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THE DISTRIBUTION AND POTENTIAL BENEFITS OF RHIZOSPHERE- ASSOCIATED BACTERIA IN CORN (Zea mays L.)

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THE DISTRIBUTION AND POTENTIAL BENEFITS OF RHIZOSPHERE-ASSOCIATED BACTERIA IN CORN (*Zea mays* L.)

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Abstract

Plant growth-promoting rhizobacteria (PGPR) are known for their potential use as biofertilizers, which can help to reduce the need and cost for synthetic fertilizers. The distribution and quantification of three PGPR in association with corn (*Zea mays* L.) was determined by the detection of *chaperonin*60 genes through real-time quantitative PCR. *Sphingobacterium canadense*, *Azospirillum zeae*, and *Gluconacetobacter azotocaptans* were detected at concentrations approaching 10^6 CFU/g in root tissues and rhizosphere soils in corn-growing regions of Ontario and Québec. Three additional bacterial isolates were recovered from corn rhizosphere soil and roots then identified via colony PCR. Greenhouse trials of all six bacteria as seed inoculants yielded no significant promotion of growth in corn. This study indicated that *S. canadense*, *A. zeae*, and *G. azotocaptans* were detected in locations where previously unknown to be, new PGPR can be isolated, and that a variety of potential PGPR inoculants failed to promote corn growth.

Keywords: *Azospirillum zeae*, bacteria, chaperonin, *Gluconacetobacter azotocaptans*, plant growth-promoting rhizobacteria, real-time qPCR, rhizosphere, soil, *Sphingobacterium canadense*, *Zea mays* L.

Dedication

To my loving family and friends who have been great supporters along the way.

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List of Abbreviations

ANOVA	analysis of variance
bp	base pair
BLAST	Basic Local Alignment Search Tool
CCM	combined carbon medium
CFU	colony-forming unit
CHU	crop heat units
*cpn*60	Type 1 chaperonin gene
C_t	threshold cycle
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphates
EDTA	ethylenediaminetetraacetic acid
FAM	6-carboxy-fluorescein
gDNA	genomic deoxyribonucleic acid
IAA	indole-3-acetic acid
kDa	kilodalton
Kb	kilo base pairs, 1000 base pairs
LGI	nitrogen-free medium
N	nitrogen
NCBI	National Center for Biotechnology Information
NFM	nitrogen-free medium
NTC	no-template control
PGPR	plant growth-promoting rhizobacteria

qPCR	real-time quantitative polymerase chain reaction
rDNA	ribosomal deoxyribonucleic acid
TAE	Tris-acetate-EDTA
UV	ultraviolet

All numerical units included in this thesis are standard SI units

Chapter 1

General Introduction

1.1 Corn as a crop

1.1.1 Scale and impact of corn production

Corn (*Zea mays* L.) is one of the most widely farmed cereals in the world. In Canada, it is estimated to currently cover 1,200,000 hectares (Statistics Canada, 2010), an area that has remained steady for the last decade. The total yield from this crop was 10,554,500 metric tonnes in 2007 for all of Canada (Statistics Canada, 2007). Corn production is highly dependent on chemical fertilizers such as nitrogen, usually applied at rates of 140-165 kg N/ha/year (Anuar *et al.*, 1995, OMAFRA, 1995). The seed is used in an extremely wide range of products such as cosmetics, plastics, adhesives, feed for humans and livestock, and, more recently, fuel alcohol. As demand for corn rises, particularly for ethanol production, intensified corn cultivation presents an economic challenge. An increase in the demand for costly fossil fuels occurs because they are a significant component in the production of synthetic fertilizers. According to the United States Department of Agriculture (USDA), the cost of nitrogen fertilizers (e.g. urea, ammonium nitrate and others) in the past 10 years has doubled, peaking at approximately $500/tonne (USDA, 2010). There is also an environmental concern related to the leaching of excess synthetic fertilizer with respect to maintaining soil and water quality within the ecosystem. Over-application of fertilizers, or application at a time when the ground is waterlogged or the crop is not able to use the fertilizer, can lead to contamination of ground- and surface waters. This can lead to rapid growth of algal species and

destruction of lake ecosystems through a process called eutrophication (Environment Canada, 2001). Excess N, above 10 ppm nitrate-nitrogen (OMAFRA, 2005), in drinking water can lead to adverse health effects such as blue-baby syndrome (Comly, 1945). For these reasons, research towards achieving the required crop fertility/nutrient status while addressing cost and environmental concerns is currently of interest.

1.1.2 Macronutrients required for corn production

Soil nutrients, particularly nitrogen, potassium and phosphate, are needed for corn growth and development. Currently, the fertilitization needs of most corn crops are met through the application of synthetic fertilizers but manures, biosolids, and crop rotations with legumes are also utilized to increase the fertility of the soil (OMAFRA, 2009).

The limiting macronutrient in the soil is usually nitrogen because it must be converted to a reduced or fixed state before it can be used by crops. Corn is a non-leguminous species so it cannot form symbiotic relationships with bacteria that fix nitrogen through nodules as is found with crops such as peas (*Pisum sativum* L.), beans (*Phaseolus vulgaris* L.), lentils (*Lens culinaris* M.), and alfalfa (*Medicago sativa* L.). These symbiotic relationships are highly valued because of the nutrients they can provide to the plant. Corn has been found to have relationships with associative and free-living bacteria that can biologically fix nitrogen. The distinction between nitrogen fixation via associative versus free-living bacteria is not completely clear because many organisms can be defined by one or both terms. Associative nitrogen fixation is commonly defined as

fixation of nitrogen by diazotrophic bacteria under the direct influence of a host, exchanging nutrients but no differentiated root structures are formed (Dalton and Kramer, 2006). Free-living nitrogen fixation occurs within the vicinity of the plant but the microbes do not rely on the plant as a host. Associative bacteria are mostly found closer to the roots of plants, whereas free-living bacteria are found further within the soil. Plant growth-promoting rhizobacteria (PGPR) are free-living bacteria that can colonize around plant roots and compete with other microflora for nutrients (Kloepper *et al.*, 1978). Some PGPR, referred to as endophytes, are capable of invading the tissues of living plants causing asymptomatic infections (Sturz and Nowak, 2000). PGPR have the potential to provide their plant hosts with fixed nitrogen, solubilized phosphates, and other micronutrients as well as growth-stimulating phytohormones, such as indole-3-acetic acid (IAA) (Podile and Kishore, 2006).

In Brazil, endophytic, nitrogen-fixing bacteria such as *Gluconacetobacter diazotrophicus*, are utilized in sugarcane production, thereby reducing the rates of synthetic fertilizer application levels to 50 kg/ha versus 100-350 kg/ha in other countries (Baldani, 2002). Tropical climates, such as that of Brazil, are more favourable for associative and free-living nitrogen fixation because of optimal temperatures and high light intensity resulting in an increased supply of photosynthate (Döbereiner *et al.*, 1972).

1.2 Rhizosphere

Regions within the soil ecosystem that are adjacent to and influenced by seeds and roots provide microenvironments with organic substrates that are

readily available for proliferation of a diverse array of soil microorganisms (Kennedy, 2005). Plants offer a wide range of habitats that support microbial growth, which include sites that are moist and rich in nutrients (Beattie, 2006). One region in particular that microorganisms inhabit is the area influenced by the root system, called the rhizosphere. This volume of soil surrounding the roots is influenced chemically, physically, and biologically by the plant roots (Sorensen, 1997). It is well known that many plants can establish rhizospheric and/or endophytic associations with various bacteria such as *Azospirillum* – a bacterial genus that can supply plants with fixed nitrogen (Beattie, 2006).

1.3 Plant growth-promoting rhizobacteria

1.3.1 Diversity of PGPR

PGPR are divided into two groups according to their mode of plant interaction, direct or indirect (Glick *et al.*, 1999). Phytohormone production and enhancement of plant nutrition are the two major mechanisms by which PGPR directly contribute to plant growth. Enhanced plant nutrition occurs mainly through iron uptake by production of iron-chelating siderophores and phosphorus uptake by solubilization of inorganic phosphates (Podile and Kishore, 2006). Indirect benefits of PGPR include suppression of harmful microorganisms that inhibit plant growth and suppression of root pathogens through parasitism and competition for nutrients and space within the plant root area (Podile and Kishore, 2006).

Plant type, soil type and the nutrients available are the largest factors affecting the diversity of PGPR. Strains of genera including *Azospirillum* (Munir

et al., 2003; Mehnaz *et al.*, 2007a), *Azotobacter* (Narula *et al.*, 1981), *Bacillus* (de Freitas *et al.*, 1997; Murphy *et al.* 2003; Jaizme-Vega *et al.*, 2004), *Enterobacter* (Mirza *et al.*, 2001), *Gluconacetobacter* (Mehnaz *et al.*, 2006a), *Klebsiella* (Iniguez *et al*, 2004), *Pseudomonas* (Arndt *et al.* 1998; Garcia *et al.* 2003; Dey *et al.*, 2004; Tripathi *et al.*, 2005), *Phyllobacterium* (Bertand *et al.*, 2001), and *Rhizobium* (Antoun *et al.*, 1998; Gupta *et al.*, 2003) have been identified as PGPR, while the search for additional strains continues.

The search for growth-promoting bacteria to enhance non-leguminous plant growth has been the focus of study in the Lazarovits' lab for multiple years. Work completed by Mehnaz (2006a; 2007a; 2007b) resulted in three promising newly discovered species in Canada, *Gluconacetobacter azotocaptans*, *Sphingobacterium canadense*, and *Azospirillum zeae*.

1.3.2 *Gluconacetobacter azotocaptans*

Gluconacetobacter species are often present in high densities in or surrounding the xylem of grasses (Hurek *et al.*, 1994), rice (You and Zhou, 1989) and sugarcane (Oliveira *et al.*, 2002). Vascular tissue provides an ideal environment for colonization because it has a relatively low pO_2 and high concentrations of photosynthate (Butz and Long, 1979). Association with the vascular elements (both xylem and phloem) facilitates nutrient exchange between the diazotroph and the host plant (Patriquin, 1983). *Gluconacetobacter diazotrophicus*, a species found in high numbers in roots and stems of sugarcane in Brazil and Australia (Stephan, 1991), was the only known nitrogen-fixing species of this genus until Jimenez-Salgado *et al.*, (1997) isolated two more

acetic-acid producing, nitrogen-fixing species, *Gluconacetobacter azotocaptans* and *Gluconacetobacter johannae*, from the rhizosphere soil of coffee plants (Fuentes-Ramirez, 2001). The original discovery of *G. diazotrophicus* and its association with nitrogen fixation in sugarcane came about after many years of attaining high yields of this crop in Brazil while using minimal amounts of N fertilizer. Brazil is the world's primary producer of sugarcane, supplying high demands for export as well as using the sucrose for ethanol production to fuel approximately half of their automobiles (Triplett, 1996). Uncovering an association with corn similar to *G. diazotrophicus* and sugarcane would have a huge impact on current agricultural practices in temperate regions.

