Characterization of timothy (*Phleum pratense* L.) -associated bacteria, their functional traits and positive interaction with the model grass *Brachypodium distachyon*

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LIST OF ABBREVIATIONS

ACC	1-aminocyclopropane-1-carboxylate
AHL	Acylated homoserine lactone
Al	Aluminum
ANOVA	Analysis of variance
BLAST	Basic local alignment search tool
bp	base pair
CAS	Chrome azurol S
CN	Colloidal chitin
CFU	Colony forming units
cs	Citrate synthase mutant
CS	Citrate synthase gene
CTAB	Cetyltrimethylammonium bromide
DAPG	2,4-diacetylphloroglucinol
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acids
DSF	*cis*-11-methyl- 2-dodecanoic acid
dpi	days post-inoculation
ELONG	Elongation
EDTA	Ethylenediaminetetraacetic acid
EPS	Exopolysaccharides
GA	Gibberellic acid

GC-MS	Gas chromatography- Mass spectrometry
GEN	Generative
GSP	Gene specific primers
GYEN	Glucose yeast extract
h	Hour
HCl	Hydrogen Chloride
HCN	Hydrogen Cyanide
IAA	Indole 3-acetic acid
idh	Isocitrate dehydrogenase mutant
IDH	Isocitrate dehydrogenase gene
ISR	Induced systemic resistance
kV	kilo volts
KMBA	2-keto-4-methylthiobutyric acid
LB broth	Lysogeny broth
LBA	Lysogeny broth Agar
MATH	Microbial adhesion to hydrocarbons
mdh	Malate dehydrogenase mutant
MDH	Malate dehydrogenase gene
MES	2-[N-morpholino]ethanesulfonic acid]
mM	Milli molar
MOX	Methoxyamine
Mbp	Million of base pairs
MPa	Megapascal

MSgg medium	Minimal medium
MTBSTFA	N-(tert-Butyldimethylsilyl)-N-methyltrifluoroacetamide
MS	Murashige and Skoog
N	Nitrogen
N.A.	Not Available
NADP+	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NCBI	National center for biotechnology information
NOD (nod)	Nodulation
nt	Nucleotide
OA	Organic acid
OPLS	Orthogonal partial least square analysis
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PGP	Plant growth-promoting
PGPB	Plant growth promoting bacteria
PIPES	Piperazine-1,4-bis (2-ethanesulfonic acid)
QS	Quorum sensing
RCR	Relative chemotactic ratio
RFI	Relative fold increase
rRNA	Ribosomal ribonucleic acid
RNA	Ribonucleic acid
ROS	Reactive oxygen species

SCER1	Putative ethylene receptor
SCERF1	Putative ethylene responsive factors
SCERF2	Putative ethylene responsive factors
sdh	Succinate dehydrogenase mutant
SDH	Succinate dehydrogenase gene
SEM	Scanning electron microscopy
Tris-HCl	Tris hydrochloride
TSA	Tryptic soy agar
USA	United States of America
VEG	Vegetative
VOC	Volatile organic compounds
α-ketobutyrate	Alfa-ketobutyrate

ABSTRACT

Plants grown in soils live in a close habitat with endophytic bacteria. These non-pathogenic microbes can increase the plant's growth, contribute to the supply of required nutrients for its development and enhance its capacity to resist harsh environmental conditions. In this study, several bacterial endophytes isolated from two cultivars of timothy (*Phleum pratense* L.), were studied for their functional attributes relating to growth promotion properties, antimicrobial and biosurfactant capacities. Bacterial endophytes were isolated from timothy grown under field conditions located at Macdonald seed farm of McGill University. Some bacterial isolates were genotype -and source- specific. The majority showed capacity to promote growth directly by the production of indole acetic acid, ACC deaminase, volatiles and siderophores, along with an array of enzymes capable of making nutrients more available, thus rendering them successful candidates for growth and plant health promotion. Others had a positive effect on plant health indirectly by the production of antimicrobials, hydrogen cyanide and biosurfactants, and through their ability to tolerate abiotic (salt and drought) and biotic stress conditions, along with the synthesis of enzymes capable of degrading fungal cell walls. Inoculation of timothy with the top performing bacteria caused a phenotypic effect as illustrated by abundant root and root hair growth promotion and were successful in intracellular colonization of timothy tissues which indicates that the host allowed successful inter-communication between the plant and its surrounding competent isolates.

Root colonization by rhizospheric bacteria wouldn't be possible without certain bacterial traits. Adaptation of the bacteria to their surrounding environment and their chemotactic capacity towards the roots to form biofilm is necessary to create a successful interaction with its host. The multispecies consortium consisting of three top-performing strains belonging to *Bacillus* and *Microbacterium* genera coexisted together and had the ability to form an extensive biofilm on

biotic and abiotic surfaces. In response to organic acids released from roots of *Brachypodium distachyon,* the multispecies consortium formed biofilm, and displayed strong chemotactic behaviour towards selective organic acids. Recognizing that the transition from single strains of bacteria to a "multicellular" system wouldn't happen without the presence of adhesion, alginate and exopolysaccharides (EPS) contents, these compounds were evaluated. The EPS amounts were comparable in single strains and consortium forms. Alginate production increased 160% in consortium subjected to drought stress (10% PEG). These findings demonstrated that bacteria-bacteria interaction is the hub of various factors that would not only affect their relation, but further could indirectly affect the balanced plant-microbe relation.

The modulation of root exudates plays a significant role in the chemotaxis of rhizospheric bacteria and biofilm formation. This study focused on the relative changes in the metabolite profile and gene transcription of organic acids released from roots and in roots of inoculated *Brachypodium distachyon* or not with a multispecies consortium. In roots, the relative amounts of malic and citric acids were significantly more abundant compared to other organic acids. Higher relative concentrations of succinic and fumaric acid were present in inoculated *B. distachyon*. In response to the consortium, the relative transcript abundance of the genes encoding malate dehydrogenase (*MDH*), succinate dehydrogenase (*SDH*), citrate synthase (*CS*) and isocitrate dehydrogenase (*IDH*) were increased but were not significantly different compared to non-inoculated plants. Taken together, this study provides the first insight into the endophytes of timothy as well as the first understanding of how they could be employed to promote the growth of other grasses.

RÉSUMÉ

Les plantes poussant aux champs ou en serres partagent leur habitat avec les endophytes bactériens. Ces bactéries non-pathogéniques sont connues pour leurs caractéristiques bénéficiaires aux plantes: elles favorisent leur croissance, contribuent à l'approvisionnement en nutriments nécessaires à leur développement, et améliorent leur chance de survie face aux conditions environnementales difficiles. Lors de ce projet, plusieurs endophytes bactériens ont été isolés de différents tissus de la fléole des prés (*Phleum pratense* L.), appartenant à deux cultivars de la fléole, présents dans différents sites de cultivation. Ces bactéries ont été étudiées pour savoir plus à propos de leur capacité de promouvoir la croissance directe et indirecte des plantes à travers leurs attributs fonctionnels, leurs relations comme stimulateur de croissance, leurs composés antimicrobiens, ainsi que leurs capacités de biosurfactant. La majorité de ces organismes produisaient l'ACC déaminase et l'hydrogène cyanide, les composés volatils et sidérophiles, ainsi qu'une gamme d'enzymes capables de rendre les nutriments plus disponibles et de dégrader les parois des cellules fongiques. Ces organismes ayant la capacité de tolérer les conditions de stress abiotiques (sel et sécheresse) et biotiques, ont été enregistrés comme provocateur de changement phénotypique au niveau des racines de la fléole des prés. Ils ont réussi à stimuler la croissance des racines et des poils de la plante, et à la colonisation intercellulaire des tissues, indiquant la réussite de la communication entre la plante et ses isolats compétents.

La colonisation des racines par les bactéries du sol n'aurait pas été possible sans certains traits des bactéries elles-mêmes. Le succès de l'interaction des bactéries avec leur hôte, est basé sur plusieurs critères tels que l'adaptation des bactéries à leur environnement et leur capacité chimiotactique avec les racines leur permettant de former un biofilm. Parmi tous les isolats bactériens de la fléole des prés, trois souches bactériennes appartenant aux genres *Bacillus* et

Microbacterium, ont été enregistrées comme les meilleures performantes. Celles-ci ont prouvé leur capacité de coexister ensemble et de former un biofilm sur les surfaces biotiques et abiotiques.

Il a été démontré que les acides organiques peuvent altérer le comportement chimiotactique des bactéries. Grâce à leur production par les racines du *Brachypodium distachyon,* le consortium composé de souches de plusieurs espèces a formé un biofilm. Cette transition du niveau planctonique au niveau multicellulaire, n'aurait pas réussi sans la présence d'un haut contenu d'adhérence, d'alginate et d'exopolysaccharides (EPS). Ces résultats ont démontré que l'interaction entre les bactéries est soumise à plusieurs facteurs qui la délimitent et qui indirectement affectent la relation équilibrée entre la plante et le microbe. En fait, lors de la soumission des bactéries à des conditions de sécheresse (10% PEG), la production d'alginate a augmenté de 160%.

Le *Brachypodium distachyon*, une plante-modèle pour les céréales et les graminées fourragères de climat tempéré, a été étudiée lorsque inoculée ou non avec le consortium. Les résultats ont démontré que les quantités relatives d'acides maliques et citriques étaient significativement abondantes par rapport aux autres acides organiques présents dans les racines et que les plantes inoculées avaient relativement des concentrations plus élevées d'acide succinique et fumarique par rapport aux plantes stérilisées. D'autre part, l'abondance relative des transcrits due à l'inoculation avec le consortium était différente, mais non significative dans les gènes codants pour le malate déhydrogénase (*MDH*), le succinate déhydrogénase (*SDH*), le citrate synthase (*CS*) et l'isocitrate déhydrogénase (*IDH*).

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PREFACE AND CONTRIBUTIONS

The following thesis was prepared according to the "Guidelines Concerning Thesis Preparation" of McGill University. The thesis contains three chapters (Chapters 3-5) representing three separate research manuscripts: Chapter 3 is published in the *Journal of Applied Microbiology* and Chapter 4 is published in the *Canadian Journal of Microbiology*. Detailed information pertaining to authors and their contributions to each chapter are mentioned in the "Connecting Text" section before each chapter. Below is a general description of the thesis topics as well as of the contributions of each author to the thesis.

Contributions of Authors

Dina Saleh was the primary researcher for each chapter. She designed and planned the experimental procedures performed the experiments and analyses under the guidance of Dr. Suha Jabaji, Plant Science Department, Macdonald Campus of McGill University. Dr. S. Jabaji provided clear supervision, guidance, technical assistance and funding for all chapters. Dr. K. Aliferis, Adjunct Professor, Plant Science, McGill University, and Assistant Professor at the University of Athens, Greece, provided expertise and insight on the analysis of the orthogonal partial least squares-discriminant data. Dr. Philippe Séguin provided partial funding for chapter 4 and assistance in field sampling of timothy grass and supplied us with timothy seeds. Mr. Joseph Jarry, a summer student funded by NSERC USRA program helped in screening bacteria for antifungal and volatile attributes. Ms. Mamta Rani provided assistance in partial sequencing of the strains and in molecular biology protocols as well as in performing certain biochemical tests. Miss Meha Sharma, PhD candidate under the supervision of Dr. Suha Jabaji, provided assistance

in the capillary chemotaxis assay performed and the T-DNA genotyping of mutant lines of *Brachypodium*.

Contributions to Knowledge

The chapters of this thesis describe original and novel findings on the bacterial endophytes of timothy (*Phleum pratense* L.), their traits leading to the formation of biofilm in multispecies consortium, and the effect of organic acids released from plant roots on the chemotactic response and biofilm formation of the bacterial consortium.

Chapter 3 represents the first evidence on the diversity, abundance and distribution of bacteria associated with different tissues of two cultivars of timothy grass (*Phleum pratense* L.) grown under field conditions, along with their biochemical and molecular characterization. Bacterial endophytes of timothy were subjected to an exhaustive screening and evaluation for their functional biochemical attributes with respect to growth promotion properties, antimicrobial, biosurfactant and emulsification activities. Data integration using partial least square analysis (OPLS-DA) was used to select the best performing bacteria based on their biochemical traits. This analysis provided insight on the isolated strains based on their potential use and application as plant growth promoters or as biological control agents. Also, this study reported for the first time that the differences between bacterial communities were cultivar specific. This finding could be useful in plant breeding strategies related to timothy. Selective breeding of plant genotypes with distinctive and specific bacterial endophytes could reflect a distinctive timothy cultivars with higher growth promotion traits as well as drought tolerance abilities.

Chapter 4 describes original findings related to the abilities of bacterial endophytes of timothy in the form of multispecies consortium to promote growth, form biofilm and display chemotactic behavior in response to different concentrations of organic acids. Three isolates of

bacterial endophytes of timothy were tested for their compatibility to coexist together as a consortium. Organic acids, such as citric and succinic acids enhanced the selective recruitment of single species in a dose-dependent manner, promoting the biofilm formation on root surfaces. Biofilm formation was visualized as a function of effective root colonization which further supports the findings of Chapter 3 related to the potential of the isolated microbes to be used as plant inoculum. Endophytes of timothy were tested for the first time against root exudates of the model grass *Brachypodium* as a strategy to identify the universality of these endophytes to be used as a multispecies consortium in grasses. In addition, results of this study are in accordance with those of the previous study, in which endophytes of timothy survived hydric stress under salt and drought-mimic conditions, proving their potential to be used in grasses to survive harsh environments. Results of this chapter provide novel evidence that associated bacteria of timothy whether in monoculture cells or as a multispecies consortium, are capable of forming a substantial biofilm under hydric stress which was linked to the significant production of alginate, an essential component of bacterial biofilm.

Chapter 5 reports for the first time that the colonization of *Brachypodium* by the multispecies consortium influenced the metabolite profile and composition of organic acids excreted by roots The abundance of succinic acid in the exudates of inoculated *Brachypodium* provides evidence of the chemotactic behavior of the multispecies consortium and further stresses on the selective production of certain organic acids based on the surrounding microbiota. This selectivity is a key factor in promoting the biofilm formation around the root surfaces of the grass as previously proven in chapter 4.

INTRODUCTION

1.1 PROBLEM DEFINITION

The current soil management strategies depend on inorganic chemical-based fertilizers (Chen 2006; Mahanty et al. 2017) which cause tremendous negative consequences to the environment. Therefore, exploitation of beneficial microbes such as plant-growth-promoting rhizobacteria (PGPR) as a biofertilizer has become of paramount importance in the agriculture sector for their potential role in food safety and sustainable crop production.

Plant-growth-promoting rhizobacteria (PGPR) are associated with plant roots and augment plant productivity and immunity. The beneficial effects of bacteria derived from the plant rhizosphere on roots and overall plant growth have been demonstrated (Ferreira et al. 2019; Hardoim et al. 2015). The significant beneficial effect of rhizobacteria on plant growth are achieved by phosphorus solubilization, more nitrogen fixation, production of siderophores and biosurfactants along with their capacity to synthesize an array of metabolites like hormones and organic acids capable of assisting the plants and increasing their productivity under biotic and abiotic stress (Expósito et al. 2017; Goswami et al. 2016; Numan et al. 2018).

Forages and grass seeds sustain a particular profile of bacterial communities (Ikeda et al. 2006), and vertical transmission from one generation to the next via the seed may be one of the major sources of bacterial endophytes in some perennial grasses including *Miscanthus*, timothy, switchgrass and bent grass (Cope-Selby et al. 2017; Gagné-Bourgue et al. 2012; Truyens et al. 2015). Despite this knowledge, reports on the diversity of bacterial communities hosted by different plant tissues of pasture and forage grasses are limited.

Successful and effective colonization of plant roots by rhizospheric PGPR depends on the exudates excreted by roots in the rhizosphere. Root exudates consisting of organic acids, free sugars and amino acids are essential components of rhizodeposits (Jones et al. 2009). It has been established that low molecular weight carbon compounds such as malic, fumaric, citric, succinic and oxalic acids from the roots represent one of the essential drivers of bacterial activity and diversity in the rhizosphere (Eilers et al. 2010) that influence the microbial community surrounding the root system in the rhizosphere (Zhang et al. 2014). Components of root exudates have been reported to mediate both positive and negative interactions in the rhizosphere chemotactic response and biofilm formation of rhizospheric bacteria (Rudrappa et al. 2008).

Brachypodium distachyon is a C3 plant and a close relative to important cultivated crops such as rice, wheat and maize, and has been widely used as a model for studies of plant–pathogen interactions and stress tolerance (Shi et al. 2015; Verelst et al. 2013). It is fully sequenced, and the plant can be easily genetically transformed and is amenable to genetic manipulation. A relatively large collection of *B. distachyon* genotypes, and mutant lines exists (Kawasaki et al. 2016), which exhibit significant phenotypic diversity (Pacheco-Villalobos and Hardtke 2012). Considering the above desirable features of *B. distachyon*, and most importantly, its capacity to grow in small volumes of soil, its root morphology which is similar to other grasses (Chochois et al. 2015), make it an excellent model to study the interaction of grass fibrous root systems such as timothy grass with rhizospheric PGPR. Very limited work is done on the microbiome diversity of *Brachypodium* (do Amaral et al. 2016; Kawasaki et al. 2016) and on the effect of root exudation on chemotactic responses and biofilm formation of rhizospheric microbiota.

Soil bacteria belonging to different genera, exist in their natural environment in close proximity where fitness of a single cell depends on the interaction and cooperation with other cells in the population (Cavaliere et al. 2017). This cooperation, referred to as syntropy, among different bacterial genera stimulates key processes that benefit plant growth and health and governs metabolism and growth among diverse microbes in natural settings (Kouzuma et al. 2015). Thus, a combination of microorganisms in the form of mixed inoculants that interact synergistically is a feasible strategy for increased activity and better viability of plant-growth-promoting rhizobacteria (PGPR). Additionally, studies reported that multispecies consortia are more effective in their biological function than monocultures of single species (Seneviratne et al. 2008). Therefore, it is essential to use selective PGPR as consortia, which are compatible and able to produce certain phytohormones and exhibit assorted biochemical functions. The understanding of application multispecies consortium capable of producing phytohormone will serve as the basis for future research to elucidate the role of bacterial communities in crop productivity and sustainable agriculture.

Therefore, the overarching goal of this study was to (i) characterize at the molecular and biochemical levels the bacterial isolates associated with tissues of timothy cultivars grown under field conditions, (ii) select the top performing isolates, in relation to phytohormone production and other growth promoting attributes, in the form of multispecies consortium, (iii) determine whether organic acids released from root exudates of the model grass *Brachypodium* act as an attractant to the multispecies consortium by inducing chemotactic response and biofilm formation of the consortium compared to single species inoculum.

1.2 OBJECTIVES AND HYPOTHESES

Global Objectives

The main objective of this research is to identify and characterize bacteria associated with timothy (*Phleum pretense* L.) that could be used as plant growth promoters of grasses by studying their functional traits and interaction with the model grass *Brachypodium distachyon*.

Specific Objectives

1- To characterize the microbial diversity associated with timothy and explore biochemical traits leading to improved plant productivity and stress tolerance (Chapter 3).

2- To investigate the effect of organic acids and root exudates of *Brachypodium* on the chemotactic behavior and biofilm formation of multispecies bacterial consortium (Chapter 4).

3- To explore the potential use of the multispecies consortium as a universal PGPB by investigating the chemical and molecular profiles of organic acids in roots and root exudates of the model grass *Brachypodium distachyon* under inoculated semi hydroponic tissue culture conditions (Chapter 5).

Hypotheses:

1- Associated bacteria of timothy have the necessary functional attributes to protect cool season grasses against biotic and abiotic stresses.

2- The success of a compatible multispecies consortium is related to several bacterial traits that allow biofilm formation and colonization of the host. Plant- microbe interaction is induced by root exudates. Root exudates of *Brachypodium* will be more similar to timothy grass than eudicot model plant *Arabidopsis*.

3- The inoculation with the multispecies consortium will alter the composition of organic acids released from *Brachypodium* roots and induce a change in the expression of organic acid genes of the TCA cycle.

LITERATURE REVIEW

2.1 FORAGE GRASSES

2.1.1 Timothy

Timothy (*Phleum pratense*) a perennial grass introduced to North America from Northern Europe during the early colonial period. It is a cool season perennial grass well adapted to humid and temperate areas having harsh winter conditions (Barnes 2003). Timothy is the most cultivated grass in Québec, Canada (Centre de référence en agriculture et agroalimentaire du Québec 2010) since it has the capacity to survive very cold winters and withstands extreme icing conditions reaching -20ºC. Due to its superficial root system, it prefers humid soil conditions and is therefore very sensitive to drought conditions (Moser et al. 1996). Timothy grass is usually planted with forage leguminous crops such as red or white clover and alfalfa (Centre de référence en agriculture et agroalimentaire du Québec 2010). Mixing timothy with leguminous crops increases its yield by utilizing some of the N fixed by the above legumes (Heath et al. 1973). Requirements of nitrogen (N) vary based on several factors including growth cycle, stage of development, environmental conditions and the cultivar planted.

Timothy grass has a unique method of regeneration when compared to other forage grasses. In the seeding year, timothy overwinters after forming its first single shoot. In the following years of growth, a seed head is formed near the base of the grass. In parallel, an onion-like bulb structure known as corms will be forming at the base of the shoot. This contains all the sugars and food reserves needed. There are three stages of timothy development based on tiller types. Stage 1 is the vegetative stage consists of vegetative tillers (VEG). At this stage, timothy can only produce the leaf primordia. Stage 2 is the generative stage known as the elongation stage (ELONG) in which timothy grass develops true stems and nodes. Stage 3 is generative stage (GEN), in which the apex becomes reproductive (Jokela et al. 2014).

Harvesting timothy is highly influenced by its stage of development. This grass uses its nutrient storage to re-emerge at the end of each growth cycle and in the spring season too. A two-system cut is the ideal method to ensure a high yield and a healthy regeneration of timothy grass. Hence, the first cut should take place at the full head stage and the second cut 45-50 days later. This is because timothy synthesizes carbohydrates at the beginning of its growth that leads to its subsequent growth and an increase in its yield later in the season (Grant and Burgess 1978). Timothy has valuable feeding capacity as a forage crop. It is an intermediate feed for livestock ruminants that has equal amounts of energy as alfalfa. It is also highly valued as a feed for cattle and horses due to its capacity to meet their mineral requirements. Several studies state a decrease in the crude protein content of timothy over its life cycle as well as its digestibility was shown to decrease with age (Fagerberg 1988; Grant and Burgess 1978). Its feeding value, digestibility as well as dry matter content decrease if it is not harvested at the heading stage. Nevertheless, its lignin content as well as its fiber components (hemicellulose, cellulose) increase in its stems as they are forming through age (Centre de référence en agriculture et agroalimentaire du Québec 2010).

2.1.2 Brachypodium

Brachypodium distachyon has been identified as a model system for grasses due to its small stature reaching approximately 20 cm, its rapid generation time and its simple growth requirements (Brutnell et al. 2015; Ozdemir et al. 2008). It belongs to the *Poaceae* family of grasses and has a very similar genomic sequence to various temperate grains including wheat, barley, rye, oats and rice (Brutnell et al. 2015; de la Peña et al. 2019). Furthermore, the diploid *Brachypodium distachyon* has a small genome size of 272 Mbp (Gordon et al. 2017) and is highly homozygous. Many accessions exist for diploid, tetraploid and hexaploid *Brachypodium* which simplifies the genomic studies as well as other sorts of research projects. This C3 plant is used in fundamental research covering plant biology

and development, abiotic stresses as well as plant- microbe interactions (Scholthof et al. 2018) and has been the interest of the DOE Joint Genome Institute that has several accessions available for gene expression studies targeting a vast umbrella of genes present in this grass. It is an annual, self-fertile plant with a life cycle of less than 4 months depending on its vernalization requirement (Ozdemir et al. 2008), which makes of it a very attractive model in research labs (McWilliams 2018).

2.2 PLANT GROWTH PROMOTING MICROBES

Endophytes are non-harmful bacterial or fungal microorganisms isolated from surface disinfected plants or from within plant tissues (Hallmann et al. 1997). They colonize the interior parts of plants like roots, stems and seeds without causing any harm to their hosts. They belong to a wide population representing more than 54 genera and hundreds of species co-existing with a vast majority of plants in different climatic environments. Many studies have been targeting the importance of endophytes to plants and their identification, source, and mode of entry. For the purpose of this work, the term endophytes will refer only to bacteria that establish a non-harmful relation with their host plant (Gaiero et al. 2013). Bacterial endophytes are either gram positive or gram negative (Lodewyckx et al. 2002). They can be classified into three main categories based on their strategies of inhabiting plants. 1. Obligate endophytes originate from plant seeds; 2. Passive endophytes (not competitive) enter plants through stomata, lenticels, wounds, lateral roots or from elongation zones of root tips and then colonize them by inhabiting the intercellular spaces (Zakria et al. 2007) of the epidermal and cortical regions as well as vascular tissues and xylem cells (Reinhold-Hurek and Hurek 2011) ; 3. Free living endophytes that live freely in the soil, and colonize plants under available opportunities (Gaiero et al. 2013; Hallmann et al. 1997; Hardoim et al. 2008). Once inside the plant, endophytes can be either localized around their point of entry or might move inside their host and begin producing enzymes

such as endoglucanases and endopolygalacturonidases to help them move inside the plant (Hardoim et al. 2008). Many reports described the distribution of endophytes once they colonize their host. The main aim was to explore whether they stay localized at their point of entry or if they spread throughout the plant. Jacobs et al. (1985) have reported that these microorganisms stay within the cells of sugar beet root, Patriquin and Döbereiner (1978) have found them in the intercellular spaces of maize, wheat, sorghum and other grasses while Bell et al. (1995) have found them in the vascular system of grapevine. They have also been found in all sorts of plants and trees including and not restricting to wheat (Díaz Herrera et al. 2016; Pan et al. 2015; Robinson et al. 2016), rice (Bertani et al. 2016; Walitang et al. 2017), maize (Mousa et al. 2015; Shehata et al. 2016), tomato (Abbamondi et al. 2016; Tian et al. 2017), cucumber (Akbaba and Ozaktan 2018; Jeong et al. 2016), pine (Anand and Chanway 2013; Madmony et al. 2005; Shen and Fulthorpe 2015), cannabis (Taghinasab and Jabaji 2020), chickpea (Brígido et al. 2019; Saini et al. 2013), turfgrass (Shehata et al. 2018) and olive trees (Müller et al. 2015). Furthermore, they were reported to belong to a vast array of genera including *Bacillus, Enterobacter, Pseudomonas, Microbacterium, Burkholderia, Pantoea, Streptomyces* (Oteino et al. 2015; Saleh et al. 2019; Yadav et al. 2018).

2.2.1 Beneficial role of bacterial endophytes

Most plants growing in the field live in symbiosis with beneficial bacteria without getting injured by their presence. This is because endophytes, when associated with their hosts, help in the crop improvement by improving the plant's growth, accelerating its development and improving its resistance to environmental stress.

2.2.1.1 Crop improvement

Several studies showed that endophyte-grass associations lead to improved grass resistance to biotic and abiotic stresses (Ravel et al. 1997; Rosenblueth and Martínez-Romero 2006). For instance,

grasses inoculated with endophytes showed an increased tolerance to drought (Gagné-Bourque et al. 2015; Tan and Zou 2001). Growth conditions and agronomic performances of ryegrasses were greatly improved in terms of their resistance to drought conditions and lack of nutrient availability (Pii et al. 2015; Ravel et al. 1997). Also, endophytes from the genus *Pseudomonas* were able to increase the tuber numbers of potato, their dry weight, stem length, and secondary root branching and induced the production of root exudates in sugar beets (Hallmann et al. 1997; Mark et al. 2005), while *Sinorhizobium meliloti* isolated from the rhizobial microbiome of *Medicago*, *Melilotus*, and *Trigonella* plant species was capable of fixing nitrogen and accumulating copper in the shoots and roots of the reported plants (Sharma et al. 2018).

These beneficial capacities of endophytes or plant growth promoting bacteria (PGPB) affect their host plants directly and indirectly. Direct mechanisms can be achieved through the production of metabolites such as plant growth regulators and phytohormones including cytokinins and plant growth promoting compounds that help them access nutritional elements like nitrogen and phosphorus (Bhattacharyya and Jha 2012; Gagné-Bourgue et al. 2012; Tan and Zou 2001), or through helping the plant uptake nutrients, while indirect mechanisms are based on the production of metabolites such as antibiotics and siderophores (Imperi and Visca 2013; Loaces et al. 2011; Saha et al. 2013).

a) Nitrogen fixation

Nitrogen fixation occurs naturally by the presence of a large group of bacteria able to fix nitrogen and provide it to plants. Rhizobia species are diazotrophs capable of fixing the atmospheric nitrogen and converting it to ammonia in the presence of nitrogenase enzyme. This is due to the interaction between the endophytic bacteria and the host legume, leading to the exchange of energy and carbon of the plant, with the nitrogen fixed by the bacteria (Sulieman and Tran 2014). A number of free-living bacteria such as *Azospirillum* sp*., Azoarcus* sp*., Herbaspirillum* sp*., Acetobacter* and *Diazotrophicus*

sp. were reported to fix nitrogen in grasses (Reinhold-Hurek and Hurek 2011; Steenhoudt and Vanderleyden 2000). Others like *Burkholderia*, *Herbaspirillum*, *Azospirillum*, and *Rhizobium leguminosarum* bv. *Trifolii* contributed to the fixation of nitrogen in rice specifically (Afify et al. 2019; Hoseinzade et al. 2016; Naher et al. 2018; Stephen et al. 2015). Nitrogenase enzymes present in both free- living and symbiotic bacteria are encoded by the *nif* genes induced in response to low fixed nitrogen concentrations as well as low oxygen concentrations (Glick 2012; Reinhold-Hurek and Hurek 2011; Steenhoudt and Vanderleyden 2000). Rhizobia have the *nod* genes that encode for the nodulation. The symbiosis of the plant and bacteria will activate the *nod* genes in the bacterium, leading to the production of NOD factors that would through their production, lead to the formation of root nodules and to the colonization of the bacteria to its attracting host (Lugtenberg et al. 2013). Among the plant growth promoting bacteria capable of fixing nitrogen in nodules are the *Rhizobium* spp. *Rhizobium* for instance, has been reported to improve the morphology of roots and the growth physiology of rice plants (Yadav et al. 2018) yet many of the non-rhizobial strains are also capable of fixing nitrogen in nodules. Sánchez-Cruz et al. (2019) have isolated endophytes from nodules of *Mimosa pudica* while Stajković et al. (2009) have isolated non-rhizobial bacteria from *Medicago sativa* and both studies showed significant improvements in the growth parameters of the inoculated plants showing that the isolated strains possess growth promoting and nitrogen fixing potential.

b) Phosphate solubilization

Phosphorus is an essential macronutrient required for healthy growth of all plants. It is available in large quantities in all soils, however a very large part of it isn't soluble and therefore not available to plants. PGPBs have the capacity to solubilize and mineralize phosphorus stocks and make them available to plants (Glick 2012). For instance, microbes having the phosphate solubilizing property have been reported after performing phosphate plate assays for the following genera *Bacillus*,

Brevibacterium, *Chryseobacterium*, *Curtobacterium*, *Methylobacterium*, *Paenibacillus*, *Pseudomonas* and *Stenotrophomonas* (Saleh et al. 2019). Rhizosphere colonizing bacteria are efficient in liberating organic phosphates through the production of phytases, C-P lyases and phosphonatases that would alter the pH, reduce metals enzymatically and form carbon dioxide (Matos et al. 2017). These microbes are also efficient in solubilizing inorganic phosphates through the production of organic acids such as gluconic, citric acid (Glick 2012), acetic, lactic, and succinic acids (Yadav et al. 2018). Among the bacteria capable of solubilizing phosphorus are the microbes belonging to the genera *Rhizobium, Agrobacterium, Burkholderia, Erwinia, Paenibacillus, Bacillus* and *Lysinibacillus* sp. (Matos et al. 2017). Oteino et al. (2015) showed that one of the ways followed by bacteria to solubilize the phosphate in their environment is through the production of low molecular weight organic acids that would acidify the soil in the rhizosphere. Accordingly, acidification seemed like a strategy followed by the strains of *Pseudomona*s to solubilize phosphate which makes of them ideal candidates for phosphorus bio-fertilizers application. The greater the GA production, the higher were the fresh and dry weights of *Pea sativum*, and the higher was the concentration of phosphate released.

c) Iron Sequestration

Iron is one of the essential nutrients for plants. However, it is not easily available to plants nor to soil microbes due to its predominant form as a ferric ion Fe^{+3}. It is essential in DNA synthesis, important in the electron transport system and in the formation of heme, is a cofactor for enzymes, takes part in the oxygen transport, synthesis of ATP and in reducing nitrite in the nitrogen cycle (Saha et al. 2013). To survive the limited supply of iron, bacteria produce small molecular weight compounds known as siderophores that have high affinity to Fe^{+3} (Loaces et al. 2011). Siderophores can be classified into three categories based on the binding of oxygen to Fe^{+} (Loaces et al. 2011; Saha et al. 2013).

