated plants in southwestern Québec (Sainte-Anne-de-Bellevue region), Canada, and their impact in alleviating common scab disease of potato.

Specific objectives:

1. To isolate and screen bacterial strains associated with undomesticated plant roots for their potential in reducing common scab disease.

2. To characterize isolated strains showing antagonistic activity towards *Streptomyces scabies* for plant growth promotion traits.

3. To investigate whether the selected bacterial strains show other important characteristics such as (i) potential for plant growth rate enhancement, (ii) compatibility of selected bacterial strains as a consortium (iii) swimming motility (iv) root ccolonization (cellulose degrading ability) (v) and resistance to antibiotics.

Hypotheses:

1. Bacterial isolates from undomesticated plant species can act as biocontrol agents, being antagonistic to the common scab pathogen of potato

2. The antagonistic selected bacterial isolates against *Streptomyces scabies* show plant growth promotion traits such as abilities to: fix nitrogen, solubilize phosphorous, produce indole acetic acid and siderophores.

3. Selected bacterial strains are fast growing and show motility characteristics in the growth medium, could perform as a multispecies microbial consortium and are resistant to other microbes in the surrounding environment.

Chapter 2: Review of Literature

2.1 Bacteria

A new microbial world was discovered by Antonie van Leeuwenhoek in the middle of seventeenth century with a simple handmade microscope (Gotō 1992). Pasteur (1876) made a significant contribution to our understanding of the role of bacteria in fermentation and decay. The isolation and description of bacteria from leguminous plants was carried out by Woronin in 1876. The association between bacteria and plant disease was established by Burbil in 1878. He showed that the bacterium, *Erwinia amylovora* can cause fire blight on pear and apple. Bacterial decay of wheat kernels was reported by Prilleaux (1879). Another investigation by Wakker (1887) indicated the bacterial nature of yellow slime disease of hyacinth (Van Tuyl and Toxopeus, 1980). The results of investigation by Arther (1885-1887) confirmed Burrill's research. Bacterial knot disease was described by Savastano (1886). American pathologist, Erwin F. Smith began to amass knowledge regarding bacterial plant diseases in 1890 and played a major role in founding plant bacteriology. He demonstrated that bacteria cause crown gall disease of plants. Further study on the crown gall bacterium was carried out by Chilton et al. (1977). Patel and his team began serious studies on bacterial plant pathogens in 1948. They reported almost 40 bacterial diseases over the subsequent 15 years.

2.2 Types of bacteria

2.2.1 Beneficial bacteria

Plant growth promoting bacteria (PGPB) and plant growth promoting rhizobacteria (PGPR) are known for their potential to promote agricultural sustainability (Martínez-Hidalgo et al., 2019; De et al., 2023). Their use as single strain inoculants and/or as consortia of multiple bacteria is established; many of these technologies exhibit symbiotic interactive plant growth promoting traits

(Backer et al., 2018; Bashan et al., 2014). Plant growth promoting bacteria (PGPB) play their roles by enhancing the viability, growth and overall productivity of crops (Yanni et al., 2001; Laabas, 2017), by showing biocontrol performance (Bach et al., 2016) against pathogens (Aziz et al., 2016), and by increasing the nutritional value of crops (De Andrade et al., 2023; Egamberdiyeva, 2007). It has been proven that PGPR can enhance plant growth and survival under adverse conditions, such as, drought, nutrient deficiency, and other environmental stresses such as xenobiotic pollutants, etc. (Vilchez and Manzanera, 2011; Wang et al., 2017). In return, plant roots increase PGPR viability by providing nutrition and a protected environment, particularly for endophytic bacteria (Vilchez and Manzanera, 2011).

2.2.2 Plant Pathogenic bacteria

Bacterial plant pathology or plant bacteriology is a branch of plant pathology dealing with plant diseases responsible for devastating major and high-value agricultural crops, including fruits and trees, and causing enormous economic losses each year. Bacterial plant pathogens cause diseases by obtaining nutrients from their host plants, often through causing plant cell death. They may use specific mechanisms to secrete proteins or other molecules to locate and parasitize their host (Davis et al., 2008).

Bacterial plant pathology had its beginnings in the late nineteenth century. Inoculation tests done by Burrill (1880) proved that fire blight disease of pear is caused by a bacterial infection. He named the causative organism *Micrococcus amylovorus*. Furthermore, *Bacterium hyacinthi* was reported by Wakker (1887) as the plant pathogenic bacterium causing yellowing disease of hyacinth (Gotō, 1992). Smith reported a description series of plant pathogenic bacteria. With regard to the validity of bacteria as plant pathogens, Smith established a clear concept for this via convincing evidence and careful analysis (Fischer, 1981).

2.3 Impact of plants and microbial communities on each other

Soil microbial communities play an important role in structuring plant communities (Bever et al., 2015; Crawford and Hawkins, 2018). Soil microbes are involved in various processes in ecosystems, such as turnover of organic matter and formation of soil aggregates. Soil microbial communities consist of plant antagonistic and plant beneficial microbes; plants respond to them in species-specific ways, and these responses influence plant community composition, relative abundance and productivity (Mangan et al., 2010).

Plants depend on their microbial partners to survive against microbial invaders in nature (Turner et al., 2013). Beneficial effects of these microbiota on plant health have been reported in several studies. They increase plant health by inducing systemic resistance, increasing nutrient uptake, increasing tolerance to biotic and abiotic stresses, suppressing disease and enhancing adaptation to other environmental variations (Naamala et al., 2023; Msimbira et al., 2022; Van der Ent et al., 2009; Haney et al., 2015). The interaction between plants and their microbial partners is not unidirectional. The host plant provides metabolic capabilities for its microbial partners, which leads to their co-adaptation. This adaptation can be positive (mutualistic), negative (pathogenic) or neutral with regard to impacts on their host (Thrall et al., 2007). The population of plant-associated microbes is the phytomicrobiome, and the phytomicrobiome plus its host plant constitutes the holobiont, which is the entity that produced crop yield and that evolution acts upon (Lyu et al., 2021). This relationship between plants and their associated microbes is like that of human beings and their microbiome. An example of the most-studied and elaborated beneficial plant-microbe interactions is the symbiotic relationship between legumes and rhizobia. In this symbiotic relationship, bacteria benefit from being hosted in an environment with reduced carbon and other metabolites from plants made available, in addition legume plants

get fixed nitrogen from root associated rhizobia (Oke and Long, 1999).

2.4 Potato

The genus including potato (*Solanum* L., Solanaceae family) is a large one with roughly 900 species. The cultivated potato emerged somewhere from central Peru to central Bolivia, probably in the Lake Titicaca region, on the border of Peru and Bolivia (Hawkes, 1990). According to the Food and Agriculture Organization of the United Nations (FAO), potato is one of the most diverse and nutritious crops; it is ranked as the world's fourth most important food crop. Because of its importance as a staple crop, the cultivated potato has attracted much attention from scientists and researchers in the time since it was brought to Europe by Spanish explorers at the end of 16th century. Potato is a very good source of vitamins, phytonutrients and health promoting compounds. As humanity's favorite root vegetable potato may improve heart health, reduce high cholesterol levels in the body and play a major role in healthy digestion (Patil, 2019). Potato is Canada's most important horticultural crop, and it is cultivated in every province. The highest rate of potato production is on Prince Edward Island.

2.5 Potato common scab

Potato common scab is one of the most drastic diseases of potato plants, found worldwide (Loria et al., 1997). *Streptomyces* means twisted fungus and it was initially thought to be a fungal pathogen because of its filamentous structure. This bacterium is classified as a gram positive, filamentous bacterium belonging to the order *Actinomycetales*. Actinomycetes are recognized as producers of two thirds of known antibiotics and *Streptomyces* species are responsible for production of 80% of these antibiotics (Wang et al., 2017). These antibiotics allow them to compete with other soil microorganisms, or to be utilized by humans for medical purposes (Burchett and Burchett, 2017). However, some species of this genus are plant pathogens, such as *Streptomyces*

scabies, which is believed to be the main organism causing potato common scab disease. The other species that cause potato common scab include *S. europaeiscabiei, S. turgidiscabies, S. acidiscabies*, *S. stelliscabiei, S. bottropensis* and *S. aureofaciens* (Leiminger et al., 2013). Although they are not the same species, they result in similar symptoms on potato and their pathogenic characteristics are determined by specific plant phytotoxins (Loria et al., 1997). Potato common scab (*Streptomyces scabies*) does not kill potato plants or stop tuber production. However, "scabby" lesions that are caused by this pathogen, affecting the marketability of potato (Burchett and Burchett, 2017).

2.6 Cultivation system versus bacterial diversity

The soil microbiome is considered a key player in agroecosystems (Bacon et al., 2015). Microbiota attributes affect various ecosystem processes such as organic matter decomposition (Emmerling, et al. 2002), soil structure (Anderson, 1991), pollutant degradation (Paeschke and Heitefuss, 1978), nutrient cycling and availability, plant productivity and quality (Ding, et al., 2018), and plant disease suppression (Savary, et al., 2006).

Several studies have proven that agricultural practices such as fertilization (Savary et al., 2006), tillage (Degrune et al., 2015), crop residue retention or removal (Degrune et al., 2015; Jimenez-Bueno et al., 2016), and continuous cropping or monoculture (Xiong et al., 2015), modify microbial communities. For instance, a study conducted by Xiong et al. (2015) demonstrated that a continuous cropping system of black pepper resulted in reduction of soil pH, organic matter, and enzymatic activity. Consequently, decline in bacterial abundance, alteration in soil microbial structure and poor growth of black paper was observed. Application of synthetic fertilizers, agrochemicals and pesticides has led to remarkably increased crop productivity, however significant soil erosion, degradation, pollution, etc. have been observed in recent years (Tilman et

al., 2002; Hartmann et al., 2015). Unsuitable agricultural practices, overuse of chemical fertilizers and their negative impacts on the broader environment have, in some cases, reduced crop productivity and biodiversity (Wu, et al., 2018).

Organic management has been proposed, to diminish these adverse effects, protect natural resources and achieve sustainable agriculture (Gomiero, et al., 2011). According to the previous studies, organic management methods improve soil structure and fertility, protect biodiversity and reduce soil degradation (Średnicka-Tober et al., 2016).

2.7 Biological control

2.7.1 Definition of biological control

Biological control, sometimes shortened to "biocontrol", is a cost-effective and environmentally safe means of disease management, which can be defined as a method using natural enemies (biocontrol agents) of plant pests, such as insects, pathogens, predators or parasitoids which suppress disease to below economically damaging levels. This suppression can be achieved in many ways. It is a long-term management solution that helps mitigate the economic losses and costs of disease control through chemicals and their side effects on humans, the environment and overall ecological health now and in the future (Bhardwaj et al., 2014).

2.7.2 History of biological control

There are historical records that trace the evolution and development of biological control and provide remarkable insights into it. Without these discoveries and conceptualizations, modern biological control would have been delayed. These concepts include symbioses between/among species, natural control and natural enemies of pests, including the role of natural enemies in abundance determination.

The recorded history of applied biological control extends back to Egyptian times, almost 4000 years ago when house adapted cats were portrayed as a useful means for controlling rodents. Learning more about small natural enemies became possible with the invention of the microscope by van Leeuwenhoek in the 1600s (Bosch et al., 1982). In the seventeenth century, in Italy, Vallisnieri, for the first time, recognized insect parasitism between *Apanteles glomeratus* (a parasitic wasp) and *Pieris rapae* (cabbage butterfly) (Bosch et al., 1982). Destruction of cabbage caterpillar infestation through the ichneumon fly was noted by Erasmus Darwin in 1800. Hartig proposed the idea of collecting and storing of parasitized caterpillars for later release (Bosch et al., 1982). Agostino Bassi, in 1835, showed that the cause of a key disease of silkworm larvae (*Bombyx mori*) was a fungal pathogen (*Beauveria bassiana*) (Hajek and Eilenberg, 2018). Inhibition of plant pathogenic microorganisms by beneficial microbes was first demonstrated by Potter and Farmer (1908).

2.7.3 Types of biocontrol agents

Biocontrol agents are fungi and/or bacteria, usually from the rhizosphere, that are capable of exhibiting antagonistic activity against plant pathogens; they are able to reduce damage by plant pathogens and may also directly cause growth stimulation (Stewart et al., 2010; Kashyap et al., 2017; Pagano et al., 2017). The microbial community can be found in every plant-based habitat, rhizosphere, phyllosphere or occurring on specific plant surfaces, or within specific elements of plant tissues, such as roots, leaves, flowers, etc. Isolation and evaluation of theses microbes and their antagonistic activity against disease agents may provide a source of effective biocontrol agents (Saleh et al., 2013; Raza et al., 2017; Zhang et al., 2017). Phylloplane antagonists such as bacteria or epiphytic filamentous fungi were found to be effective against several fungal diseases. Several studies reported the successful control of plant diseases using biocontrol agents, including

soil-borne pathogens and foliar diseases (Al-Mazroui and Al-Sadi, 2015; Lopes et al., 2015; Wang et al., 2015; Arroyave-Toro et al., 2017; Yuan et al., 2017, Zhao et al., 2017). Some of the fungal types used as biocontrol agents include *Trichoderma harzianum, T. viride, Pythium oligandrum, Aspergillus* spp*., Talaromyces* spp. and others (Madi et al., 1997; Abdelzaher et al., 2000; Mbarga et al., 2012; Naraghi et al., 2012; Vongphachanh et al., 2016; Manjunath et al., 2017; Vasumathi et al., 2017). The rhizosphere, rhizoplane and endophytic habitats are considered as rich and diverse ecological niches for microorganisms. The rhizosphere is a thin layer of soil close to the root surface and is influenced by root exudates, rhizo-deposited nutrients and mucilages (Igiehon and Babalola, 2017; Kazerooni et al., 2017). Rhizosphere microorganisms have an effect on plant diversity directly or indirectly through various mechanisms that lead to interaction between plants, symbionts and antagonists (Cesco et al., 2012; Lenc et al., 2015; Igiehon and Babalola, 2017). The endorhizosphere contains biotic agents such as arbuscular mycorrhizae fungi (for example, *Glomus intraradices* and *Rhizophagus irregularis*) that have an impact on plant growth and nutrition (Igiehon and Babalola, 2017).

Pear brown spot (*Stemphylium vesicarium*) was reduced by almost 57% by *Erwinia herbicola* and *Pseudomonas fluorescens* isolates, which were isolated from several plant roots and aerial parts (Montesinos and Bonaterra, 1996; Montesinos et al., 1996). Among many bacteria, actinomycetes isolated from roots of watermelon a number showed ability to control watermelon *Fusarium* wilt (Larkin et al., 2007). Plant growth-promoting rhizobacteria (PGPR) such as *Bacillus subtilis*, *Pseudomonas fluorescens*, *Pseudomonas putida* which are normally isolated from disease suppressive soils besides being beneficial and growth enhancing, can also be used as biocontrol agents to suppress important diseases (Gowtham et al., 2016). The other source of biocontrol agents, which has been found effective, is manure-based compost extract. Fortifying biocontrol

agents with compost mixture was demonstrated to be useful for controlling numerous soil-borne diseases (Niu et L., 2020).