Gluconacetobacter azotocaptans DS1 is a Gram-negative, acid producing, nitrogen-fixing bacterium that was originally isolated from the rhizosphere soil of corn grown in Delhi, Ontario (Mehnaz *et al.* 2006a). Analysis showed strain DS1 to produce IAA (106 ng/ml), fix nitrogen (40 nmol/h/mg bacterial protein) and solubilize phosphate (Table 1.1). Strain DS1 can also produce antibiotic compounds against several fungal pathogens and has been shown to promote the growth of three corn varieties in sterile sand and unsterilized soil under greenhouse conditions (Mehnaz and Lazarovits, 2006b). Given these findings, this isolate appears to be a promising agent for use as a biofertilizer.

1.3.3 *Sphingobacterium canadense*

Many *Sphingobacterium* species, including *S. antarcticum*, *S. bambusae*, *S. composti* and *S. anhuiense* and others, have been discovered in association with materials ranging from soil surrounding lakes (Shivaji *et al.*, 1992), compost

Table 1.1. Characteristics of *Sphingobacterium canadense, Gluconacetobacter azotocaptans,* and *Azospirillum zeae,* from Mehnaz *et al.* (2006a; 2007a; 2007b).

Characteristic	*S. canadense*	*G. azotocaptans*	*A. zeae*
Motility	–	ND	+
Growth at 5 °C	–	ND	–
Growth at 41°C	–	–	+
Gram stain reaction	–	–	–
pH range for growth	5.0-10.0	4.0-7.0	5.0-7.0
Carbon source utilization:			
D-Glucose	+	±	±
D-Galactose	+	±	+
D-Mannitol	–	–	+
Sucrose	+	+	–
Glycerol	+	+	+
Maltose	+	–	–
IAA production	+	+	+
Phosphate solubilization	–	+	–
Nitrogenase activity	–	+	+

For growth at 5 °C, growth at 41 °C, and carbon source utilization:
+, good growth; –, no growth; ± slight growth; ND, not determined

samples (Ten *et al.*, 2006), soil from forests (Wei *et al.*, 2008) and bamboo plantations (Duan *et al.*, 2009). From this, it is evident that these bacteria have an association with plants but few studies have been conducted on their ability to promote plant growth.

One recent study with *Sphingobacterium* strain 1ZP4 from a metal-contaminated site revealed significantly increased root and shoot length as well as root and shoot biomass in corn. Also, P and N levels in the roots were greater than that of the non-inoculated control (Marques *et al.*, 2010).

Sphingobacterium canadense CR11 is a free-living, Gram-negative bacterial strain isolated from corn rhizosphere soil of fields from London, Ontario in 2007. The genus *Sphingobacterium* currently contains 17 species and *S. canadense* was the first to be isolated in Canada. The cells of this bacterium are short rods; they grow between 20 and 37°C, pH ranging from 5-10 and concentrations of 0.5-3% NaCl, and produce the plant hormone, IAA (277 ng/ml) (Mehnaz *et al.*, 2007b) (Table 1.1). Recent greenhouse trials with two corn varieties inoculated with *S. canadense* CR11 in sterile sand revealed 24% and 30% increased dry shoot weights and 16% and 21% increased dry root weights after 30 days growth (Mehnaz *et al.*, 2010). Similar effects were noted in corn dry root and shoot weights when the experiment was repeated in non-sterilized field soil.

1.3.4 *Azospirillum zeae*

Azospirillum is one of the most extensively studied genera of diazotrophs. In 1925, the first *Azospirillum* isolate was discovered in the Netherlands by

Beijerinck (as cited in Tarrand *et al.* 1978) but only beginning in 1975 was its potential value in plant development recognized. Döbereiner and Day (1975) noticed grasses associated with *Azospirillum* were not as nitrogen deficient as those that lacked *Azospirillum*. Since then, *Azospirillum* has been isolated from the roots of numerous wild and cultivated plants, from moist tropics (Döbereiner *et al.*, 1976; Kirchhof *et al.*, 1997; Gunarto *et al.*, 1999) to temperate regions (Mertens and Hess, 1984; Mehnaz et al, 2007a). It has been found at densities as high as 10^5-10^7 bacteria per gram soil or root in the tropics (Döbereiner and Pedrosa, 1987). Many species of this genus are capable of fixing nitrogen and promoting plant growth through a variety of mechanisms. For example, *Azospirillum brasilense* Az39 promoted seed germination and early seedling growth in corn, especially increased dry plant weight and shoot length when compared to non-inoculated seeds in a greenhouse evaluation (Cassan *et al.*, 2009).

Azospirillum zeae N7 was isolated from corn rhizosphere soil in London, Ontario in 2007. The species is described as Gram-negative, with motile rods via single polar flagellum and has semi-helical shaped cells – a consistent characteristic of *Azospirillum* spp. This isolate grows between 20-41°C, pH 5-7 and in 0.5-1.0% NaCl (Mehnaz *et al*, 2007a). For *Azospirillum zeae* N7, Mehnaz (2007a) demonstrated IAA production in the presence of 100 mg tryptophan/L in combined carbon medium (CCM) (Rennie, 1981). The amount of IAA produced was between 6.0–6.5 mg/ml. An acetylene reduction assay was carried out as described by Mehnaz *et al.* in 2006 using semi-solid malate medium. A veil-like

subsurface pellicle formed and from this area of high nitrogenase activity, a range of 5.9–6.6 µmol ethylene/h/mg protein was observed (Mehnaz *et al.*, 2007a). *Azospirillum zeae* N7 has increased root weight in corn variety 39D82 and increased shoot weight in corn variety 39T68 (Mehnaz and Lazarovits, 2006b) but no articles on the potential growth-promoting abilities of this species have been published since this study.

1.3.5 Distribution of three potential PGPR

G. azotocaptans was first isolated from coffee plants in Mexico and subsequently discovered in association with corn plants in Delhi, Ontario, as previously mentioned. *Sphingobacterium canadense* and *Azospirillum zeae* have been identified from only one location in London, Ontario, according to current literature (Mehnaz *et al.*, 2007a; 2007b). The distributions of these bacteria are unknown and it is advantageous to determine how extensive their associations are in order to gain a broader understanding of the soil environments in which they can thrive.

1.4 Detection of plant growth-promoting rhizobacteria

1.4.1 Past and present ways to monitor PGPR

Tracking PGPR in the rhizosphere in the past involved a variety of laborious methods such as the use of spontaneous or induced antibiotic resistance markers, antibodies labelled with fluorescent markers, immunodiffusion assays, and fatty acid profiling (Podile and Kishore, 2006). Researchers relied almost entirely on direct plating on selective growth media to detect and monitor microbial populations. This posed a problem because many

intact viable cells enter into a non-culturable state after a period of time in their environment. Advances in molecular assays such as polymerase chain reaction (PCR) have become important tools for more rapid and accurate detection of bacterial species through the use of gene targets. PCR methods can detect culturable and non-culturable bacteria with high accuracy (Heid *et al.*, 1996).

1.4.2 Real-time quantitative PCR

Real-time quantitative PCR is a fast and sensitive method of detecting low concentrations of target DNA in a reliable manner. This method measures PCR product accumulation, as it occurs, using a dual-labelled fluorogenic probe such as a TaqMan probe (Figure 1.1). No post-PCR sample handling is required, which prevents potential PCR product carry-over contamination. The result is a precise and reproducible quantification of gene copies (Heid *et al.*, 1996).

The ability to quantify bacteria is also made simple with the use of chaperonin genes (Hemmingsen *et al.*, 1988). Type 1 chaperonins (*cpn*60) are present in virtually all bacteria, some archaea, and the plastids and mitochondria of eukaryotes (Hill *et al.*, 2004). Sequences of *cpn*60 genes, which encode 60 kDa chaperonin protein subunits, are more useful in quantification than 16S rDNA because they are present in a single copy within a given microbial genome, whereas 16S rDNA genes are present in multiple copies per genome (Hill *et al.*, 2004). A 549–567-bp segment of the *cpn*60 coding region can be amplified with universal PCR primers and species-specific primers can be designed from this region (Hill *et al.*, 2004).

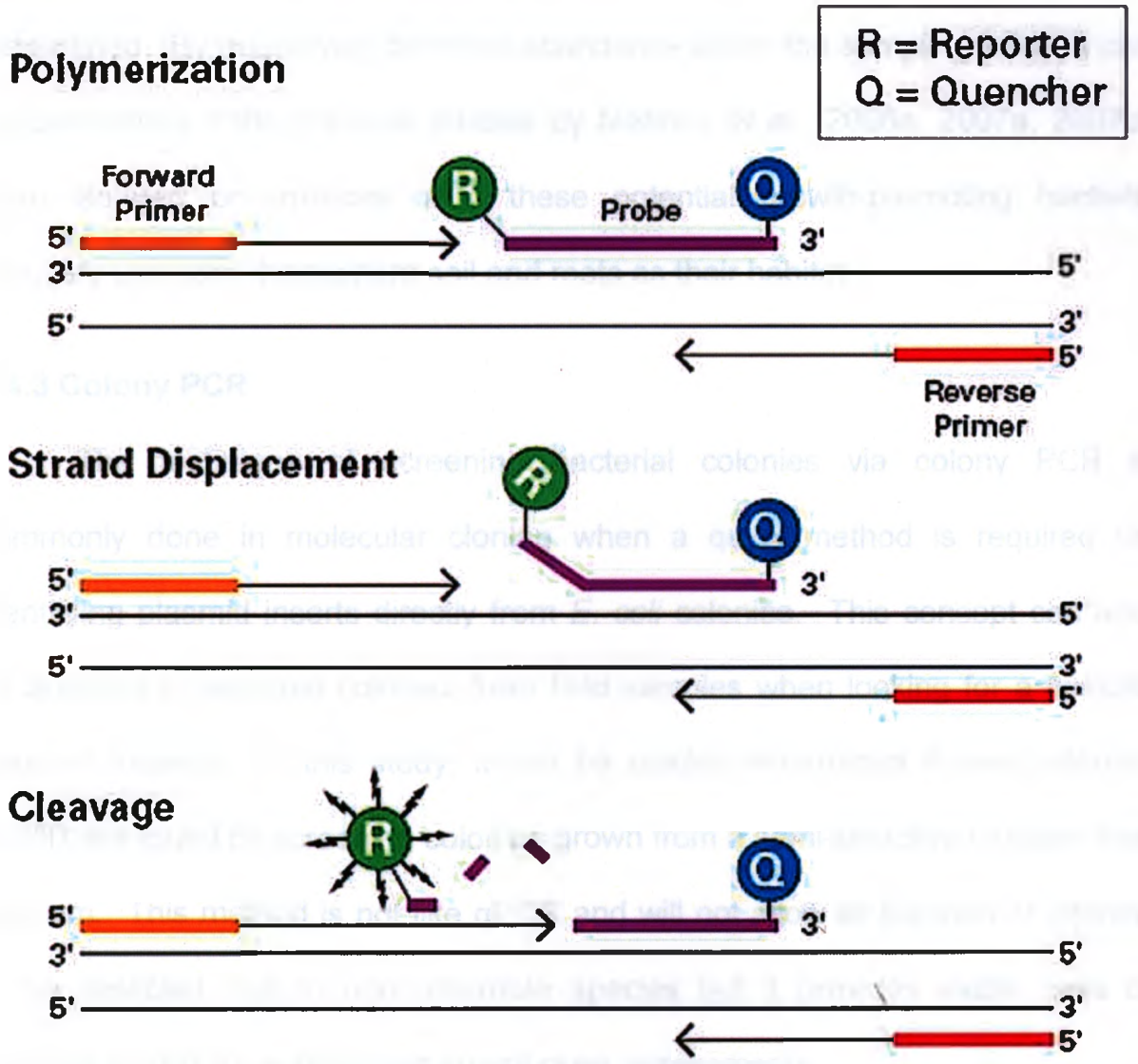

Figure 1.1 Schematic diagram of TaqMan real-time quantitative PCR.