By attracting iron present in the soil, siderophores directly contribute to plant growth. Additionally, they are known to have a role in phytoremediation by alleviating the plant stress caused by the accumulation of heavy metals (Glick 2012; Kong et al. 2017; Loaces et al. 2011). Certain strains of *Pseudomonas* were also recorded to suppress virulent pathogens by preventing them from feeding on iron. Thus, growth of soil-borne pathogens such as *Fusarium oxysporum* and *Pythium ultimum* was limited due to endophytic siderophore production (Loaces et al. 2011). In addition, it was found that iron played a crucial role in minimizing the effect of the pathogen *M. oryzae* common in rice plants. The study by Zeng et al. (2018) revealed that the endophyte *Streptomyces sporocinereus* OsiSh-2 is capable of producing siderophores under iron limited conditions, contributed to the inhibition of the fungus growth by depriving the latter from the iron source and by decreasing the antagonism of OsiSh-2.

d) Phytohormone production

i) Indole acetic acid (IAA) and ACC deaminase

IAA is the most commonly studied plant auxin. It is involved in cell division, seed and tuber stimulation, development of xylem and root system as well as in photosynthesis, pigment formation and in stress resistance (Glick 2012). In addition, IAA produced by bacteria can have direct effect on the interaction between plants and bacteria. For example, the auxin produced by *Pseudomonas putida* helped in developing the root system in canola (Patten and Glick 2002). Endophytic bacteria also have the capacity for phytostimulation through the production of ACC (1-aminocyclopropane-1-carboxylate) deaminase, an enzyme directly involved in lowering ethylene concentrations and thus promoting plant growth (Gaiero et al. 2013). After colonizing the seed or the root of a growing plant, endophytic bacteria are directly involved in the production of IAA by using the tryptophan molecule present in plants as well as some root exudates. Plants will therefore benefit from the production of

IAA through growth promotion, cell proliferation and elongation or through the synthesis of ACC synthase responsible for converting S-adenosyl methionine to ACC, the immediate precursor of ethylene in all higher plants. In parallel, a portion of ACC produced by plants is used by bacteria and converted through ACC deaminase into ammonia and α-ketobutyrate. This leads to the suppression of ethylene levels inside the host plant, enhances root development and promotes shoot growth (Glick 2012; Hardoim et al. 2008).

According to Defez et al. (2017), the overproduction of IAA by endophytes isolated from rice has resulted in an increase in the nitrogen fixation capacity of the grass, which led the authors to conclude that nitrogen fixation might not be only restricted to the symbiosis between rhizobia and legumes. Some studies on the other hand have reported a relatively negative aspect of IAA producing endophytes. Tabatabaei et al. (2016) have reported about four *Pseudomonas* isolates from durum wheat and their effect on the seed germination and the α-amylase activity of wheat. The results showed that seed germination rate and the activity of α-amylase has decreased and were dependent on the concentration of IAA.

ii) Cytokinins and Gibberellins

Cytokinins are plant growth regulators that influence physiological processes of plants such as cell division, seed germination, root development, accumulation of chlorophyll, leaf expansion or delay of senescence. Besides their production in plants, they are synthesized by soil bacteria (Arkhipova et al. 2007) and are detected in the cell-free medium of some bacterial strains like *Azotobacter* sp., *Rhizobium* sp., *Rhodospirillum rubrum*, *Pseudomonas fluorescens*, *Bacillus subtilis* and *Paenibacillus polymyxa* (Garcia de Salamone et al. 2001; Glick 2012). Several *Bacillus* spp. including the species *subtilis* or. *megaterium* strains are capable of producing cytokinins which in turn improves growth of inoculated plants (Ortíz-Castro et al. 2008; Pérez-García et al. 2011). Kudoyarova

et al. (2014) have reported that strain IB 22 of *B. subtilis* showing ability to produce cytokinins has also stimulated the deposition of amino acid exudates from the roots of wheat thus indirectly promoting its growth.

Gibberellins are plant hormones also involved in the developmental and physiological growth processes of plants (Gagné-Bourgue et al. 2012; Glick 2012). They are produced by endophytic bacteria and stimulate plant growth and crop yield, cell division and elongation (Glick 2012). Many gibberellins are produced by many endophytic bacteria such as *Acetobacter diazotropicus*, *Rhizobium faseoli*, *Bacillus pumilus* and others (MacMillan 2001). Root growth of wheat increased after the application of gibberellin producing bacteria belonging to *Azospirillum* and *Bacillus* genera (Bottini et al. 2004). The inoculation of the latter gibberellin producing endophytes was also recorded to stimulate increased ^{15}N uptake of wheat roots (Kucey 1988) but also to improve the host plant growth as reported by Shahzad et al. (2016) who concluded that the utilization of *Bacillus* isolated from the seeds not only improved the GA and SA production but further reduced ABA and JA content.

iii) Ethylene

Ethylene is a plant growth regulator important for the initiation of roots, ripening of fruits, germination of seeds, the biosynthesis of other phytohormones, and in stress signaling (Ali et al. 2014). It plays a dual role in the plant-endophyte interaction. It has the capacity to promote plant growth and development, is involved in the plant disease resistance, and helps in suppressing abiotic stress conditions; however when its concentration highly increased in the plant, it can cause suppression of root elongation (Hardoim et al. 2008; Long et al. 2010).

Endophytes can manipulate ethylene concentrations inside the plant by interfering with two mechanisms that normally take place. They either i) break down ACC i.e., endophytes degrade ACC to supply nitrogen and energy without disturbing the nutritional balance of the plant or ii) inhibit the

synthesis of ACC synthase or β-cystathionase enzymes, and therefore attenuate stress caused by the production of high ethylene levels (Hardoim et al. 2008). Some bacterial strains of *Pseudomonas, Enterobacter, Azotobacter and Azosporillum* promote plant growth through the production of plant growth regulators such as ethylene as well as auxins and cytokinins (Lodewyckx et al. 2002). Also, controlling ethylene levels by inoculating plants with PGPB-producing ACC deaminase was followed as a strategy to weaken the damage caused by several phytopathogens including *Pythium ultimum, Fusarium oxysporum, Erwinia carotovora, Agrobacterium tumefaciens, Agrobacterium vitis, Sclerotium rolfsii*, and *Rhizoctonia solani* (Glick 2012). Thus, ethylene is considered an important regulator in the plant colonization by bacteria. In fact, diazotrophic endophytes isolated from sugarcane have altered the gene expression of the SCER1, SCERF1, and SCERF2 genes involved in the ethylene signaling pathway (Cavalcante et al. 2007). Ethylene was also reported to be involved in the plant's survival under biotic and abiotic stress conditions. Under conditions of salt stress, *Enterobacter* sp. SA187 induced tolerance to salt by producing KMBA (2-keto-4-methylthiobutyric acid) known to be converted to ethylene (de Zélicourt et al. 2018). On the other hand, Kusajima et al. (2018) have reported that the involvement of the ethylene signaling pathway in the bacterial endophyte *Azospirillum* sp. B510 has induced disease resistance in rice.

2.2.1.2 Stress tolerance

Plants are subjected to several types of stress that affect their growth, development and productivity. Drought stress has serious consequences on crop plants leading to a reduction in the rate of early germination, cell division, size of leaves, lower comparative stem elongation, less root proliferation as well as closure of stomata, production of reactive oxygen species (ROS), diminished activities of enzymes and delayed flowering (Zhenzhu et al. 2010). This results in (i) increased plant

photorespiration (Noctor et al. 2002), (ii) a limited carboxylation (Reddy et al. 2004), (iii) an obstructed ATP synthesis leading to a decline in photosynthesis and crop yield (Farooq et al. 2012).

Drought stress is the result of soil water deficit and high temperature. Plants react to drought stress by either avoiding it, tolerating it or recovering from it (Malinowski and Belesky 2000). Plants have created mechanisms to tolerate the stress caused by drought. These are the physiological and biological adaptations that help plants withstand water deficit. Carbohydrates such as fructans and sucrose accumulate, are translocated in plant tissues exposed to stress and enhance tolerance to drought by maintaining its homeostasis (Chaves 2003; Malinowski and Belesky 2000). Adjusting the osmotic pressure inside the crop is helpful in drought tolerance. Osmotic pressure is controlled by the following groups of solutes including water soluble sugars, fungal metabolites such as mannitol and arabitol, amino acids such as proline, aspartic acid and glutamic acid, and alkaloids such as indole alkaloids (indoglycerol, indoleacetic acid) (Malinowski and Belesky 2000). Drought plants tend to accumulate sugar levels in their leaves helping them tolerate drought, maintain metabolic activity and interact with other signal transduction pathways (e.g., ethylene). In addition, synthesis of proline amino acid helps in preventing protein denaturation, preserving enzymes and protecting cell membranes from the activity of ROS while synthesis of alkaloids makes the plant negligibly affected by biotic and abiotic conditions. Endophyte-plants associations were found to stimulate alkaloid synthesis in for protection against any possible threat (Bacon 1993; Malinowski and Belesky 2000). In a similar manner it has been shown that bacterial strains capable of producing certain organic acids like GA alleviate different sources of stress. *Pseudomonas putida* was reported to alleviate the stress caused by salt and drought in soybean plants (Kang et al. 2014). For instance, *Sphingomonas* sp. were effective under salt stress in tomatoes (Halo et al. 2015) and *Curtobacterium* sp. SAK1 against salt stress of soybean (Khan et al. 2019).

Co-inoculation of a common bean crop with *Paenibacillus polymyxa* and *Rhizobium tropici* alleviated drought stress (Figueiredo et al. 2008). Furthermore, co-inoculating *Bradyrhizobium japonicum* with strains of actinomycetes showed prome in terms of increasing crop yield, plant nutrient uptake as well as plant dry weight of soybean (Nimnoi et al. 2014). Interestingly, *Bacillus* sp. increased the biomass of maize, its relative water content, leaf water potential, and concentrations of proline, sugars, and free amino acids (Sandhya et al. 2011). Furthermore, *Bacillus subtilis* was reported to increase tolerance to drought stress and to accelerate the growth rate of the grass *Brachypodium distachyon* (Gagné-Bourque et al. 2015). Also, PGPB producing ACC deaminase have been reported to facilitate plant growth under abiotic stress caused by high levels of salt stress by reducing levels of ethylene production inside plants (Ali et al. 2014).

2.3 FUNCTIONAL POTENTIAL OF MICROBIOME AND ITS EFFECT ON PLANT HEALTH

Plant health does not only depend on its genes or its ability to survive under stress conditions but also on the presence of microbes in its surroundings. Microbes come in different forms and belong to different communities covering bacteria, fungi, archaea and Protista (Hardoim et al. 2015). They can be neutral, can cause harm to their hosts or can be beneficial (Spence and Bais 2013) but most importantly can be found in different anatomical areas of their hosts. This divides microbes into two major groups: the epiphytes representing the group of microbes that live and multiply outside the host and the endophytes coming from within the plant. In general, epiphytes grow well outside the plant and occasionally enter its endosphere. For example, epiphytes on the surfaces of the leaves of plants are exposed to high levels of solar radiation, moisture and humidity as well as temperature fluctuations which make these communities resistant to the environmental changes or well adapted to them. In fact,

Sundin and Jacobs (1999) have reported that leaf surfaces contain pigmented bacterial species like *Pantoea agglomerans* and *Pseudomonas fluorescens.* On the other hand, endophytes live within their host for at least part of their life cycle without causing any harm to them. Many species belonging to various genera take part in the endosphere community and were reported to have plant growth promoting abilities that would make nutrients more available to plants thus acting like biofertilizers (Kumar et al. 2017). In fact, some microbes have the capacity to fix nitrogen, can make phosphorus and potassium more available to plants, thus indirectly leading to a better soil quality and a more balanced nutrient cycle (Lambers et al. 2009; Zhang et al. 2017). In this section we intend to provide insight about how plants communicate with their surrounding microbiome.

2.3.1 Factors that affect plant microbiota

The dynamics of the microbial composition of the rhizosphere is the result of a mixture of various factors that shape it. These factors include and are not restricted to the temperature, soil moisture, pH, salinity, soil texture, carbon availability, bacterial biofilm and root exudates (Fierer 2017; Spence and Bais 2013). These conditions favor the mutual selection of the microbe for its host and vice versa. It has been repeatedly reported that certain endophytes are specific to a certain anatomical source (ie. tissue) within its host or to a certain cultivar. Saleh et al. (2019) demonstrated that the genus *Arthrobacter* was specific to the cultivar Champ of timothy grass, while the genus *Pedobacter* was predominantly associated with the cultivar AC Alliance. It was also observed that the genus *Methylobacterium* was only associated with the leaves of the grass. Also, Manter et al. (2010) have reported that communities of bacterial endophytes were specific to potato (*Solanum tuberosum*) and moreover, were significantly different in their members and structures between different potato cultivars. Another study on bacterial rhizosphere communities of potato has mentioned that the microbes were linked to plant genotypes. In fact, three cultivars of potato attracted different endophytes

belonging to Pseudomonales, *Streptomycetaceae* and *Micromonosporaceae* which indirectly affected their ability to control the pathogens of the plant (Weinert et al. 2011). This pattern was also observed in wheat. Wheat cultivars were selective in their selection of bacterial endophytes allowing only DAPG- producing microbes like *Pseudomonas* sp. to enter their microbiome due to their capacity to suppress diseases (Meyer et al. 2010).

2.3.2 The basics of plant communication

2.3.2.1 Plant to plant communication

Chemical signaling is one of the most important ways through which plants communicate with their surroundings. Chemicals can either take the form of volatiles (Gagné-Bourgue et al. 2012; Saleh et al. 2019; Scott et al. 2018) or may be released from the roots of plants in the form of exudates. The composition of exudates may vary based on the plant species along with its physiological state and age (Biedrzycki and Bais 2013; Zolla et al. 2013). Root exudates are divided into two main classes: low molecular weight and high molecular weight compounds. Low-molecular weight compounds include amino acids, organic acids, sugars, phenolics, and other secondary metabolites, whereas high molecular weight exudates are composed of polysaccharides and proteins (Bais et al. 2006; Carvalhais et al. 2011; Kawasaki et al. 2016; Zolla et al. 2013). These secretions cost the plant a tremendous amount of carbon and energy. Badri and Vivanco (2009) reported that 30 to 40% of photosynthetically fixed carbon is released from the plant into the rhizosphere which highlights the huge investment of plants in rhizodepositions to secure their survival. Rhizodeposition, known as the continuous secretion and diffusion of organic substances and carbon into the soil, has been widely studied (Fischer et al. 2010). Several reports showed that the total distribution of photosynthetic carbon to roots in cereals is estimated to represent 20-30% and a little bit more (30-50%) in pasture plants (Carvalhais et al. 2011; Jones et al. 2009; Kuzyakov and Domanski 2000). Current evidence suggests that the amount of

rhizodeposition increases under stress conditions (Vishwakarma et al. 2017; Zolla et al. 2013). Moreover, phosphorus deficiency in canola and barley has led to a faster exudation of citric and malic acids, respectively (Wang et al. 2015). A complicating factor in the root exudate collection is the multitude of techniques by which they are collected. A standardization for the protocols is required because the conditions in which the roots are grown affect the outcome (van Dam and Bouwmeester 2016). Zolla et al. (2013) along with many researchers, reported that the most common technique for root exudate collection involves seedlings growing under hydroponic axenic conditions; however, some techniques have been developed for seedlings grown in solid media which are known to alter soil exudations.

Plant interactions with their surrounding environment, whether with other plants, microbes or animals could be positive or negative, and many of them are directed through root exudates. Allelopathy is one of the mechanisms that could highlight the communication of a plant with another plant. By the secretion of certain phytotoxins into the soil, one plant will suppress the growth of another (Bais et al. 2006) through affecting its metabolic and respiratory system, its root and shoot growth, thus leading to its mortality (Bais et al. 2006; Weir et al. 2004). Root exudates play a key role in the interaction between plants. This cross talk is well-understood and has been extensively studied in the field. Strigolactones for example, are plant hormones secreted in the exudates of plants and act as attractants to parasitic weeds. Pavan et al. (2016) have reported on the selection of a pea landrace showing resistance to the weed *Orobanche* due to its capacity to secrete lower concentrations of stigolactones into its microbiome. The secretion of hydrogen peroxide by *Striga* seedlings into the rhizosphere of its host play an integral role in the formation of haustoria (Kim et al. 1998).

2.3.2.2 Plant-microbe interaction

The plant interaction with its surrounding is not restricted to other plants but is also profoundly linked with its surrounding microbes. Mycorrhizal associations form one of the major aspects of this interaction in which the host plant supplies carbohydrates to the symbiont, while it provides the limiting nutrients to the plant. Plants through the secretion of specific amino acids, sugars, and secondary metabolites promote the attraction of specific fungi. For example, limonene was specifically produced from the roots of *Medicago truncatula* after being exposed to the spores of *Rhizophagus irregularis* (Dreher et al. 2019). Besides this mutualistic lifestyle, plants have created a way to defend themselves from any potential pathogenic attack by the production of antimicrobial metabolites into their surroundings (Biedrzycki and Bais 2013; van Dam and Bouwmeester 2016). Phytoalexins for example are antimicrobial secondary plant metabolites usually located around infected sites of plants following fungal or bacterial attack (Komives and Kiraly 2019). Antimicrobial phenolics are present in plant exudates and some exudates like terpenoids are involved in the plant defense mechanisms (Olanrewaju et al. 2019; Rasmann and Turlings 2016).

Many studies highlight the importance of metabolically active border cells that are detached from the root caps of plants into their surrounding soils. Border cells serve as mucilage secretions that attract certain microorganisms and inhibit the growth of others (Driouich et al. 2013; Mohanram and Kumar 2019; Sasse et al. 2018). Chemoattraction, repulsion and suppression are different ways through which border cells interact with microorganisms. For example, border cells behaved as attractant to *Agrobacterium tumefaciens* in pea but acted as repellent to the same bacterium in cotton and beans (Cannesan et al. 2012; Hawes 1990; Wen et al. 2007). In addition, these cells are active towards plant growth promoting bacteria (PGPB). Canellas and Olivares (2017) showed that the

PGPB *Herbaspirillum seropedicae* colonized the root tips of maize after the production of border cells as a result of humic acid exudation.

PGPB are soil bacteria present in the microbiome of the plant that facilitate its growth and improve its health. Two types of mechanisms help PGPB to promote growth of plants: (i) direct and (ii) indirect. (i) The direct mechanism stimulates plant growth through the production of plant hormones like auxin, cytokinins, gibberellins along with the fixation of the atmospheric nitrogen into the soil and the sequestration of iron. The relation between legumes and rhizobia is a clear example of a direct mechanism of interaction. Under limiting nitrogen content in the soil, legumes would attract the surrounding rhizobia in the soil through the secretion of flavonoids. Once flavonoids are recognized, bacterial Nod factor is produced and recognized by the plant which allows the rhizobia to infect its root hairs and form nitrogen fixing nodules (Badri and Vivanco 2009; Biedrzycki and Bais 2013). Experimental evidence has shown that nitrogen fixation by microbes does not only happen in nodules or in the presence of rhizobia due to their very selective host range. Several non-symbiotic microbes have contributed to the nitrogen fixation in the pool of their host plant and induced a better plant fitness due to their presence in their surroundings. This was reported for, *Paenibacillus* in poplar trees (Scherling et al. 2009), *Klebsiella* and *Gluconacetobacter diazotrophicus* in sugarcane (Govindarajan et al. 2007), and *Gluconacetobacter diazotrophicus* in beetroot, radish, banana and pineapple (Eskin et al. 2014). (ii) The indirect mechanism helps the plant by inhibiting the activity of potential pathogens through the production of an array of biochemical features including ACC deaminase, hydrogen cyanide, siderophores as well as cell wall degrading enzymes. Another indirect mechanism is Induced Systemic Resistance (ISR) which is a process induced by PGPB to alleviate the stress caused by pathogenic organisms. This mechanism protects the plant by activating the jasmonate and ethylene pathways. Several studies have targeted

this topic, Rudrappa et al. (2008) has studied the response of *Arabidopsis thaliana* inoculated with *Bacillus subtilis* in response to infection by the foliar pathogen *Pseudomonas syringae* pv. *tomato* DC3000. The infection by the foliar pathogen increased malic acid exudation from the roots which has led to the attraction of the endophyte and promoted its binding and biofilm formation. Liu et al. (2014) examined a similar scenario where the PGPB, *Bacillus amyloliquefaciens,* colonizing infected cucumber roots expressed a high chemotaxis and a better biofilm formation along with an increase in citric and fumaric acids secretions despite the presence of the fungus on its roots. In another study, Dudenhöffer et al. (2016) showed that barley changed the composition of root exudation in response to the pathogen *Fusarium graminearum* and promoted the production of antifungal compounds as a protection policy. These observations stress the importance of bacterial chemotaxis as a driver towards strengthening plant immunity, and how bacterial communities interact and influence plant root exudation.

The role of exudates as a signaling factor that regulates the interaction between plants and their surrounding microbes is essential for plant health maintenance (Olanrewaju et al. 2019). Exudates of plants have played a role in attracting bacteria surrounding them and have triggered their colonization with specific species (Badri and Vivanco 2009; Lareen et al. 2016; Philippot et al. 2013). Rhizosphere growth promoting bacteria are mainly attracted to carbohydrates, organic acids and amino acids produced by roots (Rasmann and Turlings 2016). The amino acid, canavanine, for instance can stimulate action of one group of bacteria while suppressing others (Cai et al. 2009) while amino acid tryptophan secreted by plant roots is a requirement for PGPB to be attracted to the host and to stimulate the synthesis of indole acetic acid (Gilbert et al. 2018).

2.4 MOTILITY, BIOFILM FORMATION AND COLONIZATION OF MICROBES

One of the most important forms of adaptation to the environment is the ease of bacterial cells to organize themselves into biofilms. Bacterial cells find themselves attached to biotic and abiotic surfaces as mixed bacterial consortium or single species, all coordinated together in an extracellular polymeric matrix composed of a mixture of different elements including water along with proteins, polysaccharides, DNA, RNA and ions (Jamal et al. 2018; Kovács and Dragoš 2019; Singh et al. 2020). This dynamic community has a multitude of various phenotypes that make it possible for organisms to survive under different environmental conditions (Annous et al. 2009; Battin et al. 2007). Cell-to-cell communication is key in biofilm formation since it plays an important role in their development, architecture and physical adaptation, thus is involved in the regulation and expression of related genes.

Microbes organize themselves into biofilms to protect themselves from harsh environmental conditions of heat, cold, changes in pH, etc. They also benefit from their entity to become less sensitive to the defense mechanisms of their surrounding hosts, along with a better absorption of nutrients and metabolites and a better transfer of the genetic material between them. This creates a communication pattern between the microbes, a method for microorganisms to control the population progress among them, this is known as “Quorum sensing” (Singh et al. 2020).

2.4.1 Biofilm on roots and in the rhizosphere

Bacterial communication with roots of plants is the subject of interest of many researchers. The complexity of this relation and how it is related to the physiological changes in plants and in the microbiome surrounding it have been analyzed (Hardoim et al. 2008; Hardoim et al. 2015). In addition to the physico-chemical changes, abiotic conditions like nutrients, temperature and humidity have played an important role in biofilm associations. In fact, bacterial cells have found ways to adapt to those changes by increasing their numbers and by colonizing roots of plants as biofilms

(Angus and Hirsch 2013). Plant exudates have the capacity to alter the soil microbiome based on their composition. A multitude of factors like the age, the nutritional status of the plant or the stressful conditions under which it is living (Ansari et al. 2017; Haichar et al. 2008; Lareen et al. 2016; Philippot et al. 2013) can also affect the microbiome compositions. For example, plants growing in an iron-limited environment can attract siderophore -producing microbes involved in the solubilization of iron in rhizosphere mediated by two different processes (Lareen et al. 2016). One process is through chelating siderophores produced by microorganisms making iron more available to plants while the second strategy is through the reduction of Fe^{3+} into Fe^{2+} across plasma membranes (Römheld 1987).

It is now apparent that bacteria utilize regulatory systems called quorum sensing (QS) to sense their population density. Such systems are dependent upon the production of signalling molecules that activate specific genes when the signal reaches a critical threshold concentration. Such QS-regulated genes produce phenotypes that require coordinated behaviour to convey competitive advantage to the population (such as biofilm formation and pathogenesis). The best-characterized QS system is that driven by acylated homoserine lactone (AHL) molecules (Hayat et al. 2017). Researchers have demonstrated that in the presence of bacterial biofilm, AHLs secreted by both *Pseudomonas* and *Serratia* species have induced resistance against *Alternaria* in tomato (Schuhegger et al. 2006).

Once the bacteria recognize the presence of each other in their surroundings, signaling molecules change the transcription of genes which would directly modify their physiological status and activities, and lead to the coordination of their community (Lareen et al. 2016). This system creates changes in the adhesion and motility of bacteria that would themselves form a biofilm. Although, it has been thought that biofilms only form between bacteria of the same species, research

findings have concluded that biofilms can form in mixed genera of bacteria. These are called mixed biofilms and they represent the interaction between different types of microorganisms through quorum sensing which would influence the development, the shape and the protection of individual microbes of the newly formed community (Ansari et al. 2017). In fact, the biofilm formation for *Bacillus subtilis* is more influenced by bacteria from distinct genera compared to bacteria from the same genus (Shank et al. 2011).

2.4.2 Biofilms on seeds, sprouts and food crops

Microbes have a profound influence on plant health and productivity. Beneficial bacteria physically interact with surfaces of plants, and seeds forming complex multicellular aggregates of multispecies assemblies in a form of biofilm (Danhorn and Fuqua 2007). Biofilms have been described on the surface of alfalfa, broccoli, clover, and sunflower sprouts as well as cotyledons and hypocotyls (Fett 2000). The microbes contained in the biofilms are attached to each other and to the plant surface by a matrix, most likely composed of bacterial exopolysaccharide (EPS). EPS-producing bacteria can play a beneficial role in nature. For instance, EPS- producing bacterial consortium of *Aeromonas* sp. and two species of *Bacilli* alleviated salt stress in wheat (Ashraf et al. 2004), and other EPS-producing rhizobacterium (strain YAS34) associated with plant growth-promoting features protected sunflower from drought conditions (Alami et al. 2000).

2.4.3 Mechanisms of biofilm formation

2.4.3.1 Diversity of mechanisms that affect the biofilm formation

a) Quorum sensing

Several signals have been associated with quorum sensing and it is speculated that signal molecules in bacteria are gram stain- or species- related. For instance, AHLs (N-acylhomoserine lactones) are mostly produced by proteobacteria and synthesized by bacteria of the following genera:

Agrobacterium, *Erwinia*, *Pantoea* and *Rhizobium*. DSFs (*cis*-11-methyl- 2-dodecanoic acid) are mainly produced by *Xanthomonas,* gamma-butyrolactones are synthesized by *Streptomyces* spp. and oligopeptides by gram-positive bacteria (Danhorn and Fuqua 2007). Host plants detect the signaling molecules and respond to them. This creates a cross talk in the microbiome and structures biofilm associations (Rudrappa et al. 2008).

b) Phosphorus

Biofilm formation and nutrient composition of the soil are related. There is a direct correlation between the amount of available phosphorus in the soil and the degree of biofilm formation (Ghosh et al. 2019). Biofilm quantification suggested that lower concentrations of phosphorus led to a stronger biofilm among different species and strains of *Burkholderia*. This limitation could have positive or negative impacts on the biofilms formed as reported by Danhorn and Fuqua (2007).

c) Phase variation

Microbes in a biofilm differentiate into a diversity of phenotypes through a phenomenon known as "phase variation" (Danhorn and Fuqua 2007). This phenomenon also reported as "oscillations in phenotype" (Chia et al. 2008), and "on-off" switches (Brooks and Jefferson 2014) represents the fast change in the phenotypes along with genetic mechanisms and rearrangements (Garcia-Pastor et al. 2019). These genetic rearrangements could be internal or external to the cell and include an array of changes such as inverted segments of DNA, expression of silent genetic recombination and mobile transposons.

2.4.3.2 Motility and chemotaxis

The rhizosphere effect is a plant derived mechanism that alters the microbial composition of the microbiome (Altaf et al. 2017). The microbial distribution varies among lateral and mature roots,

and root tips (Badri and Vivanco 2009; Faoro et al. 2017; Rudrappa et al. 2008; Sasse et al. 2018; Schmidt et al. 2018; Tovi et al. 2019). Highly motile bacteria capable of chasing root exudates are stronger and capable of reaching towards root tips and hairs (Turnbull et al. 2001) which creates a denser bacterial biofilm around them.

2.4.3.3 Surface Adhesins

Before aggregating into a biofilm structure, bacteria sense the surface that they will colonize through the production of adhesins. These are proteins that allow the attachment of the bacteria to the roots of plants (Kline et al. 2009; Mhedbi-Hajri et al. 2011; Nigmatullina et al. 2015) and come in various structures including type 1 pili, P-pili, type IV pili, curli for gram negative bacteria, as well as toll like receptors for gram positive bacteria (Kline et al. 2009).

2.4.3.4 Matrix Components

To ensure an effective colonization of the microbes into the root system of the host, the biofilm matrix includes an array of metabolites like extracellular proteins, cell surface adhesins and protein subunits of cell appendages such as flagella and pili. Proteins in the matrix are key in defining its structure and strength.

2.4.3.5 Development of biofilm

Populations of bacteria usually live freely in a planktonic state or attached together in a sessile form as a biofilm. Microbes in a biofilm produce a mixture of metabolites like proteins, DNA, exopolysaccharides and extracellular polymeric substances (Altaf et al. 2017; Solanki et al. 2020) and are affected by many environmental and ecological factors surrounding them (Altaf et al. 2017; Emanuel et al. 2010; Morris and Monier 2003; Solanki et al. 2020). The formation of a biofilm is complex and occurs in a series of developmental stages: (1) initial contact and attachment to the surface, (2) micro-colony formation and maturation, (3) the architecture formation, (4) detachment.

In the initial contact stage, biofilm formation requires the attachment of bacterial cells to the surface using pilli and flagella as means for attachment. Fimbrial adhesins are also important in the formation, attachment and stability of the biofilm (Solanki et al. 2020) as is hydrophobocity of surfaces (Liu et al. 2014). Once attachment happens, the non-flagellated bacteria increase in number and begin forming a two-dimensional strong layer composed of polysaccharides, proteins and nucleic acids. This layer is the hub of communication between bacterial cells that are connected by cohesive forces and strong bonds due to the presence of the polysaccharide intercellular adhesion polymers and the divalent cations (Singh et al. 2020). This process of multiplication and division is responsible for the formation of micro-colonies which themselves form different types of micro-communities that make the final state of the biofilm. Cells in the biofilm engage in cell signaling known as Quorum Sensing, QS. This leads to the production of autoinducers which are signaling molecules that induce the expression of genes responsible for the building blocks of the extracellular matrix (Jamal et al. 2018). The detachment of the biofilm is triggered by the depletion of nutrients and oxygen which allows the bacteria to spread and switch from the sessile mode to the motile form to compensate for the shortage of nutrients. This phenomenon happens with the help of the saccharolytic enzymes produced by the biofilm facilitating the lysis of polysaccharides and the discharging of the bacterial cells on the surface towards new colonization (Jamal et al. 2018; Singh et al. 2020; Solanki et al. 2020).

2.5 CONNECTING TEXT

Chapter 3 describes the molecular characterization and the functional biochemical traits of the microbial diversity associated with timothy. This study is of paramount importance to understand the full potential of timothy's endophytes in improving plant productivity and stress tolerance. Some of the bacterial isolates exhibited several attractive functional attributes such as growth promotion abilities, biocontrol potential and efficient colonization of timothy. These isolates represent the first evidence of bacterial endophytes that have the necessary functional attributes to protect cool season forage grasses against abiotic stress.