2.7.4 Factors affecting biological control

2.7.4.1 Abiotic factors

Various environmental factors influence microbial activities. Hence, tolerance to these abiotic factors is a key for their successful biocontrol activity. Vänninen et al. (2000) reported that the persistence of *Metarhizium anisopliae* was poor in peat compared and clay soils. Temperature is another abiotic factor that has an impact on fungal biocontrol activity. Based on previous studies, most soil borne fungal biocontrol agents are mesophyllic and normally grow well between 15-30 °C, thus their biocontrol activity may be thermally constrained in winter (Stewart et al., 2010). For example, mycoparasitism activity of *T. harzianum* against sclerotia of *Sclerotinia sclerotiorum* was reduced at 15 °C (Knudsen et al., 1991). Conversly, the optimal temperature for antagonism of *Trichoderma viride* against sclerotia of *S. cepivorum* was at 10 °C and this biocontrol activity was reduced by increasing the temperature (Clarkson et al., 2004). Soil moisture has a clear impact on fungal biocontrol activity. Spore germination, mycelial growth and conidial survival do not occur in very dry soils (Stewart et al., 2010). Besides water availability, pH is also an important variable that has an influence on mycoparasitism activity and efficiency (Kredics et al., 2004). Mycelial growth of *Trichoderma* spp. was optimum at pH 4 and water activity of a_w 0.997 (Kredics et al., 2004). Numerous factors such as agricultural practices (egs. fertilizer addition or effects on soil organic matter content) have impacts on soil pH. Acidic conditions are favorable for some fungi, such as *Metarhizium anisopliae* (Padmavathi et al., 2003). Mineral nutrition, such as nitrogen, is essential for growth, sporulation and secondary metabolite production by microbial community components, and so for disease suppressiveness by biocontrol elements (Al-Azizi et al., 2013).

2.7.4.2 Biotic factors affecting biological control

Soil augmentation with a single organism does not always lead to successful establishment as other, already established microbes, may show inhibitory activity against it. Soil microorganisms, plants and invertebrates are identified as the three major biotic factors having impact on biocontrol persistence and efficacy (Stewart et al., 2010). For example, entomopathogens persist in the soil environment and they cannot produce sufficient inoculum in the absence of their host (Padmavathi et al., 2003; Culebro-Ricaldi et al., 2017). Thus, their growth, germination and survival are affected by soil microbe metabolites. Similarly, antagonistic or competitive activity of microorganisms in the rhizosphere may lead to a decline of biocontrol agents in the soil. For instance, inhibition of conidial germination of *T. virens* by the ectomycorrhizal fungus *Laccaria laccata* has been reported (Zadworny et al., 2004).

2.7.5 Commercial biocontrol products

Given that circumstances that can follow from excessive use of chemical fertilizers and pesticides (agrochemicals), as well as concernes of some anti-pesticide and health consciousness groups, exploitation of living organisms to reduce plant pathogen issues or activity seems to be a good alternative to chemicals. Many antagonists that have shown consistent results over time have been commercialized. The United States Environmental Protection agency registered *Trichoderma harzianum* ATCC 20476 as the first fungus for plant disease control. The EPA (United states Environmental Protection Agency) has registered 12 fungi and 14 bacteria for plant disease control (Fravel, 2005). These commercialized biocontrol products account for only 1% of agricultural pathogen control measures (Fravel, 2005). In this progression, some of the nationally or internationally commercially available products that have been established are based on fungal or bacterial antagonists and have entered into world market in recent years. Some of these commercial

products, which utilize *Trichoderma* sp. as an active ingredient, are BioGuard, Soilguard, Biocon, Fstop, or Ecofit and with *Bacillus* as an active ingredient are Mycostop, Rhizoplus and Subilex (Junaid et al., 2013). Various steps are involved in the commercialization process for biocontrol products, which include searching for and screening of microorganisms with desirable traits from within their natural ecosystems (bioprospecting). This step depends on the crop, cropping system and pathogen and usually a desirable isolate will be selected from leaves, roots, and rhizosphere of healthy plants in fields infested with the target pathogen. *In vitro* and *in vivo* evaluation of the isolates is done to examine their efficiency and compatibility under a range of conditions (Al-Hinai et al., 2010; Wang et al., 2015). Formulation is considered a critical obstacle in bio-agent commercialization. A formulation must be easy to handle, stable over a specific range of temperature and have a desirable shelf life and viability (minimum 2 years at room temperature). At the end, registration and release of suitable isolates by considering its toxicity and residual activity will be carried out (Junaid et al., 2013).

2.7.6 Evaluation of biological and chemical controls

Biological control is considered as a part of long-term natural control for suppressing pest populations while chemical control can decimate localized pest populations, temporarily and immediately (Lahlali et al., 2022, Bosch et al., 1982; Al-Sadi, 2012). Pesticides can cause tremendous benefits. Improved productivity and yields can be expected from their use. Several factors may have effects leading to increased productivity, which include higher seed yield: agricultural chemicals (pesticides), fertilizers and irrigation systems. Pesticides are an inevitable and integral part of crop productivity through reducing diseases, insect pests, weeds or the other factors which can reduce crop yields meaningfully (Sukul and Spiteller, 2000; Saramanda and

Kaparapu, 2017). Conversely chemical control can also provide control of vector borne diseases through vector control (Kingdom, 2013; Queiroz et al., 2016).

However, long-term use of agricultural chemicals has led to serious health concerns for humans and the environment. Their detrimental effects on soil fertility, degradation of beneficial microbes and also the development of resistant pathogens are undeniable (Manjunath et al., 2017). Their excessive use results in non-sustainable agriculture and has led to an emphasis on the development of sustainable farming, ecofriendly agricultural methods and long-term management perspectives. Farm workers, formulators, mixers or sprayers are categorized as high and increased risk groups for exposure to potentially dangerous chemicals.

Biological control of damping-off disease caused by *Pythium* species gained increased popularity during the 1990s and is still a relatively new area of research. Suppression of damping-off disease by fungal biocontrol agents has been attained through the use of *P. oligandrum* Drechsler*, P. nunn* Lifsh, Stangh. & Baker, *Trichoderma* species and a few other fungal species (Martin and Loper, 1999). When applied to tomato seedlings before transplanting, *P. oligandrum* significantly reduced damping-off caused by *P. ultimum* and *P. aphanidermatum* and was as efficacious as metalaxyl (Martin and Semer, 1991). Paulitz and Baker, (1987) reported a decrease in population densities of *P. ultimum* and greater emergence of cucumber seeds when *P. nunn* was added to soil and seeds, respectively. In a greenhouse trial to evaluate the efficacy of *T. harzianum* Rifai in the management of damping-off of cucumbers Deadman et al. (2002) reported *T. harzianum* to give a level of control equivalent to metalaxyl. Bacterial strains of *Pseudomonas fluorescens* and some other species are the most commonly reported fluorescent pseudomonads used to suppress damping-off (Gaya et al., 2020; Mannai et al., 2020; Martin and Loper, 1999).

Although some biocontrol agents are very effective in the management of damping-off disease, their use has encountered some difficulties. A report by Fang and Tsao (1995) indicated a reduced seedling growth of sweet orange when *P. nunn* was applied at a high concentration to control *Phytophthora* root rot. Some bacterial strains have a short shelf life (Powell and Jutsum, 1993) and are affected by soil conditions (Martin and Loper, 1999). A requirement for the effective control of damping-off disease is that the biocontrol agent should be active immediately after application. Another important consideration is that the biocontrol be integrated with other disease and pest management strategies implemented in a given area.

2.8 PGPR

2.8.1 Definition of PGPR

Plant growth promoting rhizobacteria (PGPR) refer to the diverse group of beneficial and free-living soil bacteria that actively occupy rhizosphere, root surface or root itself and enhance plant growth and development directly or indirectly (Shah et al., 2021; Gray and Smith, 2005; Beneduzi et al., 2012). They promote plant growth directly by providing compounds that are synthesized by bacteria or by easing the uptake of specific nutrients, water and air from the environment (Glick, 1995; Podile and Kishore, 2006). The indirect method of plant growth promotion occurs when bacteria reduce or prevent detrimental effects of phytopathogenic organisms by producing antagonistic substances, boosting plant resilience against stress and inducing resistance to the pathogen (Khalil et al., 2022; Glick, 1995; Raj et al., 2003; Podile and Kishore, 2006).

2.8.2 History of PGPR

The discovery of rhizobia by Hellriegel and Wilfarth occurred in 1886. They reported that specific bacteria can fix atmospheric nitrogen when symbiotic biological nitrogen fixation was established between leguminous plants and bacteria. Antibiotic production in soil by *Streptomyces*, *Penicillum*, *Aspergillus* and *Trichoderma* was demonstrated by Grossbard (1948–1952) and Wright (1952–1957). The concept of the rhizosphere in plant research and its future was described by the father of the green revolution, Norman E. Borlaug in the 1960s. Kloepper (1980) determined the importance of siderophores formed by PGPR in plant growth enhancement. The ability of PGPR such as *Azospirillium* to generate plant growth promoting substances and conduct nitrogen fixation was illustrated by Dobbelaere et al. (2003).

2.8.3 Mechanisms of interactions with pathogens

Several biocontrol mechanisms have been exhibited, which involve secondary metabolite production: hydrogen cyanide, antibiotics, bacteriocins, extracellular cell wall degrading enzymes, siderophore production, materials inducing pathogen resistance (SAR, ISR), improving competition for nutrients and minerals, substances that affect microbial colonization and compounds that regulate plant ethylene level (Kloepper et al., 1980; Dobbelaere et al., 2003; Van Loon, 2007; Martinez-Hidalgo et al., 2015). All of these mechanisms can work concurrently with each other, or act independently. Martinez-Hidalgo et al. (2015) as demonstrated in the ability of a gram-positive bacterium, *Micromonospora* isolated from alfalfa roots, to generate ISR in tomato cultivars against the fungal pathogen, *Botrytis cinera*. A study conducted by Kloepper et al. (1980) presented evidence that PGPR (*Pseudomonas* strains) exert their PGPR activity by depriving native microflora of iron, making it less available to native microflora.

2.8.4 Advantage and disadvantage of PGPR biological control agents

In general, PGPR can be considered as environmentally friendly, nontoxic and naturally occurring microorganisms. From an ecological view, their application is sustainable and much less environmentally disruptive than the currently deployed technologies. Moreover, they have diverse modes of action such as volatiles, cell wall degrading enzymes, antibiosis, etc.

However, they have certain disadvantages compared to chemical control compounds. Due to their biological nature, they are more sensitive to environmental conditions, temperature, soil type, etc. Also, their shelf life is shorter than chemical compounds. The most important disadvantage is that their efficiency is variable under filed conditions. Moreover, formulating these living microorganisms as a commercial product is clearly more challenging than is the case for chemical compounds (Labuschagne et al., 2011).

Overall, various factors should be considered when attempting to achieve successful biological control by using these treatments. The biocontrol agent outcome relies on inoculation method, presence of nutrients, treatment volume, plant type and cultivar, presence of protective agents, biocontrol agent physiological state and concentration (Knudsen et al., 1997; Lugtenberg et al., 2002; Khan et al., 2006; Levenfors et al., 2008; Labuschagne et al., 2011).

2.8.5 Commercial PGPR biocontrol products

PGPR can act as biocontrol agents and ease plant protection against various pathogens including fungi, bacteria, viruses and insects (Mishra et al., 2015; Myresiotis et al., 2015). PGPR have several advantages compared to chemical pesticides, including being safe for humans and the environment, degrading more easily in soil and having lower potential to result in the development of resistance in the pathogens (Berg and Smalla, 2009).

Previous literature has indicated disease reduction in major crops, such as rice, wheat and corn by using seeds treated with microbial biocontrol agents (Heydari et al., 2007; Saravanakumar et al., 2007; Heydari and Pessarakli, 2010; Karthiba et al., 2010; Senthilraja et al., 2013) and some of them are available as commercial products in the market place.

Most of the PGPR products on the market are based on gram positive bacteria (Ding et al., 2013). This group of bacteria includes the genus *Bacillus*, which produces spores and can survive longer in soil or storage as a result. Furthermore, they are easy to formulate, and the spores are capable of enduring desiccation during formulation. Gram negative bacteria with a persistent nature, such as *Pseudomonas* and *Burkholderia*, have been included in commercial formulations (Yuan et al. 2012; Loganathan et al., 2014).

PGPR may be applied as a single rhizobacterial strain or as bacterial consortia comprised of various strains. For example, Zhang and Xue (2010) applied a single bacterial strain (*Bacillus subtilis* strain SB24) against *Sclerotinia* stem rot in soybean (*Sclerotinia sclerotiorum*). They observed that sclerotia formation and mycelial growth were reduced up to 90% and 75%, respectively. A bacterial consortium comprised of *Bacillus*, *Pseudomonas* and *Achromobacter* was used against rice blast (*Pyricularia oryzae*). Bacterial soil suspension was applied on rice roots and they observed disease reduction of up to 100% in the field and under greenhouse conditions (Suprapta et al., 2016).

Some of the commercially available PGPR based products are Actinovate AG (soil borne disease), Bio Promotor Phosphobacteria (phosphate solubilization), Paddy *Azospirillum* (nitrogen fixation), potash solubilizing liquid (nitrogen fixing, P-solubilizing, K-solubilizing), and Rice-Biofert (mineral fertilizer with PGPR that improves soil health) (Tabassum et al., 2017).

2.8.6 Impact of PGP inoculant on soil microbiome and biosafety

For decades, many microbial products have been supplied by companies in developing and emerging nations for home gardeners and commercial farms. These biological materials have been adopted as substitutes for chemical fertilizers and pesticides (Kecskés et al., 2016; Parnell et al., 2016; Menendez and Garcia-Fraile, 2017). However, a huge gap of information has been observed regarding the effectiveness and performance of plant growth promoting microbes based on laboratory studies compared to field studies.