A probe with a 5′ end fluorescent reporter dye and a 3′ end quencher dye is used in combination with a forward and reverse primer. The quencher dye decreases the fluorescence emitted by the reporter dye when the probe is intact. If the target sequence is present, the probe anneals downstream from the primer site and is cleaved by Taq polymerase as this primer is extended. This causes the reporter dye to separate the from the quencher dye, increasing the reporter dye signal.

Reference: [Online] Modified from http://www.asuragen.com/images/TaqMan%20GX%20cartoon.jpg

Using this technique, the approximate size of bacterial populations can be determined. By quantifying bacterial abundance within the sampling fields, it can be determined if the previous studies by Mehnaz *et al.* (2006a, 2007a, 2007b) were isolated occurrences or if these potential growth-promoting bacteria regularly use corn rhizosphere soil and roots as their habitat.

1.4.3 Colony PCR

The technique of screening bacterial colonies via colony PCR is commonly done in molecular cloning when a quick method is required for identifying plasmid inserts directly from *E. coli* colonies. This concept can also be adapted to bacterial colonies from field samples when looking for a specific gene of interest. In this study, it can be quickly determined if new potential PGPR are found by screening colonies grown from a semi-selective nitrogen-free medium. This method is not like qPCR and will not allow all bacteria of interest to be detected due to non-culturable species but it provides viable cells of potential PGPR for subsequent greenhouse assessments.

1.5 Hypotheses

1. *Sphingobacterium canadense*, *Azospirillum zeae*, and *Gluconacetobacter azotocaptans* are naturally associated with corn plants in Ontario and Québec.

2. New potential plant growth-promoting rhizobacteria can be isolated from corn rhizosphere soil and corn roots.

3. Corn plant colonization by new potential plant growth-promoting rhizobacteria as well as *S. canadense*, *A. zeae* and *G. azotocaptans,* will increase the rate of plant growth.

1.6 Objectives

1. To establish standard curves for *S. canadense*, *A. zeae* and *G. azotocaptans* for real-time qPCR and quantify the presence of these bacteria in rhizosphere soil and surface-sterilized roots from selected corn fields.

2. To facilitate the growth of bacteria from corn rhizosphere soil and surface-sterilized roots using nitrogen-free growth media followed by colony PCR for amplification of specific *cpn*60 gene fragments and *nifH* gene fragment.

3. To evaluate the ability of new bacterial isolates from objective 2, as well as *S. canadense*, *A. zeae* and *G. azotocaptans,* to exhibit plant growth-promoting effects on corn using a seed coating inoculation method in greenhouse trials.

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Chapter 2

Distribution and quantification of rhizosphere-associated bacteria

2.1 Introduction

Field corn is grown in every province in Canada, with approximately 96% of this crop grown in Ontario and Québec (AAFC, 2006). Corn is economically important and finding bacteria that can associate with it and provide benefits to the crop is of great interest. Potential plant growth-promoting rhizobacteria have been isolated from two fields in London, Ontario and Delhi, Ontario but current studies do not indicate if they are more widespread and associated with corn in nearby farming communities. I hypothesize that *Sphingobacterium canadense*, *Azospirillum zeae* and *Gluconacetobacter azotocaptans* are found naturally associated with corn plants. They can be detected and quantified from Ontario and Québec corn rhizosphere soil and surface-sterilized corn roots within the field studies selected.

2.2 Methodology

2.2.1 Soil field sampling

In June and July of 2008, field grown corn roots were collected from 25 farmers' fields in Ontario and Québec (Table 2.1). For each plant, the root system was collected with the adhering rhizosphere soil. Five plants were sampled from each corn field site within a 5 metre radius of a centre-point for which geographical co-ordinates were obtained. The collection occurred at an early growth stage when plants had approximately 4-8 leaves. The samples from Ontario were collected by fellow lab members and me, whereas colleagues at

Table 2.1 Characteristics of the field sites from which corn rhizosphere soil was sampled.

Town/City	Sample Code	Co-ordinates	Sampling Date	Previous Crop	Soil Type
Glencoe	F1	N 42°43.384' W 81°45.667'	June 16/08	Soybean	Sandy Loam
Glencoe	F2	N 42° 43.615' W 81°45.378'	June 16/08	Soybean	Sandy Loam
Glencoe	F3	N 42°43.478' W 81°45.778'	June 16/08	Soybean	Sand
Glencoe	F4	N 42°43.654' W 81°46.007'	June 16/08	Corn	Sandy Loam
Glencoe	F5	N 42°43.773' W 81°46.158'	June 16/08	Soybean	Sand
Glencoe	F6	N 42°44.027' W 81°46.171'	June 16/08	Hay	Sandy Loam
Rodney	R1	N 42°37.227' W 81°47.146'	June 24/08	Soybean	Sandy Loam
Rodney	R2	N 42°39.395' W 81°44.267'	June 24/08	Soybean	Loam
Rodney	R3	N 42°37.285' W 81°40.678'	June 24/08	Soybean	Sand
Mt. Brydges	L1	N 42°51.218' W 81°29.999'	June 25/08	Soybean	Loam
Mt. Brydges	L2	N 42°51.350' W 081°30.071'	June 25/08	Soybean	Sand
Mt. Brydges	L3	N 42°50.753' W 81°29.976'	June 25/08	Wheat	Sandy Loam
Mt. Brydges	L4	N 42°50.618' W 81°31.120'	June 25/08	Corn	Sandy Loam

Town/City	Sample Code	Co-ordinates	Sampling Date	Previous Crop	Soil Type
Woodstock	W1	N 43°09.227' W 80°50.663'	June 27/08	Wheat	Sandy Clay
Woodstock	W2	N 43°10.033' W 80°50.035'	June 27/08	Wheat	Loam
Woodstock	W3	N 43°06.836' W 80°50.657'	June 27/08	Soybean	Loam
London	A1	N 43° 14.760' W 81°12.390'	July 11/08	N/A	Sandy Loam
London	A2	N 43° 14.972' W 81°12.399'	July 11/08	N/A	Sandy Loam
Ste. Monique	Q1/Q6	N/A	June 17/08	Cereal	Clay
Ste. Isidore	Q2	N/A	June 27/08	Soybean	Sandy Loam
St. Bernard	Q3	N/A	June 27/08	N/A	Loamy Sand
Lévis	Q4	N/A	July 2/08	Soybean	Sandy Loam
Yamachiche	Q5	N/A	July 2/08	Wheat	Sandy Loam
St. Romain	Q7	N/A	July 4/08	Cereal	Sandy Loam
Barnston	Q8	N/A	June 26/08	Corn	Sandy Loam

Agriculture and Agri-Food Canada in Saint-Foy, Québec, collected samples from farms in the surrounding region. Québec samples were transported to my location on ice.

2.2.2 Soil analysis

Agricultural soils with different characteristics from various geographic locations were used in this study. A 250-350 g sample of soil from each harvesting site was analyzed at A&L Canada Laboratories Inc. for soil textural class, pH, potassium, phosphorus, nitrate nitrogen, and organic matter.

2.2.3 Soil DNA extractions

For each sampling site, the five root systems obtained were dried and the rhizosphere soil was removed with a sterile brush and collected in sterile conical tubes. The Soil DNA Isolation Kit from Norgen Biotek Corporation (Thorold, Ontario) was used for DNA extractions following the manufacturer's instructions. Two hundred milligrams of soil was used for each DNA extraction. Following this procedure, each DNA elution was measured using the NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, Delaware) to determine the concentration (ng/µL) and purity of DNA.

2.2.4 Root tissue field sampling

In the summer of 2009, another smaller harvest, consisting of nine sites, was completed in close proximity to some of the fields from the previous summer (Table 2.2). The plants were used to obtain fresh roots for surface sterilization. The same locations used in the previous summer could not be used due to crop

Table 2.2 Characteristics of the field sites from which corn roots were sampled.

Town/City	Sample	Co-ordinates	Sampling Date	Soil Type
Mt. Brydges	LC	N 42°51' 44.8" W 81°29'20.1"	August 19, 2009	Sand
Mt. Brydges	LM	N 42°50' 58.6" W 81°30'20.9"	August 19, 2009	Sandy Loam
Mt. Brydges	LS	N 42°50'54.2" W 81°30'35.9"	August 19, 2009	Sandy Loam
Glencoe	G1	N 42°42'36.6" W 81°40'17.8"	August 19, 2009	Sand
Glencoe	G2	N 42°42'32.5" W 81°40'12.6"	August 19, 2009	Sandy Loam
London	AC	N 43°1'51.5" W 81°12'25.2"	August 10, 2009	Sandy Loam
Rodney	R1	N 42°38'36.7" W 81°43'55.5"	August 19, 2009	Loam
Rodney	R2	N 42°37'26.7" W 81°44'7.1"	August 19, 2009	Sandy Loam
Rodney	R3	N 42°37'52.8" W 81°43'26.7"	August 19, 2009	Sand

rotations. This additional sampling was collected to determine if the bacteria were endophytic.

2.2.5 Root tissue surface-sterilization procedure

The roots were washed in distilled water until most of the soil was removed. Five grams of root tissue for each replicate, 1 g from each plant, was used for the isolation procedure. The roots were surface-sterilized using a modified version of a method by Coombes and Franco (2003), consisting of 1 minute in 70% ethanol, 5 minutes in 1.5% NaOCl, followed by 1 minute in 70% ethanol and three 1 minute washes in sterile water. The third wash solution was plated on LB agar (Appendix) to confirm the efficacy of the sterilization process. The roots were cut into small pieces using a sterile scalpel and further pulverized in liquid nitrogen using a mortar and pestle. Pulverized root tissue was kept at -80°C until ready for DNA extractions.