D. Saleh helped design the experiments, performed all experiments and analysis and wrote the first draft of the manuscript. Dr. Jabaji helped conceive the idea of the experiment as well as provided insights on the experimental design. Dr. Aliferis performed the orthogonal partial least squares-discriminant analysis of the bacterial isolates based on the recorded biochemical features. Mr. Joseph Jarry, a summer student funded by NSERC USRA program helped in performing experiments related to the production of biosurfactants, volatiles and in-vitro antagonistic activity. Mamta Rani helped in the molecular identification of the endophytes and in performing the IAA test and in preparing the microbiological media for the biochemical assays. Funding to perform the experiments was provided by Dr. Jabaji who helped edit and corrected several versions of the manuscript. The manuscript was written by D. Saleh and revised by all authors prior to publication in the *Journal of Applied Microbiology*.

CHAPTER 3

Diversity, distribution, and multi-functional attributes of bacterial communities associated with the rhizosphere and endosphere of timothy (*Phleum pratense* L.)

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3.1 ABSTRACT

Aims: To characterize the bacterial communities of the rhizosphere and endosphere of the forage grass timothy (*Phleum pratense* L.) and evaluate the functional attributes with respect to growth promotion properties, antimicrobial and biosurfactant capacities.

Methods and Results: A total of 254 culturable bacteria were identified using 16S rRNA sequencing and grouped into 16 taxa that shared high homology of 98–99% with other known sequences. A majority of the isolates were recovered from the rhizosphere soil fraction and leaf and crown tissues. *Bacillus* genus was the most abundant in the bulk and rhizosphere soil fractions. Isolates belonging to the *Methylobacterium* genus were exclusively found in leaves making them tissue specific. A majority of the bacterial isolates exhibited multi-functional growth promotion attributes and plant stress improvement related to the production of indole 3-acetic acid, VOC and siderophores and polymer degrading enzymes and 1-aminocyclopropane-1-carboxylate (ACC) deaminase activities. Some demonstrated antimicrobial properties such as hydrogen cyanide and biosurfactant production and activities of fungal cell wall degrading enzymes. The internalization and spread of selected bacterial isolates in timothy seedlings under gnotobiotic conditions was confirmed using the culture-dependent method and SEM microscopy in proof-of-concept experiments.

Conclusions: The attributes of some isolates with respect to growth promotion abilities, biocontrol potential and efficient colonization of timothy make them desirable for future development as potential biofertilizer tools.

Significance and Impact of the Study: This study provides the first evidence of bacterial endophytes that have the necessary functional attributes to protect cool-season forage grasses against abiotic stress.

Keywords: bacterial communities, endophytes, endosphere, biosurfactants, functional traits, rhizosphere, timothy.

3.2 INTRODUCTION

Forage-based dairy and livestock production are foundation segments of agriculture in the province of Québec in economic terms (Bélanger et al. 2006). Timothy (*Phleum pratense* L.) is one of the most widely grown cool-season forage grass in the region due to its demonstrated persistence and tolerance to low temperatures and ice encasement. However, its regrowth under prolonged hot and dry conditions is poor due in part to a shallow fibrous root system (Bertrand et al. 2008). With predicted global climate change, rising temperatures and atmospheric greenhouse gases, winter hardy grasses such as timothy could be severely impacted (Piva et al. 2013). These changes will likely negatively affect timothy regrowth and yield (Jing et al. 2012) and its nutritive value (Bertrand et al. 2008). Plants develop a diverse range of strategies to cope with biotic and abiotic stresses. One approach is to establish an on-going relationship with beneficial microbes such as bacterial endophytes, termed plant growth promoting bacteria (PGPB) that enhance plants' ability to manage stress as well as to facilitate their growth and development. For example, bacterial endophytes can confer fitness benefits to plants including increased root and shoot biomass, augmented yield, and improved tolerance to abiotic stress (Gagné-Bourque et al. 2016; Rolli et al. 2015; Sheibani-Tezerji et al. 2015; Su et al. 2015). Therefore, the prospect of exploring the development of endophytic bacteria as a potential strategy to address climate change associated stresses should not be overlooked.

Forages and grass seeds sustain a particular profile of bacterial communities (Ikeda et al. 2006) and vertical transmission from one generation to the next via the seed may be one of the major sources of bacterial endophytes in some perennial grasses including *Miscanthus*, timothy, switchgrass and bent grass (Cope-Selby et al. 2017; Gagné-Bourgue et al. 2012; Truyens et al. 2015).

Despite this knowledge, reports on the diversity of bacterial communities hosted by different plant tissues of pasture and forage grasses are limited.

A recent study focusing on the occurrence of endophytes in timothy showed that fungal endophytes belonging to 10 different taxa of the Ascomycota are associated with timothy blades (Varvas et al. 2013). However, studies directed at the occurrence, diversity and functional properties of bacterial communities in vegetative tissues of timothy are underexplored. Knowledge of this diversity is needed to realize the full potential of timothy's endophytes in improving plant productivity and stress tolerance. Bacterial endophytes are well known for their potential to improve plant growth by direct mechanisms involving the microbial synthesis of phytohormones and volatile organic compounds (VOC), the potential to produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase, causing a substantial alteration in ethylene levels in the plant, improving root system growth, assisting plants in acquiring nutrients such as phosphate and nitrogen and the synthesis of siderophores and the production of plant's lytic enzymes (Kandel et al. 2017; Liu et al. 2017). Indirect promotion of plant growth can involve the synthesis of enzymes that degrade cell-wall of plant pathogens and the production of antimicrobials, biosurfactants and hydrogen cyanide (Kandel et al. 2017; Liu et al. 2017). Therefore, isolation and characterization of endophytic bacteria with various promising properties from unexplored hosts such as timothy may have many applications to improve plant productivity and stress tolerance.

Taken together, this study is aimed primarily at (i) establishing the diversity of populations of culturable bacteria that are plant-associated and isolated from bulk soil and rhizosphere soil (i.e., the soil bound to roots) and from the endosphere (i.e., the internal tissues of plant) of field-grown timothy, (ii) determining their putative identities using molecular-based techniques, and (iii)

examining them for a wide array of functional traits which are involved in direct or indirect mechanisms that might increase early timothy growth (i.e., PGPB traits). A select number of isolates were validated for their internalization and systemic spread in timothy seedlings and root promotion ability.

3.3 MATERIALS AND METHODS

3.3.1 Field site selection, plant, and soil sampling

Three field sites located in Sainte-Anne-de-Bellevue, Québec, Canada were selected for sampling. Field site 1 (45’25'30″N; 73’ 55'35″W) was seeded in 2002 as a mixture of timothy (cv. Champ), tall fescue [*Schedonorus arundinaceus* (Schreb.) Dumort.], meadow bromegrass (*Bromus bibersteinii* Roem. & Schult.) and Kura clover (*Trifolium ambiguum* M. Bieb). It was managed under a three to four harvests regime per year with no fertilizer or pesticide application. Field site 2 (45'25'35″N and 73’ 55'31″W) was planted with timothy (cv. AC Alliance) in 2016. Timothy was cut and harvested three times per year and fertilized following local recommendations based on soil tests (Centre de référence en agriculture et agroalimentaire du Québec 2010); no pesticides were used. Field site 3, (45'25'57″N and 73'55'26″W) was planted in 2003 with the same mixture as described for field site 1 but was managed differently, following simulated intensive grazing with cuts every 15 days. The field was neither fertilized nor treated with pesticides.

Sampling was conducted in October 2016. A total of three replicate samples (four plants per sample), their associated rhizosphere soil (20 g) and surrounding bulk soil (20 g) were randomly collected from every field, placed separately in Ziplock bags, stored at 4^{o}C in a refrigerator and processed within 48 h. Leaves and crowns (four of each) from each sample were cut into sections of

0.5 cm in width and surface sterilized by stepwise washing procedure with ethanol and sodium hypochlorite according to Schulz et al. (1993) and processed separately. Samples of the rhizosphere and bulk soils of each field site were pooled and three subsamples (10 g each) were processed separately.

3.3.2 Isolation and maintenance of culturable bacteria

3.3.2.1 Preparation of plant tissue

Homogenates of surface sterilized leaves and crowns of each sample were prepared by grinding 300 mg of each tissue in 3 ml of sterile water, serially diluted (10^{-1} - 10^{-5} ml^{-1}). An aliquot of 100 µl from each dilution was plated on the following different culture media: glucose yeast extract, nutrient broth supplemented with 15% agar (GYEN; Germida and Casida 1980), tryptic soy agar (TSA) and Lysogeny broth Agar (LBA) (BBL, New York, NY). All plates were incubated for 2- 4 days at 22–24°C. The efficiency of the sterilization procedure was tested using the imprint method (Schulz et al. 1993). Plant tissue sections whose imprints on culture media showed epiphyte growth were discarded. An absence of growth of epiphytes on the imprinted culture medium indicates that the surface sterilization procedure was effective.

3.3.2.2 Preparation of rhizosphere and bulk soil samples

The rhizosphere is defined as the soil fraction adhered to the roots and is differentiated from the bulk soil that corresponds to the soil fraction that is non-adhering to the root and is outside the rhizosphere (Barillot et al. 2013). Rhizosphere soil adhered to the roots was collected by rinsing the roots of each sample in sterile water and shaking them in 0.15 mol l^{-1} phosphate buffer for 45 min at 2 *g* using an orbital shaker. Bulk soil of each sample (3 x 10 g) was ground using a sterile mortar and pestle in 100 ml of sterile distilled water. Aliquots (100 µl) of the buffer containing rhizosphere soil

and of bulk soil suspension were serially diluted (10^{-1} – 10^{-5} ml^{-1}), spread on GYEN, TSA and LBA media and incubated at room temperature (22 ± 2°C).

Emerging bacterial colonies from plant tissues and from both types of soils were subjected to three rounds of single cell isolation by streaking them on TSA medium to ensure purity. For long-term storage at 80°C, each bacterium was transferred into a well in a 96-well plate and grown on half-strength tryptic soy broth with 0.15% yeast at 27°C with agitation (2 *g*) for 48 h. Bacterial cells were preserved in glycerol (10% final concentration) and stored at -80°C.

3.3.3 Molecular identification

Bacterial cells were grown in LB broth at 27°C for 48 h with agitation (2 *g*) to achieve adequate growth and pelleted using centrifugation. Genomic DNA was extracted using direct colony PCR according to the procedure of Woodman (2008). Briefly, bacterial colonies were mixed with sterile water, incubated at 98°C for 10 min and subjected to sudden freezing with liquid nitrogen for an additional 10 min. Suspensions were centrifuged for 5 min and allowed to rest on ice for further use. DNA quality was confirmed on 1% agarose gel prior to subsequent reactions.

Bacterial primer pairs (27F; 5'-AGAGTTTGATCCTGG CTCAG-3' and 534R; 5'-ATTACCGCGGCTGCTGG-3') amplifying the positions of 27 and 534 of 16S rRNA genes were used in PCR assays according to published protocols (Gagné-Bourgue et al. 2012) to identify bacterial endophytes. Amplified products were sequenced at Génome Québec (Montreal, QC, Canada). Sequence results were checked for isolate identity using NCBI BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Isolates were putatively identified based on the lowest expected (E) value considered to be the most significant match.

3.3.4 Selection of bacterial isolates with putative plant growth promotion bacteria

In order to identify bacterial endophytes with potential growth promoting traits, a range of functional biochemical tests were performed in triplicate to characterize the endophytes' traits for growth promotion. Single bacterial colonies were grown in 4 ml of LB broth overnight (16– 18 h) with agitation at 2 *g*. Following appropriate dilution in LB broth, 10 µl of 10^6 CFU per ml was used in all tests unless otherwise stated.

3.3.4.1 Growth regulator production of indole 3-acetic acid and VOC

Estimation of indole 3-acetic acid (IAA) in 1 ml culture broth of each bacterial isolate was done using colorimetric assay following the method of Gordon and Weber (1951). The production of IAA from the transamination and decarboxylation reactions of tryptophan estimates the quantities of indole compounds produced by bacteria in a medium containing the precursor L-tryptophan.

The production of (volatile organic compounds VOC) associated with growth promotion ability and released by bacterial endophytes was tested following the method of Ryu et al. (2003). Briefly, polystyrene Bi-Plates (Fisher Scientific, Nepean, ON, Canada) containing 15 ml of Murashige and Skoog (MS) on one side and a mixture of LBA (5 ml) and tryptophan (5 mmol l^{-1}) on the opposite side. Timothy seeds (cv. AC Alliance) without their seed coats were surface sterilized in a stepwise fashion using hydrogen peroxide (30% w/w) for 7 min followed by three rinses of water of 5 min each. The seeds were germinated in petri plates lined with sterile filter paper that was moistened with 1 ml of sterile water containing (0.1 mg ml^{-1}) cycloheximide and placed in the dark for 2 days. Ten seeds with germ tubes of 15 mm length were placed on MS medium, while the LBA + tryptophan side was inoculated with 5 µl of bacterial inoculum (10^6 CFU per ml). Non-inoculated MS plates served as controls. Plates were parafilm-sealed and incubated for 12 days at 16 h/8 h of

light/dark at 22°C/20°C. Each bacterial inoculum was replicated 10 times and each replicate plate contained 10 pregerminated timothy seeds. Seedlings' dry weights were statistically compared to the control treatment.

3.3.4.2 ACC (1-aminocyclopropanecarboxylic acid) deaminase test

One of the efficient functioning roles of bacterial endophytes is the ability to produce 1-ACC deaminase. This enzyme degrades the ethylene precursor ACC, causing a substantial alteration in ethylene levels in the plant and improving root system growth (Glick 2005). Bacterial isolates were tested for the production of ACC deaminase as fully described by Penrose and Glick (2003) which measures the amount of α-ketobutyrate produced upon the hydrolysis of ACC. Briefly, bacterial pellets were suspended in 1 ml of Tris-HCl (pH 7.6), centrifuged at 16 000 *g* for 5 min and later resuspended in 600 ml of Tris-HCl (pH 8.5) with 30 µl of toluene. Suspensions were vortexed and incubated for 30 min at 30°C after the addition of 300 ml of 0.2% 2,4-dinitrophenylhydrazine reagent (Sigma-Aldrich Co., Oakville, ON, Canada), 2 mol l^{-1} HCl and 2 ml of 2 mol l^{-1} NaOH. ACC content was spectrophotometrically determined by measuring the absorbance at 540 nm after mixing the components.

3.3.4.3 Phosphate solubilization test

Phosphate solubilization may improve phosphorous availability to the plant (de Freitas et al. 1997). Phosphate-solubilizing efficiency assay was tested on modified Pikovskaya agar (Gupta et al. 2014) indicator plates supplemented with the following composition in g l^{-1} according to Delgado et al. (2014): glucose (10), $Ca_3(PO_4)_2$ (5), $(NH_4)_2SO_4$ (0.5), NaCl (0.2), $MgSO_47H_2O$ (0.1), KCl (02), yeast extract (0.5), $MnSO_4H_2O$ (0.002) and $FeSO_47H_2O$ (0.002), agar (15) and bromothymol blue (0.5% wt/vol). Each bacterial isolate (10 µl) was streaked in the center of the plate. Development of

clear halo zones after 48 h around the isolates exhibited their positive phosphate solubilization activity.

3.3.4.4 Urea solubilization

Some PGPB have the ability to break down urea into simple forms of nitrogen that can be readily absorbed by the plants to promote growth. A 10 µl bacterial culture was inoculated into Christensen's urea broth (Gerhardt et al. 1994). The color change of the medium from intense red to purplish characterized the positive reaction for urea hydrolysis, while the negative reaction was characterized when the medium remained with no change in color.

3.3.4.5 Calcium solubilization

Some endophytes are capable of hydrolyzing calcite (calcium carbonate) and assisting in plant growth (Puente et al. 2009). Ten microliters of test bacteria were streaked on Henderson's culture medium (Henderson and Duff 1963) at 30°C supplemented in g l^{-1}: calcium carbonate (2), yeast (2) glucose (2) and agar (15). Hydrolysis of calcite is indicated by a clear halo around the bacterial culture.

3.3.4.6 Siderophore production

Bacteria produce and secrete siderophores to sequester iron. The production of siderophores by endophytic bacteria may enhance plant growth by improving the uptake of iron or suppressing the colonization of roots by plant pathogens. Siderophore production was assessed by placing 10 µl of test bacteria on plates enriched with CAS medium prepared as follows: Chrome azurol S (CAS) 60.5 mg; hexadecyltrimetyl ammonium bromide 72.9 mg; Piperazine-1,4-bis (2-ethanesulfonic acid) (PIPES) 30.24 g; and 1 mmol l^{-1} $FeCl_3.6H_2O$ in 10 ml of 10 mmol l^{-1} HCl (Pérez-Miranda et al.

2007). Development of a yellow–orange halo around the growth was considered as positive for siderophore production.

3.3.5 Selection of bacterial isolates exhibiting plant's colonization traits

3.3.5.1 Cellulose degrading ability

Plant cell-wall degradation ability is one of the crucial traits for successful colonization of plant by endophytic bacteria (Liu et al. 2017). Rapid and sensitive screening for cellulose degrading ability of bacterial endophytes was performed on cellulose Congo-Red agar medium (Gupta et al. 2012) with the following composition in g l^{-1} of distilled water: KH_2PO_4 (0.5), $MgSO_4$ (0.25), carboxymethyl cellulose (2) g, agar (15), Congo-Red 0.2 g and gelatin (2), distilled water at pH 6.8–7.2. Colonies showing discoloration of Congo-Red were considered as positive cellulose degrading bacterial colonies.

3.3.5.2 Starch degrading ability

The ability of endophytic bacteria to degrade starch was used as the criterion for the ability to produce amylases. Bacterial cultures were inoculated on Difco Nutrient Agar amended with 0.25% starch, 0.5% peptone and 0.25% yeast and adjusted to pH 6.8–7.2. After 3 days of incubation, the plates were flooded with Lugol solution, a solution of potassium iodide (2 g) and iodine crystals (1 g) dissolved in 300 ml of double distilled water. A yellow clear zone around a colony indicates amylolytic activity.

3.3.5.3 Proteolytic degrading ability

The production of proteolytic enzymes by endophytic bacteria was tested on nutrient broth amended with skim milk (2%), Casein (0.5%), yeast extract (0.25%) and agar (15%) at pH 6.8–7.2

and incubated at 28°C for 48 h. A clear zone around the bacterial colonies indicates positive proteolytic activity.

3.3.6 Selection of bacterial isolates exhibiting antimicrobial traits

The ability of bacterial endophytes to produce cell-wall degrading enzymes, antimicrobial metabolites and allelochemicals that inhibit the growth of fungi and prevent diseases are traits of biological control potential (Compant et al. 2010).

3.3.6.1 Chitin and chitosan degrading ability

The production of chitinases and chitosanases on indicator plates was performed according to Murthy and Bleakley (2012) using colloidal chitin (CN) for chitinases or crab shell chitosan (Sigma-Aldrich) for chitosanases. The culture medium is composed of the following ingredients in g l^{-1}: colloidal chitin (15) or crab shell chitosan (5), peptone (5), yeast (0.25), glucose (1) and agar (15) at pH 6.8–7.2. Bacterial cultures (10 µl) were inoculated on the plates and incubated at 28°C for 1 week. Clearing zones produced around the colonies on colloidal chitin agar or on crab shell chitosan agar indicate the production of chitinases or chitosanases. The addition of bromothymol blue dye or Lugol iodine can intensify the clearing zone.

3.3.6.2 HCN production

One of the traits that contribute to endophyte competitiveness is the production of HCN. Bacterial cultures were grown in nutrient broth supplemented with 4.4 g l^{-1} of glycine. The production of HCN was detected after 48 h of inoculation by the color change of soaked Whatmann filter papers with picrate/Na_2CO_3 that is fixed to the inside of petri-plate lid as described in Bakker and Schippers (1987). A change in color from bright yellow to orange indicates the production of HCN.

3.3.6.3 Biosurfactant production

Several biosurfactants produced by endophytic bacteria have antimicrobial activity against plant pathogens (Santos et al. 2016). Five different assays (Table S3.1) were evaluated for biosurfactant activity of bacterial isolates. Isolates were ranked according to their performance in the oil-spreading assay (Youssef et al. 2004), while the remaining four assays were used to confirm biosurfactant production. Isolates were considered excellent biosurfactant producers if the clearing zone they produced in the oil-spreading assay was at least 1.0 cm in diameter. For all tests, bacterial cultures were grown for seven days at 30°C, centrifuged (6511 *g*) for 20 min at 4°C. Cell-free supernatant of each isolate was used in different tests. All tests had Triton X-100 as the positive control, water and LB broth as negative controls, and were replicated twice for each bacterial isolate.

3.3.6.4 In vitro antagonistic activity

The ability of bacterial isolates to inhibit radial growth of pathogenic fungi was tested using confrontation assay plates (Gagné-Bourgue et al. 2012). Duplicate plates were performed for each bacterial isolate and radial growth inhibition of the fungus was measured 5 days post confrontation.

3.3.7 Intrinsic antibiotic spectra

Antibiotic sensitivity of bacterial isolates was tested on LBA agar plates amended with antibiotics with 100 µl ml^{-1} of kanamycin, rifampicin (Sigma-Aldrich), streptomycin (Bioshop, ON, Canada) and tetracycline (Fisher Scientific) or with 125 µl ml^{-1} of ampicillin, gentamicin (Sigma-Aldrich), chloramphenicol (ICN Biomedicals, Cleveland, OH, USA) and hygromycin (Fisher Scientific). Bacteria were considered sensitive to an antibiotic at the concentration tested if no visible growth was observed on treatment plates and when there was visible growth on control plates after 48 h of incubation at 28°C.

3.3.8 Drought and salt stress tolerance of bacterial cells

Bacterial isolates were tested for their ability to grow and tolerate osmotic stress that is associated with drought and salt stress that is associated with salination. Isolates were grown in LB broth cultures (10^6 CFU per ml) supplemented with different concentrations of PEG8000: 6% (-0.81 MPa), 9% (-1.24 MPa), 10% (-1.5 MPa) or 20% (-6.34 MPa) or with 100 mmol l^{-1} of NaCl and incubated with agitation at 30°C for 24 h. Bacterial cultures with no PEG or without the addition of NaCl supplements served as controls. The ability of bacterial cells to grow in amended or non-amended culture broth was confirmed using serial dilution (10^{-1} to 10^{-8}) on LBA culture plates and estimated as CFU per ml.

3.3.9 Recolonization, internalization, and detection of bacterial isolates in plant tissues

As a proof-of-concept, we evaluated the ability of selected bacterial isolates, based on the production of phytohormones and volatile compounds, to internally colonize timothy seedlings. Timothy seeds (cv. AC Alliance) without their seed coats were surface sterilized and pregerminated on filter papers as previously mentioned. Pregerminated seeds were carefully placed on the surface of 12 g of moistened sterile sand particles with 2.5 ml of Hoagland solution in NifTAL tube system. The tubes were incubated with 16 h photoperiod, 150 µmol m^2 s^{-1} of light intensity and day/night of 22°C/20°C for 3 weeks. Timothy seedlings were soil-drenched with 400 µl (10^6 CFU per ml) of selected bacteria previously shown growth promotion capacity. Seedlings receiving 400 µl of distilled water or of suspended dead bacterial pellet served as controls. Each treatment was replicated 10 times.

Colonization of plant tissues and rhizospheric sand by bacterial endophytes was confirmed by cell counts 21 days post-inoculation (dpi). Stem and root tissues and rhizospheric sand (100 mg of

each) were homogenized and 100 µl of each sample was serially diluted and plated on LBA medium. Plates were incubated overnight at 30°C. Colony forming units (CFU) were determined and calculated per 1 ml of fresh weight of tissue or sand. Each treatment was replicated three times and each replicate contained samples of roots, leaves and rhizosphere sand of five experimental NifTAL tubes.

3.3.10 Electron microscopy for bacteria and tissue

Scanning electron microscopy imaging was conducted to visualize the external appearance of candidate bacteria and to visualize the localization of the bacteria over the surface of timothy grass roots.

Bacteria: Bacterial cells were incubated overnight in LB broth at 30 °C and later centrifuged at 8928 *g* for 5 min to recover the pellet. The pellet was fixed in 4% formaldehyde prepared in potassium phosphate buffer (0.1 mol l^{-1}, pH 7.1) for 30 min at room temperature. The pellet was washed twice with 0.1 mol l^{-1} potassium phosphate buffer and centrifuged for 5 min at 8928 *g*. The pellet was dehydrated using an increasing ethanol series ranging from 30 to 100% with the last step repeated three times and then followed by critical point drying (Leica EM-CPD300). The dried pellet film was coated with 5 nm of gold-palladium (Leica EM-ACE200) and examined using a Hitachi TM-1000 operating at 15 kV.

Roots: Colonized and noncolonized root tissues were fixed overnight in 100% methanol following the procedure of Neinhuis and Edelmann (1996). Samples were subjected to constant slow shaking at room temperature, followed by three washes of 100% ethanol. Each wash lasted 4 h. Tissues were subjected to critical point drying (Leica EM-ACE200), cut into longitudinal pieces or cross-sectioned and coated with 5 nm gold-palladium and observed with a Hitachi TM-1000 operating at 15 kV. The

sample preparation and image acquisition were performed at the McGill University Multi-Scale Imaging Facility, Sainte-Anne-de-Bellevue, Quebec, Canada.

3.3.11 Data analysis

One-way ANOVA was performed using the JMP 13.0 software to analyze biomass data of the volatile experiment and the percentage distribution of endophytes inside tissues of timothy in the NifTAL tube experiment. All experimental data were tested for statistical significance with differences between means being assessed using Tukey HSD ($P \leq 0.05$). Data were $\log_{10}$ transformed when required for normalization.

3.3.12 Orthogonal partial least squares-discriminant analysis of the bacterial isolates based on the recorded biochemical features

For the high-throughput functional classification of the bacterial isolates based on the recorded biochemical features, orthogonal partial least square analysis (OPLS) was performed using the SIMCA-P+ software version ver. 12.0.1.0 (Umetrics, Sartorius Stedim Data Analytics AB, Umea, Sweden) as previously described (Aliferis et al. 2013). The composed data matrix was composed of eleven biochemical tests (eight enzymatic assays, IAA production, siderophore and HCN) for all sequenced bacteria.

3.3.13 Accession numbers

The 16S RNA gene sequences of the bacterial isolates have been deposited in GenBank database under the following accession numbers (MH173890–MH173979; MH173981–MH174019; MH174021–MH174074; MH174184–MH174253).

3.4 RESULTS

3.4.1 Diversity, distribution, and taxonomic composition of bacterial isolates associated with timothy

A total of 476 culturable bacterial isolates were recovered from three replicate samples of bulk and rhizosphere soils and crown and leaf tissues (endosphere) of timothy grown in the different fields. Percent distribution of bacterial isolates in the three fields was comparable, with field 3 having the greater proportion of the total number of isolates (i.e., 36%; Fig. 3.1A). Interestingly, the genus *Arthrobacter* was cultivar-specific to Champ, while the genus *Pedobacter* was predominantly associated with the cultivar AC Alliance (Data not shown). More than 60% (305/476) of the culturable isolates were recovered from different fractions of the soil (bulk and rhizosphere) with a majority of them (193/476; 41%) recovered from the rhizosphere soil. Leaf and crown tissues harbored 106 and 65 isolates of culturable endophytes respectively (Fig. 3.1B).

A total of 319 out of 476 isolates were putatively identified using partial sequencing of the 16S rRNA gene using the primers 27F/534R. The remaining isolates did not yield good sequencing reads and were not pursued further. Out of 319 isolates, 254 were associated with five different phyla (Actinobacteria, α- and β-Proteobacteria, Bacteroidetes and Firmicutes) and 16 discrete genera that shared high homology (91 to > 99%) with known bacterial sequences in GenBank. The remaining isolated bacteria were grouped into others and represented 65 isolates (Table S3.2). The most frequently isolated bacteria belonged to the Gram-positive bacterium, *Bacillus* (89, 28%), the Actinobacteria *Streptomyces* (18, 6%) and the Gram-negative genera, *Pseudomonas* (23, 7%), *Pedobacter* (17, 5%) and *Pantoea* (15, 5%) (Fig. 3.1C).

Isolates belonging to *Stenotrophomonas*, *Pseudomonas*, *Chryseobacterium* and *Pantoea* genera were encountered in all plant tissues and bulk and rhizosphere soils (Fig. 3.2). More than 80% of the cultured bacteria were isolated from bulk (36/73) and rhizosphere (53/150) soil fractions and identified as *Bacillus* species (Fig. 3.2 A, B). The two other genera, *Arthrobacter* and *Brevibacterium* were recovered only from both soils and were not found in plant tissue (Fig. 3.2). Bacterial isolates originating from the rhizosphere fractions were distributed among 13 different genera, nine of which were common with the bulk soil fractions (Fig. 3.2B). *Streptomyces* (18/150), *Pedobacter* (6/150) and *Variovorax* isolates (7/150) were associated exclusively with the rhizosphere soil (Fig. 3.2B). The highest number of bacteria (4/33) isolated from the crown tissue belonged to *Pedobacter*, *Pseudomonas*, *Stenotrophomonas* and *Xanthomonas* (Fig. 3.2C). In the case of leaf tissue, isolates belonging to *Curtobacterium* (9/63), *Pantoea* (8/63) and *Pedobacter* (7/63) were the most abundant (Fig. 3.2D). Isolates belonging to *Methylobacterium* genus (6/63) were exclusively associated with leaf tissues (Fig. 3.2D).

3.4.2 OPLS-DA revealed a variable grouping among the bacterial isolates based on the recorded biochemical features

OPLS-DA analysis revealed a variable grouping among the bacterial isolates based on the recorded biochemical features. Bacteria sharing the same biochemical attributes were clustered close together (95% confidence interval) (Fig. S3.1). Typically, the isolates that had outstanding performance in terms of their biochemical attributes were detected outside the ellipse representing the 95% confidence interval. Additionally, a few other bacteria that were at the periphery of the ellipse, were also different from those clustered in the center. In total, 60 isolates that belong to 16 different genera were selected for additional assays related to volatile compounds production, antifungal activity, and biosurfactant-producing properties.

3.4.3 Assessment of plant growth promotion and plant colonization traits

All bacterial isolates were screened for their growth promoting properties and nutrient acquisition using a range of biochemical functional tests. Calcite solubilization, the production of siderophores showing varying intensities of CAS substrate hydrolysis and biosynthesis of the plant growth promoting hormone IAA, are the most commonly occurring functional traits of isolates across the 16 genera. Bacterial isolates belonging to 14 and 8 genera were related to urea and phosphorus acquisition, respectively (Table 3.1, Fig. S3.2). Quantitative measurement for VOC and ACC deaminase was done on the top performing seven bacterial isolates. The release of VOC by isolates (28, 50, 63, 120, 144, 295, 464) significantly ($P \leq 0.05$) triggered growth promotion in grass seedlings for a period of 12 days compared to the control treatments (Fig. 3.3). The best performing isolate was *Bacillus* (28) which caused a 32-fold increase in seedling's dry weight when compared to the control treatment. The same isolates were excellent IAA producers with concentrations ranging between 12.13 μg ml^{-1} for *Bacillus* (28) to 20.93 μg ml^{-1} for *Stenotrophomonas* (474) (Table 3.4). The same seven isolates showed a wide range of ACC deaminase activity ranging from 25 000 to 125 000 nmol of α-ketobutyrate mg^{-1} 30 min^{-1} (Table 3.4). Additionally, they demonstrated high tolerance to salt and drought stress conditions. All isolates were able to grow on 100 mmol l^{-1} NaCl or 20% PEG yielding 4 $\log_{10}$ CFU per ml (data not shown). Isolates belonging to all 16 genera possessed the ability to degrade cellulose. Isolates of the genus *Rahnella* failed to degrade starch and protein. Isolates of the genus *Pantoea* were not able to degrade protein (Table 3.1).

3.4.4 Assessment of antimicrobial traits

All isolates belonging to 16 genera had the ability to degrade fungal cell wall components. Isolates belonging to *Bacillus*, *Pseudomonas* and *Streptomyces* were found positive for HCN

production based on the color intensity developed on the picric acid impregnated filter papers (Table 3.1).

Involvement of diffusible substances with antifungal activity released into the culture medium by the bacterial isolates was tested in confrontation assay plates. Out of 60 isolates, many bacterial isolates exhibited varying degrees of antifungal activity against five phytopathogens of different lifestyles (Table 3.2). Many bacteria were successful in reducing the radial growth of the test pathogens, displaying a large diameter of inhibition (>1.5 < 3 cm) (Fig. S3.2). *Bacillus* (isolate 333), *Brevibacterium* (isolate 70), *Pseudomonas* (isolates 12 and 17) and *Streptomyces* (isolate 48) displayed strong antifungal activities against all or 3/5 fungal pathogens during co-culture assays (data not shown).