Moreover, there is a lack of knowledge about the effect of adding large numbers of microorganism inoculants to indigenous soil microbiomes. It is important to initiate study and create data sets regarding soil micrbiomes before and after adding soil microbial inoculants. In this way, we can assess their impacts on crops, soil health and performance (van Elsas et al., 2015). Several methods and strategies have been developed to measure and evaluate soil divergence, biochemical and microbial variables quantitively and statistically. Metagenomic sequencing is a direct way for monitoring soil changes over time in response to biological and abiotic amendments (Martínez-Hidalgo et al., 2019). A study by Dinesh et al. (2010) revealed positive influence of organic manure and biofertilizers (*Azospirillum lipoferum* and *Bacillus megaterium*) on soil quality, microbial biomass carbon, oxidoreductase enzyme production, soil respiration and N mineralization.

Sometimes theses microbial products might have untoward impacts on humans, other plants, animals, soil dwelling and other non-target organisms (O'Callaghan and Glare 2001; van Elsas et al., 2015; Lugtenberg, 2018; Martínez-Hidalgo et al., 2019). They may impact their recipient environment by chemical or abiotic modification of environment (Kowalchuk et al., 2003), by transferring genes to new hosts (horizontal gene transfer), by antagonizing, competing and cross

feeding the indigenous microbiota. Smit et al., (1991) demonstrated plasmid movement from microorganisms to indigenous soil bacteria and Lilley and Bailey (1997) observed uptake of plasmids from indigenous bacteria. Moreover, their data demonstrated that genetic and phenotypic stability of released inoculants into natural environment is unpredictable (Lilley and Bailey, 1997).

The majority of bacterial species with PGP activity such as *Pseudomonas*, *Ralstonia*, *Burkholderia*, *Enterobacter* are potentially virulent or opportunistic human pathogens (Berg et al., 2005). The plant rhizosphere harbors bacteria such as *Bacillus cereus*, *Bacillus cepacia*, *Pseudomonas* spp. and *Proteus vulgaris* which have been reported in skin, wound and urinary infections. *Pseudomonas* species such as *P. fluorescens*, *P. putida*, *P. aeruginosa* are commonly found in soil and in clinical samples and they can cause lung disease, respiratory infection (Ortega-Calvo and Saiz-Jimenez, 1998; Berg et al., 2005; Baum et al., 2009). It has been predicted that problems with rhizosphere bacteria as opportunistic human pathogens will become more serious as we deploy more bio-technologies in crop production, unless we take great care during the development of these new technologies.

To commercialize these microbial products, they should be isolated from their original sources, their identity should be determined and their traits to support plant growth should be evaluated. Furthermore, their performance, success and their potential risks need to be determined under laboratory and natural conditions. Finally, any potential negative impacts of these microbes on the natural soil microbiomes should be examined (Martínez-Hidalgo et al., 2019).

Chapter 3: Materials and Methods

3.1 Isolation of rhizobacteria from various undomesticated plants

3.1.1 Collection site and sampling

Plant root samples for this study were collected from Sainte-Anne-de-Bellevue in the southwestern portion of the province of Quebec, Canada, (lat. 45°24′22″ N, long. 73°56′44″ W, and 24 m above sea level). A total of five species of undomesticated plants at the site were sampled and assessed for the presence of cultivatable endophytic bacteria. The sampled plants include dandelion (*Taraxicum officinale*), heal-all *(Prunella vulgaris)*, milkweed (*Asclepias syriaca*), wild lettuce (*Lactuca virosa*), and coltsfoot (*Tussilago farfara).* These undomesticated plants were chosen based on at least four main criteria 1- availability at date of sampling, 2- importance in agriculture, possibly related to their vigorous growth in the habit of the area, and most importantly 4- their association with bacterial ecosystems in this particular area, as researched by Fan et al. (2018) in her study titled “Isolation and diversity of culturable rhizobacteria associated with economically important crops and uncultivated plants in Québec, Canada." Each root sample was taken directly beneath each plant at 15-20 cm of soil depth. Thereafter, much of the soil was removed and plant root samples were transported in plastic bags to the laboratory and kept overnight in the fridge at 4°C to process the next day.

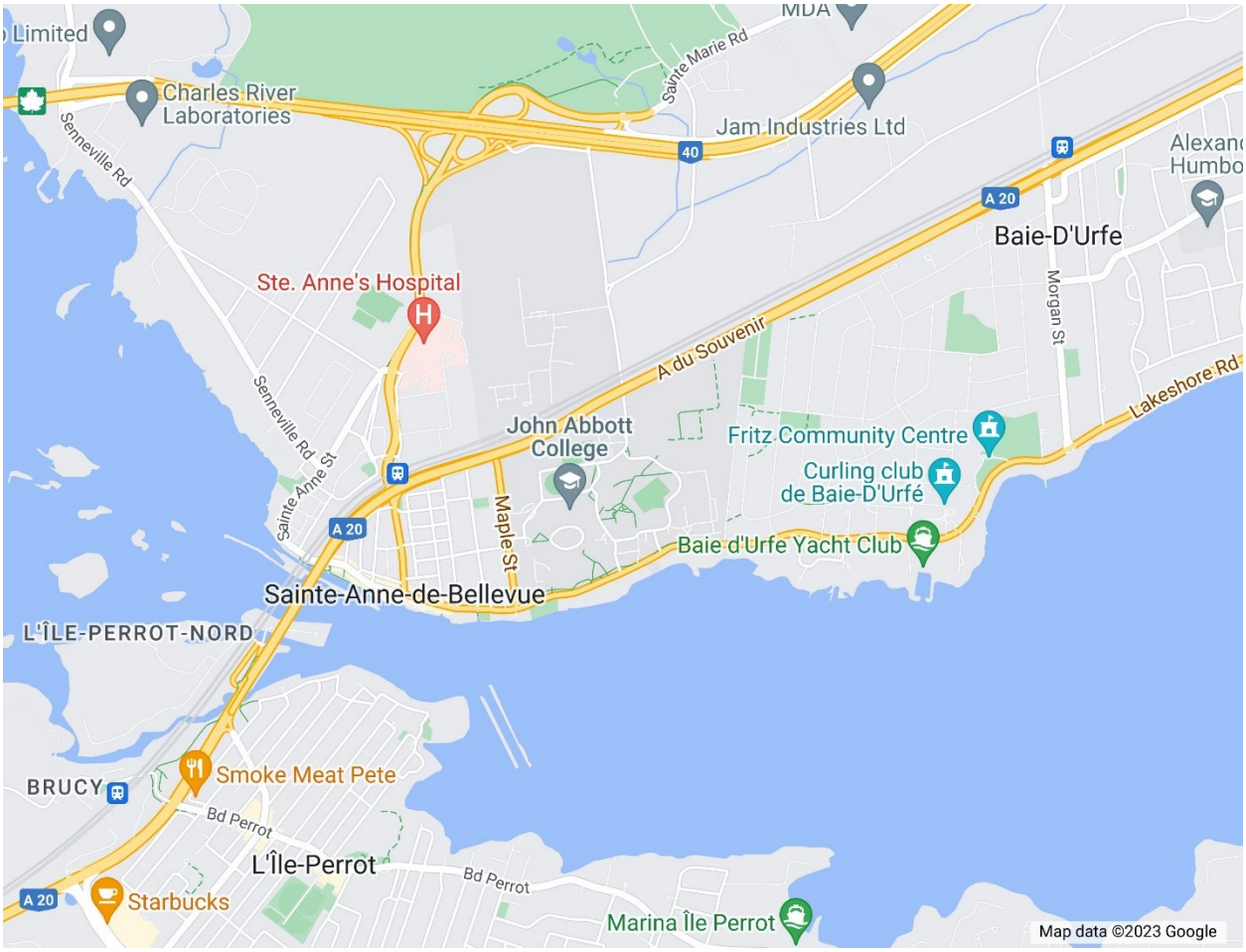


Fig. 3. 1 Location in Sainte-Anne-de-Bellevue, Canada, from which samples of plant growth-promoting bacteria were collected for isolation, using a Google Map.

Milkweed *(Asclepias syriaca)* **Heal-all *(Prunella vulgaris)***

Wild lettuce (*Lactuca virosa*)

Coltsfoot (*Tussilago farfara)* ***Dandelion*** (*Taraxicum officinale*)

Fig. 3. 2 Representative images of five undomesticated plant species collected for the isolation of endophytic bacteria.

3.1.2 Isolation of endophytic bacteria

To achieve sterility, all tools necessary for this procedure were sterilized by autoclaving at 121°C for 20 min; and the isolation protocol was conducted under a laminar flow hood. Endophytic bacteria were isolated from roots of healthy undomesticated plants, sampled from the site indicated above. Isolation of root associated endophytic bacteria were carried out by surface sterilizing of root samples to remove microbial colonizers. To surface sterilize root samples, adhering soil particles were removed by soft brushing and thoroughly washed under tap water. Subsequently, root samples were rinsed in sterile distilled water (ddH_2O) several times under aseptic conditions. Using sterile scissors, the individual root samples were then divided into smaller pieces (3 cm long) and transferred into sterile tubes (Boyle et al. 2001). Followed by surface sterilization with 70 % ethanol (v/v) for 3-5 min, washed with ddH_2O (four times), then incubated in sodium hypochlorite (NaOCl 3%, 5-10 min) and rinsed seven times with ddH_2O to remove disinfectant.

After surface sterilization, a sterile mortar and pestle were used to crush the sterilized root fragments in phosphate-buffered saline (PBS) in the laminar airflow cabinet. Supernatants from crushed root tissue were serially diluted up to 10^{-3} after which 100 µL from each diluent were plated onto nonselective nutrient media such as Trypticase Soya Agar (casein peptone (pancreatic) 15 g, soya peptone (papainic) 5 g, sodium chloride 5 g, agar 15 g, final pH 7.3 +/- 0.2 at 25°C); Nutrient Agar media (NA, Difco; 8 g L^{-1}); and King's B media (KB; 20 g proteose peptone no. 3, 10 mL glycerol, 1.0 g $MgSO_4$, 1.5 g K_2HPO_4, pH 7.2). The root contents were diluted immediately to decrease the concentration of plant enzymes and toxins released during maceration, as higher concentrations of these compounds can kill or inactivate endophytic microorganisms. The petri dishes were covered with parafilm and placed in an upright position for incubation. They were then kept at a temperature of 28°C for a period of 2 to 5 days, allowing sufficient time for bacterial

colonies to form and become visible. Individual bacterial colonies grown on the surface of plates were chosen and purified, based on morphological features, and transferred to new plates. Pure bacterial cultures were preserved in medium, containing 25 % (v/v) glycerol, and stored at -80 °C for further experimentation.

3.2 Initial screening of bacterial isolates for plant growth-promoting properties

Plant growth promotion traits refer to specific characteristics exhibited by certain microorganisms that enhance the growth and development of plants. These traits can be beneficial for agriculture, as they contribute to increased crop yields, improved nutrient uptake, enhanced stress tolerance, and overall plant health. Plant growth-promoting traits are typically associated with certain beneficial bacteria and fungi that form symbiotic relationships with plants, directly interacting with their root systems. Hence, the effect of isolated bacteria as a potential candidate, a strain producing plant growth promotion traits such as nitrogen fixation, phosphate-solubilizing capability, and indole-3-acetic acid (IAA) production were tested under *in vitro* conditions. In this initial screening assay, to identify potential PGPR from the newly isolated endophytic bacteria, a total of 75 strains were isolated. Based on the results obtained from this primary screening assay, only bacterial isolates that manifested growth stimulation effects were selected and used for subsequent experiments.

3.2.1 Bacterial strains, and growth conditions

To perform the initial screening assay of bacterial isolates maintained in ultra-cold storage, the strains were regularly produced on their appropriate growth medium such as KB, TSA, and NA. These were used as the primary growth media for the bacteria during the screening process. By maintaining the bacterial strains on these media, we can ensure the viability and stability of the strains over time. This allows for consistent access to the isolated strains, enabling repeated screenings and further analysis of their plant growth promotion traits.

For bacterization experiments, individual colonies of the bacterial strains were introduced into the liquid media indicated above in order to obtain uniform bacterial suspensions.

The bacterial cultures were then placed on a rotary shaker set at a speed of 120 rpm for incubation in the absence of light at 28 °C for up to 3 days. The purpose was to allow the bacterial cultures to reach the exponential growth phase. Prior to inoculation, the bacterial cells were collected by centrifugation at 6,000 g for 10 minutes at 4 °C. The cell pellets were washed four times using a sterile 10 mM $MgSO_4$ solution and then reconstituted in 10 mM $MgSO_4$ to achieve a final concentration of 10^7 colony-forming units (CFU) mL^{-1}. The bacterial density was determined by measuring the optical density (OD) at 600 nm and confirming the count through serial dilutions and plate counts. These standardized bacterial suspensions were used for bacterization experiments.

3.2.2 Nitrogen fixation

The nitrogen-free bromothymol blue malate method is a biochemical assay used to measure the nitrogen-fixing activity of microorganisms. It is commonly used to assess the nitrogen-fixing ability of bacteria. Nitrogen fixation was qualitatively evaluated by growing the PGPR in nitrogen-

free bromothymol blue semisolid medium (NFb) (Baldani *et al.*, 2014). Ten microliters of each isolate were added into the semisolid medium. The tubes were incubated at 27 °C for up to 7 days. A color change from green to blue in the isolate's solid culture suggests the presence of nitrogen-fixing activity. NFb medium consists of: KH_2PO_4, 0.4 g L^{-1}; 188 K_2HPO_4, 0.1 g L^{-1}; $MgSO_4.7H_2O$, 0.2 g L^{-1}; NaCl, 0.1 g L^{-1}; $CaCl_2$, 0.02 g L^{-1}; $FeCl_3$, 189 0.01 g L^{-1}; $Mo.O_4Na.2H_2O$, 0.002 g L^{-1}; sodium malate, 5 g L^{-1}; agar, 15 g L^{-1}; 190 bromothymol blue (0.5% in alcohol solution), 5 mL L^{-1}, pH 7.

3.2.3 Plate assay for phosphate-solubilizing capability

Phosphate solubilizing capability is one of the key traits of plant growth promotion (PGP) and can be easily detected by plate assay (Martínez-Hidalgo et al., 2019). For this experiment, potential bacterial isolates were qualitatively evaluated by plate assay using National Botanical Research Institute's phosphate (NBRIP) growth medium which contained tricalcium phosphate $Ca(PO_4)_2$ as the inorganic phosphate source (Nautiyal, 1999). Ten microliters of bacteria were dropped on the agar plate surface and incubated at 27 °C. After incubation, the presence of clear halo zones around the colonies was regarded as a positive indication of phosphate (P) solubilization.