2.2.6 Root tissue DNA extractions

Eighty milligram sub-samples of the pulverized root tissues were further processed using the DNeasy® Plant Mini Kit from QIAGEN for DNA extractions according to manufacturer's instructions.

2.2.7 qPCR conditions and optimization

Species-specific primers were designed by Mehnaz (not published) to amplify a 220 bp region of the *cpn*60 chaperonin gene for *S. canadense, G. azotocaptans* and *A. zeae* (Table 2.3). A Taqman fluorescent probe was also designed for each primer pair. The probes were labelled with a reporter dye,

Table 2.3 Sequences of *cpn* 60 primers and probes for real-time quantitative PCR.

Bacterium	Primer/Probe Name	Sequence	Tm (°C)
Sphingobacterium canadense CR11	NEW CR11-Forward	F: CGCATCAATCTCTGCGAATA	58.4
	NEW CR11-Reverse	R: CAATTCCGCTTCCATCTTGT	58.4
	CR11	P: AATCGGTTCATTGATCGCGCAAGC	64.6
Azospirillum zeae N7	NEWN6&N7Forward	F: AGAAGGTGACCACCAACGAG	62.5
	NEWN6&N7Reverse	R: CGAAGTAGGGCGAGGTGTAG	64.5
	N7	P: CCGCGAGATCGGCGACATGCTGGCCC	75.6
Gluconacetobacter azotocaptans DS1	DS1N-F	F: CGAGGAGCTGAAGAAGAACAC	62.6
	NEW DS1-Reverse	R: CGTGATGAAGTACGGGGAGA	62.5
	DS1	P: CGCAGGTCGGCACGATCTCGGCCAA	72.8

F, forward; R, reverse; P, probe
Tm (°C), melting temperature

FAM (6-carboxy-fluorescein), at the 5' end and a quencher dye, TAMRA (6-carboxy-tetramethylrhodamine), at the 3' end (Table 2.3). Quantitative PCR was performed in 20 µL reaction volumes for each sample within a 36-well rotary plate of the Corbett Rotor-Gene™ 3000 (Corbett Life Science, Concorde, NSW, Australia). Each 20 µL reaction mixture contained 10 µL EXPRESS qPCR Supermix. This product contained Platinum® *Taq* DNA polymerase (Invitrogen, Carlsbad, California), $MgCl_2$, heat-labile uracil DNA glycosylase (UDG), dNTPs, and stabilizers. In addition, 0.5 µM forward primer, 0.5 µM reverse primer, 0.15 µM probe, approximately 20 ng of template DNA, and 6 µL filter-sterilized, deionized water were incorporated into each reaction.

The reaction efficiency was evaluated using a gradient of annealing temperatures, DNA template concentrations, as well as the length of annealing time. Optimal cycle conditions for *S. canadense* probe/primers and *A. zeae* probe/primers consisted of: 50°C for 2 minutes, 95°C for 2 minutes, followed by 40 cycles of 95°C for 10 seconds, and 56°C for 30 seconds. *G. azotocaptans* probe/primers performed best with the protocol of 50°C for 2 minutes, 95°C for 2 minutes, followed by 40 cycles of 95°C for 15 seconds and 55°C for 50 seconds. To confirm DNA contamination was not present, each group of reactions processed included a no-template control (NTC), replacing the DNA template with sterilized milli-Q water. Positive controls in each setup included bacterial gDNA, extracted gDNA from soil spiked with a known concentration of bacteria, and bacterial cell suspensions from either*S. canadense, A. zeae*, or *G. azotocaptans.*

2.2.8 Standard curves for absolute quantification

To quantify results, standard curves were developed for *S. canadense, A. zeae*, and *G. azotocaptans*. For each species, a loop of colony material was transferred into 30 mL of Rich broth (Appendix) and incubated in a 30°C chamber overnight, shaking at 150 rpm. From this suspension, a 10-fold serial dilution series (10^{-1} – 10^{-6}) was prepared and bacteria were enumerated by spreading 100 µL of each dilution on combined carbon medium (CCM, Appendix) plates in triplicate, followed by incubation at 30°C for 1 - 3 days. One microlitre of each dilution was used as template DNA in a real-time qPCR reaction. The conditions for each standard curve PCR reaction were identical as specified in section 2.2.6. Three biological and two technical replicates were used to produce each standard curve. The relationship between bacterial cell number and threshold cycle (C_t) value was determined using the Corbett Rotor-Gene™ 3000 software (version 6). The standard curve was produced by plotting the C_t value obtained in the qPCR against the number of CFU (log CFU) in the standards. From this linear relationship, the amplification efficiency was calculated as follows: amplification efficiency = $10^{(-1/\text{slope})} - 1$.

2.2.9 Detection of *cpn*60 fragments in soil and surface-sterilized root samples using real-time qPCR

Target DNA from soil and surface-sterilized root extractions was amplified in triplicate using the protocol and conditions in section 2.2.7. Most DNA extracts were diluted to achieve the optimal concentration (20 ng/µL) and to reduce the presence of organic compounds that can inhibit qPCR. In each qPCR run, a

standard with known DNA concentration was used as a comparison to calculate the DNA concentrations of all samples based on the standard curve generated from the results obtained in section 2.2.8.

2.3 Results

2.3.1 Chemical and physical analyses of corn rhizosphere soil field samples

Soils sampled from the root zone (rhizosphere) of corn plants collected from different fields were highly variable in their chemical and physical composition as indicated from the data provided by A&L Laboratories Canada (Table 2.4). The pH values ranged from 5.5 – 7.8 with the most acidic soil being from Ste. Isidore, Québec and the most alkaline from an Agriculture Canada field site in London, Ontario. The majority of soils were classified as sandy loam followed by loam, sand, and loamy sand. Soils from two fields were classified as clay and sandy clay.

Results of soil chemical compositions were compared to a general soil test interpretation guide to determine the range of values (Marx *et al.*, 1999). The available nitrate nitrogen ranged from very low, 2 parts per million (ppm), in London, Ontario at Agriculture Canada to very high, 150 ppm, in Glencoe, Ontario. All field samples contained high to very high amounts of phosphorus ranging from 40 - 537 ppm. The amount of potassium ranged from low to excessive, 72 - 380 ppm. The amount of organic matter present in the rhizosphere soil samples was near the normal range of 1-4% for cultivated fields (Magdoff and Weil, 2004). The field samples ranged from 1.6 - 8.3% organic matter.

Table 2.4 Analysis of corn rhizosphere soil from 25 field sites in Ontario and Quebec.

Sample	Nitrate (ppm)	Phosphorus (ppm)	Potassium (ppm)	Organic matter (%)	pH	Soil texture
F1	150	50	84	4.9	7.2	sandy loam
F2	101	54	101	3	7	sandy loam
F3	80	55	93	3.6	7.4	sand
F4	131	132	159	8.3	7.4	sandy loam
F5	25	189	144	3.1	6.5	sand
F6	71	40	113	3.7	7.5	sandy loam
R1	6	42	159	3.6	7.2	sandy loam
R2	5	98	214	3.3	7.7	loam
R3	16	70	161	3.8	7.2	sand
W1	18	122	147	4.1	7.3	sandy clay
W2	25	65	123	3.5	7.4	loam
W3	4	147	90	5	6.9	loam
L1	26	104	121	7.7	7.7	loam
L2	88	537	134	4.5	7.2	sand
L3	14	92	91	4	5.9	sandy loam
L4	6	96	133	4.1	6.8	sandy loam
A1	3	71	99	3	7.8	sandy loam
A2	2	74	149	3.1	7.7	sandy loam
Q1	118	55	380	2.9	7.6	clay
Q2	109	136	89	1.6	5.5	sandy loam
Q3	18	49	137	5.4	7.4	loamy sand
Q4	30	50	130	6	5.8	sandy loam
Q5	4	81	160	5	6.6	sandy loam
Q7	6	87	72	5.5	7.4	sandy loam
Q8	46	73	133	7.1	6.4	sandy loam

Analysis provided by A&L Canada Laboratories (2008).

2.3.2 Specificity of primers

Primers and probes were tested using commonly occurring soil bacterial species to ensure that amplification of non-specific DNA would not occur. Bacterial species tested included: *Pseudomonas fluorescens*, *Pseudomonas chlororaphis*, *Bacillus cereus*, *Bacillus amyloliquefaciens*, *Bacillus subtilis*, *Streptomyces scabies* and *Burkholderia phytofirmans*. None of these species resulted in any amplification using *S. canadense, A. zeae*, or *G. azotocaptans* specific primers.

2.3.3 Efficiency of soil and root tissue extraction process

Spiked soils and root tissue were included as positive controls for each qPCR run to ensure the detection of bacteria was possible after the DNA extraction process. After inoculating soil and root tissue with approximately 10^2 - 10^3 CFU of *S. canadense, A. zeae*, or *G. azotocaptans*, detection of 10^2 - 10^3 CFU was consistently achieved.

2.3.4 Standard curves

Overnight cultures of the *S. canadense, A. zeae*, and *G. azotocaptans* were used to create standard curves with cell numbers ranging from 10^1 to 10^7 CFU/mL (Figure 2.1, 2.2, 2.3). Linear relationships ($R^2 = 0.99$) were observed for all standard curves between the log CFU and the threshold cycle (C_T) values of each reaction. The amplification efficiency (E) of each run was calculated using the slope of the standard curve. The slopes of the standard curves ranged from

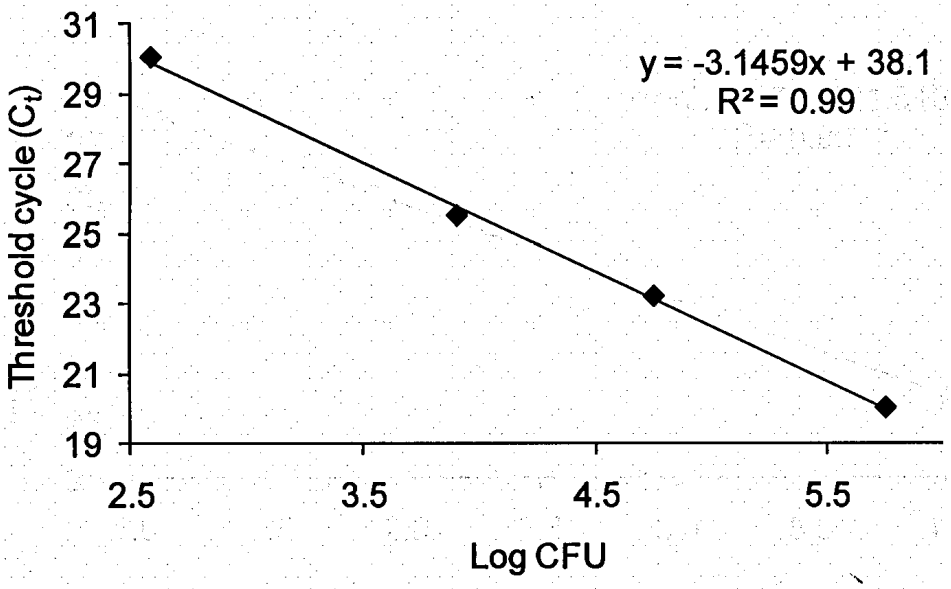

Figure 2.1 Quantitative PCR standard curve for *Sphingobacterium canadense*.