Depending on the biosurfactant assay (Table 3.3), the number of positives ranged from nine to 23 isolates with response levels varying from weak (+) to moderate (++) to strong (+++) clearing diameters. Twenty-three isolates were identified as biosurfactant-producing bacteria using the oil-spreading test (Table 3.3, Fig. S3.2). Surprisingly, the same isolates (isolates 33, 70, 12, 17 and 48) that displayed strong antifungal activities in co-culture plate assays consistently displayed a strong clearing diameter (data not shown). Additionally, isolates 48, 17 and 12 displayed moderate clearing diameters using the drop collapse test. The same isolates except for *Pseudomonas* (isolate 17) displayed a positive reaction in the CTAB test.

Most of the bacterial isolates were susceptible to rifampicin and did not grow (data not shown). Many were highly affected by the remaining tested antibiotics. Isolates were more resistant to ampicillin; however, the highest resistance was detected with hygromycin (data not shown) which

means that these microbes are capable of competing with the surrounding bacteria in the microbiome without being suppressed by their presence.

3.4.5 Recolonization, internalization, and detection on bacterial isolates in tissues of timothy

Reisolation and quantification of *Pseudomonas* (isolate 234), *Bacillus* (isolate 28) and *Chryseobacterium* (isolate 120) by the plating method demonstrated that the isolates can develop sustaining populations in the rhizosphere sand and in root and leaves tissues (Table 3.5). Population numbers of isolates 234 and 120 were consistently higher in roots than in the rhizosphere. Population numbers of all isolates in leaves were generally lower than in roots indicating that successful translocation from roots to the upper timothy tissues had occurred. No bacterial colonies were present in tissues of timothy seedlings receiving distilled water or dead bacterial pellet (Control treatments). Phenotypically, timothy seedlings inoculated with live bacterial isolates had more root mass compared to the control treatments (Fig. S3.3).

3.4.6 Microscopy

Phenotypically, timothy seedlings inoculated with live bacterial isolate (120) had more root mass compared to the control treatments (Fig. 3.4A; Fig. S3.3). This observation is supported by SEM images of the same roots (Fig. 3.4B, C). Substantially more hair roots are formed in bacterized roots compared to non-bacterized roots (Fig. 3.4B). SEM micrographs revealed bacterial presence over the surface and inside of the root (Fig. 3.4D). The presence of bacterial cells adhered to the cell walls was observed either as isolated (Fig. 3.4D; white arrows) or forming groups of bacterial cells surrounded by a self-produced matrix possibly biofilm (Fig. 3.4D; white stars). Regarding their shape, bacterial cells were predominantly rod shaped (Fig. 3.4E).

3.5 DISCUSSION

This is the first report describing the diversity, abundance and the distribution of culturable bacteria associated with timothy. The microbial isolate richness of plant-associated bacteria depends on the type of culture media used for their isolation. Bacterial isolates from both bulk and rhizosphere soils were successfully isolated on different non-defined complex microbiological media containing amino acids, carbon and salts with yeast extract, tryptone or starch to support the growth of the largest possible number of bacterial isolates (Eevers et al. 2015). Such culture media may not support the isolation of microbial isolates that are slow-growing or unable to grow. Full estimation of the abundance and diversity of unculturable bacteria of timothy grass is now possible by high-throughput next generation sequencing (Akinsanya et al. 2015).

Our results show that a greater portion of the relative abundance of the bacterial microbiome (64%) is associated with the bulk and rhizospheric soil fractions than the plant tissue. The microbiome profiling of these fractions was distributed among Actinobacteria, α- and β-Proteobacteria, Bacteroidetes and Firmicutes phyla. The similar distribution of microbial phyla of the rhizosphere and bulk soils was also reported for all soil samples of plant-free pots (Bulgarelli et al. 2013). Our results also show that the proportion and diversity of the genera inhabiting the rhizosphere soil (41%) was greater when compared with the surrounding bulk soil (24%). Of particular interest, are the enrichment and sole presence of rhizosphere competent isolates such as *Curtobacterium*, *Pedobacter*, *Streptomyces* and *Variovorax*, indicating that selectivity of timothy root to certain rhizosphere bacterial taxa could be influenced by root exudation and host-derived metabolites leading to differences in the bacterial community structure between rhizosphere and bulk soil. This phenomenon known as the rhizosphere effect (Berendsen et al. 2012) has been reported in

several studies (Bulgarelli et al. 2012; Lundberg et al. 2012; Tan et al. 2013). Root exudates are not the only key determinants of the rhizosphere's microbiome structure, but the composition can also be influenced by many other factors including plant genotype, developmental stage and cultivation practices (Edwards et al. 2015; Johnston-Monje et al. 2016; Wemheuer et al. 2017; Yu et al. 2015). Compared with field site 2, field sites 1 and 3 in this study are well-established fields that were planted with timothy cultivar Champ 15 and 16 years ago respectively but were managed differently. *Arthrobacter* isolates were associated with Champ cultivar and not with the AC Alliance cultivar (Field site 2). It is believed that bacterial endophytes adapt well to their host over time and might even become specific to a certain cultivar (Sturz et al. 2000). This genotype-dependent taxonomic distribution is also reported in other crops like wheat and cannabis (Winston et al. 2014; Yanni et al. 2016). Taken together, it seems that the genotype and stand age are likely more important than management practices in influencing timothy's microbial diversity. Another contributing factor that shapes endophytic diversity is plant tissue type. For example, *Methylobacterium* isolates originated exclusively from the leaves of timothy, making this genus tissue specific. This is not surprising since members of this genus are one of the most associated genera in the phyllosphere of plants (Knief et al. 2010).

The plant's interior is colonized by a range of bacterial endophytes which are mostly derived from the rhizosphere or vertically transmitted from seeds, with the majority of the bacterial taxa detected inside the seeds being similar to common soil isolates (Compant et al. 2010). In this study, we did not determine the presence and diversity of endophytic bacteria in timothy seeds. Therefore, it is not possible to discount the likelihood that the results reflect a subset of the bacterial population originated from the seed. Members of the majority of bacterial genera were likely derived from the

soil, migrated to the rhizosphere and eventually found their way to the internal tissues of timothy. These results signify the ability of bacterial isolates to enter the roots either passively via cracks or actively by releasing cell wall degrading enzymes and exist as endophytes (Hardoim et al. 2015). Thus, it is not unexpected that the endosphere of the aerial part of the plant has significant overlap with those found in the rhizosphere (Chi et al. 2005). Organ and tissue type have been reported as determinants of endophyte distribution and colonization. In this study, the crown and leaf tissues of timothy had six genera in common, however, each was exclusive to either one taxon in crown tissue or to a few in leaf tissue. The distribution pattern of bacterial endophytes in different plant organs is supported by several studies of other plants including rice and industrial hemp (Compant et al. 2011; Scott et al. 2018; Walitang et al. 2017).

Rhizospheric and endophytic bacteria can enable plant growth promotion through the acquisition of resources from the environment and enhancing the availability of many nutrients by the production of hormones, VOC, enzymes capable of solubilizing unavailable elements and hydrolytic enzymes capable of degrading plant cell walls. In this study, isolates of the 16 different genera were successful in producing hydrolytic enzymes, rendering them efficient colonizers due to their capacity to degrade cellulose and systemically colonize plant tissues (Hardoim et al. 2015; Zhang et al. 2017). Fourteen bacterial genera demonstrated their capacity to produce proteases, a recently established enzyme known for its ability to suppress a wide range of plant pathogens (Singh and Chhatpar 2011). Interestingly, isolates belonging to 15 different taxa demonstrated their ability to produce amylases. Endophytes associated with papaya fruit also possessed the ability to produce amylases (Krishnan et al. 2012). From biotechnology point of view, amylolytic and proteolytic enzymes of endophytes are promising sources for the detergent industry (Zaferanloo et al. 2013).

Microbial phosphate accessibility is a vital attribute in PGPB. Bacterial isolates that were derived from the rhizosphere and the endosphere of timothy (this study) and from different crops (Dinić et al. 2014; Gagné-Bourgue et al. 2012; Khan et al. 2014; Oteino et al. 2015) belong to *Bacillus*, *Brevibacterium*, *Chryseobacterium*, *Curtobacterium*, *Methylobacterium*, *Paenibacillus*, *Pseudomonas*, *Stenotrophomonas* and *Xanthomonas* and had the capacity to solubilize mineral phosphate and potentially making it available to the plant. This attribute along with the ability to access nitrogen forms from urea and also produce cellulose degrading enzymes is indicative of their nutrient delivering capacity while interacting with plant hosts and represents a promising biofertilizer tool.

In addition to providing plants with nutrients, rhizospheric and endophytic bacteria are capable of boosting growth through the production of phytohormones and siderophores (Ahmed and Holmström 2014; Pieterse et al. 2009). Generally, bacterial isolates associated with timothy are good producers of indole acetic acid (IAA) and siderophores. IAA production is a common feature of endophytic bacteria, boosting plant growth by stimulation of root and shoot cell division and elongation (Radhakrishnan et al. 2017). The production of siderophores by bacteria supports plant growth, by complexing iron in the soil and producing soluble compounds that can be absorbed by the plant (Nadeem et al. 2012). In this study, seven isolates belonging to *Bacillus* (28, 144), *Brevibacterium* (63), *Chryseobacterium* (120, 295), *Microbacterium* (50) and *Stenotrophomonas* (474) recovered from the soil and the endosphere were identified as the top producers of IAA and siderophore in our bacterial collection. Taken together, these functional traits of PGPR effectively supply plants with nutrients and reduce the need of inorganic fertilizers and promote low-input agriculture.

Beneficial bacteria such as *Bacillus*, *Pseudomonas*, *Burkholderia* and *Serratia* produce complex blends of VOC that indirectly help the promotion of plant growth and trigger plant immunity (Chung et al. 2016). One of the most studied volatile compounds that conferred plant resistance is 2,3-butanediol (Ryu et al. 2003). In our study, we did not attempt to identify the volatile compounds. Instead, we have conducted an exhaustive screening of the rhizospheric and endospheric bacteria for their capacity to produce volatiles and their functional effect on timothy seedlings. A significant growth promotion of timothy was displayed by the same seven isolates that exhibited high IAA and siderophore production capacity. Similar growth promotion patterns using the same experimental setup were reported in switchgrass and industrial hemp seedlings that were exposed to volatiles emitted by bacterial endophytes (Gagné-Bourgue et al. 2012; Scott et al. 2018).

These results indicate that bacterial volatiles are not limited to endospheric isolates but are also present in rhizospheric isolates (D' Alessandro et al. 2014). Therefore, volatiles are potentially effective to serve as biostimulant of plant growth and inducer of plant defense. It remains to be determined what specific compounds pertaining to our isolates stimulated timothy seedling's growth.

Another strategy that soil organisms employ to facilitate plant growth and development is the production of ACC deaminase which prevents plant ethylene signaling resulting in plants more tolerant to environmental stress such as drought (Glick 2005). It has been reported that bacterial isolates with ACC deaminase activity of $\geq$ 20 nmol mg^{-1} h^{-1} of α-ketobutyrate are considered growth promoters (Penrose and Glick 2003). All the seven tested isolates had a high enzyme activity with more than 20 nmol mg^{-1} h^{-1} of α-ketobutyrate, most notably isolate 28 exhibiting an activity of 0.05 mmol l^{-1} α-ketobutyrate. It remains to be seen whether these select bacteria can promote timothy root development and elongation under environmental stressed conditions.

The production of siderophores and HCN by microorganisms represents a winning functional feature that makes them successful competitors in several environments (Loaces et al. 2011). HCN is a potent secondary metabolite, when produced in sufficient quantities by microorganisms it may suppress plant pathogens and can indirectly enhance plant growth. In our study, HCN producers were members of *Bacillus*, *Pseudomonas* and *Streptomyces* that represent the largest taxa with the highest numbers of isolates. These genera have been previously reported as potent HCN producers (Ahmad et al. 2008; Anwar et al. 2016) and may have competitive advantages to colonize plant tissues and to exclude other micro-organisms from the same ecological niche.

Soil microbes and endophytes produce a variety of antimicrobial compounds including enzymes, antibiotics and biosurfactants making them potentially ideal biological control agents. In this study, isolates belonging to all 16 genera had the capacity to degrade chitin and chitosan which helps in the suppression of fungal growth (El Hadrami et al. 2010; Quecine et al. 2008). Also, a majority of the isolates displayed inhibition zones against tested plant pathogens under in vitro confrontation assays probably as a result of their capacity to produce antibiotic and low molecular surface-active compounds such as biosurfactants (Santos et al. 2016). To the best of our knowledge, most studies dealing with the microbiome of the rhizosphere and endosphere of plants focus on screening the culturable isolates for HCN and siderophore production and rarely for biosurfactant production. In this study, the screening of 60 bacterial isolates for biosurfactant production and emulsification activities using several well-established methods (Walter et al. 2010) confirmed the production of biosurfactants. It is noteworthy mentioning that the top five biosurfactant-producing isolates are also among the best siderophore producers and the most effective isolates with potent antifungal activity against plant pathogens. These isolates were derived from the rhizosphere of

timothy and are members of the genera *Bacillus* (333), *Brevibacterium* (70), *Pseudomonas* (12, 17) and *Microbacterium* (48). These results indicate that these isolates produce a mixture of surface-active compounds including lipopeptides and they can be explored as effective biocontrol agents against important plant pathogens.

One of the goals of this study was to confirm the internal colonization and spread of select bacterial isolates in timothy seedlings under gnotobiotic conditions. Population numbers of isolates belonging to *Bacillus* (28), *Pseudomonas* (234) and *Chryseobacterium* (120) were sustained at reasonable densities in the rhizosphere and endosphere (roots and leaves) of timothy. These results confirm that timothy grass recruited them to their root surfaces and allowed them to enter the root interior and move upwards to the leaves. For these isolates to colonize plant tissue and become endophytic, they either entered the root through passive penetration sites or through active penetration sites using cell wall degrading enzymes such as cellulases and pectinases (Naveed et al. 2014; Truyens et al. 2015). It is highly probable that the isolates actively penetrated the roots because of their capacity to degrade cellulose, protein, and amylose.

Our results are in agreement with previous reports that some bacteria, mostly from the rhizosphere, are able to colonize the internal tissue of the plant and migrate to the upper tissues of the plants (Gagné-Bourgue et al. 2012; Kandel et al. 2017). Colonization of plant interior by endophytes has been found to increase plant growth (Compant et al. 2010). Our isolates are strong producers of IAA and phenotypically promoted not only shoot and root lengths but also showed through scanning electron microscopy a qualitative increase in the number of root hairs of timothy seedlings in soil drench experiments. These results clearly indicate that enhancement of timothy grass growth is a direct effect caused by the isolates.

In summary, this study presents the first evidence on the distribution and diversity of taxonomic groups of bacteria associated with the bulk and rhizosphere soil fractions and with the endosphere region of timothy. Their biochemical traits allowed us to focus on a subset of isolates that have the potential to be excellent growth promoters of grasses due to a combination of several functional traits including IAA, ACC deaminase and siderophore production and other nutrient providing activities. The combination of growth promotion abilities and efficient colonization of timothy grass seedlings with notable increase in root growth by tested isolates (28, 120 and 234) make them desirable in the future development of PGPR inoculant consortium for timothy. Future experiments will focus on the effect of multiple endophytes on the growth of timothy and their effect on the synthesis and release of low molecular weight organic acids from timothy roots.

3.6 ACKNOWLEDGEMENT

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3.7 AUTHOR CONTRIBUTIONS

D.H. and S.H.J. conceived and designed the experiments and wrote the paper. D.H. performed the experiments. J.J. helped in the antifungal and biosurfactant assays. M.R. helped in 16S rRNA sequencing, antibiotic resistance and biochemical assays. K.A.A. performed the PCA analysis. P.S. provided logistical support and helped in field sampling.

3.8 CONFLICT OF INTERESTS

The authors declare no conflict of interests.

Table 3. 1 Biochemical attributes of major bacterial genera associated with timothy grass.

		Plant colonization traits				Plant growth promotion traits				Antimicrobial traits		
Number of genera	**Genus (No. of strains)**	**Protease**	**Amylase**	**Cellulase**	**Calcite hydrolysis**	**Ca Phosphatase**	**Urease**	**Siderophores**	**IAA ¶ (µg/ml) ± S.D.**	**Chitinase**	**Chitosanase**	**HCN**
1	*Arthrobacter* (9)	++	+	+	+	-	+	+++	13.52 ±0.52	+	+	-
2	*Bacillus* (89)	++	+	+	+	+	+	++	13.27±0.17	+	+	+
3	*Brevibacterium* (6)	++	+	+	+	+	+	+++	14.42 ±0.94	+	+	-
4	*Chryseobacterium* (10)	++	+	+	+	+	+	+++	14.07 ±1.09	+	+	-
5	*Curtobacterium* (10)	++	+	+	+	+	+	+++	16.23 ±1.68	+	+	-
6	*Microbacterium* (11)	+	+	+	+	-	+	+++	13.29 ±0.41	+	+	-
7	*Methylobacterium* (6)	++	+	+	+	+	+	+++	13.31 ±0.8	+	+	-
8	*Paenibacillus* (8)	+	+	+	+	+	+	+++	13.35 ±0.62	+	+	-
9	*Pantoea* (15)	-	+	+	+	-	-	+++	13.67 ±0.48	+	+	-
10	*Pedobacter* (17)	+	+	+	+	-	+	+++	14.27±1.09	+	+	-
11	*Pseudomonas* (23)	++	+	+	+	-	+	+++	12.38 ±0.19	+	+	+
12	*Rahnella* (6)	-	-	+	+	-	-	+++	12.44 ±0.41	+	+	-
13	*Stenotrophomonas* (14)	++	+	+	+	+	+	+++	13.71±0.68	+	+	-
14	*Streptomyces* (18)	++	+	+	+	-	+	+++	13.50 ±0.11	+	+	+
15	*Xanthomonas* (5)	++	++	+++	+++	+	++	+++	17.39 ±4.51	+++	+++	-

16	*Variovorax* (7)	+	+	+	+	-	+	+++	13.15 ±0.78	+	+	-
Total		14	15	16	16	8	14	16		16	16	3

* '-' negative reaction; '+', positive reaction showing a clearing zone >0 ≤0.5cm; '++', positive reaction showing a clearing zone 0.5 ≤ 1cm; '+++', positive reaction showing a clearing zone 1 ≤ 1.5 cm.

† Siderophores: '-', negative reaction; '+', positive reaction showing a clearing zone >0 ≤0.5cm; '++', positive reaction showing a clearing zone 0.5 ≤ 1cm; '+++', positive reaction showing a clearing zone 1 ≤ 1.5 cm, '++++', positive reaction showing a clearing zone 1.5 ≤ 2 cm.

HCN: + genus majorly producing HCN; - genus not producing HCN.

¶ IAA, average values based on total number of strains in each genus ± standard deviation (SD). All tests were done in replicates of three.

Table 3. 2 Comparison of radial growth inhibition of phytopathogens in confrontation diffusion assays with 60 bacterial strains.

	Positive endophyte strains by fungus		
Phytopathogen	**Number of positives**	**Number (%) of strains with identical response**	**Response level†**
Fusarium graminearum	27	10 (16.66%)	+++
Rhizoctonia solani AG3	39	20 (33.33%)	+++
Botrytis cinerea	32	12 (20%)	+++
Fusarium solani	19	10 (16.67%)	+++
Sclerotinia sclerotiorum	23	8 (13.33%)	+++

† '+++', Positive response level showing a clearing zone > 1.5 $\leq$ 3 cm.

Table 3. 3 Comparison of methods for the detection of biosurfactant production by 60 bacterial strains.

	Positive by method			Negative by method		
Method	**Number of positives**	**Number (%) of strains with identical response**	**Response level**	**Number of negatives**	**Number (%) of negatives that were positives with other methods**	**Method number**
1) Drop Collapse*	17	10 (16.66%)	+	43	2 (3.33%)	2, 3
		7 (11.66%)	++			
2) Microplate	13	13 (21.66%)	+	47	2 (3.33%)	1, 3, 4,5
3) Oil Spreading†	23	15 (25%)	+	37	1 (1.66%)	1, 2, 4, 5
		3 (5%)	++			
		5 (8.3%)	+++			
4) Emulsification	10	10 (16.67%)	+	50	2 (3.33%)	1, 2, 3, 5
5) CTAB	9	9 (15%)	+	51	2 (3.33%)	1, 2, 3, 4

* Response level: '+' the drop size > 0.35 and ≤ 0.55cm, '++' the drop size > 0.55 and ≤ 0.7cm

† Response level: '+' the diameter of clearing between > 0.2 ≤ 1cm, '++' the diameter of clearing >1and ≤ 3cm, '+++' the diameter of clearing >3 ≤ 7cm.

Table 3. 4 Biochemical attributes of the top bacterial strains.

Strain number	Genera	Siderophores (cm)	ACC deaminase activity (nmol α-ketobutyrate. mg^{-1} $30min^{-1}$)*± S.E.	IAA (µg/mL)
28	*Bacillus*	0.6	54,188 ± 6,427	12.13
50	*Microbacterium*	1.3	125,151 ± 42,905	15.57
63	*Brevibacterium*	1.8	74,222 ± 1,507	17.21
120	*Chryseobacterium*	1	25,947 ± 4,624	17.89
144	*Bacillus*	1	28,120 ± 2,720	17.79
295	*Chryseobacterium*	0.9	25,465 ± 6,797	12.57
474	*Stenotrophomonas*	1	20,6976 ± 11,989	20.93

*Values represent the average of 3 replicates ± standard error of the mean (S.E.).

Table 3. 5 Colony forming units (CFU/mL) abundance of bacterial strains in rhizosphere soil, roots and leaf

Strain number	Soil/Tissue	Log_{10} CFU/ml ± S.D.*
234	Leaves	3.9 ± 0.4
	Roots	6.3 ± 0.1
	Rhizosphere	5.2 ± 0.1
28	Leaves	3.9 ± 0.2
	Roots	3.5 ± 0.1
	Rhizosphere	4.8 ± 0.3
120	Leaves	4.3 ± 0.0
	Roots	4.7 ± 0.4
	Rhizosphere	3.9 ± 0.2

*Values represent the average of 5 replicates ± standard deviation (S.D.).

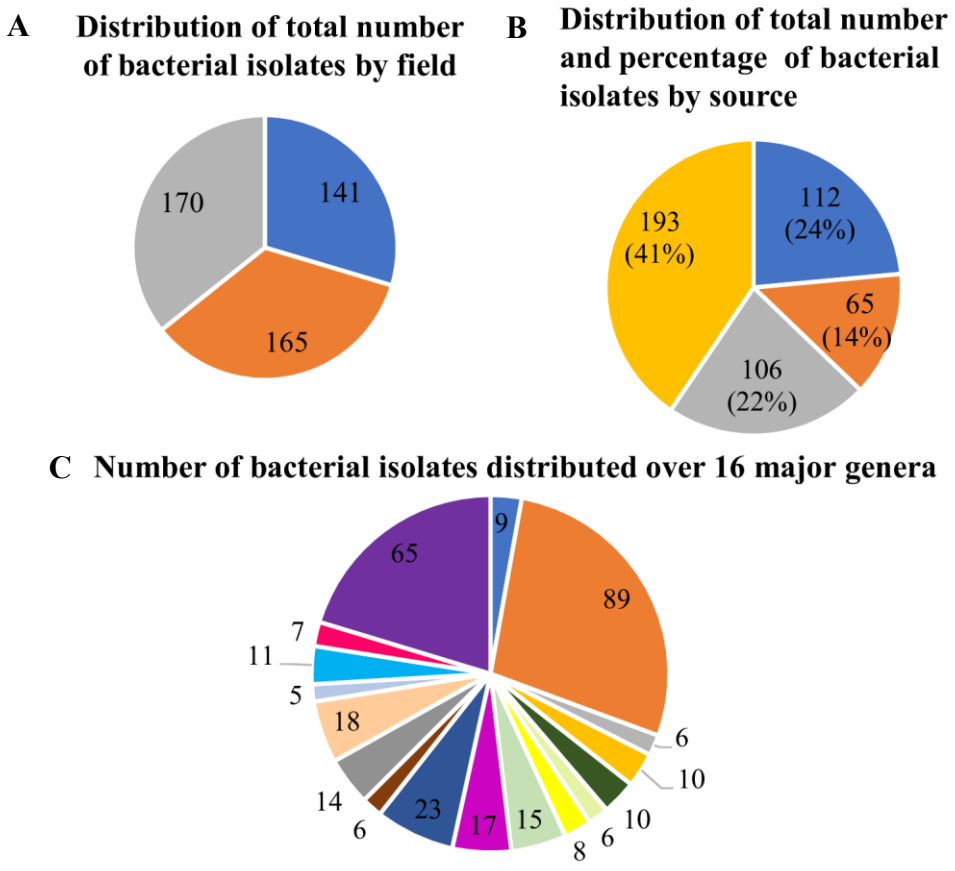

Figure 3. 1 The distribution and frequency of bacterial isolates recovered from timothy. (A) Number of bacterial isolates distributed across the three sampled fields (■) F1, (■) F2, (■) F3, and in (B) soil and internal tissues (■) Bulk, (■) Crown, (■) Leaf, (■) Rhizosphere. (C) Number of bacterial isolates distributed over 16 major genera identified based on partial sequencing based on 16S rRNA. Key of genera: (■) *Arthrobacter*, (■) *Bacillus*, (■) *Brevibacterium*, (■) *Chryseobacterium*, (■) *Curtobacterium*, (■) *Methylobacterium*, (■) *Paenibacillus*, (■) *Pantoea*, (■) *Pedobacter*, (■) *Pseudomonas*, (■) *Rahnella*, (■) *Stenotrophomonas*, (■) *Streptomyces*, (■) *Xanthomonas*, (■) *Microbacterium*, (■) *Variovorax*, (■) Others. Data represent the average of three-replicate samples.

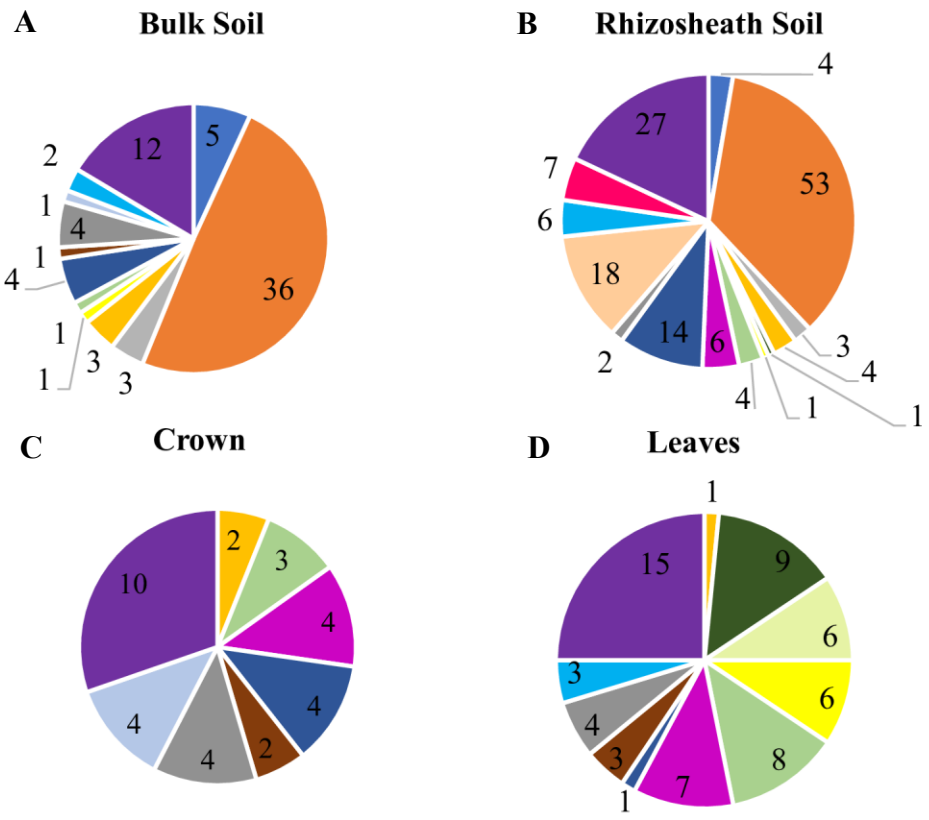

Figure 3. 2 Total number of culturable isolates in each taxon associated with timothy. (A) Bulk Soil, (B) Rhizosphere soil, (C) Crowns, (D) Leaves. Total number was calculated from three-replicates of each sample. Key of genera: (■) *Arthrobacter*, (■) *Bacillus*, (■) *Brevibacterium*, (■) *Chryseobacterium*, (■) *Curtobacterium*, (■) *Methylobacterium*, (■) *Paenibacillus*, (■) *Pantoea*, (■) *Pedobacter*, (■) *Pseudomonas*, (■) *Rahnella*, (■) *Stenotrophomonas*, (■) *Streptomyces*, (■) *Xanthomonas*, (■) *Microbacterium*, (■) *Variovorax*, (■) Others.

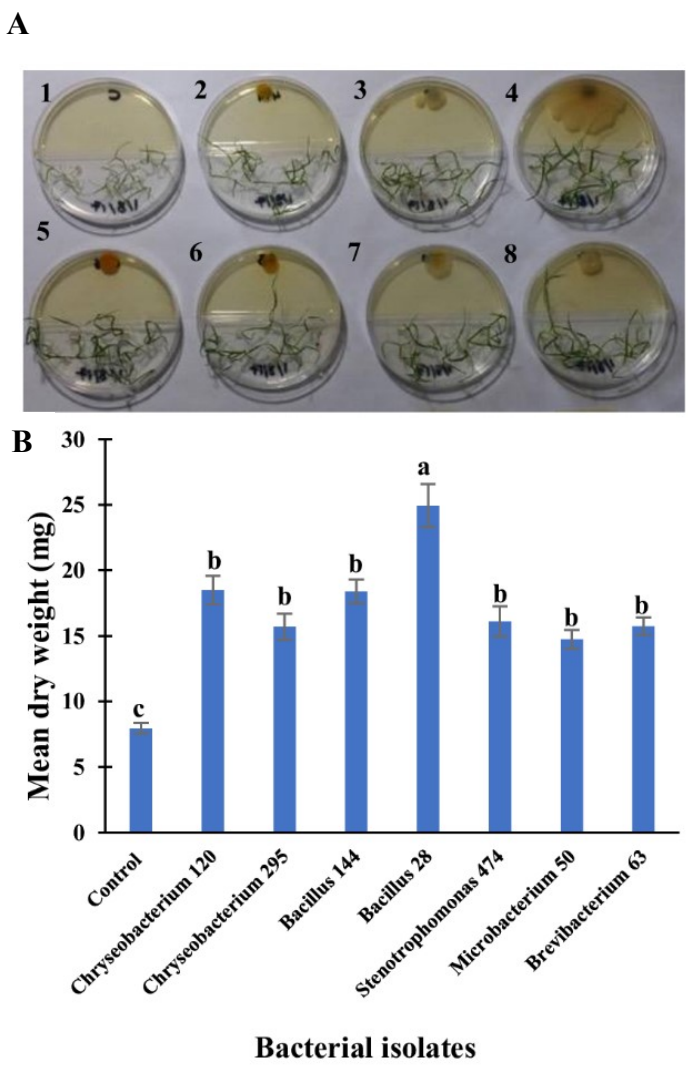

Figure 3. 3 Quantification of growth promotion in timothy seedlings with exposure to volatile chemicals released from seven bacterial isolates compared with control treatment. (A) Representative examples of timothy seedlings after 12-day exposure to bacteria. Control (1), *Stenotrophomonas* T474 (2), *Microbacterium* T50 (3), *Bacillus* T28 (4), *Chryseobacterium* T295 (5), *Brevibacterium* T63 (6), *Chryseobacterium* T120 (7), *Bacillus* T144 (8). (B) Mean dry weight, a measure of seedlings' growth after 12-day exposure of pregerminated seeds of timothy grass to volatile chemicals released from selected endophytes and compared with control seedlings. There were ten replicates per bacterial isolate per treatment. Each replicate had ten seedlings. Superscript letters on top of each bar represent significance according to Tukey's test ($p \leq 0.05$).