3.2.4 Indole-3-acetic acid (IAA) detection

IAA production was determined using the method of Gordon and Weber (Gordon and Weber, 1951) with minor modifications. One milliliter of bacterial cultures (2-d old fresh culture in KB liquid media) was used to inoculate 5 milliliters of KB medium. The KB medium was either supplemented with L-tryptophan at concentrations of 200 or 500 μg mL^{-1} (serving as a precursor for indole-3-acetic acid, or IAA), or left without supplementation as the control. The incubation took place at a temperature of 27°C in the absence of light. Indole acetic acid (IAA) production by

each strain was determined at 24 h intervals after inoculation. In preliminary experiments, we found that maximum IAA production generally occurred 24 h after inoculation. In this experiment, after 48 and 72 h, 1.5 milliliters of the sampled culture broth were extracted and transferred to a test tube. These cultures were then subjected to centrifugation at 10,000 g for 10 minutes at a temperature of 20°C. Following centrifugation, 1 mL of the resulting supernatant was carefully transferred into a fresh test tube. Thereafter, one droplet of orthophosphoric acid, along with 2 mL of freshly prepared Salkowski's reagent, which consists of 98 mL of 35% perchloric acid and 2 mL of 0.5 M $FeCl_3$, were introduced into the mixture; the resulting solution was gently vortexed, then incubated at room temperature in the dark for 30 min. The presence of IAA was indicated by pink color; the quantity of IAA was measured by reading the OD_{530}. IAA production was estimated by comparing a standard curve using an IAA solution with concentrations ranging from 0 to 100 µg mL^{-1}.

3.3 Molecular identification of bacterial isolates

Sanger sequencing is a widely used method for DNA sequencing, including the sequencing of the 16S rRNA gene for bacterial identification to the genus level, and tentative identification at the species level. The selected bacterial isolates from primary screening assay (section 3.2) were identified taxonomically, to the species level, by standard polymerase chain reaction (PCR). Sanger sequencing was carried out at Genome Québec Innovation Center to analyze the 16S rRNA region of the bacterial isolates. The target region was amplified and sequenced using universal primers, 27F (5'-AGRGTTYGATYMTGGCTCAG-3') and 1064R (5'-CGACRRCCATGCANCACCT-3'). This approach allows for the identification and characterization of the bacterial strains based on its genetic information encoded in the 16S rRNA gene. To analyze the samples, they were diluted in water at a ratio of 1:10. From the diluted

samples, 1 µL was utilized for PCR using the Fast HotStart enzyme from Kapa Biosystems. Sanger sequencing using Applied Biosystems' Big Dye Terminator V3.1 was performed on the purified PCR products. Purified PCR samples underwent Sanger sequencing utilizing the Big Dye Terminator V3.1 kit (manufactured by Applied Biosystems). The procedure initiated with an initial denaturation at 96°C for one minute, followed by 25 cycles of 96°C for 10 seconds, annealing at 50°C for 5 seconds, extension at 60°C for 4 minutes, and finally held at 4°C. A 3730xl DNA Analyzer from Applied Biosystems was used to examine the resulting sequencing reactions. The sequences obtained were compared to previously published 16S rRNA sequences by using NCBI BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.4 Secondary screening assay of bacterial isolates against *Streptomyces scabies,* a common scab causing pathogen

3.4.1 Bacterial growth conditions

Ten isolates of endophytic rhizobacteria were chosen from the initial screening assay (section 3.2) based on their performance in a plant growth promotion assay. These bacterial isolates were grown in liquid broth containing their appropriate medium, as indicated above, and incubated at 28 °C, with shaking at 120 rpm. The cell concentration was determined by measuring the optical density (OD). Two strains (44B and EF-35) of the pathogenic species of *Streptomyces scabies* were used in this study. These strains are specifically associated with potato scab, a disease that affects potatoes and other root vegetables. *Streptomyces scabies* strains were provided by Carole Beaulieu, Professor at University of Sherbrook. The *S. scabies* strains (44B and EF-35) were grown, respectively, on agar plates containing Tryptic Soy Agar (TSA: Pancreatic digest of casein 15 g, peptic digest of soybean meal 5 g, sodium chloride 5 g, agar 15, final pH 7.3 +/- 0.2 at 25°C) and

ISP-2 medium (yeast extract 4 g, malt extract 10 g, dextrose 4 g, agar 15 g, final pH 6.2 +/- 0.2 at 25°C). *Streptomyces scabies* were then incubated at 28 °C, with shaking at 120 rpm up to 3 days.

3.4.2. Antagonistic activity (disk diffusion assay)

Through an initial screening assay, elaborated in section 3.2, the ten strains that demonstrated consistent to capacity to enhance plant growth through a specific set of traits were selected for a second-round screening. The objective of the second-round screening was to further analyze and assess the antagonistic activity of the selected bacterial strains related to their activity against pathogenic *Streptomyces scabies.* The disk diffusion assay, also known as the Kirby-Bauer test, was used in this experiment to evaluate the antimicrobial activity of the strains against *Streptomyces scabies* on agar plates.

Initially, *Streptomyces scabies* cells (100 µL, 10^7 CFU mL^{-1}) were uniformly spread across the surface of their appropriate agar plates using a sterile spreader. Sterilized filter paper disks (6 mm diameter) were placed on the surface of each plate immediately after the plates became dry on the surface, followed by placing 10 µL of bacterial isolate on each filter paper disk, or KB medium in the case of controls. Plates were incubated at 28°C for 3-5 days for observation of an inhibition zone (area in the plate where growth or the plant pathogen is lacking due to proximity to the strain being evaluated) by the unaided eye. The clear growth inhibition zones surrounding the disks of paper were measured in millimeter (mm). This experiment was performed three times to confirm the antibacterial activity. In this study, bacterial isolates that demonstrated antimicrobial activity against the target pathogen were selected once again and used for further experiments. The selection of these isolates suggests that they have demonstrated significant potential in effectively suppressing the growth of *Streptomyces scabies* and improving plant growth.

3.5 Mechanisms of action of selected bacterial strains exhibiting antimicrobial traits

Plant Growth-Promoting Rhizobacteria (PGPR) exert their antagonistic effects against pathogens through various mechanisms. These beneficial rhizobacteria can promote plant growth while simultaneously providing protection against pathogenic microorganisms. Three isolates exhibiting potent antimicrobial activities against *Streptomyces scabies* were selected from secondary screening (section 3.4) based on their antimicrobial activities against *Streptomyces scabies*. These three strains were used for the remainder of the experiments. The objective of this section was to gain deeper insights into the mechanisms employed by these three bacterial isolates to effectively suppress the pathogen. The study investigated several essential mechanisms employed by bacterial isolates to effectively counteract the pathogen.

3.5.1 Siderophore production assay

Bacteria have developed various mechanisms to acquire essential nutrients, including iron, for their survival and growth. In addition to actively acquiring iron for themselves, bacteria also produce and secrete siderophores as a strategy to suppress pathogens. Siderophores can play a dual role by not only facilitating iron uptake for the producing bacteria but also limiting the availability of iron to competing pathogens in the environment. Thus, to determine the production of siderophores by our isolated and selected anti-*Streptomyces scabies* strains, we used a Chrome Azurol S (CAS) medium, following the methodology described by Alexander and Zuberer (1991). The bacterial isolates chosen through the secondary screening assay were grown in KB medium, and incubated at 28°C with shaking at 120 rpm for a duration of 3 days. A sterile filter paper disk was placed on a prepared CAS agar plate. Subsequently, 10 µL of the culture was spotted onto the filter paper disk. After applying parafilm to seal the plates, it was subjected to incubation at a temperature of 28 °C for a minimum duration of 2 days. After incubation, the presence of a yellow-

orange halo surrounding the bacterial growth area was interpreted as a positive sign indicating the production of siderophores.

3.5.2 Hydrogen cyanide production

For endophytes, microorganisms that live inside plant tissues without causing apparent harm, HCN production can be a crucial trait contributing to their competitiveness amongst each other. The production of HCN by bacterial isolates was evaluated according to Ahmad et al. (2008) with slight modification. Since some bacteria can use glycine as a precursor in the production of hydrogen cyanide (HCN). Bacterial cultures were cultivated in a nutrient broth (KB) enriched with 4.4 g L^{-1} of glycine. One hundred µL of each bacterial strain were spread on agar plates containing KB medium. Afterwards, the alkaline picrate solution, comprised of 2% sodium carbonate in a 0.5% picric acid solution, was applied to filter paper and placed on the underside of the lids of of Petri dishes. The plates were placed in an incubator at 28 °C and left to incubate for a period of 3-5 days. During this time, if the filter paper on the plates underwent a color change from bright yellow to orange and/or red brown, this indicated the production of hydrogen cyanide (HCN).

3.5.3 Biosurfactant production

Biosurfactants are surface-active compounds that are synthesized by a range of microorganisms, including bacteria. These compounds possess the ability to reduce surface tension and enhance the interaction between microorganisms and their environment. Biosurfactants produced by bacteria have shown promising potential as plant protection agents. Several biosurfactants produced by endophytic bacteria contributed to plant health by promoting growth, suppressing plant pathogens, and enhancing plant ability to withstand environmental stressors (Santos et al., 2016).

Hence, testing the ability of our isolated endophytic bacterial strains to produce biosurfactants is a crucial step in evaluating their potential as plant protection mechanism. An oil-spreading assay was performed to evaluate the biosurfactant activity of bacterial isolates (Ibrahim et al., 2013). The oil displacement test was conducted using polystyrene petri dishes (100 mm × 15 mm) containing 20 µL of crude oil layered over 20 mL of distilled water. The filtered supernatant of bacterial culture, amounting to around 10 µL, was precisely pipetted onto the middle of the oil layer in the petri dish. The diameter of the resulting clear zone on the oil layer's surface was measured and compared to the measurements obtained from the negative control samples. As a negative control, uncultured broth medium was included.

3.6 Growth rate

The growth rate of the chosen strains was evaluated using a CytationTM 5 imaging reader (Inc. BioTek headquartered in Winooski, Vermont, USA) () which determined their growth curves. Initially, tubes of liquid KB medium were used to grow bacteria. The tubes were placed on a rotary shaker and then incubated at 28 °C on a spinning shaker at a speed of 120 rpm. When the bacterial cultures were growing exponentially they were diluted in KB broth to reach an optical density at 600 nm (OD600) of approximately 0.001. This diluted culture was inoculated into each well of a microplate containing 96 wells. Microplates were kept in the dark at 28 °C for the duration of the incubation period, and spectrophotometric readings were recorded at regular intervals (every 2 h) to generate the growth curves. Serial dilutions followed by plate counts were used to examine the CFU. In the logarithmic growth phase, the average generation time was determined as described by Stanier et al. (1970).

3.7 Bacterial motility test

The ability of bacteria to swim or move is crucial for their survival and allows them to adapt to new environments. By studying bacterial motility, we can learn more about how bacteria behave in diverse situations, such as when they colonize surfaces, create biofilms, or cause diseases. In this experiment, petri plates (polystyrene, 82 mm in diameter) were used for the motility tests. The swimming motility of the selected bacterial strains was assessed using KB plates containing 0.5% agar, as described by Be'er and Harshey (2011). Swim plates were then inoculated with 3 µL of each strain of bacterial culture into the center of the semisolid agar plates and incubated under appropriate conditions for bacterial growth (48 h at 28 ºC) to determine the movement of bacterial cells by measuring the swarm diameter.

3.8 Antagonistic/compatibility activities between selected bacterial strains

Conducting a compatibility test between bacterial strains as a consortium is important for various applications in agriculture. This involves assessing whether different strains of bacteria can coexist or interact with each other in a specific environment, which is crucial for understanding microbial interactions, studying disease management applicatins, and assessing the potential of using specific strain combinations for other specific applications.

3.8.1 Cross streak method

The cross-streak assay is a method used to assess antagonism or inhibition between bacterial strains and/or their compatibility. In this study, selected bacterial isolates from the secondary screening assay (section 3.4) were streaked perpendicular to each other on an agar plate, creating an intersection of growth. The purpose of this study was to investigate whether the strains exhibit antagonism or compatibility when grown in close proximity. The growth patterns and interactions

between the strains can provide insights into their relationship whether the bacterial strains are compatible, antagonistic, or have other types of interactions when cultured together.

To do so, *in vitro* assays were carried out according to Fitriatin et al. (2020). Bacterial strains were cultured in KB liquid media as described in previous experiments. Using a sterile inoculating loop, the first strain was inoculated onto a fresh nutrient agar plate by streaking as a single straight line across the center of the agar plate and incubated at 28 °C for 2 days. Thereafter, other strains were cross streaked perpendicular, at a 90◦ angle to the first streak line, that we made previously, to ensure that they were in direct contact with the agar surface and each other. The plates were then incubated for 1 to 2 days at 28 °C. After incubation, the plates were observed by looking for any zones of inhibition or visible effects between the test strains. A clear zone or reduced bacterial growth around the bacterial strains indicates inhibitory effects on the bacterial isolates to each other.

3.8.2 Co-culture method

The co-culture method, also known as mixed culture or co-cultivation, is a technique used to study interactions between two or more different microorganisms grown together in the same culture environment. The purpose of this study was to investigate various biological phenomena, such as mutualism, competition, and antagonism, among the co-cultured beneficial bacterial strains.

The co-culture plating method was utilized to evaluate the compatibility between the strains according to (Kumar et al. 2016) with minor modifications. Equal volumes of freshly cultured bacteria (5 µL) from each strain were placed equidistantly on KB culture plates, ensuring 0.5 cm between the strains. The plates underwent incubation at a constant temperature of 28 ºC for up to

4 days. The growth compatibility and/or inhibition among strains were observed. All tests were done on triplicate plates, and each overall evaluation was conducted twice to ensure the accuracy of the results.

3.9 Intrinsic antibiotic sensitivity/resistance

A disk diffusion assay was conducted to study the susceptibility or resistance of the selected three bacterial strains against antibiotics. Six antibiotics were used: 1) gentamycin, 2) streptomycin, 3) kanamycin, 4) ampicillin, 5) chloramphenicol, and 6) tetracycline. The concentration of antibiotics varied between 0.5 and 256 μg mL^{-1}. In brief, homogenous bacterial suspensions (100 μL) of each bacterial strain were spread/inoculated uniformly on KB agar plates. A disk paper containing the above-mentioned antibiotics was applied onto agar plates and labeled with the specific antibiotics being tested. The plates were incubated for 48 h at 28°C to allow bacterial growth. After incubation, bacteria were classified as susceptible to an antibiotic at the tested concentration if no visible growth was detected on the treated plates, whereas visible growth indicated that the bacteria were resistant to the antibiotics.