A standard curve showing the linear relationship between threshold cycle (C_t) values and cell concentrations for serially 10-fold diluted *Sphingobacterium canadense* CR11 pure culture. Linear regression coefficient was $R^2 = 0.99$ and amplification efficiency was E = 99%. Data points are the mean of three replicates.

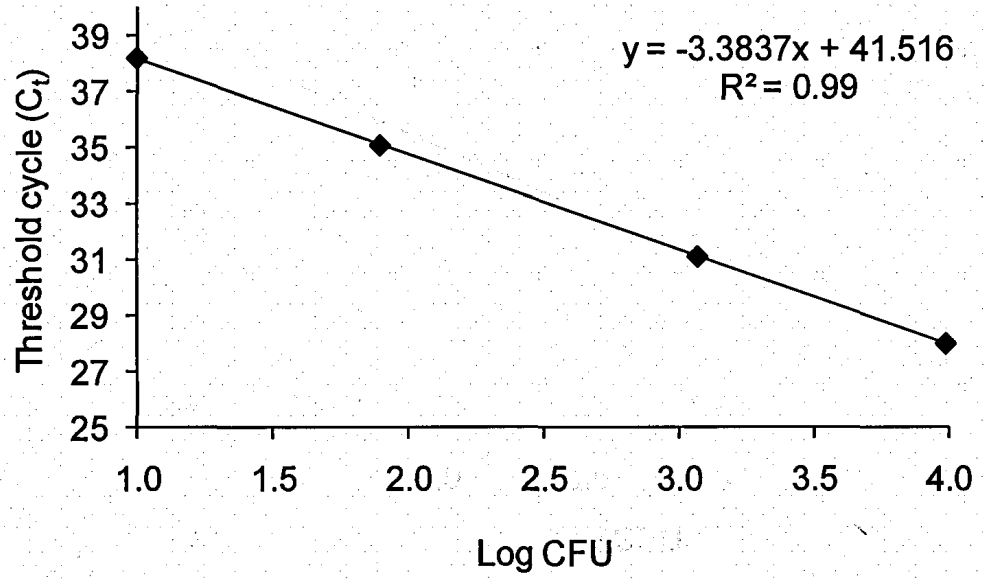

Figure 2.2 Quantitative PCR standard curve for *Azospirillum zeae.*

A standard curve showing the linear relationship between threshold cycle (C_t) values and cell concentrations for serially 10-fold diluted *Azospirillum zeae* N7 pure culture. Linear regression coefficient was R^2 = 0.99 and amplification efficiency was E = 97%. Data points are the mean of three replicates.

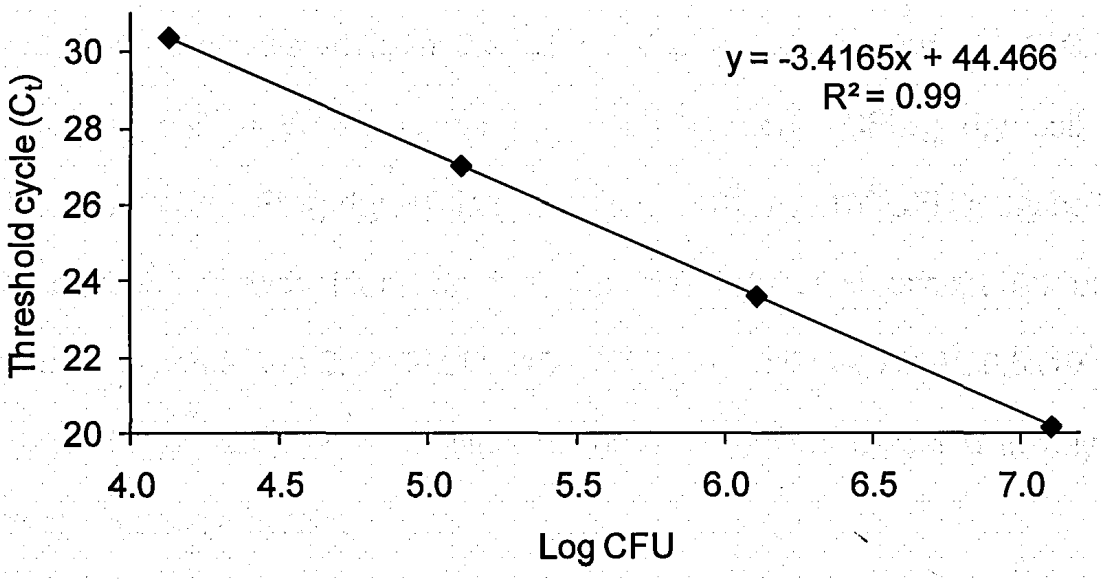

Figure 2.3 Quantitative PCR standard curve for *Gluconacetobacter azotocaptans*.

A standard curve showing the linear relationship between threshold cycle (C_t) values and cell concentrations for serially 10-fold diluted *Gluconacetobacter azotocaptans* DS1 pure culture. Linear regression coefficient was $R^2 = 0.99$ and amplification efficiency was E = 96%. Data points are the mean of three replicates.

– 3.1 to – 3.4 and the amplification efficiencies were 96%, 97% and 99% ($E=10^{-1/slope}-1$).

2.3.5 Detection of *S. canadense*, *A. zeae*, and *G. azotocaptans cpn*60 fragments in soil and surface-sterilized root tissue using real-time qPCR

For enumeration of microorganisms, I used quantitative real-time PCR. Detection of *S. canadense* within corn rhizosphere soil was found in three Ontario fields and one Québec field (Figure 2.4). The highest abundance detected was calculated from the standard curve to harbour $1.2x10^5 \pm 2.1x10^4$ CFU/g dry soil in W1, followed by $1.2x10^4 \pm 2.9x10^3$ CFU/g dry soil in R1, $7.0x10^3 \pm 1.2x10^3$ CFU/g dry soil in R2, and $7.0x10^3 \pm 1.7x10^3$ CFU/g dry soil in Q2. *G. azotocaptans* was identified in all of the field sites except A1 and Q1. Detection ranged from $2.1x10^4 \pm 1.6x10^3$ CFU/g dry soil to $4.7x10^5 \pm 8.5x10^4$ CFU/g dry soil (Figure 2.5). No amplification of *A. zeae* was detected in any of the rhizosphere soil extracts.

In surface-sterilized root tissue DNA extractions, *A. zeae* was found in R3 and AC samples in quantities estimated to be $4.6x10^5 \pm 8.0x10^4$ CFU/g fresh root and $1.3x10^5 \pm 7.0x10^4$ CFU/g fresh root, respectively (Figure 2.6). In AC, LC and R3 samples only low amounts of *G. azotocaptans* were detected, ranging from $1.2x10^2 \pm 5.2x10^1$ CFU/g fresh root to $2.3x10^2 \pm 5.6x10^1$ CFU/g fresh root (Figure 2.7). None of the surface-sterilized root tissues sampled contained detectable *S. canadense*.

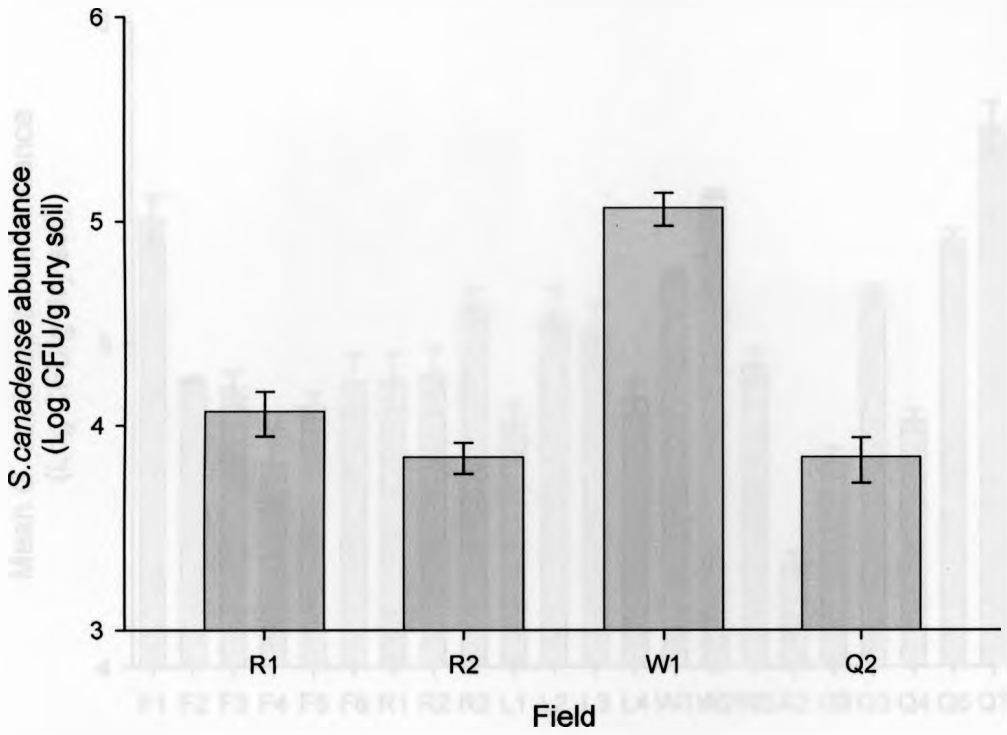

Figure 2.4 Mean (± standard deviation) abundance of *Sphingobacterium canadense* in corn rhizosphere soil.

Real-time quantitative PCR detection of a 220 bp *S. canadense chaperonin*60 (*cpn*60) gene fragment in four corn fields in Ontario and Québec. Twenty-one additional fields were tested which resulted in no detection. Sampling occurred in 2008.