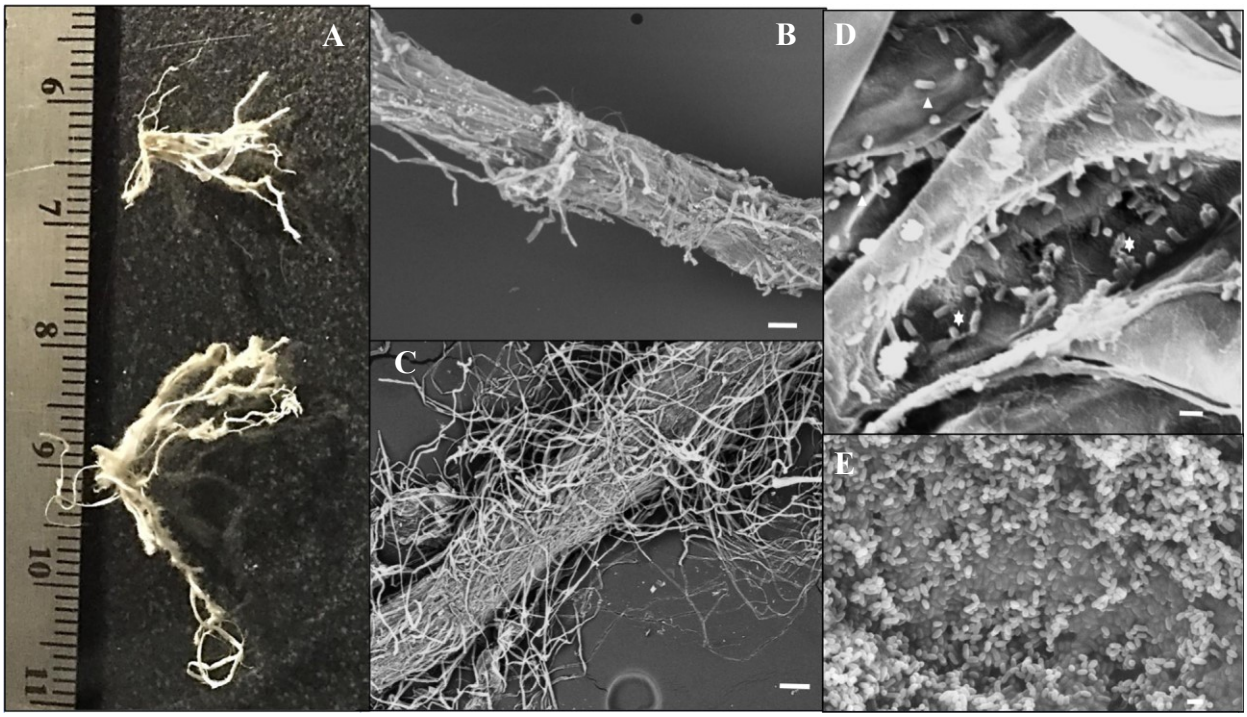

Figure 3. 4 (A) Photographs of non-colonized (upper) and colonized roots (lower) of timothy showing differences in root mass. (B) SEM micrographs of a non-colonized root showing sparse root hairs (bar = 500 µm), and (C) colonized root section with increased number of root hairs (bar = 500 µm). (D) SEM micrograph showing the presence of endophytic bacteria as single cells (white arrows) or as aggregates with possible biofilm (white star) on the surface and inside the root (D; bar=10 µm). (E) Cultured pure rod-shaped bacteria (E; bar = 10 µm).

3.9 CONNECTING TEXT

Chapter 4 investigated the effect of individual organic acids and root exudates released from the model grass *Brachypodium distachyon* (Brkljacic et al. 2011) inoculated with the competent and compatible multispecies consortium composed of the bacterial strains; *Bacillus* species strains 28 and 144, and *Microbacterium* sp. strain 50. These strains were selected because of their growth-promoting abilities in timothy through the production of IAA, volatile organic compounds that increased timothy root biomass, the production of siderophores and antibiotic resistance. We also hypothesized that root exudates of *Brachypodium* would be more similar to timothy grass than to the eudicot model *Arabidopsis* as grasses develop fibrous root system. As expected, the chemotactic activity and biofilm formation by single species and/or multispecies consortium were triggered by root exudates of *Brachypodium distachyon* and by individual component of the root exudates.

This decision to use the model grass *Brachypodium distachyon* in chapter 4 instead of timothy grass is dictated by several reasons: 1. Unsuccessful attempts to design primers (Degenerate) and amplify the *nif* H gene and DREB genes that are known to play an important role in the resistance of plants to abiotic stresses (Ishizaki et al. 2013), even after gene alignment of *Brachypodium distachyon*, wheat, rice, sorghum, and *Arabidopsis thaliana* 2. The inability of performing molecular studies on a partially sequenced genome of timothy grass. 3. Poor germination and variation in growth due to heterozygosity.

Dina Saleh wrote the first draft of the manuscript. She conceived the experimental design and performed most of the experiments. Meha Sharma, a PhD candidate under the supervision of Dr. S. Jabaji, helped in performing the capillary chemotaxis assay. Dr. Jabaji helped in designing the strategies for each experiment, corrected several drafts and did an extensive revision on the

discussion. Dr. Séguin provided assistance in reviewing the manuscript and corrected several drafts of the manuscript. Dr. Jabaji provided full funding to carry out the work in chapter 4 and Dr. Seguin provided partial funding for this chapter.

CHAPTER 4

Organic acids and root exudates of *Brachypodium distachyon*: Effects on chemotaxis and biofilm formation of endophytic bacteria

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4.1 ABSTRACT

Root colonization by plant growth-promoting (PGP) bacteria could not be useful without the beneficial properties of the bacterium itself. Thus, it is necessary to evaluate the bacterial capacity to form biofilms and establish a successful interaction with the plant roots. We assessed the ability of growth-promotion bacterial strains to form biofilm and display chemotactic behaviour in response to organic acids or to root exudates of the model plant *Brachypodium distachyon.* This assessment was based on the evaluation of single strains of bacteria and of multispecies consortium. The strains coexisted together and formed biofilm under biotic (living root) and abiotic (glass) surfaces. Citric acid stimulated biofilm formation in all individual strains, indicating a strong chemotactic behaviour towards organic acids. Recognizing that the transition from single strains of bacteria to a "multicellular" system wouldn't happen without the presence of adhesion factors, alginate and exopolysaccharides (EPS) contents, were evaluated. The EPS amounts were comparable in single strains and consortium forms. Alginate production increased 160% in consortium subjected to drought stress (10% PEG). These findings demonstrated that bacteria-bacteria interaction is the hub of various factors that would not only affect their relation, but further could indirectly affect the balanced plant-microbe relation, and that root exudates could be very selective in recruiting highly qualified multispecies consortium.

Keywords: Endophytes, organic acids, biofilm, chemotaxis, multispecies consortium.

4.2 INTRODUCTION

Bacterial endophytes are vital contributors to plant fitness and productivity (Sturz et al. 2000). Interaction of plants with competent endophytic bacteria is a complex process that begins with the recognition of root exudates and motility towards the roots, attachment to the root surface, formation of biofilm, penetration, and colonization of internal tissues (Danhorn and Fuqua 2007). The rhizosphere is an active zone of root exudation in which abundant amounts of photoassimilates are released into the rhizosphere. Organic acids, free sugars and amino acids are essential components of rhizodeposits (Jones et al. 2009). One of the significant drivers of bacterial activity and diversity in the rhizosphere is the release of low molecular weight carbon compounds such as malic, fumaric, citric, succinic and oxalic acids from the roots (Eilers et al. 2010) that influence the microbial community surrounding the root system in the rhizosphere (Zhang et al. 2014). It has been demonstrated that specific organic acids released from plant roots can attract and recruit specifically single species of bacteria in the rhizosphere. For example, malic acid released from plant roots selectively attracted *Bacillus subtilis* present in the rhizosphere (Rudrappa et al. 2008), while malic and citric acids in watermelon root exudates recruited *Paenibacillus polymyxa* (Ling et al. 2011).

Chemotaxis is defined as the ability of motile bacteria to guide their movement across a nutrient gradient formed by plant-released root exudates including some components that may function as chemoattractant for plant beneficial microbes (Badri and Vivanco 2009; Scharf et al. 2016). This represents the initial step of microbial recruitment and colonization process of diverse plant hosts (De Weert et al. 2002; Scharf et al. 2016; Zhang et al. 2014), and enhances the ability of bacteria to colonize the roots of plant hosts (Bais et al. 2006; Berendsen et al. 2012; Feng et al. 2018).

The development of bacterial biofilms in nature is a highly regulated and coordinated process involving the transition from planktonic bacteria to differentiated communities embedded in a self-produced matrix that facilitates the exchange of metabolites, genetic material and signaling molecules. The trademark of bacterial biofilms is the production of exopolysaccharides (EPS), swarming and swimming motility, cell surface hydrophobicity, and alginate production (Flemming and Wuertz 2019). Plant roots and the rhizosphere are the preferential sites for colonization by soil bacteria, since significant amounts of nutrients are exuded from plant roots. Chemotaxis towards plant roots and the formation of biofilm are part of the mechanisms determining colonization (Molina et al. 2003; Seneviratne et al. 2011). The development of a mature biofilm begins from the initial attachment of bacteria to a surface followed by the accumulation of large microcolonies (Jefferson 2004; Ren et al. 2015).

We previously isolated three competent rhizospheric bacterial strains 28, 50 and 144 from the *Poaceae* forage grass timothy (*Phleum pratense* L.). Partial sequencing of these strains putatively identified strain 28 as *Bacillus* sp.(accession MH587690.1), strain 144 as *Bacillus* sp. (accession MK484341.1) and strain 50 as *Microbacterium* sp. (accession KT803429.1) (Saleh et al., 2019). These strains displayed potential growth-promoting abilities in timothy through the production of IAA, volatile organic compounds that increased timothy root biomass, the production of siderophores and antibiotic resistance, as well as the ability to colonize timothy (Saleh et al. 2019). Populations of the Gram-positive bacteria *Bacillus* and *Microbacterium* species are able to form biofilms either on abiotic surfaces or on living tissue (Esteban and García-Coca 2018; Majed et al. 2016; Ryan-Payseur and Freitag 2018; Vlamakis et al. 2013). Normally, biofilms formed on roots are composed of multiple bacterial species with an elevated prevalence of synergy in biofilm formation among the species and higher adhesive capacity on living tissues,

implying that cooperation among the strains exists (Ren et al. 2015). The formation of multispecies biofilms in the soil has several benefits, among others are maintenance of ecological balance in the soil and increased resistance to antibacterial compounds (Burmølle et al. 2014).

Since it is reasonable to hypothesize that the role of bacterial motility in root colonization is to reach the nutrients released by the roots (Hardoim et al. 2008), the overall objectives of this study were to investigate whether (i) the above competent rhizospheric strains applied singly or as multispecies consortium display biofilm formation and the associated traits including, hydrophobicity, EPS and alginate production, and (ii) organic acids and root exudates released from the model grass *Brachypodium distachyon* trigger chemotactic response by single species and/or multispecies consortium. Since *Brachypodium* shares a high degree of synteny with several *Poaceae* grasses, we assume that root exudates of *Brachypodium* would be more similar to timothy grass than to the eudicot model *Arabidopsis* as grasses develop a fibrous root system. Therefore, Brachypodium distachyon was selected as the model plant to study the plant-microbe interaction. This study provides valuable information on the selection of competent and compatible strains for application in agricultural production.

4.3 MATERIALS AND METHODS

4.3.1 Bacterial strains and culture conditions

A multispecies consortium composed of *Bacillus spp. strains 28 and 144*, and *Microbacterium sp. strain 50* isolated from the rhizosphere of timothy was used in the present study. The selection of the strains in the consortium was based on their ability to demonstrate potential growth-promoting abilities in timothy through the production of IAA and siderophores and the ability to colonize timothy (Saleh et al. 2019). Single bacterial colonies of monoculture of

each strain were grown in LB broth overnight on a rotary shaker (1.5 x g) at 28 ºC achieving 10^6 CFU/ mL. The adjustment of cell density was based on standard curves relating absorbance at 600 nm (A_{600}) to plate counts on LBA plates. A multispecies consortium was made of 1:1:1 ratio of strains 28, 144 and 50, unless otherwise stated.

4.3.2 Compatibility tests

The compatibility and interaction among the three bacteria (strains 28, 50, 144) of the consortium was tested using the Burkholder agar diffusion assay (Burkholder et al. 1944). Mid-log phase of individual strains of 28, 50, 144 were pelleted, suspended in 60 µL distilled and mixed with 5 mL of molten half-strength LB agar, and poured into culture plates containing 15 mL of solidified LBA. A mixture of two bacteria at 1:1 ratio (10 µL) was spotted in the centre of the bacterial lawn and plates were incubated at room temperature (24 °C) for 24–48 hours. Control plates were inoculated with 10 µL of LB broth. Zones of growth-inhibition adjacent to the spotted inoculum is an indication of incompatibility between the tested strains.

Assessment of compatibility among the strains was also studied by the co-culture plating method (Kumar et al. 2016) that allows the growth of one of the three strains and limits the growth of the others. Aliquots of freshly grown bacterium (5µL) of each strain were equidistantly placed on LBA culture plates with a distance of 0.5 cm between the centres of the initial cell suspensions. The plates were incubated at 28 ºC for one week, and the growth inhibition of the isolates was measured at 24 and 48 hours as well as after a week. All assays were performed in triplicate plates, and the assay was repeated twice to confirm the findings.

4.3.3 Plant material and growth conditions

Brachypodium distachyon Bd-21 seeds, (DOE Joint Genome Institute, CA) were surface sterilized according to Gagné-Bourque et al. (2015) and germinated in Petri plates lined with sterile filter papers moistened with 1 mL of sterile water containing (0.1 mg/ mL) of benomyl and placed in the dark for 2 weeks at 4 ºC.

For root exudate collection, Magenta tissue culture boxes (7.6 × 7.6 × 10.2 cm) were filled with a mixture of glass beads of 1.7 –2.0 mm diameter (low alkali glass beads) (Ceroglass, USA) up to 2 cm in height and saturated with 4 mL of ¼ strength Hoagland's solution (pH 6.0, buffered with 2 mM MES (2-[N-morpholino]ethanesulfonic acid]). Five pre-germinated seeds were placed on the surface of the glass beads, and Magenta boxes were incubated for 40 days in a controlled growth cabinet (Conviron, Canada) set under the following conditions: 16 hours photoperiod, 150 µmoles m^2s^{-1} of light intensity, and day-night temperature of 25°C/23°C. In total, there were 12 Magenta culture boxes in which each Magenta box represents an experimental unit. Whenever needed, mostly after two to three weeks of growth, experimental units received an extra 500 µl of sterile ¼ strength Hoagland's solution. To assess the sterility of the Hoagland solution, 100 µl were plated on LBA medium before plant harvest.

4.3.4 Root exudates collection

Forty days after cultivation, six experimental units were inoculated with multispecies consortium with 3 mL of consortium/experimental unit, and the remaining six units (control) received each 3 mL of sterile phosphate buffer (0.06M, pH 6.8). All units were incubated in a controlled growth chamber under the previously described conditions. After 48 hours, inoculated and control *Brachypodium* seedlings from every two experimental units were pooled (total 10 seedlings) to make 3 biological replicates per treatment. Prior to exudate collection, roots of

intact plants from each replicate were rinsed off once in 20 mL of ultra-pure water for 2 hours to remove cell debris and nutrient solution. The root system was placed in a 150 mL glass beaker so that the root system were fully immersed in 20 mL of ultra-pure water with gentle agitation for 24 hours under the same growth chamber conditions. The plants were removed, and the solution (20 mL) was filter-sterilized using 0.22 μm filter, freeze-dried, concentrated at 50x in ultra-pure sterile water, and stored at -20 °C for downstream applications.

4.3.5 Chemotaxis assays

It is established that organic acids including malic, citric, fumaric, and succinic produced by plants are reported to act as chemotactic agents to recruit beneficial bacteria to the rhizosphere (Tan et al. 2013), and could provide nutrients for the microbial community in the rhizosphere and act as chemo-attractant representing the initial step for microbial recruitment and colonization process (Sasse et al. 2018). The chemotactic response of the multispecies consortium to organic acids was established using three different methods.

4.3.5.1 Method 1-Capillary chemotaxis assay

The response of the multispecies consortium to organic acids was quantified using a modified capillary chemotaxis assay (Mazumder et al. 1999). Briefly, the chemotactic system consisted of a 200 μL pipette tip as the chamber for holding a 150-μL bacterial suspension (OD_{600} =1.0). A disposable $25^{1/8}$-gauge needle was used as the chemotaxis capillary and was attached to a 1-mL syringe containing 500 μL of one of the following filter-sterilized organic acids: malic, citric, fumaric and succinic, prepared at concentrations of 10, 25, 50 μM/L, respectively. Syringes containing sterile distilled water served as control for each of the chemoattractants. The needle-syringe capillary system was tightly inserted into the pipette tip which allowed the bacterial suspension to be in direct contact with the organic acid of interest. Syringes were left undisturbed

for 30 min, and the liquid was collected and serially diluted, plated on five plates of LBA medium, and incubated overnight at 30 ºC. The accumulation of the attracted bacteria towards the organic acids was calculated as the average colony forming units (CFU) obtained on five plates. Relative chemotactic ratio (RCR) was calculated, which represents the ratio of the CFU in response to the chemoattractant at a certain concentration, to the CFU of the control (sterile water). An RCR greater or equal to 2 is considered significant (Mazumder et al. 1999).

4.3.5.2 Method 2-Drop assay

To trigger a chemotactic response by bacterial cells, the drop assay described by Yuan et al. (2015) was performed. Pelleted cells of multispecies consortium were resuspended in 12 mL of sterile chemotaxis buffer (100 mM potassium phosphate [pH 7] with 20 uM EDTA) and 1% (v/v) of hydroxypropylcellulose solution. The cell suspension was poured in a 60 mm diameter petri plate and 10 µL of 50 mM of each of the concentrated organic acids (succinic, fumaric, citric, oxalic, malic) or 10 µL of 50 x concentrated root exudates originating from inoculated and control roots were added to each Petri plate. Petri plates without organic acids containing the chemotaxis buffer alone were used as control. Rings of turbidity that started to appear in the next 30 min were recorded as an indication of the chemotactic response.

4.3.5.3 Method 3-Chemotactic response of multispecies consortium to attractants in carbon-free medium

Pelleted cells of multispecies consortium were resuspended in potassium phosphate buffer (0.06 M, pH 6.8), and placed in the centre of a 60 mm petri plate containing: potassium phosphate buffer and 0.3% agar as described by Kadouri et al. (2003). Two surface-sterilized *Brachypodium distachyon* Bd-21 seeds, and 5 µL of glucose (0.5%) or 5 µl of water (control) were placed near the border of the petri plate, in such a way that all are equidistant from

the bacterial consortium. The extent of movement of the bacterial cells towards the attractant (seed or glucose) was considered a chemotactic response after 24 h at 25 ºC. The experiment was replicated ten times, and results were collected after 24 and 48 h. To further confirm the findings, this test was repeated by substituting the Bd-21 seeds with 5 µL of 50 x concentrated root exudates originating from inoculated and control plants, respectively.

4.3.6 Biofilm quantification and associated traits

4.3.6.1 Biofilm quantification assay

The effect of root exudates and organic acids on the formation of bacterial biofilm was determined in 96 microtiter plates following the procedure of Yuan et al. (2015). Single species and multispecies consortium were prepared as previously described. Bacterial cells were centrifuged at 8668 x*g* for 10 min, washed twice with ½ MSgg medium (Branda et al. 2001), resuspended in the same volume (5 mL) as the culture medium. Each well contained 200 µl of ½ MSgg medium mixed with 200 µL of the bacterial suspension (single or consortium). The negative control consisted of wells with culture medium only, while positive control consisted of single bacteria only. Root exudates (50 x) or concentrated organic acids were added to wells to obtain a final concentration of wells (10 µM, 25 µM, and 50 µM). The number of replications varied with the treatment. Wells in microtiter plates studying the effect of organic acids on biofilm formation of individual strains in monoculture were replicated 4 times, while wells in microtiter plates studying the effect of organic acids on biofilm formation of multispecies consortium were replicated 12 times. Wells studying the effects of exudates on biofilm formation were replicated 5 times. The biomass of biofilm formation was quantified following an incubation of plates for 24 and 48 hours at 37 ºC and were later stained with crystal violet dye and quantified following the procedure of Yuan et al. (2015) using a Synergy HT plate reader

(Bio-TEK, Vermont, USA) at OD_{570}. Data were presented as relative fold increase (RFI) of biofilm formation. RFI ratio was calculated based on the ratio of the treated to consortium alone. Exopolysaccharides quantification of bacterial strains.

4.3.6.2 Exopolysaccharides quantification of bacterial strains

Following the procedure of Krithiga et al. (2014), individual strains and multispecies consortium were separately grown in 200 mL of LB medium and incubated with shaking at 28 ºC for five days at 1.5 x *g*. Bacterial growth was collected and centrifuged at 9167 x *g* for 20 min at 4 ºC. The resulting supernatant was filter sterilized using a 0.45 µm (Millipore Sigma-Aldrich, USA) to which 600 mL of chilled ethanol were added. After 24 h, EPS were collected and dried overnight at 55 ºC.

4.3.6.3 Alginate quantification assay

It has been reported that alginate protects cells by maintaining cellular hydration (Chang et al. 2007). Single species cultures and multispecies consortium were grown on 10%; -1.5MP PEG-amended LB broth medium. Cell-free supernatant was collected after centrifugation at 9167 x *g* for 10 min. The isolation of alginate from the culture supernatant was performed following the method of Knutson and Jeanes (1968). Alginate quantification was performed by measuring the uronic acid content from a standard curve of alginic acid of brown algae (Sigma Aldrich) ranging from 10 to 1000 µg/mL according to the method of May and Chakrabarty (1994). Absorbance at A_{530} is indicative of a positive uronic acid test.

4.3.6.4 Hydrophobicity test

The Classical MATH assay (Rosenberg et al. 1980) was performed with the hydrocarbon n-hexadecane (Alfa Aesar, United States). Bacterial suspensions (5 mL) of single species and the multispecies consortium (A_{600}= 1.0) were centrifuged, at 6868 x *g* for 10 min, and the pellets were

resuspended in phosphate magnesium buffer (pH 7.4). Three -hundred microliters of n-hexadecane (Alfa Aesar, United States) were added to the bacterial suspension, incubated for 10 min at 30 ºC, vortexed, and left undisturbed to allow for phase separation. The adherence of bacteria to the hydrocarbon was retrieved, and cell density absorbance was measured at 600 nm. The adhesion of bacteria to the hydrocarbon phase, *FPc* was calculated using the formula established by Zoueki et al. (2010): $FPC= (1-Af/A0) x100$

Where *Af* is the final absorbance after the addition of the hydrocarbon, *A0* is the original absorbance of bacterial cells prior to addition the hydrocarbon. The experiment was performed in triplicates for each treatment.

4.3.6.5 Swimming and swarming motility assay

Swimming and swarming motility of single species or multispecies consortium were performed in LB plates containing 0.3 and 0.5% agar, respectively (Be'er and Harshey 2011). Each plate was inoculated with 3 µL of individual strains or with the multispecies consortium and incubated for 24 and 48 hours at 28 ºC to determine the swarm diameter of bacterial movement (mm). Assay plates were performed in triplicates for each treatment.

4.3.6.6 Scanning electron microscopy for biofilm formation in vitro and on root surface

Biofilm formation in vitro*:* individual strains (28, 50 and 144) of the consortium were grown separately as previously described. For mixed biofilm formation on a glass surface, 1 mL of the consortium was placed on L-poly-lysine treated glass coverslips. Coverslips were incubated without shaking in an incubator set at 37 ºC for 48 h and subjected to successive washing in 0.1M phosphate buffer (pH 7.2). Biofilms formed on the coverslips were fixed overnight at 4 ºC in 4% formaldehyde solution (v/v) buffered with 0.1M phosphate buffer (pH 7.2). Slides containing the biofilm –forming bacteria were dehydrated in an increasing series of ethanol (30 –100%) with the

last step repeated three times. This was followed by critical point drying of the slides using Leica EM-CPD300 (Leica, Vienna, Austria). The dried biofilm containing slides were coated with 4 nm of gold-palladium (Leica EM-ACE200) and examined using a Hitachi TM-1000 operating at 15 kV (Hitachi, Tokyo, Japan).

Biofilm formation on root surface: Bd-21 seedlings were grown in Magenta boxes filled with glass beads for two weeks as described above. An aliquot of 500 µL of each bacterial strain (28, 50 144) or mixed consortium at the ratio of 1:1:1 was delivered on the surfaces of the glass beads. Boxes that received phosphate buffer were considered as control. Colonized and uncolonized root tissues were fixed overnight in 100% methanol following the procedure of Neinhuis and Edelmann (1996) as described in Saleh et al. (2019). The sample preparation and images acquisition were performed at the McGill University Multi-Scale Imaging Facility, Sainte-Anne-de-Bellevue, Québec, Canada.

4.3.7 Statistical Analysis

Data from all experiments were analyzed using JMP (Version 13.0.0). The data of the chemotaxis response of multispecies consortium towards attractants using the capillary method was performed with 5 replications, while data of the drop assay method was replicated three times and the chemotactic response in carbon-free medium was performed with 10 replicates. Difference between bacterial treatment means and the control was assessed using Dunnett's test ($p \leq 0.05$) when the model and treatment effects were significant ($p \leq 0.05$) according to ANOVA. The data for EPS, alginate production, and also the effects of root exudates on biofilm formation were replicated 5 times, while swimming and swarming motility and hydrophobicity were replicated 3

times and analyzed using one-way ANOVA with significant differences among the means assessed using Tukey HSD ($p \leq 0.05$). Experiments related to the effect of organic acids on biofilm formation of single species in monoculture were replicated 4 times, while the effect of organic acids on biofilm formation of multispecies consortium was replicated 12 times.

4.4 RESULTS

4.4.1 Characteristics of strains and compatibility assays

The compatibility of the three strains (i.e., 28, 50 and 144) was assessed using two methods: the Burkholder agar diffusion assay and the co-culture method. Results showed that the three strains were compatible with each other (Fig. 4.1), as all grew similarly whether inoculated alone or in combination with one or two strains. When strains were combined, there was no clearing zone observed indicating that the three strains are not antagonistic to each other. Other traits were also evaluated like EPS production, swarming and swimming characteristics (Table 4.1).

4.4.2 Exopolysaccharide and alginate production

The amount of EPS produced by individual strains in monoculture and when co-cultured in a multispecies consortium was similar ($p \leq 0.05$). In the absence of hydric stress, alginate production of strain 28 was significantly higher than strain 144 and the multispecies consortium (Table 4.1). Increased alginate production with the hydric stress treatment led to substantial increases in alginate ranging from 160% increase in multispecies consortium to 80 % in *Microbacterium* sp. 50 (Table 4.1) compared to media without PEG.

4.4.3 Swimming and swarming motility of individual strains and the consortium

The swimming and swarming motility of each of the bacteria was quantitatively measured at 24 and 48 hours of incubation (Table 4.1). After 24 h of incubation, strains 28, 50 and 144 displayed similar swimming and swarming motility ($p \leq 0.05$). After 48 hours of incubation,

swimming and swarming motility of bacteria increased. Strains 28 and 50 showed a significant swimming motility with an average swimming diameter of 40.83 and 38.33, respectively. The swimming motility of the multispecies consortium was significantly higher after 48 h compared to that of each individual strain. However, the swarming mobility pattern of the consortium was similar to that of the three tested strains (Table 4.1; Fig. 4.2**).**

4.4.4 Hydrophobicity of bacterial strains

The hydrophobicity of the bacterial strains as monoculture and as multispecies consortium was quantified as the fraction of bacteria adhered to the hydrocarbon phase. All strains were similar in their hydrophobic capacity. When the three strains were mixed in a 1:1:1 ratio, there was a slight increase in the hydrophobic capacity with a mean of 90% for the consortium (Table 4.1**)**.

4.4.5 Chemotaxis response of consortium towards different organic acids

The capillary chemotaxis assay revealed that the multispecies consortium was attracted to a variety of organic acids (Table 4.2). Generally, the consortium cells migration into the syringe were positively correlated to certain organic acid at specific concentrations. Significantly high numbers of cells compared to their relative control were observed in both concentrations of fumaric acid at 25 and 50 μM/L, being as high as 6.74 and 6.77 $\log_{10}$ CFU/mL, respectively was observed, followed by malic acid concentrations at 25 and 50 μM/L with 3.77 $\log_{10}$ and 4.10 $\log_{10}$ CFU/mL, respectively. Cell numbers that migrated to citric acid were not different from those in sterile distilled water (control) solution. Equally, a negative effect on attraction was reflected in the RCR ratio (ratio of bacteria attracted towards an organic acid) observed for the multispecies consortium at succinic acid concentration ranging from 10 to

50 μM/L. The RCR, the ratio of bacteria attracted towards an organic acid to that of water was 53 for malic acid and 6.9 fumaric acid, respectively indicating that there was significant chemotaxis with increasing malic and fumaric acid concentrations ($p \leq 0.05$).

4.4.6 Consortium response to *Brachypodium* exudates and organic acids as measured by chemotaxis

In the qualitative drop assay, all tested organic acids (succinic acid, fumaric acid, citric acid, oxalic acid and malic acid) and concentrated root exudates initiated a chemotaxis response on cells from the multispecies consortium compared to bacteria and chemotaxis buffer (Fig. 4.2). Compared to the buffer solution that served as control, consortium cells formed an unusual large ring of turbidity near the center of each organic acid within the first 30 min indicating that a chemotactic response of the consortium was triggered. The *Brachypodium* root exudates (concentrated 50 times) had a similar but intense pattern. The ring of turbidity formed indicating that exudates were actively attracting bacteria.

In parallel, chemotaxis experiments with *Brachypodium* seeds as attractant showed turbid bands composed of bacterial cells were visible after 24 h of inoculation and intensified after 48 hours (Fig. 4.3 A, B). The absence of *Brachypodium* seeds or presence of water and carbon source such as glucose failed to produce visible turbid bands (Fig. 4.3 C, D**)** indicating that seed exudates play a major role in bacterial chemotaxis. A substantially larger turbid band was formed by the multispecies consortium when they were exposed to root exudates originating from inoculated plants and control but with a larger turbidity ring formed as a result of root exudates associated with inoculated plants (Fig. 4.3F) as compared to root exudates associated with control plants (Fig. 4.3E).

4.4.7 Biofilm formation in response to organic acids and root exudates on microtiter plate

The biofilm formation by single species and by the multispecies consortium was evaluated in microtiter plates at 24 and 48 h. The biofilm formation by single species depended on the organic acid type and concentration, and time of incubation. After 24 hours of incubation, the biofilm production by *Bacillus.* sp. 144, had a significant increase of 1.5 and 1.3-fold at concentration of 25 µM of citric acid and malic acid, respectively compared to the control (Table 4.3). There was no difference in biofilm production of *Bacillus.* sp.144 in response to any of the tested organic acids after 48 h when compared to the control. At 48 h, the biofilm production in *Bacillus* sp. 28 significantly increased by 1.88 and 1.42, in response to 50 µM of citric acid and succinic acid, respectively compared to the control. Interestingly, at concentration of 10 µM of fumaric acid, a significant fold decrease of biofilm production of *Bacillus.* sp. 28 compared to the control was observed (Table 4.3). Among all the organic acids, malic acid at 50 µM significantly decreased biofilm production of the multispecies consortium at 24 h compared to the control. At 48 h, organic acids had no effect on biofilm production of the consortium.

In parallel, the effect of root exudates on biofilm development in the multispecies consortium was also studied on microtiter plate. Biofilm development increased at 10 µL of root exudates (50× concentrated) originating from *Brachypodium* seedlings inoculated with the consortium by 32% compared to the control at 48 h (Table 4.4).

4.4.8 Bacterial biofilm development on glass and root surfaces

Bacterial strains were visualized for their single and mixed biofilm formation on glass coverslips and on the root surfaces of *Brachypodium*. Strains 28, 50 and 144 formed strong but different biofilm phenotypes as well as when mixed in the consortium (Fig. 4.4 A-D). Formation and adherence of biofilm on *Brachypodium* roots was observed in all strains and also in the mixed

consortium (Fig. 4.4 E-H). The aggregation of bacterial cells of *Microbacterium* sp. 50 which are embedded in a polymer matrix resulted in a special biofilm phenotype (Fig. 4.4F).