Chapter 4: Results

4.1 Strain isolation

A total of 75 bacterial endophytes were successfully isolated from the roots of five different undomesticated plant species: dandelion (*Taraxicum officinale*), heal-all *(Prunella vulgaris)*, milkweed *(Asclepias syriaca)*, wild lettuce *(Lactuca virosa)*, and coltsfoot *(Tussilago farfara)*. The isolation process involved using three culture media, namely King's B (KB), Tryptic Soy Agar (TSA), and Nutrient Agar (NA). Among the isolated endophytes, most of the isolates (40) were obtained using the KB medium, followed by 22 strains isolated on TSA medium, and 13 strains isolated on NA medium. This diverse set of culture media enabled the successful cultivation and isolation of a wide range of bacterial endophytes from the root samples. The effectiveness of the decontamination procedures was confirmed by the water-washing step, which failed to transfer any microbial colonies onto the agar medium. This result provided strong evidence that the decontamination process successfully eradicated microbes from the root surface, ensuring that the isolated endophytes originated from within the plant tissues. The findings highlight the richness and diversity of bacterial endophytes inhabiting the roots of these undomesticated plant species. The diverse community of endophytes has the potential to contribute significantly to the ecological dynamics and overall health of the plants.

4.2 Assessment of selected bacterial isolates for plant growth promotion traits

The study aimed to explore the potential of newly isolated endophytic bacteria as PGPR. A total of 75 bacterial strains were isolated from the roots of wild, undomesticated plants (Chapter 3). Subsequently, an *in vitro* analysis was conducted to evaluate their ability to enhance plant

growth and nutrient acquisition. The analysis focused on essential PGPR traits, including nitrogen fixation, phosphorus solubilization, and the production of indole-3-acetic acid (IAA).

From the initial pool of 75 strains, ten bacterial isolates were selected for a second-round screening test. These ten isolates were chosen based on their significant capabilities in demonstrating traits associated with plant growth promotion. The *in vitro* assays provided valuable insights into the growth-promoting properties of these isolates in a controlled environment. The study's findings indicate that the ten selected bacterial isolates have promising potential as PGPR. To achieve this, we used a series of the following biochemical function tests.

4.2.1 Nitrogen fixation

The nitrogen-free bromothymol blue (NFb) semisolid medium was utilized in this study to assess the nitrogen fixation ability of 75 bacterial isolates. Nitrogen fixation is a vital process wherein certain bacteria have the ability to convert atmospheric nitrogen into a biologically usable form, contributing to soil fertility and supporting plant growth. Out of the 75 bacterial strains tested, only ten exhibited positive nitrogen fixation effects with varying levels of nitrogen fixation. The NFb medium served as an effective indicator of nitrogen fixation activity. When bacteria successfully fixed nitrogen, the medium's color changed from green to blue due to the increase in alkalinity caused by the release of ammonia or other nitrogenous compounds. The ten selected bacterial strains displayed varying levels of nitrogen fixation. Table 1 presents the genus and species of these strains along with the corresponding levels of nitrogen fixation they exhibited. The levels of potential nitrogen fixation were likely represented by different shades of blue in the medium, reflecting the extent of nitrogen fixation achieved by each bacterial strain.

4.2.2 Solubilization of inorganic phosphorus

The objective of this experiment was to evaluate the ability of the 75 isolated bacterial strains to solubilize the insoluble form of phosphate, using National Botanical Research Institute's phosphate (NBRIP) agar medium. The results demonstrated that out of the total 75 bacterial isolates tested, ten strains exhibited the capability to solubilize phosphate. Upon inoculating the isolated bacteria onto NBRIP plates containing tri-calcium phosphate, the presence of clear halo zones around the bacterial inoculation disks was observed. The formation of clear halo zones around the bacterial inoculation disks provides evidence that these ten bacterial strains have the capacity to solubilize the inorganic phosphorus present in the tri-calcium phosphate (TCP).

Table 1 Characterization of selected strains for traits related to plant growth promotion in vitro

Strain code	Species	P solubilization	N fertilization
S1	*Bacillus velezensis*	+	+
S2	*Pseudomonas chlororaphis*	+	+
S3	*Bacillus amyloliquefaciens*	+	++
S4	*Pseudomonas koreensis*	+++	+
S5	*Pseudomonas fluorescens*	++	+++
S6	*Pantoea agglomerans*	+	+
S7	*Pseudomonas sp.*	+++	++
S8	*Bacillus subtilis*	+++	+
S9	*Bacillus amyloliquefaciens*	++	+++
S10	*Bacillus siamensis*	+	++

+, low activity; ++, moderate activity; +++, strong activity

4.2.3 Indole-3-acetic acid (IAA) production

IAA, the primary auxin in plants, plays a crucial role in various physiological processes such as cell division, elongation, tissue differentiation, and light responses (Patten and Glick, 2002). In this experiment, 75 bacterial strains isolated from the roots of undomesticated plants were screened to assess their ability to produce IAA using two concentrations of tryptophan (200 and 500 µg mL^{-1}) as a precursor. IAA production was measured at two time points: 48 and 72 h after incubation. Out of the 75 isolates, ten strains were found to produce varying levels of IAA. The production of IAA was observed when L-tryptophan was present in the medium, but no IAA production was detected when L-tryptophan was absent. After 72 h of incubation with 500 µg mL^{-1} L-tryptophan in the growth medium, most strains showed the highest IAA production. However, some isolates exhibited peak IAA production at the 48-h mark, regardless of the tryptophan concentration in the medium. Notably, *Pseudomonas* spp. strains displayed the highest IAA production at 48 h, using either 200 or 500 µg mL^{-1} of L-tryptophan, indicating their faster response to tryptophan and ability to produce significant amounts of IAA earlier in the incubation period.

Table 2 IAA production

	200 ug (ug mL^{-1})		500 ug (ug mL^{-1})	
Strain	36 h	48 h	36 h	48 h
S1	11.71	14.01	28.80	40.15
S2	14.60	25.11	29.60	35.16
S3	14.18	24.94	40.73	33.70
S4	10.09	16.24	32.63	39.70
S5	15.75	12.49	19.86	39.18
S6	11.43	20.20	16.15	19.86
S7	16.27	23.34	34.05	43.88
S8	15.41	17.49	30.50	31.71
S9	11.94	10.81	23.67	30.71
S10	8.64	12.37	18.58	25.13

4.3 Identification of bacterial isolates by 16S rRNA sequencing

Among the total of 75 bacterial isolates originally obtained from the plant roots, a subset of ten were selected, based on their clear ability to promote plant growth (sections 3.2.2, 3.2.3, and 3.2.4). To acquire a comprehensive understanding of the taxonomic composition of these ten selected isolates, partial sequencing of the 16S rRNA gene was employed. The main objective of this sequencing was to identify the taxonomic classification of these ten isolates at the genus level, and some indication of possible identity at the species level. For the taxonomic identification, the nucleotide sequences obtained from the selected ten isolates were compared with sequences deposited in the GenBank database using the Mega BLAST algorithm. This comparison allowed us to find similarities between the 16S rRNA gene sequences of the selected isolates and those of known bacterial species already in the GenBank database. The comparison results revealed that most of the potential isolates from the ten selected strains belong to the genera *Bacillus* and *Pseudomonas* (Table 3). These genera are recognized for their ability to promote plant growth through various mechanisms, and their prevalence among the selected isolates underscores the importance of their ability in the context of plant growth promotion in the specific plant root environment under study.

Table 3 Molecular identification of bacterial isolates using16S rDNA as query sequences.

Strain code	Closet relative (NCBI)	% Similarity (EzTaxon)	Plant Origin
S1	*Bacillus velezensis*	100%	*Taraxicum officinale*
S2	*Pseudomonas chlororaphis*	100%	*Asclepias syriaca*
S3	*Bacillus amyloliquefaciens*	99.93%	*Lactuca virosa*
S4	*Pseudomonas koreensis*	100%	*Tussilago farfara*
S5	*Pseudomonas fluorescens*	100%	*Asclepias syriaca*
S6	*Pantoea agglomerans*	100%	*Prunella vulgaris*
S7	*Pseudomonas sp.*	99.71%	*Tussilago farfara*
S8	*Bacillus subtilis*	100%	*Taraxicum officinale*
S9	*Bacillus sp.*	100%	*Lactuca virosa*
S10	*Bacillus siamensis*	100%	*Taraxicum officinale*

4.4 *In vitro* bacterial screening against *Streptomyces scabies* (disk diffusion assay)

The ten bacterial isolates, selected from the primary screening assay, were assessed for their antagonistic activity against two strains of *Streptomyces scabies* (44B and EF-35), the pathogen responsible for causing common scab disease in potato. Among the ten isolates, several strains exhibited inhibitory effects on one or both of the pathogen strains, while three strains demonstrated strong inhibitory effects against both *Streptomyces scabies* strains. The three strains, tentatively identified as *Bacillus velezensis* (S1), *Pseudomonas chlororaphis* (S2), and *Bacillus amyloliquefaciens* (S3), displayed significant antagonistic activity against both *Streptomyces scabies* strains (44B and EF-35). This was evident from the clear inhibition zones observed around the disks on the petri plates (Fig. 4.1). During the disk diffusion assay, these three bacterial isolates effectively reduced the radial growth of the test pathogens, resulting in substantial inhibition zones with a diameter greater than 1.5 cm but less than 3 cm.

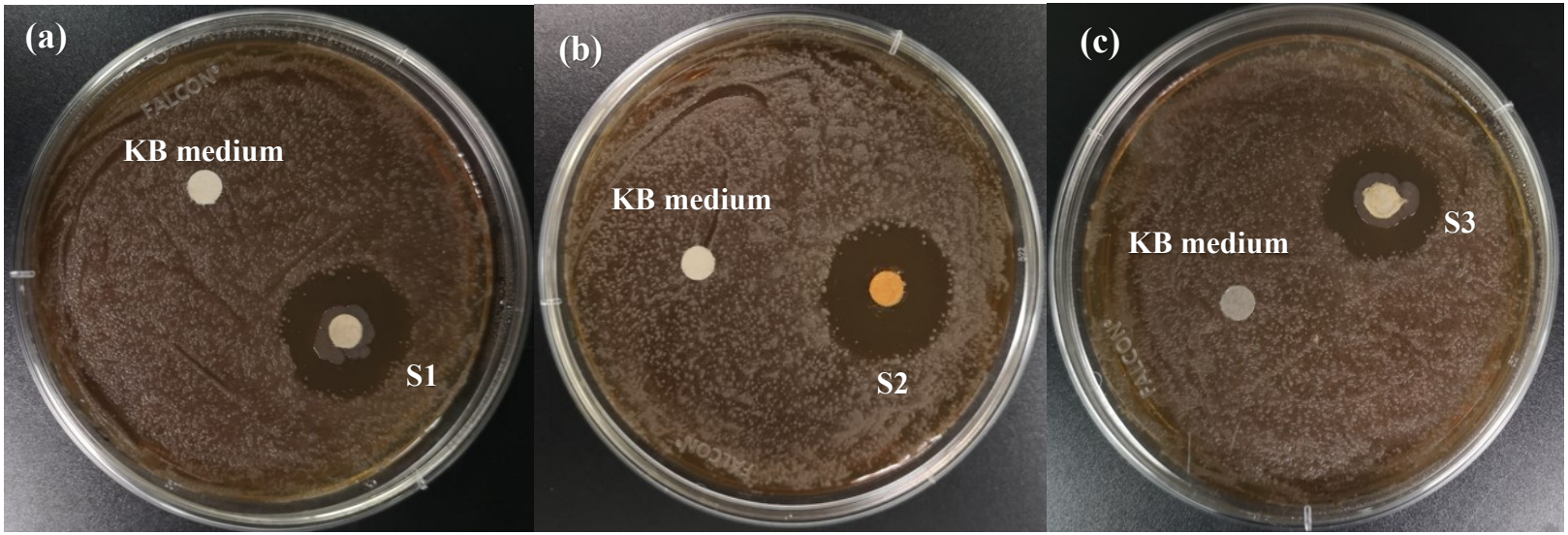


Fig. 4. 1 The antagonistic activity of the three most effective isolates against Streptomyces scabies (strain 44B) was assessed using the disk diffusion assay (a) Bacillus velezensis (S1); (b) Pseudomonas chlororaphis (S2); and, (c) Bacillus amyloliquefaciens (S3).

One hundred µL of *Streptomyces scabies* (44B) culture was spread on TSA agar plates, and 10 µL of KB media (on the left) and 10 µL of each three anti-*Streptomyces scabies* strains were placed on each desk (on the right). The plates were incubated at 28 °C for 3 days.

The zones of inhibition observed on the plates demonstrate the antagonistic activity of strains S1, S2, and S3 against *S. scabies*. To ensure the reliability of the results, the tests were repeated three times; only representative plates are presented here.

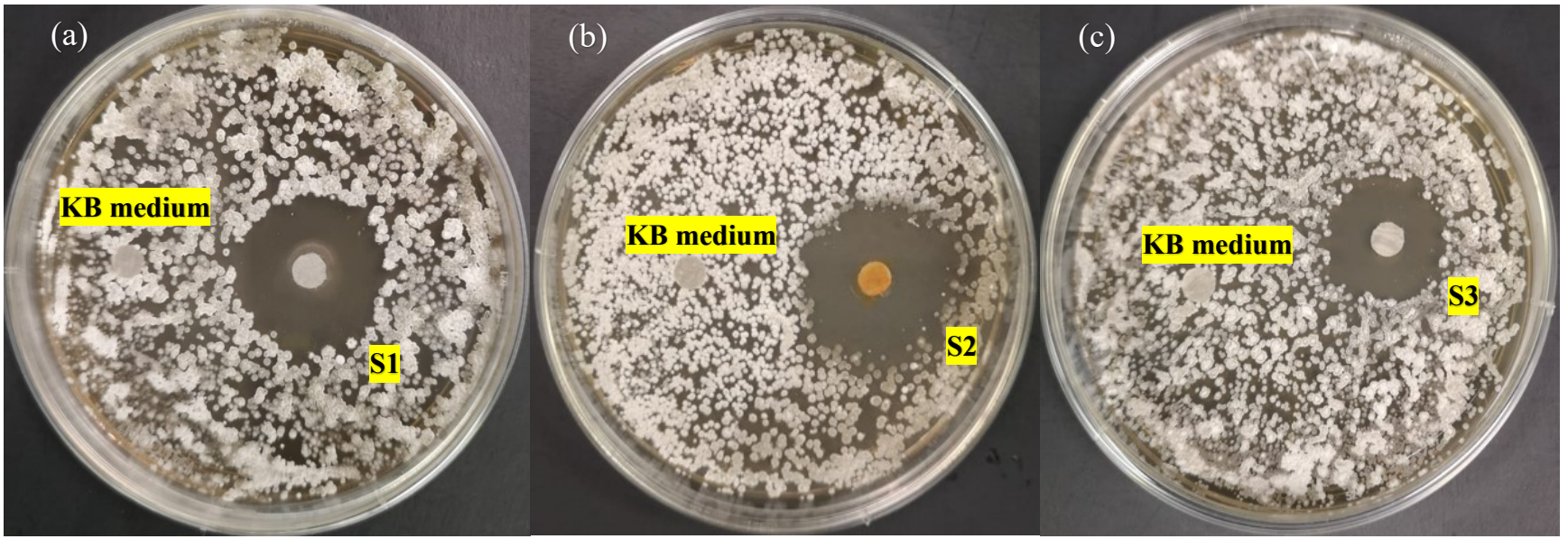


Fig. 4. 2 The antagonistic activity of the three most effective isolates against Streptomyces scabies (strain EF-35) was evaluated using the disk diffusion assay (a) Bacillus velezensis (S1); (b) Pseudomonas chlororaphis (S2); and, (c) Bacillus amyloliquefaciens (S3).