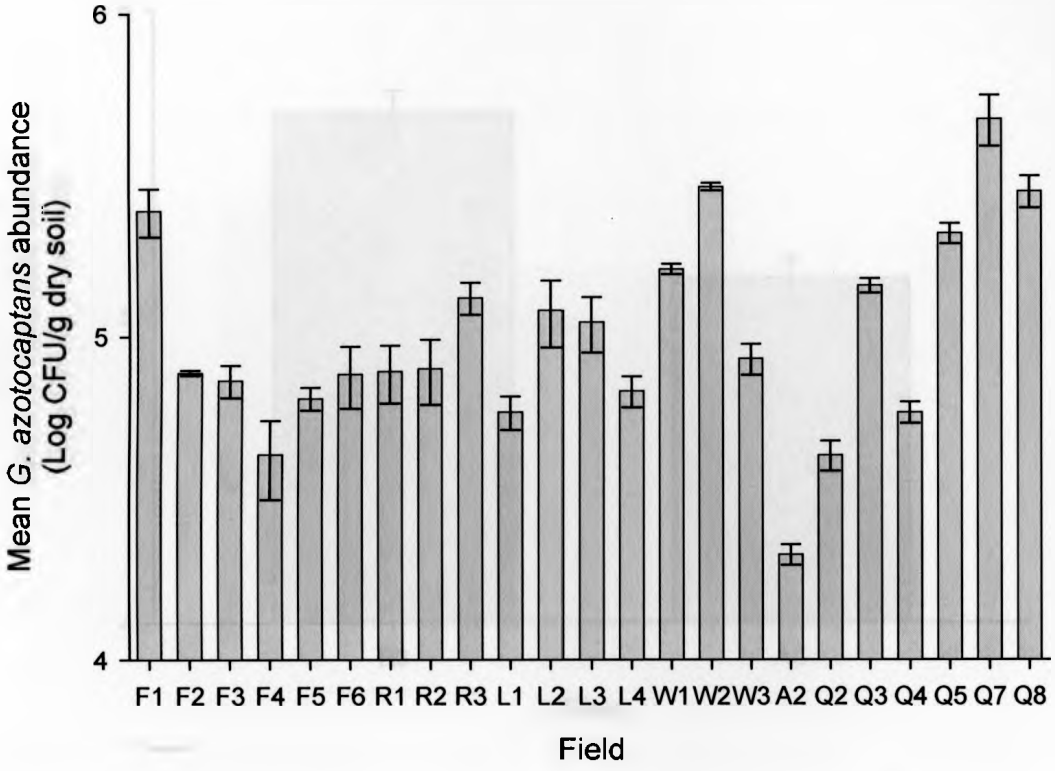

Figure 2.5 Mean (± standard deviation) abundance of *Gluconacetobacter azotocaptans* in corn rhizosphere soil.

Real-time quantitative PCR detection of a 220 bp *G. azotocaptans chaperonin*60 (*cpn*60) gene fragment in 23 corn fields in Ontario and Québec. Two additional fields, A1 and Q1, were tested and did not result in any amplification. Sampling occurred in 2008.

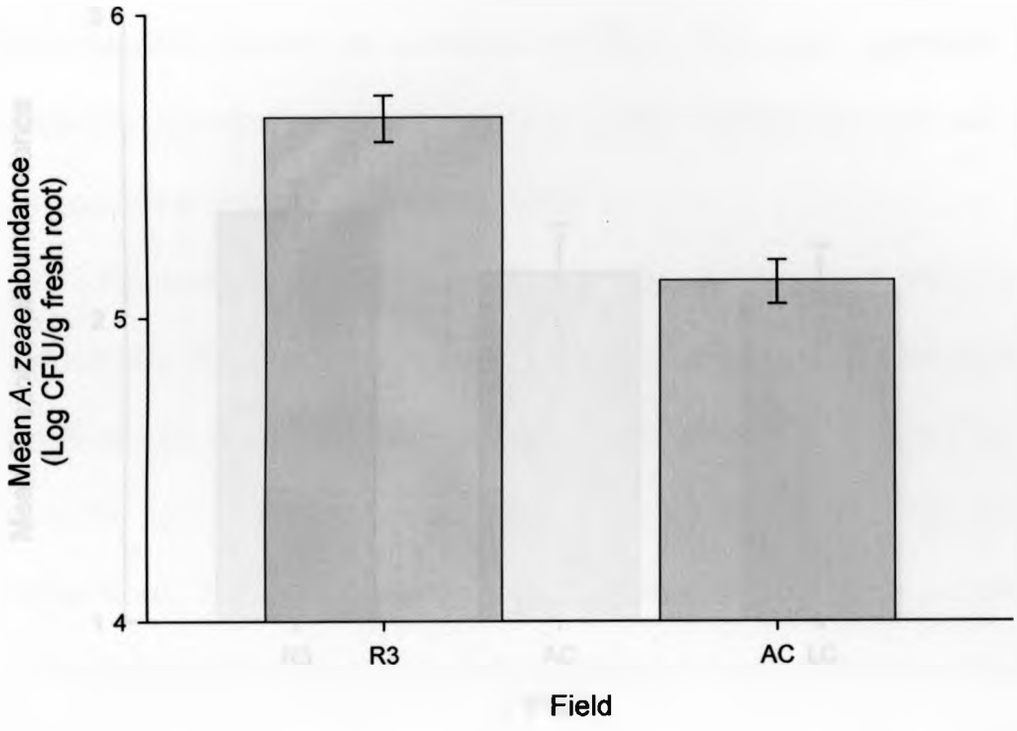

Figure 2.6 Mean (± standard deviation) abundance of *Azospirillum zeae* in corn root tissue.

Real-time quantitative PCR detection of a 220 bp *A. zeae chaperonin*60 (*cpn*60) gene fragment in two corn fields in Ontario. Seven additional fields were tested which resulted in no detection. Sampling occurred in 2009.

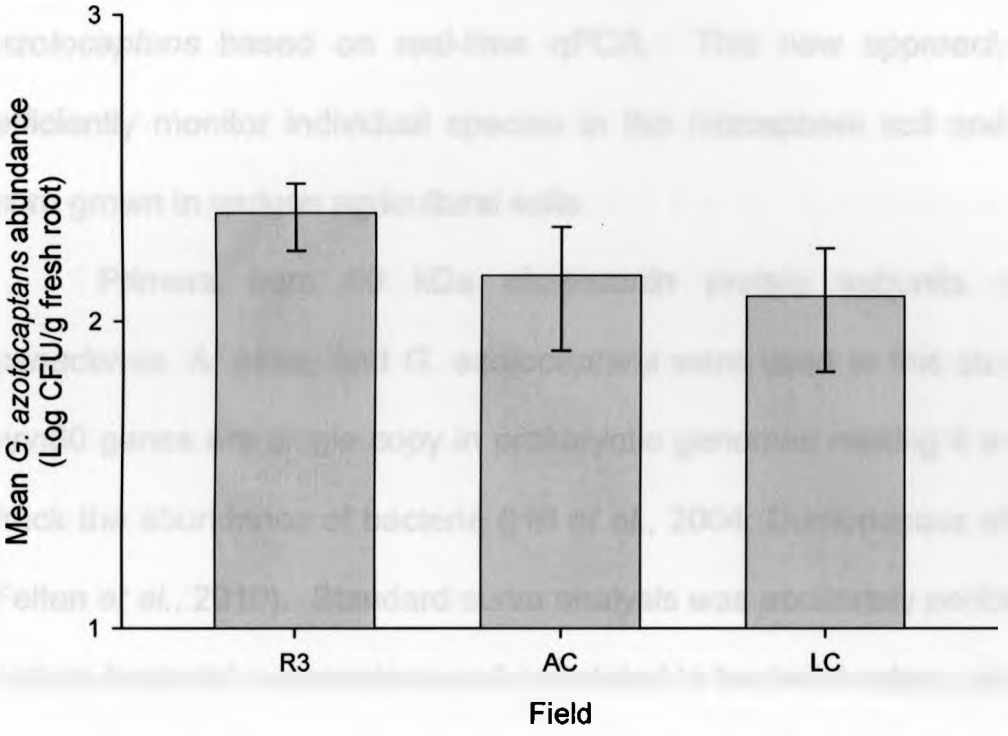

Figure 2.7 Mean (± standard deviation) abundance of *Gluconacetobacter azotocaptans* in corn root tissue.

Real-time quantitative PCR detection of a 220 bp *G. azotocaptans chaperonin*60 (*cpn*60) gene fragment in three corn fields in Ontario. Six additional fields were tested which resulted in no detection. Sampling occurred in 2009.

2.4 Discussion

2.4.1 Real-time qPCR

In this study I described the development of a culture-independent, species-specific quantification approach for *S. canadense, A. zeae*, and *G. azotocaptans* based on real-time qPCR. This new approach was used to efficiently monitor individual species in the rhizosphere soil and root tissue of corn grown in various agricultural soils.

Primers from 60 kDa chaperonin protein subunits (*cpn*60) of *S. canadense, A. zeae*, and *G. azotocaptans* were used in this study. Generally, *cpn*60 genes are single copy in prokaryotic genomes making it an ideal gene to track the abundance of bacteria (Hill *et al.*, 2004; Dumonceaux *et al.*, 2006; von Felten *et al.*, 2010). Standard curve analysis was accurately performed with pure culture bacterial suspensions and correlated to bacterial colony counts.

In order to quantify bacteria present in soil and roots, the DNA extraction method had to be thorough to ensure that the maximum amount of DNA was collected and detected while removing soil impurities such as humic acids that could interfere with PCR amplification. The extraction process was verified in the study by spiking soil and root tissue with known concentrations of bacterial cells and using real-time qPCR to detect the DNA of these cells. At the approximate concentration added to soil, 10^2 - 10^3 CFU, *S. canadense, A. zeae*, and *G. azotocaptans*, were detectable. This level of detection is consistent with other studies that used spiked soils for real-time qPCR identification (Trabelsi *et al.*, 2009).

2.4.2 Detection of *S. canadense, A. zeae,* and *G. azotocaptans* in rhizosphere soil using real-time qPCR

The distribution of the bacteria examined in this study has not been previously monitored in Canada as the bacteria were identified from only a few corn plants collected in London and Delhi, Ontario (Mehnaz, 2006; 2007a; 2007b). The extent of their association with the corn rhizosphere soil and root tissues was also unknown. I hypothesized that *S. canadense*, *G. azotocaptans,* and *A. zeae* were detectable by real-time qPCR methods in Ontario and Québec agricultural fields. This was confirmed from our results, which revealed varying quantities present in the field soils and roots sampled, ranging from zero to almost 10^6 CFU/g of fresh root.

Both *S. canadense* and *G. azotocaptans* were detected in rhizosphere soil but *A. zeae* was not detected in any soil samples. This indicates that two of the three bacterial species are more abundant than previously known in the rhizosphere soil of corn from the sites studied at the time of sampling.

S. canadense was found in high abundance from a rhizosphere soil in Woodstock (W1) with approximately 10^5 CFU/g of dry soil. It was also detected at three other locations but at lower concentrations of approximately 10^4 CFU/g of dry soil. As of yet, there have not been any other studies describing either the identification or the quantification of *Sphingobacterium* in Canadian soils. *Sphingobacterium* strain 1ZP4 has been found in rhizosphere soils in Portugal (Marques, 2010) and in a variety of environments from samples collected in Japan and China (Duan *et al.*, 2010; He *et al.*, 2010; Ten *et al.*, 2007;

Matsuyama *et al.*, 2008; Wei *et al.*, 2008). These previous studies identified but did not quantify each species.