4.5 DISCUSSION

One of the essential components for successful performance of multispecies microbial consortia is the compatibility of strains in the planktonic mode (Bradáčová et al. 2019; Santiago et al. 2017). Our study demonstrated the *in vitro* compatibility of the three strains implying that these strains can coexist together in which cooperation can occur among the strains without inhibiting the growth of others. Similar results were found with multispecies consortium (Jha and Saraf 2012; Ren et al. 2015). The results of the *in vitro* compatibility prompted us to investigate the formation of biofilm on an abiotic surface (glass) as well as on *Brachypodium* root surfaces. The hydrophobic properties of microbial surfaces are conducive to adhesion to abiotic and biotic surfaces and to penetration of host tissues. Strains 28, 50 and 144 formed strong biofilms individually, and also in mixed co-culture as multispecies consortium on glass surfaces and equally on root surfaces. Such compatibility in multispecies consortium was recently demonstrated in other studies (Burmølle et al. 2014; Ren et al. 2015). In agricultural soils where rhizospheric microbial communities are exposed to spatial and nutrient limitation, bacterial strains are likely to develop into multispecies biofilms instead of single-species biofilm (Narisawa et al. 2008). Such conditions will facilitate the close relationships between the different microbial species.

Root exudates are composed of amino acids, sugars, organic acids and secondary metabolites, and it is believed that plant root exudates could supply nutrients for rhizospheric microbes as they act as chemoattractant and help bacteria to colonize the surface of plant roots by inducing chemotactic responses of rhizospheric bacteria (Canarini et al. 2019; Ryan et al. 2001;

Yaryura et al. 2008). The formation of biofilm by bacteria is a visualized function of effective root colonization and is influenced by root exudates. Stimulation of biofilm formation in rhizospheric PGPR in response to root exudation and to specific organic acids has been reported in *Bacillus subtilis* BF17 to *Arabidopsis thaliana* (Rudrappa et al. 2008), *B. amyloliquefaciens* T-5 to tomato (Tan et al. 2013), *Paenibacillus polymyxa* SQR-21 to watermelon (Ling et al. 2011) and in *Brachypodium distachyon* root exudates (Kawasaki et al. 2016). In agreement with previous studies, here we showed that citric and succinic acids enhanced selective recruitment of single species in a dose-dependent manner thereby, promoting biofilm formation on root surface as demonstrated in SEM micrographs. Conversely, malic acid (25 uM) positively induced biofilm production in *Bacillus* sp. 144. These results indicate a strong chemotactic response to these organic acids and imply that the chemotactic property of the single species in the consortium is not only concentration-dependent but organic acid-specific (Zhang et al. 2014). Although biofilm formation of strain 28 (bacteria alone) marginally decreased at 48 h, the relative fold increase (RFI) of biofilm of strain 28 to control was higher at 48 h. We do not know why there is marginal decrease at 48 h for this particular strain. Indeed, this merits further investigation. Recently, it has been reported that the chemotactic property in response to organic acids is not only strain specific but can vary with pH and time of incubation (Bushell et al. 2019).

Moreover, root exudates originated from *Brachypodium* seedlings inoculated with the consortium caused a significant stimulation of biofilm formation of the multispecies consortium compared to root exudates from plants that were not inoculated. Although we did not attempt to characterize the organic acids in *Brachypodium* root exudates, it should be noted that the stimulation of biofilm of the multispecies consortium in response to root exudates could be due to

sugar, amino acids and phenolic compounds, all are important components of the plant root exudates (Badri and Vivanco 2009).

Additionally, qualitative demonstration of the consortium taxis by the drop assay (Yuan et al. 2015) towards concentrated root exudates of *Brachypodium* and its individual organic acid components clearly showed that concentrated root exudates and various organic acids induced a drastic response. Equally, under conditions of carbon-free medium, the motility of the multispecies consortium in presence of *Brachypodium* seeds had almost doubled in the presence of concentrated root exudates. These findings indicate that seed exudates sustained bacterial growth in the absence of any other external compounds and reinforce the notion that concentrated root exudates as well as certain individual organic acid components of root exudates can initiate a chemotactic response in the consortium leading to biofilm formation.

In addition to chemotaxis, swarming motility, which is stimulated by high growth and require energy–rich conditions, has been identified and characterized as an essential trait of *Bacillus* and *Microbacterium* strains to survive in various environments (Kearns and Losick 2004; Pang et al. 2005). The swarming motility of multispecies consortium benefits from improved resistance to eukaryotic engulfment and enhanced nutrition and competitiveness from secreted surfactants. In this study, swarming of multispecies consortium was achieved and the consortium colony grew into a featureless mass, one type of swarming patterns reported in literature (Kearns 2010).

Collectively, the EPS and alginate are an integral part of bacterial biofilm and contribute to the attachment and many advantages to bacterial cells including shelter and homeostasis when residing within a biofilm (Davey and O'Toole G 2000). In our study, the production of EPS was comparable among single species and multispecies consortium. Smaller amounts of alginate

compared to EPS were similar in strains 28 and 50 but significantly higher than those produced by strain 144 and the multispecies consortium. We also provided direct evidence that when individual bacterial cells in monoculture or as multispecies consortium are under hydric stress, the alginate production substantially increased by 160% in consortium and in individual strains by an increase ranging from 80% to 142%. These results suggest that alginate, a component of the biofilm is implicated in desiccation tolerance. These results are encouraging as it demonstrates that multispecies consortium could promote plant growth even in the presence of hydric stress. Our findings are in agreement with reports demonstrating that in response to water limiting conditions and salt stress, certain bacteria produce EPS and alginate influencing the biofilm development which leads to providing tolerance to plants against abiotic stress (Chang et al. 2007; Enebe and Babalola 2018; Kasim et al. 2016; Marsden et al. 2017).

Based on the above *in-vitro* traits of biofilm associated functions such as EPS, alginate, hydrophobicity and swimming and swarming motility of multispecies consortium, the biofilm formation was further investigated on a glass surface. A strong biofilm formation on glass surface by single strains and multispecies consortium was also demonstrated in other soil bacteria (Burmølle et al. 2014). Furthermore, the finding of root SEM analysis revealed that multispecies consortium adhered to the root surface and formed microcolonies and biofilm.

In conclusion, our results show that there is high occurrence of synergy in biofilm formation in multispecies consortium isolated from the rhizosphere, and that interspecific cooperation among the strains occurs indicating that all individual strains benefit from their presence in the multispecies community. The multispecies consortium with desired diverse components including EPS, alginate, hydrophobicity and swimming and swarming motility seems to have clear advantages for survival and is positively enhanced by root exudates. We also conclude that

intermediate products of the tricarboxylic acid cycle such as succinic, citric and malic acids, all products of root exudates, can recruit the single strains and increase their populations. These results provide evidence for better understanding of the role of organic acids in plant – microbe interaction. Future studies should investigate the qualitative and quantitative composition of organic acids and other compounds excreted by *Brachypodium* roots in response to multispecies colonization and biofilm formation.

4.6 ACKNOWLEDGMENT

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Table 4. 1 Motility characteristics, EPS, and alginate production of the bacterial strains in their single and consortium forms (Strains: 28, 50, 144).

Strain	Hydrophobicity (%) [#]	EPS* ($ug.ml^{-1}$) [+]	Alginate ($ug.ml^{-1}$) [#]		Diameter of swimming (0.3% agar) motility (mm) [#]		Diameter of swarming (0.5% agar) motility (mm) [#]	
			LB Media Alone	LB + 10% PEG	After 24 hours	After 48 hours	After 24 hours	After 48 hours
28	98	820 ± 0.01 [a]	87.31 ± 3.27 [a]	211.21 ± 8.34 [a]	5.67 ± 0.33 [b]	40.83 ± 5.02 [b]	6.33 ± 0.17 [a]	8.17 ± 0.17 [a]
50	87	800 ± 0.01 [a]	83.66 ± 5.59 [a]	151.21 ± 11.32 [b]	5.00 ± 0.00 [b]	38.33 ± 6.60 [b]	5.83 ± 0.17 [a]	8.50 ± 0.29 [a]
144	85	730 ± 0.01 [a]	68.05 ± 2.13 [b]	151.21 ± 11.32 [b]	5.17 ± 0.17 [b]	5.50 ± 0.00 [c]	6.00 ± 0.00 [a]	8.50 ± 0.29 [a]
Consortium	90	750 ± 0.01 [a]	67.18 ± 2.62 [b]	174.99 ± 5.76 [a,b]	19.00 ± 5.00 [a]	80.50 ± 4.25 [a]	6.25 ± 0.14 [a]	9.00 ± 0.00 [a]

Numbers represent the average of three replicates. *EPS, exopolysaccharide production. [+] Numbers represent average of five replicates.
Means with different superscript letters within a column differ significantly according to Tukey's test ($p \leq 0.05$).

Table 4. 2 Chemotaxis of a bacterial multispecies consortium towards different organic acids.

Chemoattractant	Chemoattractant concentration (µM/L)	CFU/ml *	Log_{10} /ml	RCR
	Sterile Water	$0.24 \times 10^3 \pm 0.075 \times 10^3$ **c**	2.38	
Malic acid	10	$0.4 \times 10^3 \pm 0.25 \times 10^3$ **c**	2.60	1.67
	25	$6 \times 10^3 \pm 1.64 \times 10^3$ **b**	3.77	25
	50	$12.8 \times 10^3 \pm 1.77 \times 10^3$ **a**	4.10	53.33
	Sterile Water	$217.5 \times 10^3 \pm 76.5 \times 10^3$ **a**	5.33	
Citric acid	10	$1590 \times 10^3 \pm 1468 \times 10^3$ **a**	6.20	7.31
	25	$210 \times 10^3 \pm 138.14 \times 10^3$ **a**	5.32	0.96
	50	$157.5 \times 10^3 \pm 150.85 \times 10^3$ **a**	5.19	0.72
	Sterile Water	$860 \times 10^3 \pm 131.03 \times 10^3$ **b**	5.93	
Fumaric acid	10	$100 \times 10^3 \pm 65.3 \times 10^3$ b	5.00	0.12
	25	$5548 \times 10^3 \pm 534.48 \times 10^3$ a	6.74	6.45
	50	$5914 \times 10^3 \pm 206.7 \times 10^3$ a	6.77	6.87
	Sterile Water	$3080 \times 10^3 \pm 1382.2 \times 10^3$ **a**	6.48	
Succinic acid	10	$800 \times 10^3 \pm 176.07 \times 10^3$ **a**	5.90	0.25
	25	$2060 \times 10^3 \pm 1195.2 \times 10^3$ **a**	6.31	0.67
	50	$180 \times 10^3 \pm 86.25 \times 10^3$ **a**	5.25	0.05

*, Numbers represent the mean of 5 replicates ± SE. RCR: Relative Chemotactic ratio, RCR ratio is calculated based on the ratio of the CFU in response to the chemoattractant at a certain concentration, to the CFU of the control (sterile water). CFU: Colony-forming Units. Means of specific chemoattractant with different superscript letters within a column differ significantly according to the Dunnett's test ($p \leq 0.05$).

Table 4. 3 Effect of various organic acids with different concentrations on biofilm formation of single bacterial strains and a multispecies consortium in 1/2 MSgg medium.

	Treatment*	Concentration[$]	Biofilm formation (OD_{570}, 24 h)	RFI (OD_{570}, 24 h)	Biofilm formation (OD570, 48 h)	RFI (OD_{570}, 48 h)
Strain 28	Citric Acid	Bacteria alone	0.40 ± 0.01 [a]		0.26 ± 0.01 [b]	
		10uM	0.37 ± 0.01 [a]	0.93	0.43 ± 0.03 [a,b]	1.65
		25uM	0.36 ± 0.02 [a]	0.90	0.38 ± 0.02 [a,b]	1.46
		50uM	0.39 ± 0.01 [a]	0.98	0.49 ± 0.08 [a]	1.88
	Fumaric Acid	Bacteria alone	0.40 ± 0.01 [a]		0.26 ± 0.01 [a]	
		10uM	0.33 ± 0.01 [b]	0.83	0.26 ± 0.02 [a]	1.00
		25uM	0.36 ± 0.01 [a,b]	0.90	0.27 ± 0.01 [a]	1.04
		50uM	0.38 ± 0.01 [a,b]	0.95	0.31 ± 0.01 [a]	1.19
	Malic Acid	Bacteria alone	0.40 ± 0.01 [a]		0.26 ± 0.01 [a]	
		10uM	0.36 ± 0.03 [a]	0.90	0.41 ± 0.07 [a]	1.58
		25uM	0.41 ± 0.07 [a]	1.02	0.40 ± 0.06 [a]	1.54
		50uM	0.35 ± 0.02 [a]	0.88	0.34 ± 0.01 [a]	1.30
	Succinic Acid	Bacteria alone	0.40 ± 0.01 [a]		0.26 ± 0.01 [b]	
		10uM	0.42 ± 0.05 [a]	1.05	0.32 ± 0.01 [a,b]	1.23
		25uM	0.39 ± 0.01 [a]	0.98	0.31 ± 0.02 [a,b]	1.19
		50uM	0.41 ± 0.02 [a]	1.02	0.37 ± 0.03 [a]	1.42
Strain 50	Citric Acid	Bacteria alone	0.31 ± 0.01 [a]		0.36 ± 0.01 [b]	
		10uM	0.28 ± 0.01 [a]	0.90	0.50 ± 0.03 [a]	1.40
		25uM	0.29 ± 0.02 [a]	0.94	0.43 ± 0.04 [a,b]	1.19
		50uM	0.30 ± 0.02 [a]	0.97	0.49 ± 0.03 [a]	1.36
	Fumaric Acid	Bacteria alone	0.31 ± 0.01 [a]		0.36 ± 0.01 [a]	
		10uM	0.30 ± 0.01 [a]	0.97	0.44 ± 0.05 [a]	1.22
		25uM	0.30 ± 0.02 [a]	0.97	0.42 ± 0.02 [a]	1.17

		50uM	0.31 ± 0.01 [a]	1.00	0.40 ± 0.02 [a]	1.11
	Malic Acid	Bacteria alone	0.31 ± 0.01 [a]		0.36 ± 0.01 [b]	
		10uM	0.30 ± 0.03 [a]	0.97	0.65 ± 0.05 [a]	1.80
		25uM	0.27 ± 0.01 [a]	0.87	0.61 ± 0.08 [a]	1.70
		50uM	0.30 ± 0.02 [a]	0.97	0.50 ± 0.03 [a,b]	1.40
	Succinic Acid	Bacteria alone	0.31 ± 0.01 [a]		0.36 ± 0.01 [a]	
		10uM	0.32 ± 0.01 [a]	1.03	0.39 ± 0.03 [a]	1.08
		25uM	0.29 ± 0.01 [a]	0.94	0.42 ± 0.04 [a]	1.16
		50uM	0.30 ± 0.02 [a]	0.97	0.34 ± 0.03 [a]	0.94
Strain 144	Citric Acid	Bacteria alone	0.30 ± 0.01 [b]		0.35 ± 0.01 [a,b]	
		10uM	0.37 ± 0.04 [a,b]	1.23	0.36 ± 0.01 [a]	1.02
		25uM	0.45 ± 0.06 [a]	1.50	0.31 ± 0.01 [b]	0.88
		50uM	0.35 ± 0.02 [a,b]	1.16	0.35 ± 0.01 [a,b]	1.00
	Fumaric Acid	Bacteria alone	0.30 ± 0.01 [a]		0.35 ± 0.01 [a]	
		10uM	0.34 ± 0.01 [a]	1.13	0.40 ± 0.01 [a]	1.14
		25uM	0.35 ± 0.03 [a]	1.16	0.38 ± 0.01 [a]	1.08
		50uM	0.35 ± 0.02 [a]	1.16	0.35 ± 0.02 [a]	1.00
	Malic Acid	Bacteria alone	0.30 ± 0.01 [b]		0.35 ± 0.01 [a]	
		10uM	0.38 ± 0.03 [a,b]	1.27	0.34 ± 0.01 [a]	0.97
		25uM	0.39 ± 0.03 [a]	1.3	0.36 ± 0.04 [a]	1.03
		50uM	0.35 ± 0.02 [a,b]	1.17	0.36 ± 0.01 [a]	1.03
	Succinic Acid	Bacteria alone	0.30 ± 0.01 [a]		0.35 ± 0.01 [a]	
		10uM	0.32 ± 0.01 [a]	1.06	0.40 ± 0.03 [a]	1.14
		25uM	0.33 ± 0.03 [a]	1.10	0.37 ± 0.01 [a]	1.05
		50uM	0.29 ± 0.02 [a]	0.97	0.34 ± 0.01 [a]	0.97
Consortia	Citric Acid	Consortium alone	0.26 ± 0.00 [a,b]		0.29 ± 0.01 [a]	

	10uM	0.24 ± 0.01 [b]	0.92	0.31 ± 0.02 [a]	1.06
	25uM	0.26 ± 0.01 [a,b]	1.00	0.29 ± 0.01 [a]	1.00
	50uM	0.27 ± 0.01 [a]	1.03	0.32 ± 0.02 [a]	1.10
Fumaric Acid	Consortium alone	0.29 ± 0.01 [a]		0.48 ± 0.03 [a]	
	10uM	0.27 ± 0.01 [a]	0.93	0.37 ± 0.04 [a]	0.77
	25uM	0.28 ± 0.00 [a]	0.96	0.42 ± 0.06 [a]	0.87
	50uM	0.28 ± 0.00 [a]	0.96	0.39 ± 0.02 [a]	0.81
Malic Acid	Consortium alone	0.30 ± 0.01 [a]		0.40 ± 0.03 [a]	
	10uM	0.27 ± 0.02 [a,b]	0.90	0.48 ± 0.06 [a]	1.20
	25uM	0.26 ± 0.01 [a,b]	0.86	0.42 ± 0.03 [a]	1.05
	50uM	0.25 ± 0.00 [b]	0.83	0.38 ± 0.04 [a]	0.95

*, Numbers represent the mean of 4 replicates for single bacterial strains and 12 replicates for multispecies consortium. Data are expressed as mean ± SE. Means with different superscript letters within a column differ significantly according to Tukey's test ($p \leq 0.05$). RFI: Relative Fold Increase, RFI ratio calculated based on the ratio of the treated to bacteria alone or the bacterial consortium alone.

Table 4. 4 Effect of roots exudates[γ] of inoculated and control *Brachypodium* on biofilm formation of the bacterial multispecies consortium (in ½ MSgg medium).

Treatment*	Volume[$]	Biofilm formation of consortia (OD570, 24 h)	RFI (OD570, 24 h)	Biofilm formation of consortia (OD570, 48 h)	RFI (OD570, 48 h)
Control plants	Consortium alone	0.33 ± 0.01 [a,b]		0.53 ± 0.02 [a]	
	4ul	0.31 ± 0.02 [b]	0.95	0.58 ± 0.01 [a]	1.10
	6ul	0.34 ± 0.01 [a,b]	1.05	0.56 ± 0.03 [a]	1.07
	8ul	0.35 ± 0.01 [a,b]	1.07	0.53 ± 0.03 [a]	1.01
	10ul	0.36 ± 0.01 [a]	1.10	0.57 ± 0.02 [a]	1.09
Inoculated plants	Consortium alone	0.33 ± 0.01 [a]		0.53 ± 0.02 [b]	
	4ul	0.34 ± 0.02 [a]	1.04	0.64 ± 0.04 [a,b]	1.21
	6ul	0.32 ± 0.01 [a]	0.97	0.63 ± 0.07 [a,b]	1.20
	8ul	0.30 ± 0.01 [a]	0.91	0.57 ± 0.04 [a,b]	1.09
	10ul	0.31 ± 0.01 [a]	0.95	0.70 ± 0.04 [a]	1.33

* Figures represent the mean of 5 replicates. Data were expressed as mean ± standard error of the mean. Superscript letters within a column represent significance according to Tukey's test ($p \leq 0.05$). [$] Consortium (Strains: 28, 50, 144) at $OD_{600} = 1.0$. RFI: Relative Fold Increase, RFI ratio calculated based on the ratio of the treated to Consortium alone. [γ] Previously (Kawasaki et al., 2016), have chemically characterized by HPLC and GC-MS the root exudates of *Brachypodium distachyon*.

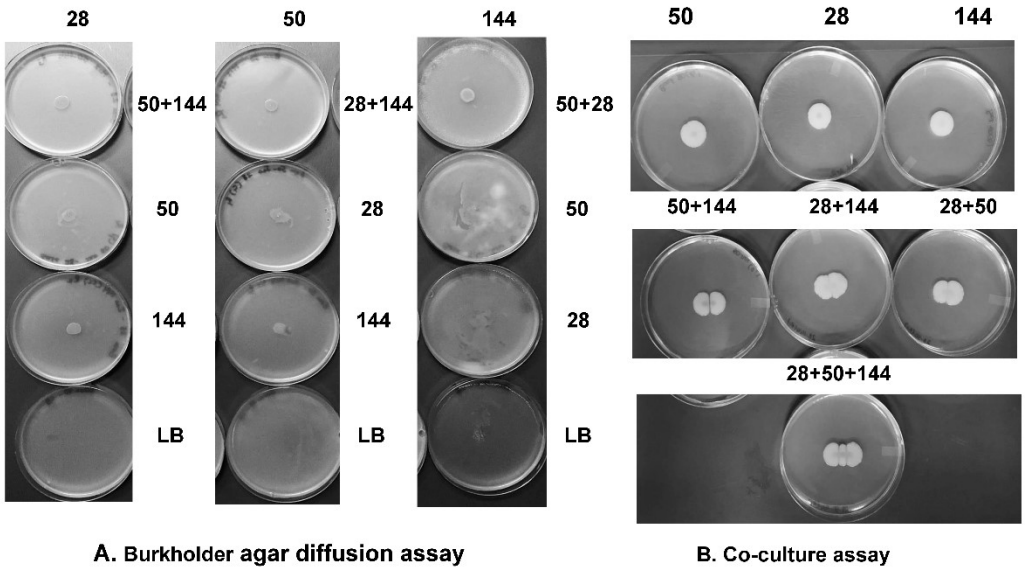

Figure 4. 1 Positive interaction between bacterial strains 28, 50 and 144 on LBA media using Burkholder and co-culture assays after 24h. A. Individual bacterial inoculum present in the molten half-strength LB agar base, and the horizontal labeling represents the spot of 10 ul inoculum (single or 1:1 mixture of two strains) inoculated in the center of each plate. B. The co-culture plate method one week after inoculation. The centers of the initial suspensions were placed 0.5 cm away from each other.

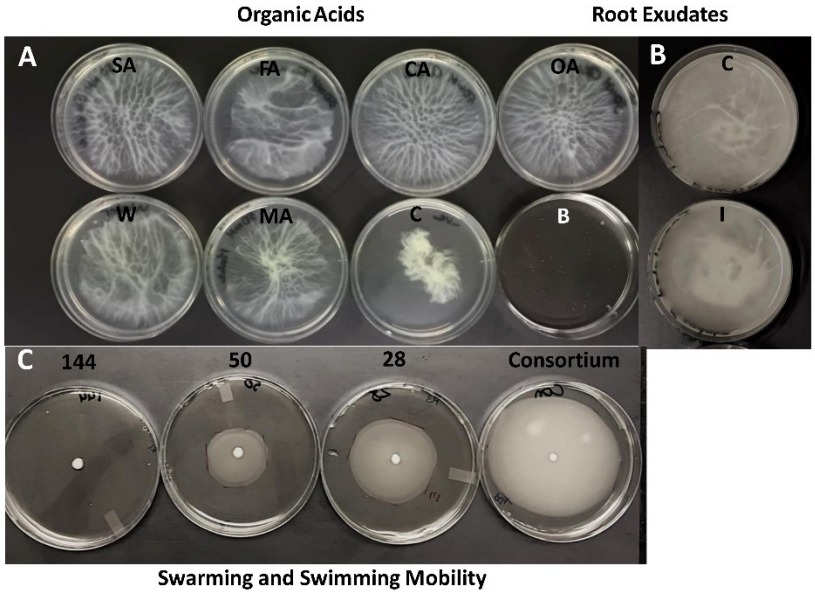

Figure 4. 2 (A) Chemotactic response of multispecies consortium towards concentrated organic acids (50 mM). SA, succinic acid; FA, fumaric acid; CA, citric acid; OA, oxalic acid; W, water; MA, malic acid; C, bacteria alone; B, buffer alone. (B) Chemotactic response of multispecies consortium towards root exudates; C, exudates of control plants; I, exudates of inoculated plants. (C) Swimming motility of individuals strains of bacteria (from left to right: strains 144, 50, 28) and multispecies consortium.

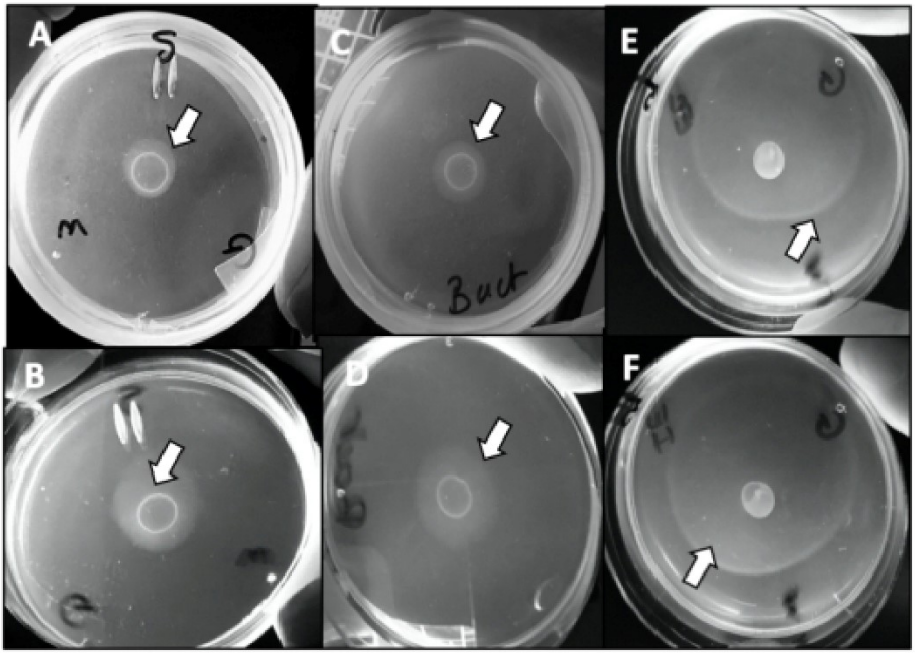

Figure 4. 3 Chemotactic responses of multispecies consortium to attractants (seeds and 5 µl of 50 x concentrated root exudates). (W) water; (G) glucose; (S) seed of Bd-21. The experiment was performed ten times, and similar results were obtained at 24 (A and C) and 48 hours (B and D) after inoculation. (E) exudates of control plants; (F) exudates of inoculated plants. Arrows represent the direction of the inoculum towards its attractant.

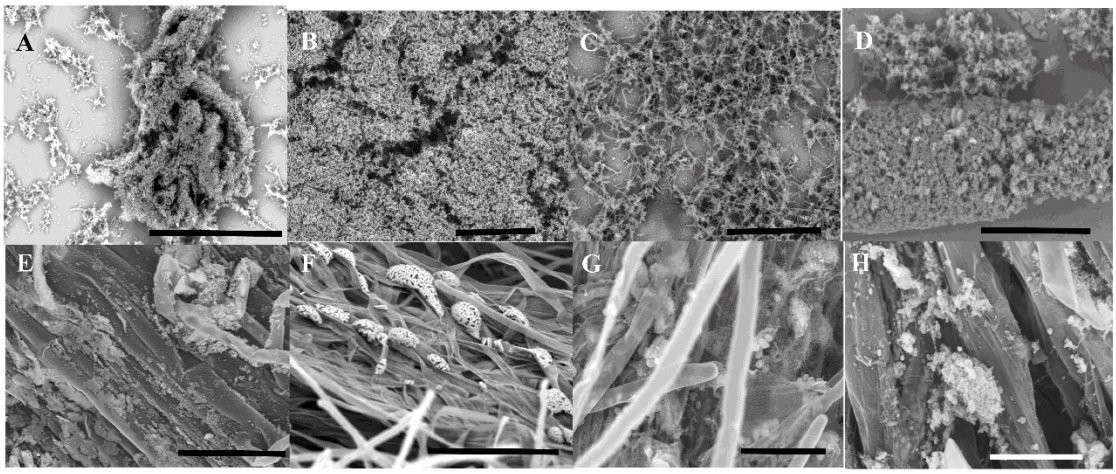

Figure 4. 4 Scanning electron micrographs of biofilm formation on cover slips by strains 28, 50 and 144. (A) Strain 50 (bar = 50 µm); (B) Strain 28 (bar = 50 µm); (C) Strain 144 (bar = 50 µm); (D) Consortium BM (bar = 50 µm). Bd-21 seedlings roots colonized by (E) Strain 28 (bar = 50 µm); (F) Strain 50 (bar = 100 µm); (G) Strain 144 (bar = 50 µm) and (H) Consortium BM (bar = 25 µm).

4.7 CONNECTING TEXT

In the previous sections, we established that organic acids and root exudates triggered a chemotactic response and biofilm formation on *Brachypodium* roots grown in semi hydroponic system. Several studies had reported that certain organic acids can act as chemoattractant for beneficial bacteria or chemorepellent for parasitic microbes (Zhang et al. 2017). In the last chapter we investigated the profile of organic acid in roots and root exudates of *Brachypodium* in response to the multispecies consortium and compared them to those present in non-inoculated *Brachypodium*. Chapter 5 explores the interaction between the multispecies consortium and the model grass *Brachypodium distachyon.* The choice of moving to the model grass was motivated by our interest to further understand the chemical and molecular profiles induced by the presence of the microbes and the capacity of these microbes to create changes in the model grass which could facilitate their universal use as plant growth promoting bacteria. Using a chemical and molecular approach, we provided evidence that inoculating with the multispecies consortium did not only alter organic acid exudations of *Brachypodium* but further increased the gene expression of four tested genes of the TCA cycle.

D. Saleh wrote the first draft of the manuscript and she conceived the experimental design, performed the majority of experiments and conducted the statistical analysis. Miss Meha Sharma, PhD candidate under the supervision of Dr. Jabaji performed the T-DNA genotyping for the mutants and provided some assistance in the maintenance and seed multiplication of the mutant lines. Dr. Séguin provided assistance in reviewing the manuscript. Dr. Suha Jabaji took part in the experimental design and corrected several drafts of the manuscript.

CHAPTER 5

Relative concentration and gene transcription of organic acids in *Brachypodium distachyon* roots in response to multispecies bacterial consortium

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5.1 ABSTRACT

Root-secreted chemicals such as organic acids modulate the dialogue between root and soil microbes. Root exudates play a significant role in the chemotaxis of rhizospheric bacteria and biofilm formation. The aim of this study is to study the relative changes in the metabolite profile and gene transcription of organic acids released from *Brachypodium distachyon* roots and in roots inoculated or not with a multispecies consortium. The profiles of organic acids of inoculated and control *B. distachyon* were similar. However, differences were observed in their concentrations. In roots, the relative amounts of malic and citric acids were significantly more abundant compared to other organic acids. Higher relative concentrations of succinic and fumaric acids were present in inoculated *B. distachyon*. Inoculation of *B. distachyon* with the multispecies consortium caused a change in the relative transcript abundance but that change was not significant in genes encoding malate dehydrogenase (*MDH*), succinate dehydrogenase (*SDH*), citrate synthase (*CS*) and isocitrate dehydrogenase (*IDH*).

Keywords: Endophytes, organic acids, root exudates, overexpression, multispecies consortium.

5.2 INTRODUCTION

Root exudation is an essential process that determines plant interaction with its surrounding environment including the rhizosphere and the root microbiome (Canarini et al. 2019). Root tips of plants exude a wide range of secondary metabolites and release high molecular weight compounds referred to as rhizodeposits into the soil, including root border cells and mucilage. These rhizodeposits have an essential function in the soil as they represent a crucial nutrient source for rhizosphere microbes and influence root -microbe interactions (Haichar et al. 2014). The quantity and quality of root exudates depend on the plant species, the age of individual plants and external biotic and abiotic factors (Carvalhais et al. 2011; Jones et al. 2009; Oburger and Jones 2018). The composition of root exudates is a complex combination of extracellular enzymes along with simple and complex sugars, amino acids, organic acids, phenolics, and other secondary metabolites like vitamins, as well as nitrogenous and gaseous molecules (Dakora and Phillips 2002; Rekha et al. 2018) modulated by their surrounding conditions. Thus, root exudations are a major driving force to the microbial root colonization (Ling et al. 2011; Zhang et al. 2014). Typically root exudates produced by plants will vary in their composition based on their communication with their surrounding microbiome.