One hundred µL of *Streptomyces scabies* (EF-35) culture was spread on ISP-2 agar plates, and 10 µL of KB media (on the left) and 10 µL of each three anti-*Streptomyces scabies* strains were placed on each disk (on the right). The plates were incubated at 28 °C for 3 days.

The zones of inhibition observed on the plates demonstrate the antagonistic activity of strains S1, S2, and S3 against *S. scabies*. To ensure the reliability of the results, the tests were repeated three times, and only representative plates are presented here.

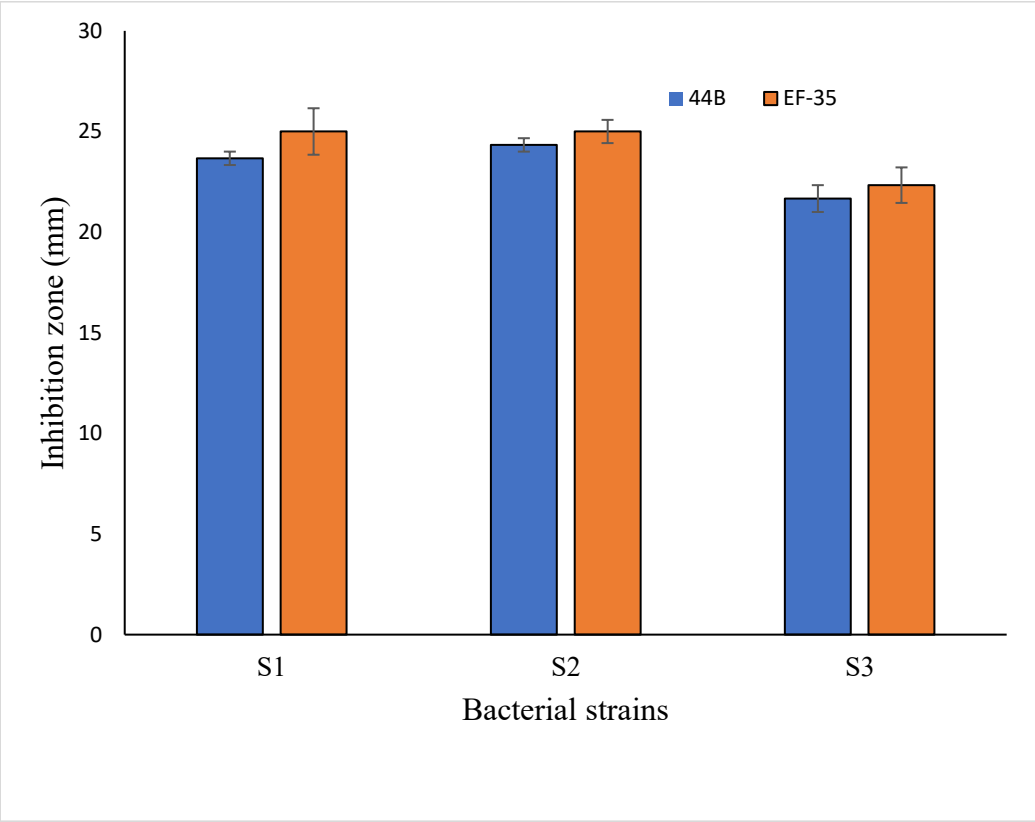


Fig. 4. 3 Bar chart showing the zone of inhibition (mm) of the selected strains (SI, S2, S3) against Streptomyces scabies (44B, EF-35)

4.5 Mechanisms of action utilized by selected bacterial isolates possessing antimicrobial Properties

4.5.1 Anti-streptomyces scabies isolates produced siderophores

Some bacteria have a competitive advantage over their rivals may have been due to the ability to sequester iron. Upon testing strains S1, S2, and S3 on the CAS agar medium, it was observed that all three of them exhibited a clearly distinguishable orange halo around their colonies. This halo serves as an indicator of hydroxamate type siderophore production, as illustrated in Fig 4.4.

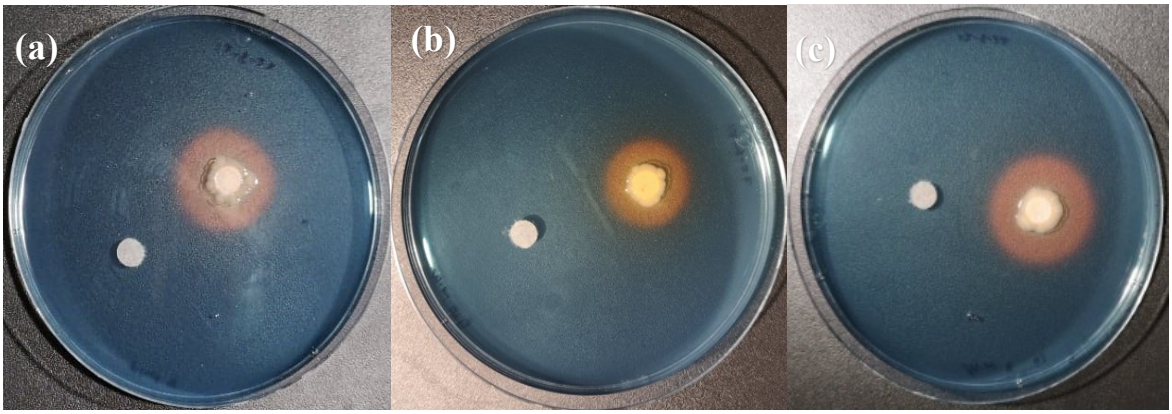
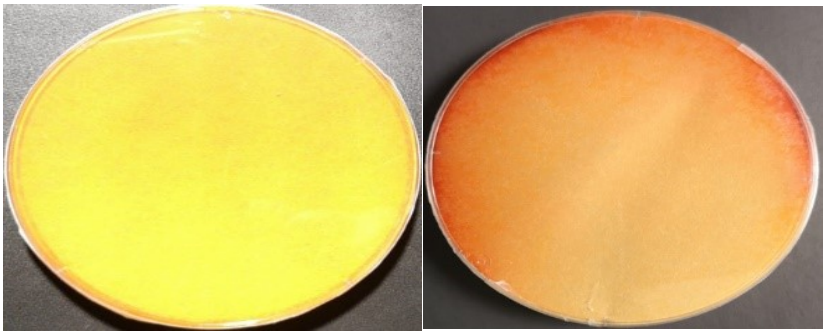


Fig. 4. 4 Siderophore production assay of plates a , b, and c tested for strains S1, S2, and S3 on a chrome azurol S (CAS) agar plate.

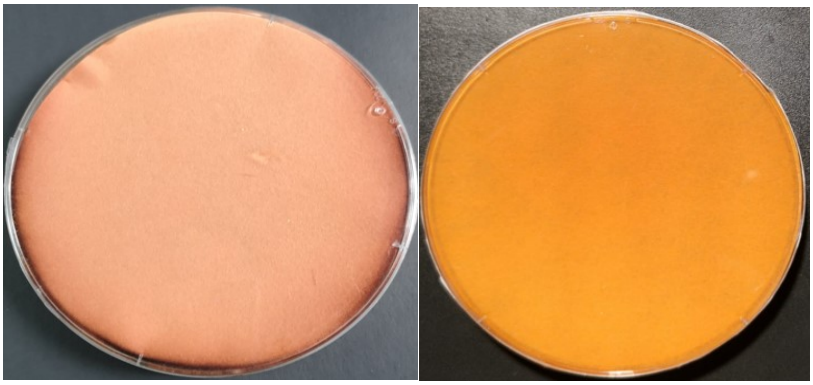
On a CAS plate, 10 µL of KB media (on the left), and the three bacterial strains culture (on the right) were placed on each disk paper (6 mm diameter).

4.5.2 Anti-streptomyces scabies strains produced hydrogen cyanide (HCN)

The bacterial isolates' ability to produce hydrogen cyanide (HCN) was examined using a method based on Ahmad et al. (2008), with some minor adjustments made to the procedure. Each of the three bacterial strains showed positive results for hydrogen cyanide production, as evidenced by the color development from yellow to orange or reddish-brown (as shown in Fig. 4.5). The negative control plate, where only the media was applied, exhibited a bright yellow color. This bright yellow color observed in the negative control plate with KB media indicates no HCN production.



(a) KB media (control) (b) S1

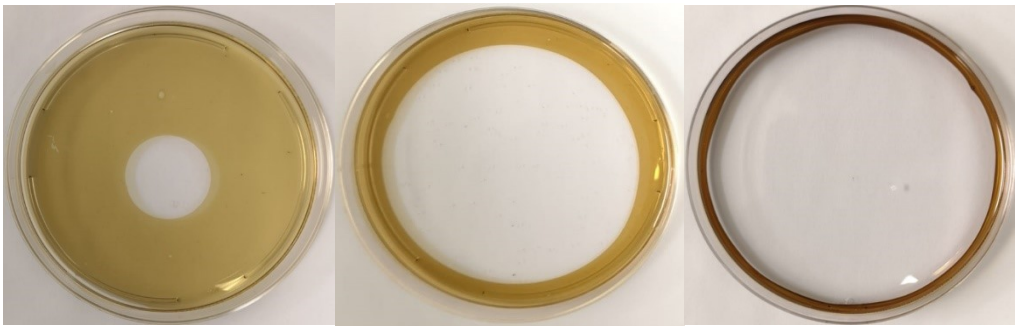


(c) S2 (d) S3

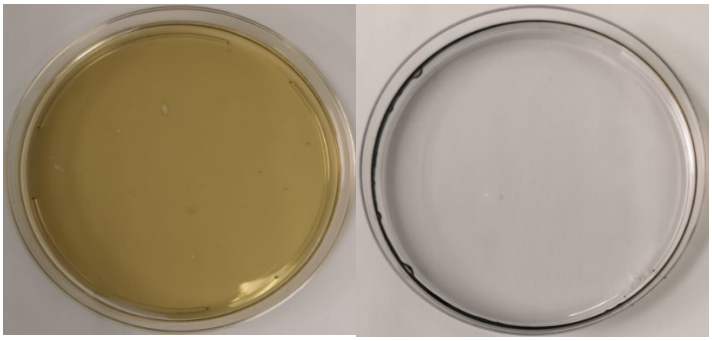
Fig. 4. 5 Hydrogen cyanide (HCN) production by selected bacterial strains (a) Kings B media (negative control), (b) Bacillus velezensis (S1); (c) Pseudomonas chlororaphis (S2); (d) Bacillus amyloliquefaciens (S3).

4.5.3 Biosurfactant production

Among the three strains tested, two strains, specifically S2 and S3, were identified as biosurfactant-producing bacteria based on the results of the oil-spreading test (Fig. 4.6). Strain S3 exhibited a particularly large clearing diameter in the oil-spreading test (Fig. 4.5c), indicating a strong biosurfactant production. Strain S2 also displayed good clearing diameters in the same test, suggesting significant biosurfactant production. On the other hand, Strain S1 showed the lowest production of biosurfactant, as evidenced by the smallest clearing zone, indicating that this strain did not produce significant amounts of biosurfactant under the test conditions.



(a) S1 (b) S2 (c) S3



(d) Negative Control (petri plate with only crude oil) (e) Positive Control (Triton X-100)

Fig. 4. 6 Bacterial biosurfactant production assessed using the oil-spreading test method on a solid hydrophobic surface to evaluate the extent of oil clearing or spreading.

4.6 Growth curves

The growth rates of the three chosen bacterial strains were established by developing growth curves (Fig. 4.7).

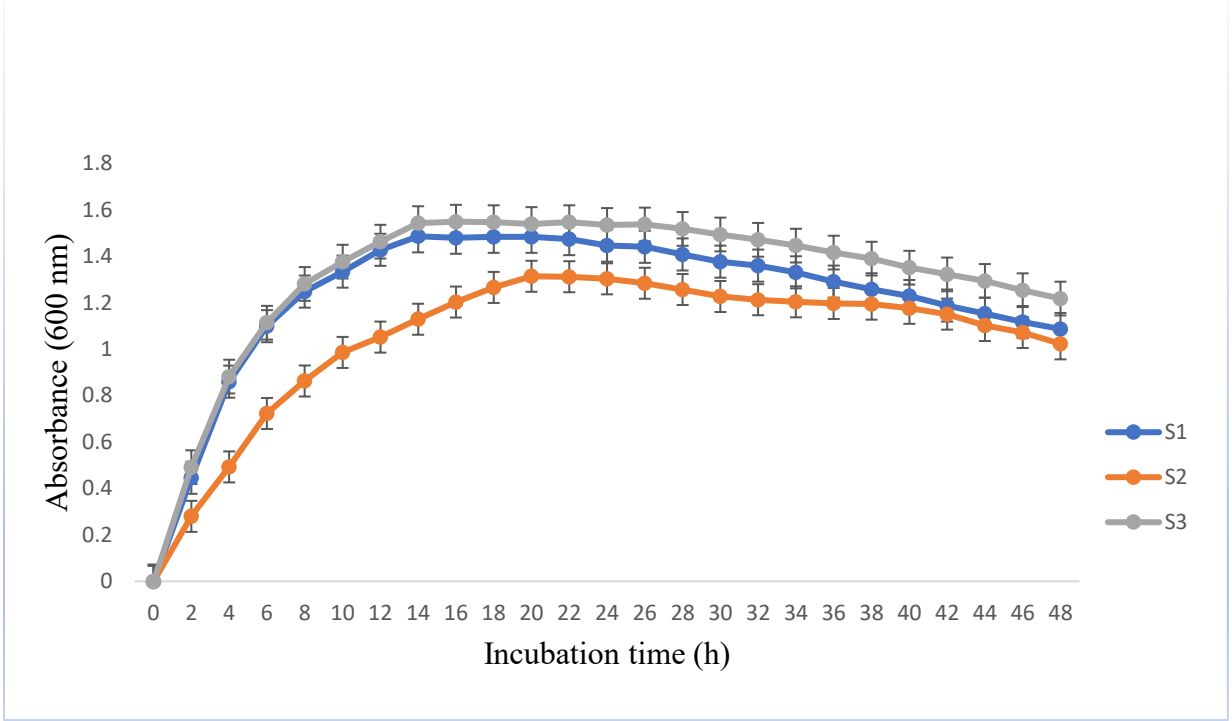


Fig. 4. 7 Growth of the selected three strains cultivated in King's B broth at 28 °C.