G. azotocaptans was found in 23 of the 25 rhizosphere soils. This was unexpected as *G. azotocaptans* is a very slow growing bacterium that is expected to be a poor colonizer of soil due to competition with fast-growing species (Podile and Kishore, 2006). *Gluconacetobacter* species are most commonly known for their affinity towards endophytic environments (Cavalcante and Döbereiner, 1988; Loganathan *et al.*, 1999). However, *G. azotocaptans* was first isolated from rhizosphere soil of coffee plants (Fuentes-Ramírez *et al.*, 2001). The fact that *G. azotocaptans* was present at relatively high levels, approximately 10^4 CFU/g dry soil, indicates that there might be, with corn, an interesting association that could be further explored.

A. zeae was not detected in any rhizosphere soils even though a number of studies have shown that species in this genus are closely associated with grasses (Döbereiner and Day, 1975; Jacoud *et al.*, 1999; Umali-Garcia *et al.*, 1980). It may be that *A. zeae* is not as abundant as other *Azospirillum* species in temperate regions. In the temperate zones of Brazil and in the US, the occurrence of *Azospirillum* spp. in soil was significantly lower than in the tropics (Döbereiner *et al.*, 1976).

Rhizosphere soil samples that had detectable levels of *S. canadense* and *G. azotocaptans* were compared with their chemical and physical components to determine any apparent trends. No trends were evident. *S. canadense* and *G.*

azotocaptans appear to be able to survive in soil environments with a range of pH, nitrate nitrogen, and organic matter values.

2.4.3 Detection of *S. canadense, A. zeae,* and *G. azotocaptans* in surface-sterilized root tissue using real-time qPCR

From the nine fields where I collected corn and tested surface-sterilized roots for the three bacteria, only *G. azotocaptans* and *A. zeae* were found in high numbers at two locations in Rodney and London, Ontario. *G. azotocaptans* was also detected in corn roots in one other site outside of London.

The isolation of microorganisms from within the root tissue of healthy corn suggests that the host plant derives some benefit, potentially from secondary metabolites, from harbouring endophytes. Also, the bacteria most likely are benefiting from the root environment due to the closer proximity to carbon substrates supplied by the plant (Döbereiner *et al.*, 1995). They can establish themselves within niches protected from oxygen, which is necessary for the expression and activity of nitrogenase so their potential to fix nitrogen can be expressed at the maximal level (Baldani *et al.*, 1997).

For *G. azotocaptans,* the levels of bacteria detected in root tissue were lower than in the rhizosphere (approximately 10^2 CFU/g of fresh root). This finding is consistent with other *Gluconacetobacter* species such as *G. diazotrophicus,* which was previously isolated from surface-sterilized plant tissue (Döbereiner *et al.*, 1972). However, the abundance was lower compared to studies in the tropics with sugarcane plants that had levels as high as 10^7 CFU/g fresh root weight of *G. diazotrophicus* (Muthukumarasamy *et al.*, 2002).

For the two sample sites with *A. zeae*, detection of over 10^5 CFU/g of fresh root indicates an established population that could be contributing to plant growth promotion. The presence of *A. zeae* in corn root tissue suggests that this species has endophytic abilities but *A. zeae* is not exclusive to root tissue because its original isolation location was from corn rhizosphere soil (Mehnaz *et al.*, 2007a).

S. canadense was not detected in the sterilized root tissue, which could indicate that it is not an endophytic bacterium or at least that it was not present in root tissue within the fields sampled. This is consistent with *S. canadense*'s original detection location, which was corn rhizosphere soil (Mehnaz *et al.*, 2007b).

When compared to existing literature, this is one of the first studies to use real-time qPCR to track and enumerate bacterial populations in rhizosphere soil and root tissue population of corn. Currently, there has been only one other study performed by von Felten *et al.* (2010) using a similar approach, but using different primer technology combined with real-time qPCR to quantify *Pseudomonas fluorescens* strains in corn.

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Chapter 3

Detection and evaluation of bacterial isolates with potential growth-promoting properties

3.1 Introduction

The strains of *S. canadense, G. azotocaptans*, and *A. zeae* identified by Mehnaz *et al.* (2006a, 2007a, 2007b) have beneficial characteristics related to plant growth promotion. Identifying new strains of these species may uncover more advantageous traits useful in enhancing corn production. Also, detecting bacteria with the *nifH* gene would indicate the presence of the enzyme, nitrogenase reductase, used in biological nitrogen fixation. Bacteria able to provide useable nitrogen for corn plants to exploit could reduce the need for synthetic nitrogen fertilizers.

Using selective nitrogen-free growth media eliminates a large portion of bacteria that do not contain the trait of interest, in turn, making the colony PCR process more efficient. I hypothesize that new potential plant growth-promoting rhizobacteria can (1) be isolated from corn rhizosphere soil and surface-sterilized corn roots using selective media and (2) be detected by colony PCR. If bacterial isolates are found, the next objective of this experiment is to evaluate the growth of corn inoculated with the bacteria. Preliminary experiments to determine minimum sample size and optimal treatment (CFU/mL) are required. I hypothesize that corn colonization of new potential growth-promoting rhizobacteria as well as *S. canadense, A. zeae*, and *G. azotocaptans,* will increase the rate of plant growth.

3.2 Methodology

3.2.1 Isolation of new potential plant growth-promoting rhizobacteria

Bacterial colonies were isolated from corn rhizosphere soil and surface-sterilized corn roots collected in 2008 and 2009, as outlined in sections 2.2.1 and 2.2.3. The bacteria were screened by colony PCR for the presence of either the *cpn*60 gene or the *nifH* gene.

To remove adhering rhizosphere soil from the corn roots, representative root samples from each site were cut and added to 70 mL of 10 mM phosphate buffer in thick plastic bags and shaken on a Gyrotory ® Shaker at 250 rpm for 30 minutes. Each bag was then placed individually in a Laboratory Blender Stomacher-400 and further blended on the "normal" setting for 30 seconds. One hundred microlitres of the soil suspension was pipetted into a 25 mL glass vial in duplicate. Vials contained 10 mL of nitrogen-free LGI semi-solid medium (Appendix) with 100 µg/ml of cycloheximide to inhibit fungal growth (Cavalcante and Dobereiner, 1988). Inoculated vials were incubated for 7 days at 30°C. A cloudy pellicle of bacteria formed within the semi-solid medium. The pellicle was removed and diluted 10-fold in a dilution series from 10^{-1} to 10^{-8} in sterile H_2O. One hundred microlitres of each dilution was pipetted and spread on LGI agar plates (Appendix). Random single colonies were picked and streaked onto new plates to obtain pure cultures, which were made into glycerol stocks and kept at -80°C for colony PCR. If isolates were ready to be used immediately, the freezing was skipped.

When fresh corn root samples were collected in 2009, they were surface-sterilized according to the method in section 2.2.5. One centimetre sections of

surface-sterilized root tissue were placed into 25 mL glass vials containing 10 mL of nitrogen-free, semi-solid CCM, NFM, or malate medium, with 100 µg/mL of cycloheximide (Appendix). The vials with root tissue were then incubated at 30°C until a pellicle was visible. Single colonies were generated for screening using the same method as specified for rhizosphere-associated bacteria mentioned in the previous paragraph with the exception of different media. Different nitrogen-free selective media were used for isolating bacteria as they grew faster on these types of media.

To confirm the ability of the selective media to allow growth of nitrogen-fixing bacteria only, nine random unknown isolates from field sample glycerol stocks had their 16S rDNA amplified using universal 16S primers - 27f (AGAGTTTGATCMTGGCTCAG) and 1492r (TACGGYTACCTTGTTACGACTT) (Eden *et al.*, 1991). Each bacterial isolate was grown in Rich broth overnight from a single colony. Bacterial gDNA was extracted using the SIGMA GenElute ™ Bacterial Genomic DNA Kit (Sigma-Aldrich, St. Louis, Missouri) and diluted to use as DNA template in PCR. Each 50 µL reaction contained 1x PCR buffer, 3.75 mM $MgCl_2$, 200 nM forward primer, 200 nM reverse primer, 0.2 mM dNTPs, 2.5 units *Taq* polymerase, 0.1 mg/mL BSA, 2% DMSO, 1µL bacterial gDNA and sterile Milli-Q H_2O. The cycle conditions were 95°C for 5 minutes, followed by 35 cycles of 95°C for 1 minute, 56°C for 1 minute and 72°C for 2 minutes, with a single final extension at 72°C for 7 minutes. Amplification was assessed by gel electrophoresis using a 1.0% (w/v) agarose gel in 1X TAE Buffer [40 mM Tris (pH 9.5), 0.5 mM EDTA (pH 8.0)] for approximately one hour at 100

V. The expected PCR product size (1500 bp) was verified and the amplicon was extracted from the gel. The QIA Gel Extraction Kit (Qiagen, Mississauga, Ontario) was used to purify the PCR products according to the manufacturer's instructions. The isolated amplicons were sequenced at Robarts Research Institute, London, ON and the sequences were compared in a nucleotide BLAST search within the NCBI database.

3.2.2 Bacterial colony preparation for PCR

To prepare bacterial colonies for PCR, glycerol stocks were plated on agar of the appropriate medium for older samples. For new samples bacteria were taken directly from dilution plates. Single colonies were picked from agar plates with a sterile toothpick or pipette tip and suspended in 100 µL of sterile H_2O and 50 mg of 0.1 mm sterile glass beads. Suspensions were placed in the FastPrep-24 for 40 seconds at 6.0 m/s to lyse the cells and centrifuged at 12,000 rpm for 1 minute. The supernatant from each tube was transferred into new tubes and was stored at -20°C until needed for PCR as DNA template.

3.2.3 Detection of new isolates containing *S. canadense, A. zeae*, or *G. azotocaptans cpn*60 genes using qPCR

The primers and probes from section 2.2.7 were used to detect new strains of *S. canadense, A. zeae*, and *G. azotocaptans* (Table 2.3). Unknown bacterial isolates were screened with each set of primers in qPCR reactions using the BioRad CFX-96™ Real-Time System. This method was used as a rapid way to screen bacteria for the presence or absence of the specific *cpn*60

genes, and not for quantification. This real-time qPCR machine was a new addition to the laboratory facility at that stage of experimentation therefore it was used instead of the Corbett Rotor-Gene™ 3000. The apparatus was fast, could process 96 samples at once instead of 36, and smaller total reaction volumes were permitted. Each 10 µL reaction contained 5 µL EXPRESS qPCR Supermix, 0.75 µM forward primer and 0.75 µM reverse primer, 0.15 µM probe, 1 µL bacterial colony suspension, and 2.0 µL filter-sterilized Milli-Q water. The cycle conditions for all three primer/probe sets were 50°C for 2 minutes, 95°C for 2 minutes, followed by 40 cycles of 95°C for 10 seconds, and 55°C for 30 seconds.