Chemotaxis especially to plant root exudates is one of the major factors of root colonization (Saleh et al. 2020; Sasse et al. 2018; Scharf et al. 2016). Exudates produced by plants will recruit certain bacteria to the plant while not attracting others. For example, different doses of malic and citric acids exuded from the roots of watermelon significantly activated the chemotaxis of *Pseudomonas polymyxa* (Ling et al. 2011). Citric acid in the exudates of cucumber specifically induced the attraction of *B. amyloliquefaciens* SQR9 and provoked its biofilm formation (Zhang et al. 2014). L-malic acid secreted by *Arabidopsis thaliana* specifically selected *Bacillus subtilis*

FB17 present in its root microbiome (Rudrappa et al. 2008) and was also reported as the sole signaling molecule in the exudates of tomato triggering root colonization and biofilm formation of *Bacillus subtilis* (Chen et al. 2012).

Brachypodium distachyon is a C3 plant and is the model grass of monocots due to its small genome size, its short life cycle and its small stature (Brutnell et al. 2015; de la Peña et al. 2019). This species along with others of the same genus have been widely studied for the last decade which led to the full genome sequencing, the compilation of many accessions and the identification of several T-DNA mutants (Kawasaki et al. 2016). Considering the above desirable features of *B. distachyon*, and most importantly, its capacity to grow in small volumes of soil, and its root morphology which is similar to other grasses (Chochois et al. 2015), *Brachypodium* is an excellent model to study grass root systems function and interaction with soil microbiota (Watt et al. 2009).

We previously isolated (Saleh et al. 2019) three rhizospheric bacteria (i.e., strains 28, 50 and 144) from the *Poaceae* forage grass timothy (*Phleum pratense* L.) that are able to work in synergy as a multispecies consortium (Saleh et al. 2020). These strains displayed all the desired functional traits of plant growth promoting bacteria, coexisted together, formed biofilm, and were able to display chemotactic behaviour in response to organic acids or to root exudates of the model plant *Brachypodium distachyon*. The addition of organic acids stimulated biofilm formation in all individual strains, indicating a strong chemotactic behaviour towards selective intermediates of the tricarboxylic acid cycle such as succinic, citric and malic acids (Saleh et al. 2020).

Based on the previous results, we hypothesized that the multispecies consortium will modulate not only the the composition and concentration of organic acids, but also the transcript abundance of genes encoding organic acids. The objectives of this study were to investigate (i) the relative composition of organic acids in root exudates and roots of *Brachypodium*, and (ii) the

quantitative gene expression of organic acids in root tissues of *Brachypodium* in response to the multispecies bacterial consortium under semi hydroponic tissue culture system.

5.3 MATERIALS AND METHODS

5.3.1 Bacterial strains and culture conditions

A multispecies consortium composed of *Bacillus* sp. strains 144, and 28 and *Microbacterium sp. strain* 50 were isolated from the rhizosphere of timothy (Chapter 1). Single bacterial colonies of monoculture of each strain were grown in LB broth overnight on a rotary shaker (120 rpm) at 28 ºC achieving 10^6 CFU ml $^{-1}$. The multispecies consortium was prepared by mixing 1:1:1 ratio of strains 28, 144 and 50, unless otherwise stated.

5.3.2 *Brachypodium* wild and mutant lines growth conditions

Seeds of the wild type *B. distachyon* accession Bd 21, cultivar AC Alliance and T-DNA mutant lines of Bd 21-3 were sourced from the DOE Joint Genome Institute, CA. Seeds of wild type and mutant were imbibed overnight and subjected to different growth conditions as described in the following sections.

Wild type accession line Bd 21: seeds were dehusked by removing the lemma, and later surface sterilized by treating them with 70% ethanol for 30s, followed by 1.3% bleach for 4 min and three separate washes in sterile distilled water for five min each. Surface-sterilized *Brachypodium* seeds were germinated in petri plates lined with sterile filter papers moistened with 1 ml of sterile water containing (0.1 mg ml^{-1}) of benomyl and placed in the dark for 2 weeks at 4ºC.

Five pre-germinated seeds of wild type of *B. distachyon* were placed on the surface of inert low alkali glass beads mixture (1.7-2.0 mm diameter) (Ceroglass, USA) up to 2 cm in height in

Magenta tissue culture boxes (7.62×7.62×10.16 cm). Glass beads were saturated with 30-35 ml of ¼ strength Hoagland's solution (pH 6.0, buffered with 2 mM MES (2-[N-morpholino] ethanesulfonic acid]). A total of 12 magenta boxes were used in this experiment, and each box represents an experimental unit. Boxes were transferred to a growth cabinet (Conviron, Canada) with light intensity of 150 µmoles m^2 s^{-1}, 16 hours light and 8 hours dark at day/night temperatures of 25°C /23°C. Whenever needed, mostly after two to three weeks of growth, experimental units received an extra 500 µl of sterile ¼ strength Hoagland's solution. To assess the sterility of the Hoagland solution, 100 µl were plated on LBA medium before plant harvest.

Accession lines of mutant line seeds: Seeds of mutant lines (Table 5.2) were sown in pots (6.35×6.35×7.62 cm) containing G2 Agro Mix® (Plant Products Co. Ltd) and were watered to field capacity. Three equidistant holes were created per pot with about 1 cm depth. Single mutant line seeds were placed in each hole with the awn up and were covered with soil. Pots were wrapped with cling film and aluminum foil to preserve the moisture and block any source of light and were left undisturbed to allow vernalization at 4ºC in the dark. A week later, pots were removed and placed in a growth chamber set under the following conditions: 16 hours photoperiod, 150 µmoles m^2 s^{-1} of light intensity, and day/night temperature of 25°C/ 23°C. When the grass had reached 4-5 leaf stage, two to three leaves of each mutant line were retained for genotype profiling, and their respective seedlings were allowed to grow to full maturity until the seeding stage. Each seedling of the mutant lines was assigned a number based on its corresponding tagged gene and T-DNA line (Table 5.2). Mature seeds from the first generation with successful mutations of their specific genes (Fig. S5.3), were sown in pots for seed multiplication. Out of 11 seeds obtained from post seed multiplication (Table 5.2), four seeds tested homozygous in the mutant line JJ19999 (Fig. S5.3). Mutant line JJ19999-3 and mutant line JJ19999-4 were pregerminated and transferred to

Magenta boxes containing inert glass beads and were inoculated with the bacterial consortia as previously described for the wild type accession line. The Bd 21-3 mutant line JJ19999 contained a T-DNA insertion in the 5' upstream region of the Bradi2g45420 isocitrate dehydrogenase gene (*IDH*), 898 nt upstream the start of the gene.

5.3.3 Collection of root exudation

Following 40 days of growth of the wild type Bd 21, six boxes were inoculated with 3 ml of the multispecies consortium inoculum (10^6 CFU.ml^{-1}) suspended in phosphate buffer (1M, pH 7) and six control boxes received 3 mL of phosphate buffer alone. The *idh* mutant seedlings of line JJ19999 were inoculated with the multispecies consortium as previously described, and the remaining boxes served as control. All boxes were incubated in a controlled growth cabinet under the previously described conditions.

Forty-eight hours after inoculation and prior to exudate collection, seedlings of inoculated and control wild type *Brachypodium* Bd-21 accession line from every two experimental units were pooled (total 10 seedlings) to make 3 biological replicates per treatment. Roots of intact plants of wild type accession line from each replicate were rinsed off once in 20 mL of ultra-pure water for 2 hours to remove cell debris and nutrient solution. The root system was placed in a 150 mL glass beaker so that the root system was fully immersed in 20 mL of ultra-pure water with gentle agitation for 24 hours under the same growth chamber conditions. The solution (20 mL) was filter-sterilized using 0.22 µm filter, freeze-dried, concentrated at 50x in ultra-pure sterile water, and stored at -20°C for downstream applications. The roots of each treatment were immersed in liquid nitrogen and stored at -80°C for mass spectrophotometric analysis and transcription of organic acids.

In the case of *idh* mutants of line JJ19999, inoculated and control seedlings were separated into three sub-samples because of insufficient number of seedlings, root exudates were collected but were not subjected to metabolite profiling.

5.3.4 Organic Acid Analysis using GC-MS

Crushed root samples as well as lyophilized root exudates of wild type accession line were transferred to 2 ml Eppendorf microtubes containing 80% methanol and were kept cold on ice. Ceramic beads (32.8 mm) were added to the samples and the latter were processed in a homogenizer (Analytikjena SpeedMill Plus) three times for 45 sec each. Samples were centrifuged at 4°C and 15000 rpm for 10 minutes and their supernatants were transferred to 1.5 ml Eppendorf tubes containing 1 µl of 800 ng/µl Myristic-d27 in pyridine. The samples were prepared for GC/MS analysis by adding 30 µl of MOX (10 mg Methoxyamine:HCl per 1 ml anhydrous pyridine) and were later derivatized with MTBSTFA. Data acquisition was done in Scan and in SIM modes.

5.3.5 T-DNA Genotyping of mutant lines

5.3.5.1 DNA Extraction

DNA was isolated from the leaf tissue of *Brachypodium* seedlings of mutant lines following the CTAB method (Porebski et al. 1997). Two to three leaves of fresh tissue samples were placed in 1.5 mL micro-centrifuge tubes and pulverized to powder, to which a previously warmed (60ºC) mixture of 800 µl of CTAB, 16 µl of β-mercaptoethanol and 5 µl of RNase were added to each sample. Samples were left for incubation for an hour at 60 ºC and were frequently vortexed. After incubation, 600 µL of iso-amylalcohol chloroform (1:24) were added per sample and the mixture followed by centrifugation at 10000 rpm for 5 min. The resulting supernatant was transferred to clean microcentrifuge tubes to which equal volumes of ice cold iso-propanol were added. The

mixture was held at -20 ºC for 60 min and later centrifuged at 14000 rpm for 8 min. The resulting aqueous phase was discarded, and the pellet rinsed in 75% ethanol, centrifuged at 14000 rpm for 5 min, air dried and re-suspended in 100 µL of DEPC water.

5.3.5.2 Bioinformatics Analysis and Genotyping of mutants

The sequenced genome of *Brachypodium distachyon* v3.1.1 (Bd21-3) was downloaded from Phytozome (Phytozome v12.1, https://phytozome.jgi.doe.gov/pz/portal.html) and uploaded to Geneious Prime software package (version 2019.2.1) to have a better localization of the T-DNA inserts. The sequences flanking the T-DNA inserts (500 bases on either side of the putative insertion site of each tested gene) were retrieved to accurately design gene specific primers (GSP). GSP (Table 5.1) for genotyping were designed using Primer 3 web tool (version 4.0.0) to test for homozygosity of the mutant lines. Another set of primers (Table 5.1) was also designed to undergo qPCR reactions for gene expression studies.

The T-DNA insertional mutants were genotyped following a PCR-based method using a set of gene specific primers (CS-IN, MD-IN, SD-IN, ID-IN; Table 5.1) for the putative sequences of citrate synthase (BdiBd21-3.3G0119500), malate dehydrogenase (BdiBd21-3.3G0165100), succinate dehydrogenase (BdiBd21-3.3G0184500) and isocitrate dehydrogenase (BdiBd21-3.2G0578900) in *Brachypodium distachyon* (Bd 21-3). PCR products were amplified in Bio-Rad T100 Thermal Cycler using BioRad Kit (California, USA) following the instructions of the manufacturer under the following conditions: initial denaturation for 5 min at 95°C and subsequent 35 cycles: denaturation for 15 s at 95°C, annealing for 30 s at the respective annealing temperature for each primer set (Table 5.1), elongation for 30 s at 72°C and final elongation for 5 min at 72°C. Genotyping of all mutant lines was performed following the protocol of DOE Joint Genome Institute, CA. Putative products were confirmed by sequencing. Only mutant lines (4 seeds) for

isocitrate dehydrogenase gene (*IDH*) were confirmed homozygous (Fig. S5.3) and were kept for downstream applications.

5.3.6 Gene Expression analysis

5.3.6.1 Expression analysis of Organic acid genes

To validate the observed trends of organic acids in root exudation, we examined the expression of genes encoding for the organic acids in the citric acid cycle in wild type and mutant accession lines. Total RNA was isolated from 100 mg of frozen roots of *Brachypodium* (Bd 21) and extraction was done using the TRIZOL reagent (Generay Biotech, Shanghai, China) following the manufacturer's instructions. RNA concentration and purity were evaluated using ND1000 (Nano-Drop, Wilmington, Delaware), and its quality was verified by gel electrophoresis. RNA (500ng) was reverse transcribed using BioRad cDNA Kit (California, USA). Real time PCR analysis was conducted on four target genes malate synthase (*MS*), citrate synthase (*CS*), isocitrate dehydrogenase (*IDH*) and succinate dehydrogenase (*SDH*) (Table 5.1) and two reference genes UBC 18 and Actin 2 using Stratagene Mx3000 (Stratagene, Cedar Creek, USA). Primer sets were designed based on sequences retrieved from Phytozome (Phytozome v12.1, https://phytozome.jgi.doe.gov/pz/portal.html) and were further checked for their specificity to amplify their target gene. The conditions of qRT-PCR were optimized for each set of primers, by using the appropriate annealing temperature and the resulting products were confirmed by sequencing (XM_003571799.3, XM_003558641.4[1]). The assays were performed on treated samples 48 hours post-inoculation, with three biological replicates and two technical replicates including routine negative and positive controls in each run. No template control served as negative

[1] Due to Covid19 Pandemic, acession numbers for succinate dehydrogenase *(SDH)* gene and isocitrate dehydrogenase *(IDH)* gene are in the pipeline of sequencing in Génome Québec, Québec, Canada.

control. Positive control consisted of the genomic DNA of *Brachypodium*. The relative transcript abundance levels of the genes were calculated then normalized against their reference genes showing the least variations across treatments. The best reference gene was chosen based on the lowest coefficient of variation shown using the statistical tool BestKeeper (Pfaffl et al. 2004).

To test the expression of the genes of the *idh* mutants lines JJ19999-3 and JJ19999-4 compared to their respective wild type control, RNA was extracted from the whole seedling using Spectrum Plant Total RNA Kit (Sigma- Aldrich, Canada) and respective primers (Table 5.1) were used to perform qRT-PCR under the previously mentioned conditions.

5.3.7 Statistical Analyses

All experiments were performed using JMP (Version 13.0.0). GC-MS analyses were performed with three replications. Comparison of the mean was performed by conducting analysis of variance (ANOVA) using the t-test ($p \leq 0.05$) to determine the statistical significance of the treatments compared to their controls (Sterile Water).

For gene expression studies, the relative transcript abundance of the genes was tested for significance between treatments and controls at each time of inoculation. Analysis was performed conducting an analysis of variance (ANOVA) using the t-test ($p \leq 0.05$).

5.4 RESULTS

5.4.1 Chemical analysis of root and exudate content

The organic acid profiles of roots and root exudates of inoculated Bd 21 and controls were similar with the following organic acids identified: pyruvic, isocitric, lactic, succinic, fumaric, oxaloacetic, 2-ketoglutaric, malic, aconitic and citric acid (Fig. 5.1 and Fig. 5.2**)**. In roots**,** the

relative amounts of malic and citric acids /mg of inoculated and control roots were significantly higher than those of other organic acids (Fig. S5.1). Inoculation of roots with the multispecies consortium did not induce accumulation of organic acids (Fig. 5.1). The relative amounts of organic acids per mg of roots were similar (Fig. 5.1**)** in inoculated (R+); and control roots (R-). The relative amounts of lactic acid and succinic acid in root exudates were the highest among the remaining organic acids (Fig. S5.1). Inoculation of roots by multispecies consortium significantly induced the accumulation of succinic (3525.5/ml of exudate) and fumaric acids (1876.4/ml) in root exudates of inoculated roots (E+) compared to control (E-) treatments (Fig. 5.2).

5.4.2 Gene Regulation of Organic Acids

In response to the multispecies consortium, the relative transcript abundance of citrate synthase (*CS*), isocitrate dehydrogenase (*IDH*), malate dehydrogenase (*MDH*) and succinate dehydrogenase (*SDH*) encoding were slightly upregulated in roots, although not statistically significant, compared to control roots **(**Fig. 5.3 and Fig. S5.2**)**.

5.4.3 Selection of mutants

To confirm that the upregulation of the tested genes was due to the presence of the multispecies consortium, *cs* mutant seeds of the knocked out gene of citrate synthase (*CS*) and other *idh*, *mdh*, *sdh* mutant seeds overexpressing their respective genes of isocitrate dehydrogenase (*IDH*), malate dehydrogenase (*MDH)* and succinate dehydrogenase (*SDH*) were ordered from the DOE Joint Genome Institute, CA. The designed primer pairs ID-IN-F2 and ID-IN-R2 successfully amplified a PCR product of 496 bp which was confirmed by sequencing to be the isocitrate dehydrogenase gene *(IDH)* (wild type in Fig. S5.3). Seedlings generated from mutant lines were tested for their homozygosity. The designed primer pairs ID-IN-F2 and ID-IN-R2 along with T3 primer successfully amplified multiplex PCR products of 600 bp for

homozygous mutants overexpressing isocitrate dehydrogenase gene (*idh* mutant line JJ19999-1,2,3 and 4 present in lanes 1, 4, 6 and 7 (Fig. S5.3) yielding a putative PCR product of 600 bp.

To validate whether the bacterial consortium influenced the expression of isocitrate dehydrogenase gene (*IDH)*, the relative transcript abundance of the gene was assessed in overexpressed *idh* mutants line JJ19999- 3 and 4 as well as in the wild type plants. Relative transcript abundance of the gene was almost similar in overexpressed *idh* mutant no. 3 with 1.15-fold increase compared to the control and 0.90-fold increase in *idh* mutant no.4 and 0.95-fold in the wild type plants (Fig. 5.4).

5.5 DISCUSSION

Rhizodeposits including root exudates are one of the main carbon sources for rhizospheric microorganisms, and the components of root exudates modulate the composition of the colonizing microbes surrounding plants (Hartmann et al. 2009; Kawasaki et al. 2016). Therefore, in this context, in order to have a better understanding of the root-microbe interaction, we aimed to investigate whether the multispecies bacterial consortium has the ability to enhance the accumulation of organic acids in *Brachypodium* roots and in their root exudates.

Sugars and organic acids found in roots and exudates generally have high concentrations, but values can be highly variable (Adeleke et al. 2017; Hartmann et al. 2009; Kawasaki et al. 2016). Examples include the release of citric acid from zea mays cutivars (Pellet et al. 1995), malic acid from Al-tolerant wheat genotypes (Ma et al. 1997), and oxaloacetate and glutaric acid from roots of *Brachypodium* (de la Peña et al. 2019). These results suggest that each component of root

exudates, including OA content, is dependent on the soil environmental condition and also varies with plant genotype as well as the developmental stage of the plant (Badri and Vivanco 2009).

It has been demonstrated that root exudates including organic acids are an important component of plant roots which selectively invite mutualistic endophytic microbes (Dennis et al. 2010). For example, Baldani et al. (2014) reported that the bacterial family Oxalobacteraceae utilizes oxalate as a source of carbon and promotes its exudations, while Kuiper et al. (2002) demonstrated that the growth of the bacterium *Pseudomonas putida* was promoted due to the presence of specific sources of carbon like citric and succinic acids. In agreement with the above studies, our results demonstrated that among the ten organic acids, fumaric and succinic acids were secreted in abundance in root exudates of *Brachypodium* roots inoculated with the multispecies consortium. These results do not only show evidence that multispecies consortium can recruit a selective organic acid, but also that plant-bacteria interactions are mediated by root exudations. Furthermore, the fact that these two organic acids increased in root exudates is not surprising since *Brachypodium* roots have in abundance fumaric and succinic acids (de la Peña et al. 2019).

Despite the fact that citric and malic acids represented the highest concentrations of organic acids in the inoculated and control roots of *Brachypodium,* the composition of root exudates had a different profile with lactic acid as the most abundant. The excessive accumulation of lactic acid in root exudates could be the result of oxygen deprivation (hypoxia) of the roots (Ryan et al. 2001). Under anoxic conditions, plants undergo anaerobic respiration producing lactic acid as an end-product that some plants release into the rhizosphere in order to avoid accumulation inside the roots (Xia and Roberts 1994). Consistent with this notion, in our study, *Brachypodium* seedlings were grown in semi-hydroponic culture system in which the roots were always in constant contact

with nutrient solution to avoid dryness of roots. This could have minimized the access of *B. distachyon* roots to oxygen thus making the surrounding rhizosphere of inoculated and control plants in it, a sink area for the lactic acid built up in the cellular metabolic pool.

The significance of citric acid production by roots of *Brachypodium* could be linked to the higher expression of its related genes (isocitrate dehydrogenase *IDH* and citrate synthase *CS*). The relative transcript abundance levels of both genes were higher but not significantly in inoculated roots of *Brachypodium* compared to the control treatment. It is possible that the differential gene expression could be influenced by time period of sampling after inoculation of the multispecies consortium (i.e., 24 hours vs 48 hours). The expression of malate dehydrogenase *MDH* (2.92-fold) and citrate synthase *CS* (3.48-fold) were differentially upregulated compared to the control when *Brachypodium* was inoculated with the consortium for 24 hours (data not shown). Future studies should focus on the temporal changes of gene transcription of *Brachypodium* roots inoculated in response to multispecies consortium at different time intervals.

To validate that transcript abundance levels of NADP+ dependent isocitrate dehydrogenase *(IDH)* gene are linked to the presence of the consortium, *idh* mutants line JJ 19999 no. 3 and 4 using the activation tagging construct pJJ2LBA were inoculated with the multispecies consortium This construct is designed to increase the transcription of the *IDH* gene. The expression of the tested *idh* mutants of isocitrate dehydrogenase gene *(IDH)* was similar to the wild type. We are not sure why the results did not produce the expected results. We can only speculate that since these mutations did not produce a phenotype, future experiments must be performed to prove that the mutation occurred in a regulatory region of the gene (Riethoven 2010). Also, in our study we

had insufficient number of generated mutant seeds, leading to very limited number of biological replicates which may affected the transcription levels.

In conclusion, our results show that the multispecies consortium was capable of inducing changes in the exudation profile of *Brachypodium*. A minimal upregulation occurred at the molecular level after 48 hours of inoculation, yet further studies need to be performed to confirm the findings.

5.6 ACKNOWLEDGMENT

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Table 5. 1 List of primers used in this study.

Genotyping[$]					QRT-PCR*				
Gene of interest	**Sequence (5'→3')**		**T(°C)**	**Size (bp)**	**Gene of interest**	**Sequence (5'→3')**	**T(°C)**	**Size (bp)**	**Source**
Citrate synthase (*CS*)	CS-IN-F3 CS-IN-R3	CTGAGGCATTACACCCCTGT TTCAGCAGTGAGAAGCCAGA	56	427	CS-Q-F CS-Q-R	CTCCCGTCCTTCCTTCAAATAA GATATCTAGAACCCGAGCAAGTC	55	226	This study
Malate dehydrogenase (*MDH*)	MD-IN-F1 MD-IN-R1	AAAAATGGGGCAGATCATCA CATTGCAGGGTCGGTTACTT	56	443	RT-MD-F RT-MD-R	TGCCAAGTGCTGTCCTAATG AGCACTTCAGCCACAAAGGT	55	171	This study
Succinate Dehydrogenase (*SDH*)	SD-IN-F1 SD-IN-R1	TGTCTTTCATGCGATTCAGC CACCTGGAAGGAGGAATGAA	56	480	SD-Q-F2 SD-Q-R2	CACGTCTTAGAAACCGCTGTA CCCATGACTTCGCCCTTATT	60	112	This study
Isocitrate Dehydrogenase (*IDH*)	ID-IN-F2 ID-IN-R2	ACTAATGGCGGATCTGA GGTTCCCGGTGTTTGATTTA	56	496	ID-Q-F2 ID-Q-R2	TACCCGTCATTTCCGTGTTC TGTGTGCAAGTCCTCTTGTC	60	92	This study

$To determine the homozygosity of mutants, primers T3 (AGCTGTTTCCTGTGTGAAATTG) and R9 (GATAAGCTGTCAAACATGAGAATTCAG) were used in a multiplex PCR along with the gene specific primers at 56 ºC. This multiplex PCR will produce two bands if seedlings are heterozygous and will produce single bands of different sizes if seedlings are homozygous.

*Two reference genes were used for QRT-PCR: Ubiquitin (60 ºC) and Actin (55ºC). UBC18-F (GGAGGCACCTCAGGTCATTT); UBC18-R (ATAGCGGTCATTGTCTTGCG); BdACTIN2-F (GTCGTTGCTCCTCCTGAAAG); BdACTIN2-R (ATCCACATCTGCTGGAAGGT).

Table 5. 2 General information about the mutant seeds ordered.

Mutant	T-DNA line	Gene Tagged	Construct	Insert Class	No. of seeds from DOE Genome Institute	No. of seeds from first generation	No. of homozygous seedlings
Malate Dehydrogenase (*mdh*)	JJ27103	Bradi3g12460.1	pJJ2LBA[^]	exon	10	11	N.A.
	JJ11635				10	7	
	JJ11665				10	14	
	JJ11645				10	11	
Succinate Dehydrogenase (*sdh*)	JJ11605	Bradi3g13980	pJJ2LBA[^]	5' UTR	10	8	N.A.
	JJ11687				10	7	
	JJ11574				10	10	
	JJ11675				10	10	
	JJ11621				10	9	
Citrate Synthase (*cs*)	JJ2510	Bradi3g08910	pJJ2LB [γ]	Exon	10	9	N.A.
Isocitrate Dehydrogenase (*idh*)	JJ19999	Bradi2g45420	pJJ2LBA[^]	Near[*]	10	11	4

[*] Near means within 1000 bp of the 3' or 5' end of the gene.

[^] The pJJ2LBA vector contain transcriptional enhancers within the T-DNA sequence. This "activation tagging" construct is designed to increase the transcription of nearby genes. It is particularly well suited to assign function to genes with redundant functions where knockouts in an individual family member do not produce a phenotype.

[γ] The pJJ2LB vector has the potential of creating a gene knockout.

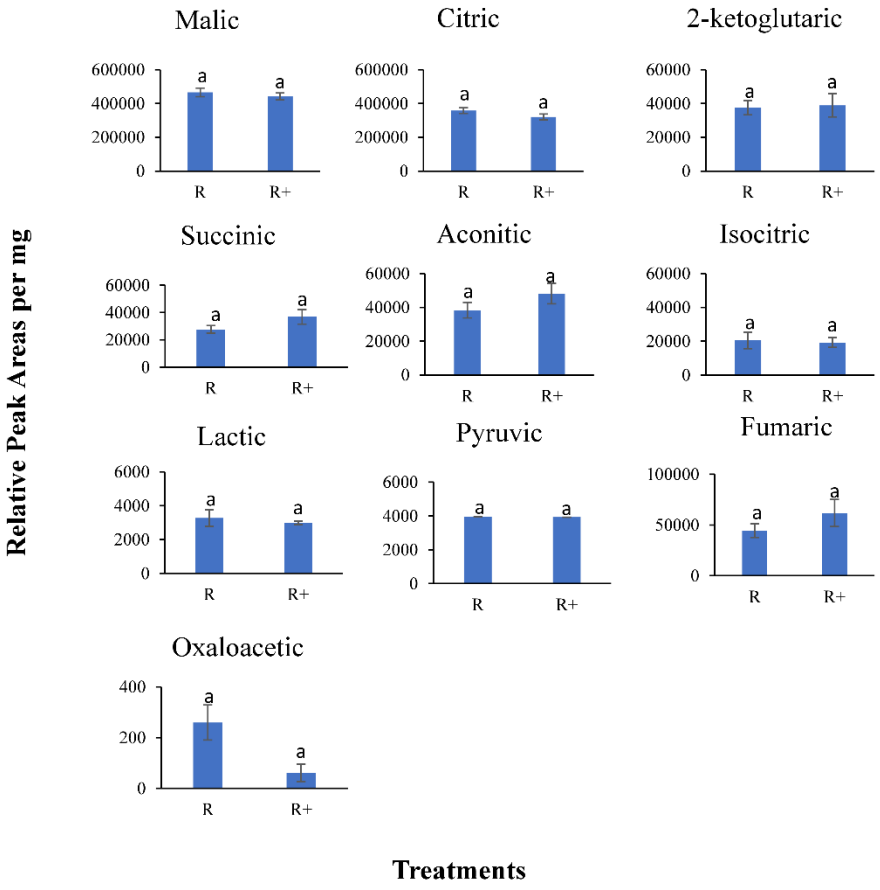

Figure 5. 1 Organic Acids and their corresponding relative peak areas per mg in the roots of the wild type *Brachypodium distachyon* Bd 21. R, Relative peak areas in control roots; R+, Relative peak areas in inoculated roots. Bars represent the average relative peak areas of three biological replicates ± standard error of the mean. Bars with the same superscript letters between inoculated and non-inoculated roots suggest that they are not statistically different according to Tukey's test ($p \leq 0.05$).

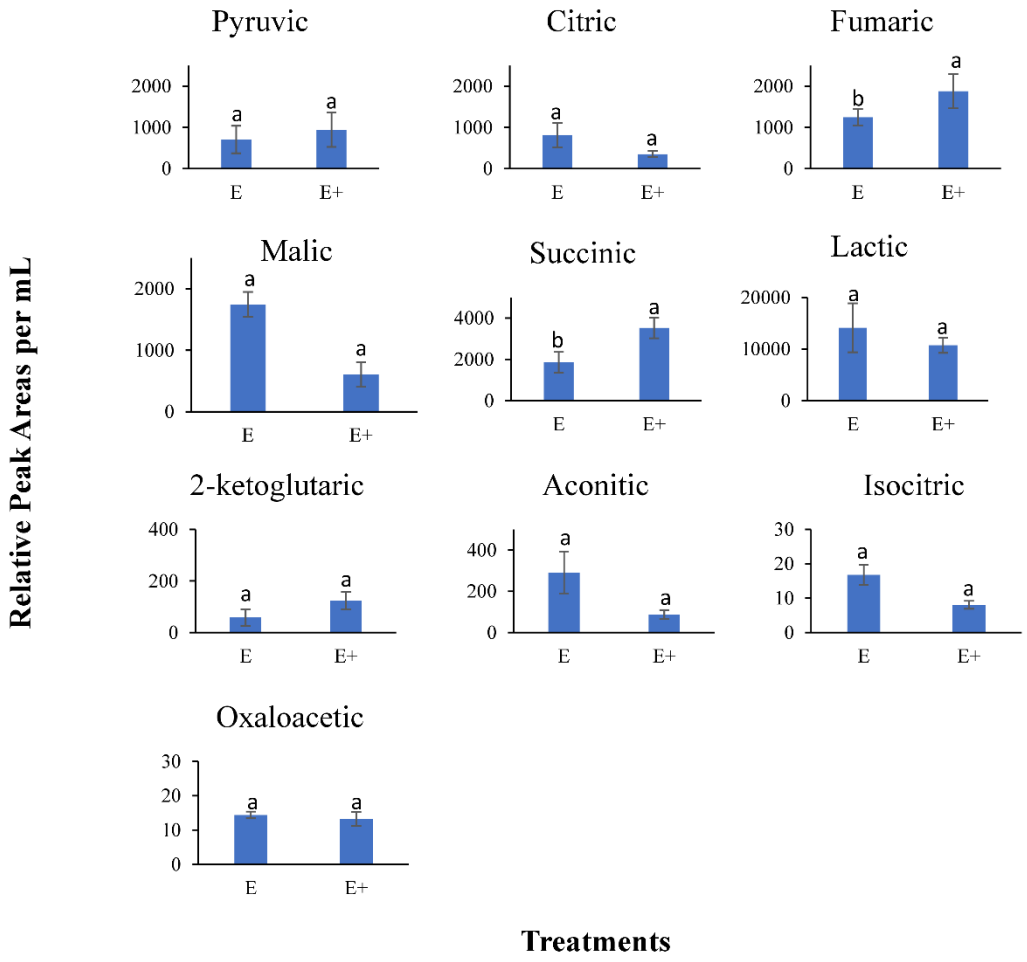

Figure 5. 2 Organic Acids and their corresponding relative peak areas per mL in the roots of the wild type *Brachypodium distachyon* Bd 21. E, Relative peak areas in the exudates of control plants; E+, Relative peak areas in the exudates of inoculated plants. Bars represent the average relative peak areas of three biological replicates ± standard error of the mean. Bars with different superscript letters between inoculated and non-inoculated root exudates differ significantly according to Tukey's test ($p \leq 0.05$).