Bacillus velezensis (S1), *Pseudomonas chlororaphis* (S2), and *Bacillus amyloliquefaciens* (S3) were grown in KB and the optical density (OD) at 600 nm was measured every 2 h for 48 h.

4.7 Swimming motility of strains

The swimming motility of the selected bacterial isolates was assessed quantitatively after 24 and 48 h of incubation, as shown in Table 4 and Fig. 4.8. After 24 h of incubation, strain S1 showed a remarkable increase in swimming motility compared to strains S2 and S3. However, after 48 h, S1's motility declined, while strains S2 and S3 displayed a noticeable rise in their swimming diameters.

Table 4 Motility characteristics of the bacterial strains in their pure forms (Strains: S1, S2, S3).

	Diameter of swimming (mm)	
Strain	24 h	48 h
S1	30	40
S2	20	70
S3	17	62

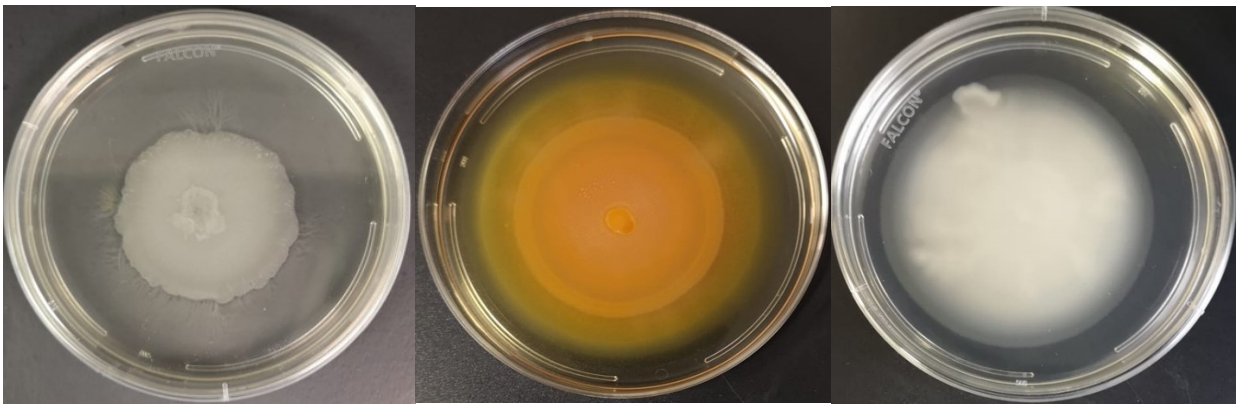


Fig. 4. 8 Swimming motility of individual bacterial strains (from left to right: strains SI, S2, S3).

4.8 Compatibility/antagonistic test of the selected bacterial strains

To test the compatibility and interactions among the selected bacterial strains cross-streak (Fig. 4.9) and co-culture (Fig. 4.10) methods were performed. The results obtained from the cross-streak method showed that strains S2 and S3 both inhibited S1, as indicated by clear zones observed around the intersection where colonies meet. In contrast, no inhibition zones were observed between S2 and S3 when they were streaked close to each other. This lack of clearing zones suggests that S2 and S3 are compatible with each other. The co-culture method (Fig. 4.10) provided similar results, confirming the antagonistic interactions between S2 and S3 and S2 and S1, and the compatibility between S2 and S3.

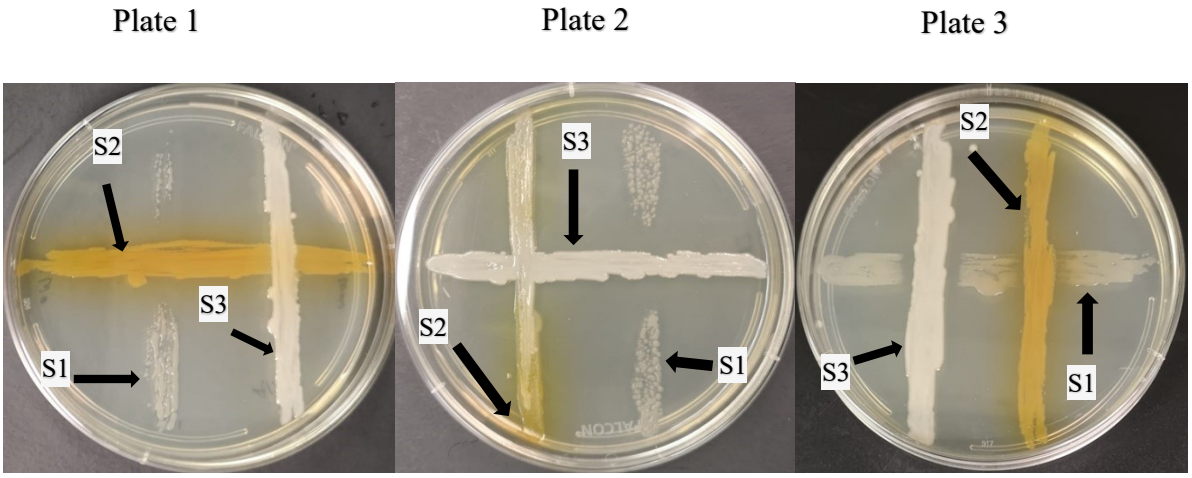


Fig. 4. 9 Antagonistic or compatibility evaluation of selected bacterial strains against each other using the cross-streak method

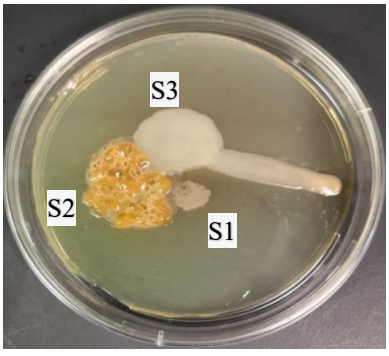


Fig. 4. 10 Co-culture assay

4.9 Anti-*Streptomyces scabies* strains (S1, S2, S3) exhibited a high level of resistance against most common antibiotics

An investigation was carried out to determine the susceptibility or resistance of three selected bacterial strains against a set of antibiotics. The six antibiotics were utilized for this purpose: gentamycin, streptomycin, kanamycin, ampicillin, chloramphenicol, and tetracycline. The results of the study showed that all the three bacterial strains were resistance to the tested antibiotics. Specifically, strains S2 and S3 exhibited a high level of resistance to all six antibiotics, as shown in Figures 4.11b and 4.11c. In contrast, strain S1 showed resistance only to streptomycin, ampicillin, chloramphenicol, and tetracycline, while being susceptible to gentamycin and kanamycin, as indicated in figure 4.11a. These findings suggest that these bacterial strains can effectively compete with other bacteria in the microbiome, even in the presence of these antimicrobial agents.

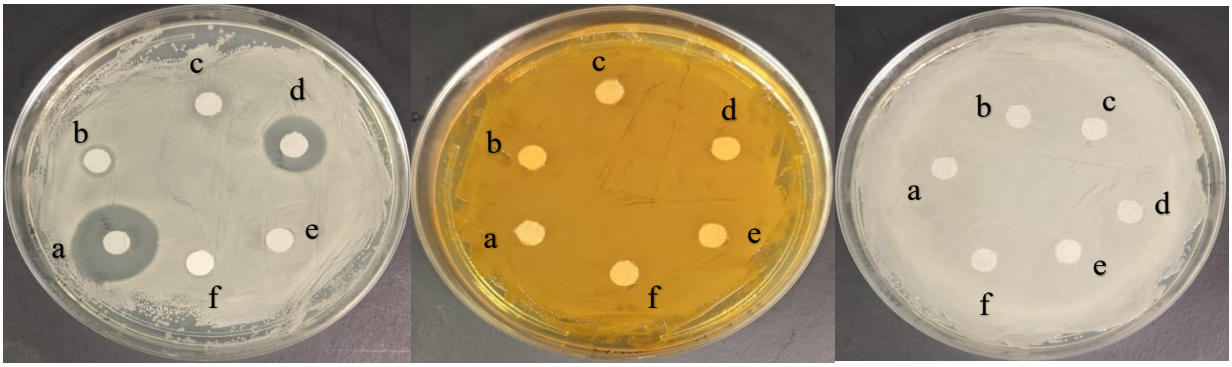


Fig. 4.11a (S1) Fig. 4.11b (S2) Fig. 4.11c (S3)

Fig. 4. 11 Effects of antibiotics on the growth of bacterial strains (S1, S2, S3). gentamycin, (b) streptomycin, (c) ampicillin, (d) kanamycin, (e) chloramphenicol, (f) tetracycline.

Chapter 5: Discussion

Plant roots have been shown to harbor valuable beneficial microorganisms, including effective biocontrol agents, as supported by current scientific literature (Saeed et al., 2021; Murthy et al., 2021). Of particular interest within the plant-associated microbiota are the endophytic bacteria, which hold significant promise for the development of innovative biotechnological tools. These tools can control plant diseases and enhance plant growth more efficiently. While the understanding of the capacity of numerous bacterial strains has advanced significantly (Vessey, 2003; Etesami et al., 2023; Nandi et al., 2018), the extent of bacterial diversity within undomesticated Quebecois field plants has remained largely undetermined. Research by Di Fan brought the potential, at least, to the forefront, unveiling insights into this previously unexplored area (Fan et al., 2018). This study provided significant insights into the rhizobacterial communities in the region. However, our current understanding of the uncultivated plant-associated microbiota remains limited, underscoring the need for further research to fully comprehend their implications and potential benefit. Numerous studies have explored the diversity of bacteria present in plant roots, providing insights into the correlation between bacterial communities linked to different plant types and the strategies used for agricultural crop management (Xia et al., 2015). In the present study, we applied a culture-dependent technique to isolate bacteria residing within the roots of a number of undomesticated plant species. The plant samples were collected from relatively undisturbed field environments, with most of them being perennial plants. While discovering and characterizing unculturable strains are, indeed, fascinating, their practical application in roles such as plant growth-promoting technologies is currently limited due to the challenges associated with their inability to be cultured in laboratory settings (Fan et al., 2018). To isolate various types of endophytic bacteria, we utilized three different types of media: NA (Nutrient Agar) medium, TSA

(Tryptic Soy Agar) medium, and KB medium (King's B medium). NA medium provides a general nutrient-rich environment, TSA medium supports the growth of diverse microorganisms, and KB medium specifically enriches *Pseudomonas* and some other bacteria. These carefully selected culture media facilitated the successful isolation and identification of diverse endophytic strains.

The study of endophytic bacteria taken from the roots of five undomesticated plant species resulted in isolation of total of 75 strains, signifying the abundant habitat for interactions between plants and microbes. The outcome aligns with the findings of Santoyo et al. (2016), underscoring the diverse community of microorganisms residing within plant roots, known as endophytes, and their potential significance in fostering beneficial interactions with the host plants.

These 75 bacterial strains, originally isolated from the roots of five undomesticated plant types, underwent a primary *in vitro* screening assay to assess their potential effects on plant growth promotion traits such as, nitrogen fixation, phosphorus solubilization and indole-acetic acid production. Among them, ten strains exhibited excellent growth promotion traits in the initial screening phase. Consequently, these promising bacterial isolates were chosen for more comprehensive and thorough investigations. Following the selection of the ten strains, their 16S rRNA gene sequences were determined, revealing that 90% of them exhibited the highest similarity in sequence with organisms from the *Pseudomonas* and *Bacillus* genera. Plant Growth-Promoting Rhizobacteria (PGPR) have been discovered in various ecosystems. Among these beneficial bacteria, *Pseudomonus* and *Bacillus* are the most dominant genera, standing out as highly promising taxonomic groups due to their aggressive root colonization abilities (Weller, 2007) and wide range of Plant Growth-Promoting (PGP) traits.

The current study demonstrated that ten of the isolated bacterial strains exhibited numerous beneficial plant growth-promoting attributes. The first trait investigated was nitrogen fixation. Nitrogen fixation was detected in all ten of these more extensively investigated isolates, albeit at varying levels. Plant growth-promoting rhizobacteria capable of fixing N_2 can form non-obligatory interactions with host plants, which is especially important for non-leguminous plants (Glick and Patten, 1999). Using Plant Growth-Promoting Rhizobacteria (PGPR) either alone or in combination with nitrogen (N) fertilizer have been demonstrated to improve nitrogen uptake and decrease losses of N fertilizer in agricultural crop systems (Arif et al., 2017). Consequently, plant growth-promoting bacteria that possess N_2-fixing capabilities are highly desirable. While the symbiotic relationship between legumes and α-proteobacteria accounts for roughly 80% of biological nitrogen fixation (Garg and Geetanjali, 2009). There are other nitrogen-fixing bacteria that are not plant species specific and can therefore be found in conjunction with a wider array of plant species, such as those with agricultural importance. Various strains of *Bacillus* (Ding et al., 2005; Miljaković, et al., 2020), *Paenibacillus* (Puri et al., 2015), and *Pseudomonas* (Mirza et al., 2006) strains have been recognized for their ability to fix nitrogen.

Phosphorus is a fundamental macronutrient for plants, playing a critical role in their growth and development. However, it is often scarce in soil and can be found in insoluble forms, making it much less available for plant uptake (Rawat et al., 2021; Wang et al., 2009). Using traditional fertilizers can be expensive and environmentally harmful, especially in the case of phosphate fertilizers. However, microorganisms with phosphate solubilizing ability offer a promising solution. These microorganisms can convert soil-bound phosphorus into a form that plants can readily use, reducing the need for chemical fertilizers (Dandessa and Bacha, 2018). In this research, ten isolates, primarily from *Pseudomonas* and *Bacillus* strains, demonstrated the ability to

solubilize phosphate in an initial screening conducted in a laboratory setting (*in vitro* study). Although these findings are promising, additional investigations, *in planta* studies, are necessary to evaluate the practical effectiveness of these isolates as biofertilizers in real agricultural conditions. The results obtained from the *in vitro* study imply that the isolates with phosphate solubilizing activity could serve as an environmentally safe and economically viable option to enhance crop production in soils with low phosphorus levels. This suggests that using these biofertilizers as production agricultural inputs could be a practical and sustainable approach to address phosphorus deficiency in soil and ultimately improve crop yields.

According to the report, approximately 80% of microorganisms isolated from the rhizosphere of various crops have the capacity to produce and release auxins, specifically indole-3-acetic acid (IAA), as secondary metabolites (Patten and Glick, 1996, Ratnaningsih et al., 2023). In this experiment, all the potential isolates studied in the initial screening assay (ten strains) produced IAA *in vitro*, and the quantity of IAA was influenced by the concentration of its precursor, tryptophan. This aligns with previous research, which found that the addition of tryptophan to culture media enhances IAA production by most rhizobacteria (Barazani and Friedman, 2000; Idris et al., 2007; Spaepen and Vanderleyden, 2011). Bacteria-produced IAA has been linked to increasing root surface area and length, which, in turn, allows plants to access more soil nutrients, leading to potential enhancements in plant development and growth (Bhattacharyya and Jha, 2012). Many rhizobacteria can convert the root exudate tryptophan to IAA, making it a highly effective substance that promotes plant growth (Mohite et al., 2013). The study's *in vitro* assay revealed variations in IAA production among the isolates, with *Pseudomonas* isolates demonstrating the highest IAA production at both 200 and 500 μg mL^{-1} concentrations of tryptophan. The practical implications of these findings could be significant, but further field

experiments are needed. Such findings might have practical applications, but these should be based on further field experiments, as it is unreasonable to conclude definitively that the greater IAA producer would benefit the plants more. Studies have revealed that plant growth-promoting rhizobacteria (PGPR) are capable of producing indole-3-acetic acid (IAA). This compound has the potential to boost root growth in situations where plant IAA levels are inadequate, but it could also hinder root development if plant IAA levels are already optimum (Spaepen et al., 2007). Furthermore, there is variation in how different plants respond to distinct auxin levels, encompassing diverse plant species, cultivars, and plants at different developmental stages (Cheng et al., 2013). As a result, field experiments focusing on specific plant types and growth conditions are necessary before drawing definitive conclusions and identifying suitable applications.

Many bacterial genera, including *Rhizobium*, *Azotobacter*, *Pseudomonas*, and *Bacillus*, have shown significant potential as biocontrol agents for controlling plant diseases (Ahemad and Kibret, 2014). One such disease, common scab, is a significant potato disease worldwide (Sarwar et al., 2018; Kopecky et al., 2021; Lin et al., 2018; Kang et al., 2022; Biessy and Filion, 2022). Using PGPR for the purpose of biological control and promoting plant growth holds promise as a sustainable strategy for ensuring continuous agricultural production in the long term. This research evaluated the capabilities of endophytic bacteria derived from undomesticated plants (dandelion, heal-all, milkweed, wild lettuce, colts foot) in pathogen control and growth promotion. Based on initial *in vitro* growth promotion testing, the top ten isolates were further assessed against *Streptomyces scabies*, the bacterium responsible for potato common scab disease. Disk diffusion assay results revealed that three isolates (designated as S1, S2, and S3), from dandelion, milkweed, and wild lettuce, belonging to the genera *Bacillus* and *Pseudomonas*, and tentatively to the species *Bacillus velezensis, Pseudomonas chlororaphi*s, and *Bacillus amyloliquefaciens,* respectively,

demonstrated strong antagonistic activity against the potato scab causative organism *in vitro*. This study is the first, to our knowledge, to report on the antibacterial properties of endophytic bacteria from wild plants against economically important crop diseases in Quebec. These findings underscore the potential of utilizing rhizobacteria from wild plants in the fight against crop diseases.

In the present study, we selected three strains (designated as S1, S2, and S3) for their ability to inhibit potato scab by producing siderophores. The utilization of siderophores produced by *Pseudomonas* and *Bacillus* species has been widely studied as effective biological agents for disease control (Swarnalatha et al., 2022; de Villegas et al., 2002; Prema and Selvarani, 2013). These siderophores play a crucial role in sequestering ferric iron, particularly when it is scarce in the environment. By capturing Fe^{3+} ions in the rhizosphere; they effectively limit the availability of iron to phytopathogens, thereby safeguarding the health of plants (Shen et al., 2013). Numerous plant growth-promoting rhizobacteria (PGPR) have been identified for their capacity to enhance plant development. This is achieved through the production of potent extracellular siderophores, which serve as a means of controlling various plant diseases. This strategy involves starving pathogens of essential iron nutrients, ultimately leading to increased agricultural yields (Radzki et al., 2013; Sharma and Johri, 2003). The ability of a substantial number of PGPR to generate ferric chelators, such as siderophores, results in competitive interactions within the rhizosphere, both with other rhizobacteria and potential pathogens. This dynamic competition contributes to the superior adaptability of PGPR, enabling them to efficiently colonize root systems and enhance overall plant growth. Notably, the synthesis of siderophores serves as a natural biocontrol mechanism, effectively suppressing the growth of harmful phytopathogenic fungi and bacteria (Prema and Selvarani, 2013; Shanmugaiah et al., 2015).

Microorganisms that produce hydrogen cyanide (HCN) possess a valuable functional trait that contributes to their success in various environments, as highlighted by Loaces et al. (2011). HCN possesses inherent antimicrobial characteristics that impede the proliferation and maturation of numerous plant pathogens. It achieves this by interfering with the function of cytochrome oxidase, a pivotal enzyme crucial for the transportation of electrons within cellular respiration. Consequently, this interference disrupts the pathogen's capacity to generate energy through the respiratory chain, ultimately this disrupts the energy production of the pathogen and can lead to its death (Abdelaziz et al., 2023; Brimecombe et al., 2001). When microorganisms produce substantial quantities of hydrogen cyanide (HCN) as a secondary metabolite, it becomes a potent compound capable of both inhibiting plant diseases and, therefore, indirectly enhancing plant growth. In our investigation, *Bacillus* and *Pseudomonas,* which constitute the predominant and most abundant taxa, demonstrated the capability to produce HCN. Our research results highlight that all three strains demonstrated the ability to produce HCN. Due to being regarded as excellent HCN producers, these genera may have an advantage over other microbes competing for the same ecological niche space when it comes to colonizing plant tissues (Ahmad et al., 2008; Anwar et al., 2016). Research has revealed that the *Pseudomonas* spp. strain LBUM330, capable of producing HCN, exhibited the ability to hinder bacterial pathogens (Lanteigne et al., 2012). The production of HCN could potentially contribute to the partial understanding of the antagonistic behavior observed in our study, where strains S1, S2, and S3 displayed activity against *Streptomyces scabies.*

Soil microbes, plant endophytes, yeast, and even oil well microorganisms release biosurfactants, substances with antimicrobial properties. These biosurfactants have the potential to serve as effective biological agents for controlling plant diseases caused by pathogens (Serrano et

al., 2021; Rani et al., 2020). Two of the three strains studied, *Pseudomonas chlororaphis* (designated as S2) and *Bacillus amyloliquefaciens* (designated as S3), were able to produce a biosurfactant that inhibited the growth of *Streptomyces scabies*. As far as our current understanding goes, most investigations into the phytomicrobiome of a plant's root endosphere have primarily concentrated on evaluating culturable isolates to detect the presence of hydrogen cyanide (HCN) and siderophore production, often overlooking the assessment of biosurfactant production. In contrast, this study adopted a distinct approach in this regard, and meticulously examined the potential for biosurfactant production in three bacterial isolates using a widely recognized method (Walter et al., 2010). This assessment demonstrated the presence of biosurfactant production within these isolates. Notably, the two primary biosurfactant-producing isolates were also strong siderophore producers and displayed significant anti *Streptomyces scabies* activity. These results suggest that these strains produce a combination of surface-active compounds, including lipopeptides, and could potentially be harnessed as effective agents for controlling important plant pathogens.

The ability of endophytic bacteria to move within the plant's tissues, whether through swimming or other means, indeed plays a crucial role in their overall effectiveness as beneficial partners (Ansari & Ahmad, 2017). In our study, we conducted observations of motility in the chosen bacterial strains (S1, S2, and S3) using a petri plate *in vitro*. Interestingly, all three strains exhibited clear swimming mobility. Additionally, they showed a strong ability to produce a substantial quantity of siderophores, suggesting that the presence of flagella and the effective production of siderophores could play important roles in colonizing and possibly triggering responses such as Induced Systemic Resistance (ISR) by these strains. The observation of motility on Petri plates serves as an initial indicator of the bacterial strains' inherent ability to move. This

characteristic holds significant importance in unraveling the potential dynamics of their interactions with plants as endophytes. Such motility not only underlines the bacteria's capability to navigate their environment but also hints at their potential to colonize and engage with plant tissues, laying the foundation for further exploration into their role as beneficial partners for plant growth and health.

For multi-species microbial consortiums to function optimally, strain compatibility in the planktonic phase is crucial (Santiago et al. 2017; Bradáová et al. 2019). Our investigation has revealed that, in the context of *in vitro* analysis, two of the three strains (S1 and S2) exhibit a distinct compatibility with each other. This suggests that these two strains can coexist in a balanced manner, promoting potential collaboration without hindering each other's growth. Similar findings have been seen in research exploring interactions among diverse species (Jha and Saraf, 2012; Ren et al., 2015). Importantly, within the set of three selected strains, S2 and S3 demonstrated a particularly evident level of compatibility. This became apparent through techniques such as cross-streaking and co-culturing, where their interactions closely paralleled those observed in multi-species consortia. This emphasizes the potential of these strains as contributors to efficient cooperative systems, as their demonstrated compatibility suggests the possibility of synergistic interactions within a complex microbial community.

All three strains (S1, S2, and S3) exhibited strong resistance to the antibiotics used in this study, with the exception that for strain S1 that showed susceptibility to the gentamycin and kanamycin. It has been reported that many plant growth promotion bacteria are naturally resistant to antibiotics (Gilbert et al., 1993). There are ecological benefits to having plant growth-promoting rhizobacteria (PGPR) in the rhizosphere that are resistant to several antibiotics (Döbereiner and Baldani, 1979; De Brito et al., 1995). As a means of protecting themselves against environmental

threats, PGPR have developed mechanisms to resist antibiotics. Antibiotic resistance is just one aspect of the ecological benefits of PGPR in the rhizosphere. It is important to monitor and control the spread of antibiotic resistant PGPR so that their beneficial effects on plant health and ecosystem sustainability can be preserved while hazards are kept to a minimum. Indeed, numerous antibiotics, such as penicillin, originated from soil microbes and fungi (Alexander, 1977; Yasmin et al., 2009), or are generated by soil microbes, like chloramphenicol, kanamycin, streptomycin, and tetracycline. These discoveries imply that the selected three strains, possessing resistance to various antibiotics, could exhibit strong competitiveness upon introduction to a new environment through inoculation.

Chapter 6: General Conclusions and Future directions

6.1 Conclusions

This research project has provided valuable insights into the potential of newly discovered bacterial strains that live inside plants (endophytic) to address the challenges posed by common scab disease in potato and promote plant growth. After conducting a series of experiments and screenings, we have identified and characterized three bacterial strains: *Bacillus velezensis* (S1), *Pseudomonas chlororaphis* (S2), and *Bacillus amyloliquefaciens* (S3), where the species designations are tentative. These strains have shown promising abilities to control *Streptomyces scabies* and also exhibited characteristics that can enhance the growth of plants.

The initial screening tests helped us identify strains that potentially can produce plant growth hormones, solubilize inorganic phosphate, and fix nitrogen. This suggests their potential as biofertilizers. The taxonomic identification of these strains confirmed their classification, at least to the genus level and tentatively to the species level, providing a foundation for further investigations. Subsequent laboratory screenings demonstrated that these selected strains can mitigate the effects of *Streptomyces scabies*, the causal agents of common scab disease, in potatoes.

The subsequent *in vitro* screenings revealed the antagonistic effects of these selected strains against the pathogenic *Streptomyces scabies*, the causative agent of common scab disease. The mechanisms through which these strains suppress the pathogen, involving hydrogen cyanide production, siderophore synthesis, and biosurfactant generation, have been elucidated. These findings enhance our understanding of their biocontrol potential and pave the way for targeted applications in agricultural practices.

Moreover, the mobility and compatibility of the selected bacterial strains were explored, demonstrating their rapid growth, antibiotic resistance, and ability to move efficiently on Petri plates. The potential for coexistence of strains S2 and S3 in multispecies consortia was observed, suggesting potential synergistic effects that could be harnessed for more effective disease control and plant growth promotion.

The significance of this research lies in its contribution to the development of sustainable agricultural practices. The potential utilization of these endophytic bacterial strains offers an eco-friendly approach to managing common scab disease in potatoes while also enhancing plant growth. By understanding the intricate interactions between these beneficial bacteria and the pathogens, as well as their mobility and compatibility traits, we have laid the groundwork for future studies and applications in real-world agricultural settings.

In essence, this study underscores the substantial potential of the isolated endophytic bacteria, specifically strains S1, S2, and S3, as viable alternatives for mitigating common scab disease and promoting overall plant health. The comprehensive insights gained from this research pave the way for further investigations, field trials, and the development of practical applications that can contribute to more sustainable and productive agriculture.

6.2 Future directions

Moving forward, there are several promising avenues for advancing the insights gained from this research. These future directions encompass diverse aspects, ranging from practical application to deeper mechanistic understanding. The selected endophytic bacterial strains, particularly *Bacillus velezensis* (S1), *Pseudomonas chlororaphis* (S2), and *Bacillus amyloliquefaciens* (S3), hold significant potential for transforming agricultural practices. By exploring these suggested pathways, researchers can contribute to the realization of more

sustainable and effective solutions for combating common scab disease and promoting plant growth.

Here are some suggested future research directions to consider:

1. Field Trials and Validation: It would be beneficial to conduct field trials in actual environmental conditions to validate the effectiveness of the chosen strains.

2. Understanding Mechanisms: Thoroughly probing the mechanisms, especially their antibiotic production, is crucial to grasp how the selected strains effectively counter common scab disease.

3. Exploring Synergistic Formulations: Explore combinations of the selected strains with other biocontrol agents or practices for enhanced efficacy.

4. Studying Microbiome Interactions: A holistic understanding can be achieved by studying the interactions between these selected strains and the plant microbiome.

5. Developing Commercial Products: It's worth considering the feasibility of developing products based on these selected strains.

6. Integrated Pest Management (IPM); Integrating these strains into IPM strategies can lead to pest and disease control.

7. Assessing Environmental Impact: It is crucial to evaluate any effects of these strains on target organisms and ecosystems.

8. Crop Specific Applications: Considering the applicability of these strains in crops is vital for broader impact.

9. Enhancing Traits through Gene Editing: Utilizing gene editing techniques can help enhance crop-beneficial traits within these strains.

10. Long Term Persistence Studies: Studying how these strains persist and remain stable in soil over growing seasons is essential for their long-term effectiveness.

11. Community Engagement and Education: Developing outreach programs will allow us to share research findings and promote practices effectively.

12. Global Adaptation: Investigate the adaptability of strains to diverse geographic regions and climates.

These directions collectively contribute to a reasonably comprehensive roadmap for further research and practical implementation of the isolated bacterial strains in agriculture.

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