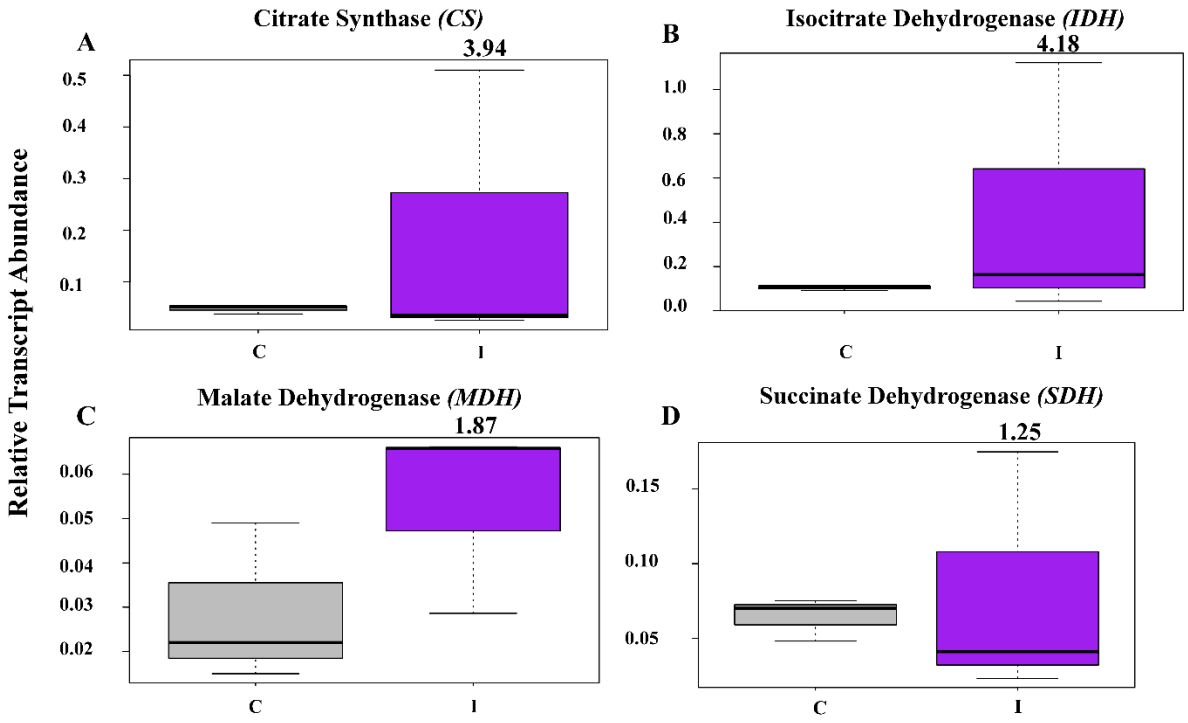

Figure 5. 3 Effect of multispecies consortium inoculation on the expression of different genes of organic acids after 48 hours of inoculation. (A) Citrate Synthase *(CS)*; (B) Isocitrate dehydrogenase (*IDH)*; (C) Malate dehydrogenase *(MDH)*; (D) Succinate dehydrogenase (*SDH)*. The relative transcript abundance of gene expression was normalized with appropriate housekeeping genes (Actin2 and UBC18). C, Control roots; I, Inoculated roots. Asterisk indicates significant relative transcript abundance between the control and the inoculated plants using t-test ($P < 0.05$). Bars represent the average relative transcript abundance of three biological replicates ± standard error of the mean. Numbers represent the fold change which was calculated by normalization of inoculated samples with their appropriate controls at each time point.

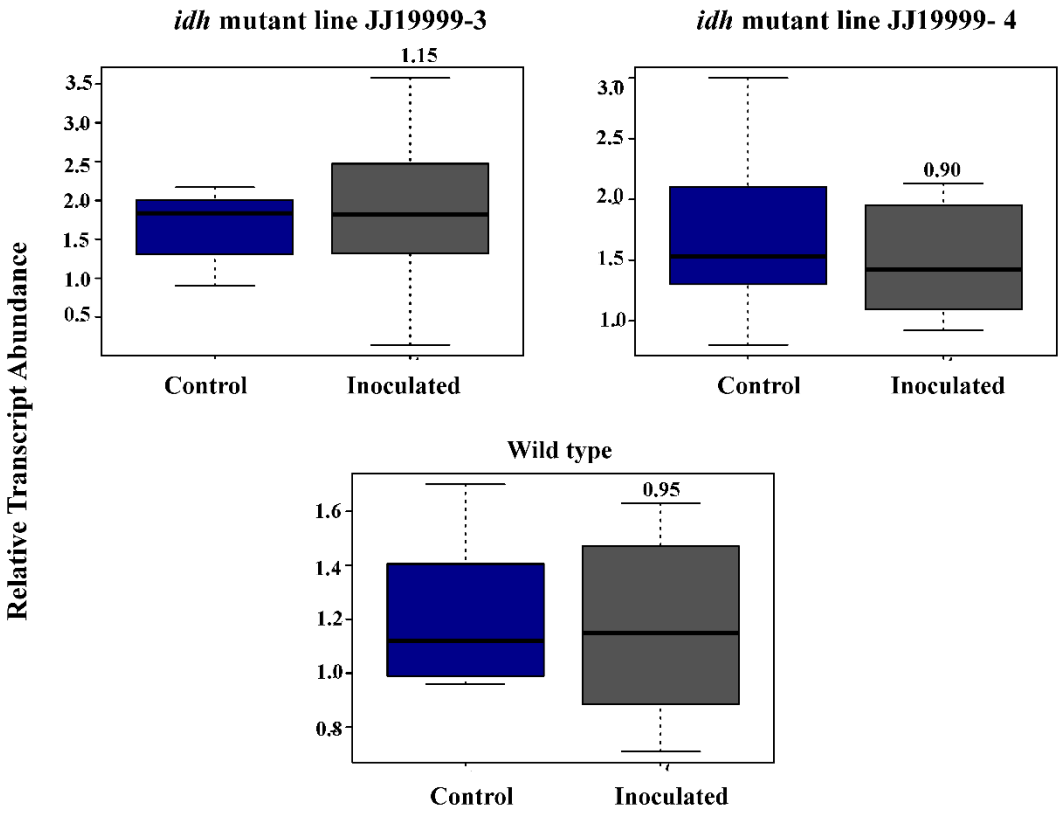

Figure 5. 4 Box-plot representation of isocitrate dehydrogenase gene *(IDH)* expression in wild type (Bd 21) and *idh* mutant seedlings (Bd 21-3) of *Brachypodium distachyon.* (A) *idh* mutant seedling no.3 from mutant line JJ19999; (B) *idh* mutant seedling no.4 from mutant line JJ19999; (C) Wild type. Bars represent the average relative transcript abundance of three biological replicates ± standard error of the mean. Numbers represent the fold change which was calculated by normalization of inoculated samples with their appropriate controls.

GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

6.1 CONCLUSIONS

This thesis was written having two broad objectives in mind; to identify associated bacteria of timothy grass that could be used as plant growth promoters in grasses under biotic and abiotic stress conditions, and to understand the microbe-microbe as well as the plant-microbe interactions, the required traits that could potentially lead to a successful communication, along with the potential of certain multispecies consortia to be universally used in grasses.

To the best of our knowledge, this study reported for the first time the abundance, diversity and distribution of bacteria associated with different tissues of two cultivars of timothy grass (*Phleum pratense* L.) grown under field conditions, along with their biochemical and molecular characterization. Despite several studies related to bacteria associated with grasses, an in-depth study on the use of bacteria on cool season grasses, such as timothy is lacking. Hence, studies such as ours using strains isolated from timothy on the model grass *Brachypodium* help to bridge the gaps leading to a possible overview for the kind of relation between bacterial isolates and forages.

Following a series of cultural and biochemical tests, bacterial strains were studied for their growth promoting attributes and the capacity to sustain biotic and abiotic stress conditions through the production of indole-acetic acid, siderophores, HCN and a large array of enzymes that are capable of making nutrients more available to the grass, as well as enzymes capable of degrading cell walls of fungi. These attributes have accelerated the selection of efficient strains successfully colonizing the internal parts of plants tissues.

This study allowed us to gain insight on the effect of individual organic acids and root exudates released from *Brachypodium distachyon* on the multispecies consortium. Features, like the competency and compatibility of single free cells behaving like a multicellular entity were

documented. In addition, the study provided information on bacterial biofilm traits, including motility, alginate and exopolysaccharide production and their chemotactic behavior towards pure organic acids and root exudates. All these traits enhanced the chances of rhizospheric bacteria to successfully colonize the host.

This thesis presented knowledge on specific organic acid(s) released from *Brachypodium* roots which may have modulated the chemotactic response and biofilm formation of multispecies consortium, and also induced changes in the expression of the genes coding for organic acid synthesis in colonized *Brachypodium*.

Overall, this research provides novel insights into the importance of bacterial microbes in forages and will help pave the way for breeding programs of timothy and the development of effective universal consortia for forages.

6.2 FUTURE WORKS

6.2.1 Genomic study of the multi-species consortium

Understanding the genetic profile of each microbe of the consortium would provide us with knowledge on possible applications of the consortium due to the availability of a vast amount of data ready to be processed for downstream applications.

6.2.2 Biochemical and genetic studies

Root-secreted chemicals mediate multi-partite interaction in the rhizosphere. In this thesis we have profiled the organic acids released from *Brachypodium* root exudates. Additionally, metabolite profiling of bacteria is required to identify which organic acids are secreted from the bacterial strains and how they could affect their host. Organic acids are one component. Future

studies should address how other components including carbohydrates, amino acids and enzymes of root exudates not only affect the colonization of *Brachypodium* by the multispecies consortium but alter their environment.

6.2.3 Research on the role of grasses in the interaction with microbes

This study has proved that the inoculation with the bacterial strains can promote the growth of grasses by favouring growth promoting traits. To further understand this relation, it is essential to have solid understanding about the mechanisms leading to this interaction. What are the mechanisms activated in the presence of endophytes and whether the same mechanisms are triggered in the model grass *Brachypodium* compared to other grasses of the *Poaceae*.

Photosynthesis may have an impact on the production of root exudates and the potential observable changes that could occur in phenotypes of grasses such as flowering and seed development under inoculated conditions. Furthermore, Bushell et al. (2019) have shown the effect of different concentrations of organic acids and low pH on the pathogen *Pseudomonas aeruginosa*. Thus, concerns should arise regarding the effect of the plant growth medium, its nutritional content and pH, and how this could alter the microbiome around it and indirectly its exudation profile and expression of related genes.

6.2.4 Large scale field study

For successful application of the consortium large scale studies under field conditions are an absolute requirement. This is crucial to evaluate the capacity of the consortium to survive and to compete with other microorganisms present in the field.

6.2.5 Approaches for microbe application

A huge effort should be invested on how to apply the microbes. Encapsulation of the product for soil-based applications should be considered. Studies focusing on the optimum number of cells, storage and shelf-life are essential. The multispecies consortium in this study was isolated from the rhizosphere of timothy; it would be important explore whether the consortium could be applied by foliar application. Endophytes are known to have different routes of entry to plants (Hardoim et al. 2008). Stomata and leaf openings could be one of them. Thus, by having a foliar application strategy, the multispecies consortium could have multifaceted applications for several purposes.

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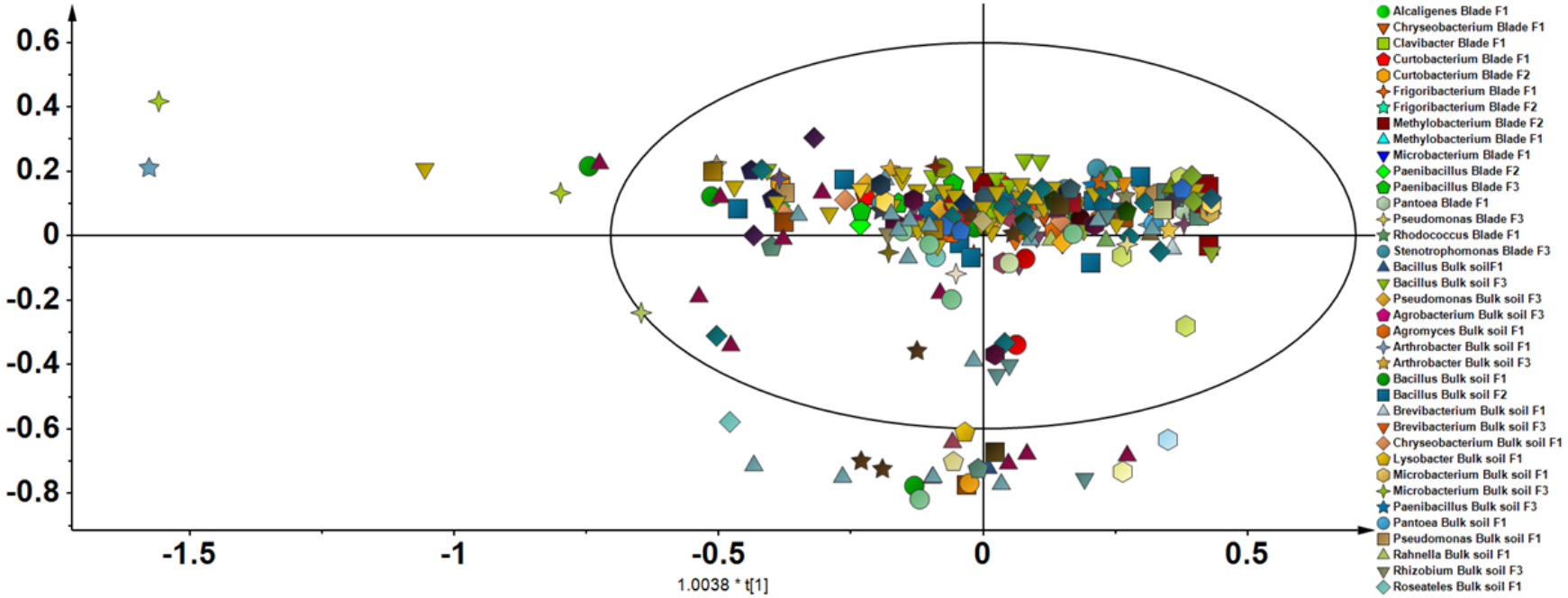
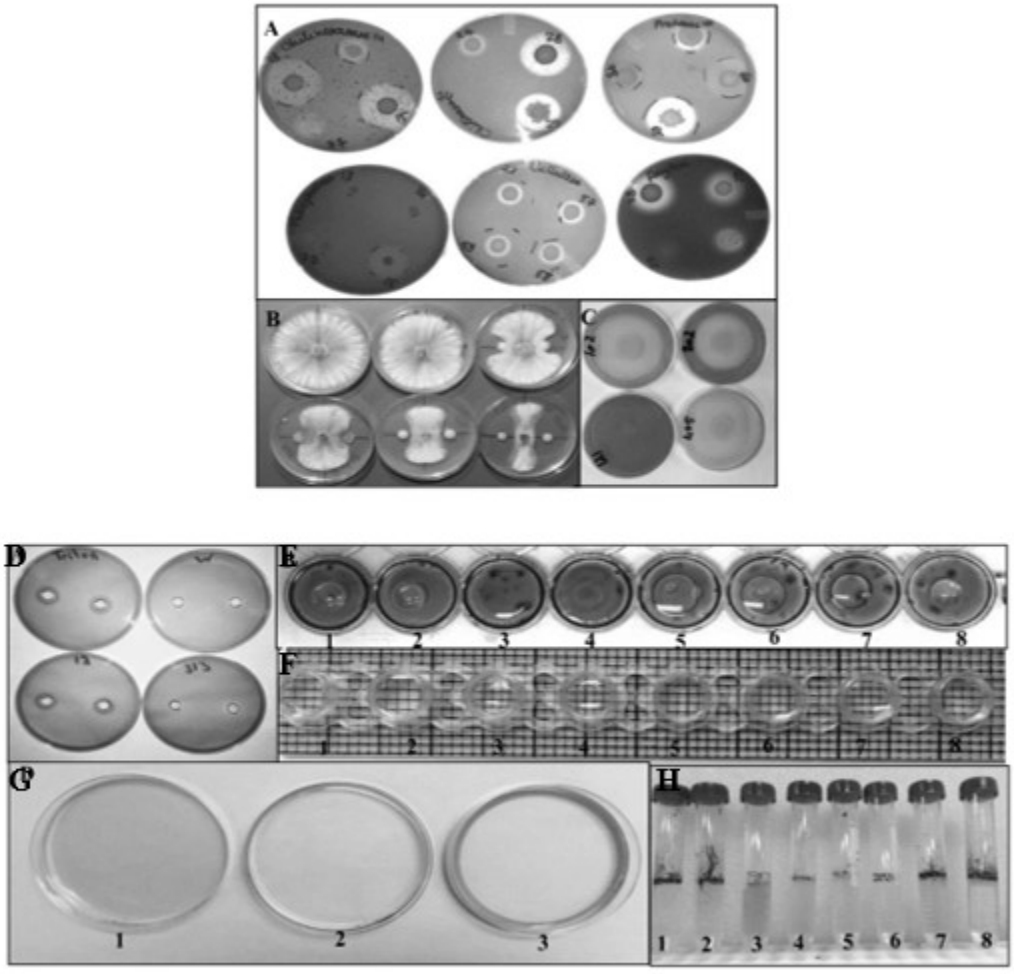
APPENDICES

8.1 SUPPLEMENTARY FIGURES AND TABLES

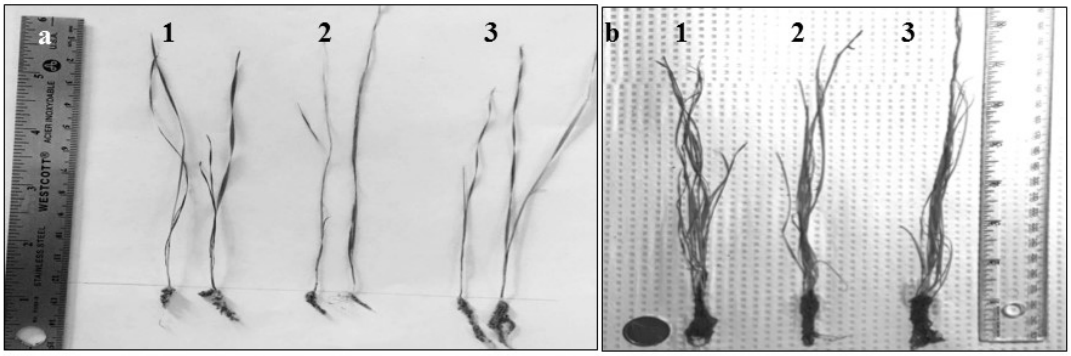
Supplementary Figure 3. 1 Orthogonal Partial Least Square-Discriminant analysis (OPLS-DA) PC1/PC2 score plot of 320 bacterial isolates. Analysis is based on their recorded biochemical attributes. The ellipse represents the Hotelling's T^2 with 95% confidence interval.



Supplementary Figure 3. 2 Representative examples of culturable bacterial isolates exhibiting biochemical antagonistic and biosurfactant activities. (A) Enzymatic activities of representative bacteria. From left to right: chitosanase, chitinase, protease, phosphatase, cellulase, amylase. (B) Antifungal activity against *Sclerotinia sclerotiorum.* (C) Siderophore production. (D) CTAB assay with representative plates of Triton X-100 as a positive control, water (W) as a negative control, and isolates 12 and 313. (E) Drop Collapse test; (1) water, (2) LB broth, (3) triton X-100, (4) isolate 70, (5) isolate 49, (6) isolate 234, (7) isolate 178, (8) isolate 109. (F) Microplate Test; (1) water, (2) LB broth, (3) triton X-100, (4) isolate 39, (5) Isolate 8, (6) isolate 144, (7) isolate 23, (8) isolate 28. (G) Oil Spreading Test; (1) LB broth, (2) Triton X-100, (3): Isolate 12. (H) Emulsification Test; (1) water, (2) LB broth, (3) triton X-100, (4) isolate 11, (5) isolate 90, (6) isolate 18, (7): isolate 70, (8) isolate 28.



Supplementary Figure 3. 3 Timothy grass seedlings inoculated with isolates *Bacillus* isolate 28 and *Pseudomonas* isolate 234. (a). Seedlings drenched with (1) dead pellet of isolate 28; (2) water; (3) live pellet of isolate 28. (b). Seedlings drenched with (1) water; (2) dead pellet of isolate 234; (3) live pellet of isolate 234.



Supplementary Table 3. 1 Screening assays for biosurfactant-producing bacteria.

Cell-free supernatant	Method	Method of Assessment	Ingredients	Reference*
150 µl	CTAB	Formation of dark blue Halo	Cetyltrimethylammonium bromide (CTAB), Methylene blue	1,2
5 µl	Drop collapse	Drop spread and collapse	Crude oil (2µl) as coating material of well covers of 96 well microplate	3
10 µl	Oil spreading	Oil displacement and diameter of clearing zone on the surface of the oil layer	Crude oil (20µl) layered over 20 ml of water in a petri plate	4
1000 µl	Emulsification capacity	Colorimetric at OD_{600}	Layer of crude oil /50mM Tris buffer pH 8.0	5
45 µl	Microplate	Optical distortion of the grid image	PCR caps placed on 1mm x 1mm squares	3

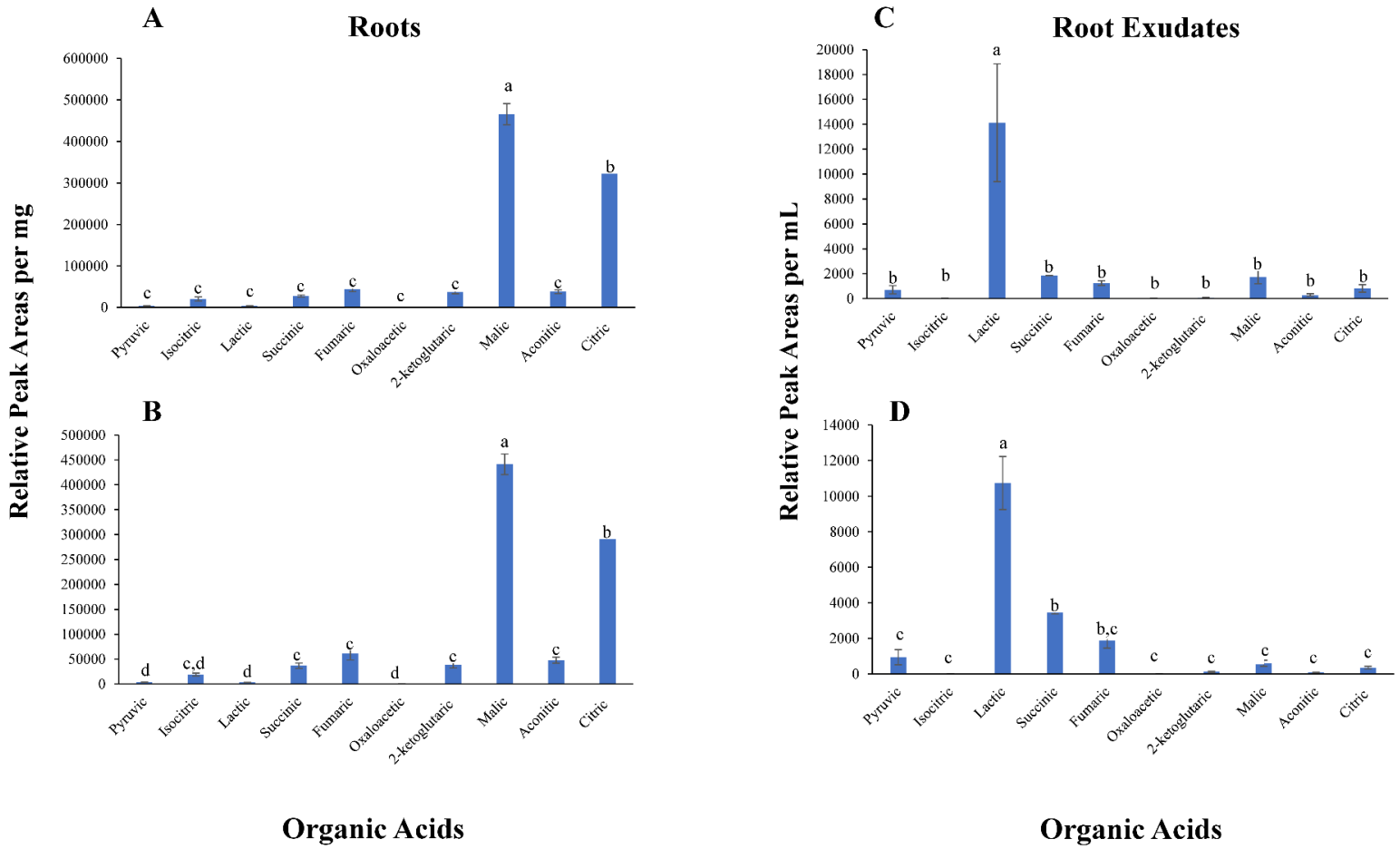
*Methodology for each test is fully described in listed references.
1Tahzibi et al. (2004); 2. Walter et al. (2010); 3. Jain et al. (1991); 4. Ibrahim et al. (2013); 5. Muthezhilan et al. (2014).

Supplementary Table 3. 2 Minor genera isolated from timothy.

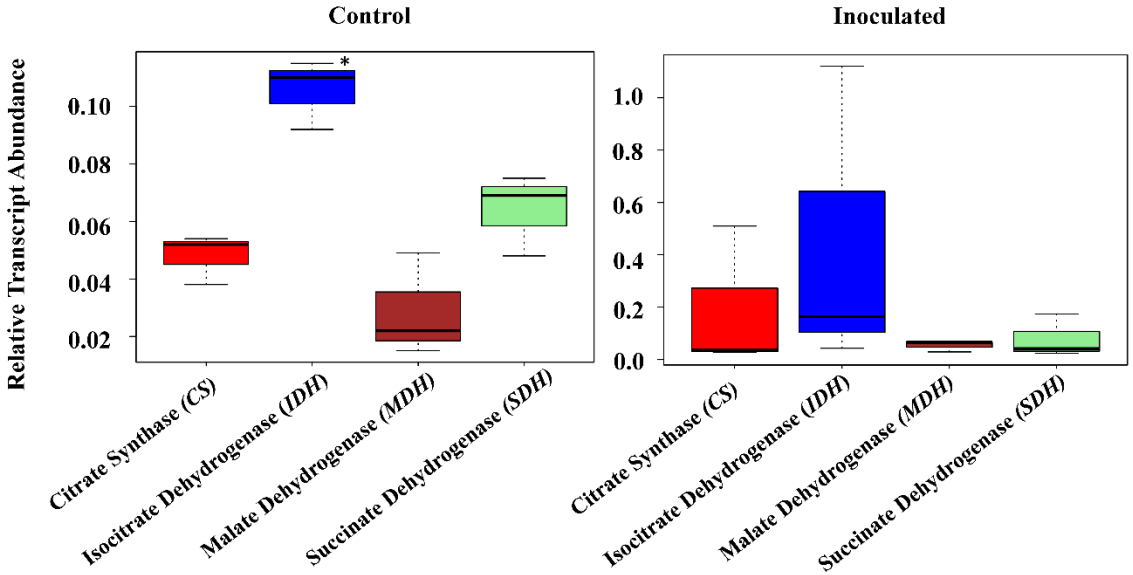
	Sources*			
Genus	**Bulk Soil**	**Rhizosheath soil**	**Crown area**	**Leaves**
Acidovorax	0	0	1	0
Acinetobacter	1	1	0	0
Actinobacterium	1	0	0	0
Aeromicrobium	1	0	0	0
Agreia	0	0	1	0
Agrobacterium	1	0	0	0
Agrococcus	0	0	0	1
Agromyces	2	0	0	0
Alcaligenes	0	0	0	1
Burkholderia	0	0	1	0
Buttiauxella	0	1	0	0
Caulobacter	0	0	2	0
Citrobacter	0	1	0	0
Clavibacter	0	1	0	2
Enterobacter	0	3	0	0
Escherichia coli	0	0	0	1
Flavobacteriaceae	0	0	1	0
Flavobacterium	0	0	3	0
Frigoribacterium	0	0	0	3
Lysobacter	1	0	0	0
Microbacteriaceae	0	0	0	2
Mitsuaria	0	1	0	0
Nocardia	0	1	0	0
Novosphingobium	0	1	0	1
Plantibacter	0	1	0	0
Proteobacterium	0	1	0	0
Raoultella	0	1	0	0
Rathayibacter	0	0	0	1
Rhizobium	1	0	0	1
Rhodococcus	1	1	0	1
Roseateles	1	2	0	0
Sphingobacteriaceae	0	1	0	0
Sphingobacterium	1	2	0	0
Sphingopyxis	0	1	0	0
Sporosarcina	0	1	0	0
Subtercola	1	0	0	0
Terrimonas	0	1	0	0
Williamsia	1	1	0	0
Xanthomonadaceae	0	0	1	0
Xenophilus	0	1	0	1
Sphingobium	0	1	0	0
Sphingomonas	0	1	0	1

*The counts represent the number of isolated endophytes that are three or less in total number.

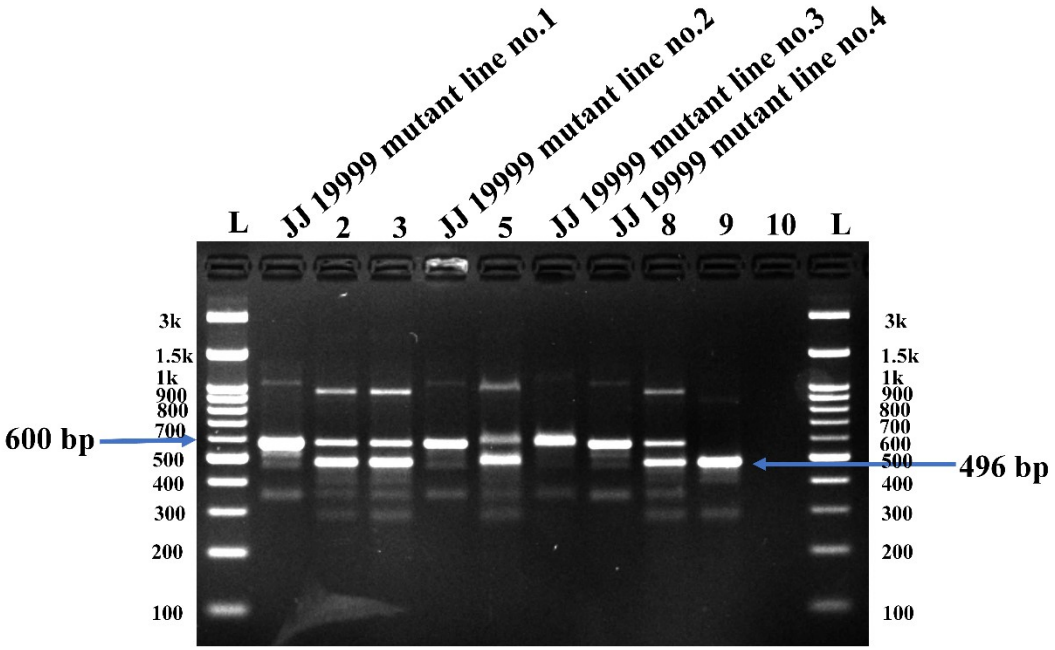
Supplementary Figure 5. 1 Organic Acids and their corresponding relative peak areas per mg in (A, B) Roots; and per mL in (C, D) Exudates. (A) Organic Acids in roots of control plants; (B) Organic Acids in roots of inoculated plants; (C) Organic Acids in exudates of control plants; (D) Organic Acids in exudates of inoculated plants. Bars represent the average relative peak areas of three biological replicates ± standard error of the mean.



Supplementary Figure 5. 2 Expression of organic acid genes under control and inoculated conditions. (A) Gene expression of organic acids under control conditions; (B) Gene expression of organic acids under inoculated conditions. Asterisk indicates significant relative transcript abundance between organic acid genes. Bars represent the average relative transcript abundance of three biological replicates ± standard error of the mean.



Supplementary Figure 5. 3 Gel of a multiplex PCR using the isocitrate dehydrogenase *(IDH)* primers along with T3 primer. The gel helps with the identification of mutant, heterozygous and wild type *Brachypodium* seedlings based on the difference in band sizes. L, 100 bp +3K DNA Ladder; The numbers represent samples from seedlings of the first generation of Bd21-3 seeds ordered from the DOE Joint Genome Institute. Lane numbers 1, 4, 6 and 7 represent homozygous plants from mutant line JJ19999 and represent *idh* mutants from seedlings no. 1, 2, 3 and 4 respectively. Lanes: 9, Wild type (positive control); 10, negative control. The the other lanes represent heterozygote seedlings.



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