3.2.4 Detection of new isolates containing the *nifH* gene using conventional PCR

An alternative colony PCR method was also utilized for the detection of new potential plant growth-promoting rhizobacteria. Using "universal primers" designed by Poly *et al.* (2001), detection of bacteria with the gene encoding nitrogenase reductase, *nifH*, was possible. These primers detect a wide range of bacteria containing the *nifH* gene. The selected primers, PolF and PolR (5' TGC GAY CCS AAR GCB GAC TC 3' and 5' ATS GCC ATC ATY TCR CCG GA 3', respectively), were used to amplify a 360 bp region between sequence positions 115 and 476 (referring to the *Azotobacter vinelandii nifH* coding sequence [M20568; Poly *et al*, 2001). Each *nifH* reaction consisted of 1x PCR buffer, 2.5 mM $MgCl_2$, 400 nM forward primer, 400 nM reverse primer, 200 µM dNTPs, 2.5 units *Taq* polymerase, 1µL bacterial colony suspension and sterile Milli-Q H_2O to bring the volume to 25 µL. The optimal protocol used with the Eppendorf

Mastercycler ® pro Thermal Cycler was 95°C for 5 minutes, followed by 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute, ending with a single final extension of 72°C for 5 minutes. At the end of the program, PCR reactions were maintained at 4°C until the PCR products were run on a 1.0% (w/v) agarose gel in 1X TAE Buffer. The gel was run at 5V/cm for approximately 45 minutes to confirm the presence of the PCR product at the expected base pair size.

3.2.5 Determination of optimal sample size

A preliminary growth experiment was conducted to assess the natural variability of corn plant growth with respect to shoot height, fresh plant weight, and dry plant weight. Corn seeds were supplied by Dekalb Canada. The variety selected was DKC 50-20 YieldGard® Corn Borer/ Roundup Ready® Corn 2 and the crop heat units (CHU) rating was 2975. Crop heat units are part of an indexing system to assist farmers in choosing suitable hybrids and varieties for their area (OMAFRA, 1997).

Seeds were surface-sterilized before planting by soaking in a 0.1% NaOCl solution for 2 minutes, rinsed three times in sterile dH_2O and dried on a sterile paper towel in a laminar flow hood for 20 minutes. Each pot contained 8 seeds with 6 replicates giving 18 pots or 144 seeds altogether. Surface-sterilized seeds were planted 3 cm below the surface in pasteurized Promix BX soil (60°C for 4 hrs), using sterile forceps. The plants were grown using a 15/9hr (light/dark, 23°C/19°C) photoperiod, with light supplied by fluorescent bulbs. Every second

day, pots were given equal aliquots of water ranging from 100 – 200 mL depending on soil dryness.

The variability was assessed over a three week period, each week removing 48 plants for measurements. The maximum variability determined the sample size needed to ensure significant results for testing bacterial inocula with corn seed for their potential benefits. The maximum mean variance observed was used in a sample size equation to calculate the minimum sample size needed for further experimentation.

The following equation was used to determine sample size (Gomez and Gomez, 1984):

$$n = \frac{(Z)^2 (u)}{(d)^2 (\bar{x})^2}$$

n = minimum sample size
Z = 1.96 (at p = 0.05)
u = sampling variance
d = 0.05
$\bar{x}$ = sample mean

Growth was assessed based on shoot height and total dry plant weight (dry root mass and dry shoot mass).

3.2.6 Determination of optimal inoculation treatment

Bacteria applied as a corn plant biofertilizer can have detrimental effects if used at too high of a concentration (CFU/mL). The optimal concentration was determined for *S. canadense* CR11, *A. zeae* N7, and *G. azotocaptans* DS1, as well as strains Q2-104, LC2, and LC9.

Each Pyrex jar contained approximately 350 g of pasteurized sand (4 hrs at 60°C), 50 mL distilled water, 53 mg urea, 18 mg of phosphate, and 18 mg of potassium added to mimic half-strength fertilizer compared to recommended corn fertilizer rates for Ontario (OMAFRA, 1996).

Corn seeds were surface-sterilized by soaking in 0.1% sodium hypochlorite for 5 minutes, followed by three rinses of sterile distilled water and drying in a laminar flow hood. Groups of 30 seeds were counted into 50 mL conical tubes in preparation for the inocula.

Bacterial inocula were prepared by growing overnight rich medium liquid cultures of *S. canadense* CR11, *A. zeae* N7, and *G. azotocaptans* DS1, as well as strains Q2-104, LC2, and LC9. Cell suspensions were pelleted by centrifugation at 4000 rpm for 20 minutes at room temperature. The supernatant was removed then 3 mL of sterile distilled water was added to wash the pellets, and re-suspend them. The suspensions were centrifuged again and the supernatant was removed. Suspensions were adjusted with distilled water until a concentration of 10^8 CFU/mL was attained. This was used as the highest treatment of inocula, followed by 10^7, 10^6, and 10^5 CFU/mL. The number of CFUs for each inoculum was confirmed by performing a 10-fold dispersion series in sterile H_2O and plating 100 µL of the dispersions on CCM agar plates in duplicate followed by counting individual bacterial colonies.

Application of inocula to seeds was carried out by submerging surface-sterilized seeds in 10 mL of the bacterial suspension for 5 minutes while they were being agitated on a rotary shaker. The control group was submerged and

agitated in 10 mL of sterile H_2O. Ten seeds per jar were planted at a depth of 3 cm using sterile forceps. Growth chamber conditions were 25°C and a photoperiod of 8 hrs of light/16 hrs of dark. Each inoculum had 3 replicates of 10 seeds per jar and consisted of three separate trials. After seven days of growth, corn plants were removed from the sand and were evaluated based on emergence, shoot height, fresh weight, and dry weight.

Data were analyzed using SPSS Statistics 18 software. Analyses of variance (ANOVA) were performed to compare the differences among inoculum treatments for each bacterium and if p values indicated a significant difference ($p < 0.05$), means were separated by Least Significant Difference (LSD) test.

3.2.7 Evaluation of new potential growth-promoting rhizobacteria

The inoculum treatments with the best performance in the previous experiment were used in the following trials. Each inoculum had 6 replicates of 8 seeds per pot and consisted of three trials.

Sand preparation involved pasteurization at 60°C for 4 hrs after which approximately 2700 g was measured into each of the 42 pots. Half-strength fertilizer concentrations of N-P-K were added to each pot at a rate of 75 kg/ha for N, and 25 kg/ha for P and K, compared to recommended corn fertilizer rates for Ontario (OMAFRA, 1995). Pots were arranged in a randomized design on the greenhouse bench.

Corn seeds were surface-sterilized by soaking them in 0.1% sodium hypochlorite for 5 minutes, followed by three rinses of sterile water and drying in

a laminar flow hood. Groups of 50 seeds were counted into 50 mL conical tubes in preparation for the inoculum.

Bacterial inocula were prepared using the same method as in section 3.2.6. The number of CFUs for each inoculum was confirmed by performing a 10-fold dispersion series in sterile H_2O and plating 100 µL of the dispersions on CCM agar plates in duplicate followed by counting individual bacterial colonies. Application of inocula to seeds was done by submerging seeds in 10 mL of the suspension for 5 minutes while they were being agitated. The control group was submerged and agitated in 10 mL of sterile H_2O. Eight seeds per pot were planted at a depth of 3 cm using sterile forceps. Average daily daytime maximum temperatures in the greenhouse were 28°C. After 15 days of growth, corn plants were harvested and were evaluated based on shoot height, fresh weight, and dry weight. Statistical analyses were completed as outlined in section 3.2.6 to determine if any significant differences existed between plants from the 6 treatments and those of the control group.

3.3. Results

3.3.1 Determination of the selectivity of the culture medium for nitrogen fixing bacteria

To determine the selectivity of various culture media for growth of nitrogen fixing bacteria, nine isolates selected randomly were sequenced and their identities were determined from the sequence data. Based on BLAST searches against the NCBI data libraries for similarities of the 16S rDNA sequences, the isolates were all identified as genera of bacteria commonly found in soil. Four of the isolates were identified as *Enterobacter* sp., *Klebsiella* sp.,

Burkholderia sp., and *Pseudomonas* sp., which could potentially be nitrogen-fixing species (Elmerich and Newton, 2007). Three other isolates were identified as *Stenotrophomonas* sp., *Comamonas* sp., and *Xanthomonas* sp. (Table 3.1).

3.3.2 Colony PCR using *cpn*60 primers for *S. canadense*, *G. azotocaptans*, and *A. zeae*.

Bacterial colonies grown on a selective medium were prepared for PCR screening. All colonies were screened with primer/probe sets for *S. canadense, A. zeae and G. azotocaptans.* Three hundred colonies were screened using real-time PCR methods which resulted in one positive amplicon with *S. canadense cpn*60 primers. This bacterial colony, named Q2-104, was then further verified by sequencing a portion of its *cpn*60 gene fragment. After a BLAST search, the fragment was found to be 97% similar to the *S. canadense* CR11 homolog. This bacterium was isolated from sandy loam rhizosphere soil in Saint Isidore, Québec.

3.3.3 Detection of the *nifH* gene using conventional PCR

Three hundred and fifty-five colonies were screened for the *nifH* gene. When testing colonies isolated from surface-sterilized root tissue, two colonies resulted in positive amplification with the *nifH* primers, named strain LC2 and strain LC9 (Figure 3.1). These positive isolates were also screened using the real–time primers/probe sets to determine if they were closely related to any of the three established bacteria: strains CR11, N7, and DS1. No amplification was detected with strain LC2 or strain LC9.

Table 3.1 BLAST search results of randomly selected strains isolated from nitrogen-free semi-solid medium.

Strain	BLAST Search Result	Maximum Identity (%)[a]
F2-2	*Enterobacter aerogenes*	95
Q2-10	*Stenotrophomonas maltophilia*	96
L2-1	*Stenotrophomonas maltophilia*	96
R2-4	*Xanthomonas translucens*	96
W1-5	*Xanthomonas translucens*	96
W3-2	*Klebsiella oxytoca*	94
L4-5	*Burkholderia caledonica*	95
Q7-2	*Pseudomonas fluorescens*	96
Q8-4	*Comamonas testosteroni*	94

[a] Maximum identities show how closely an unknown bacterium matched with a given sequence in Genbank as a percentage of identical base pairs.

Identification was based on 16S rDNA gene analysis of rhizosphere-associated bacteria grown in nitrogen-free semi-solid medium.

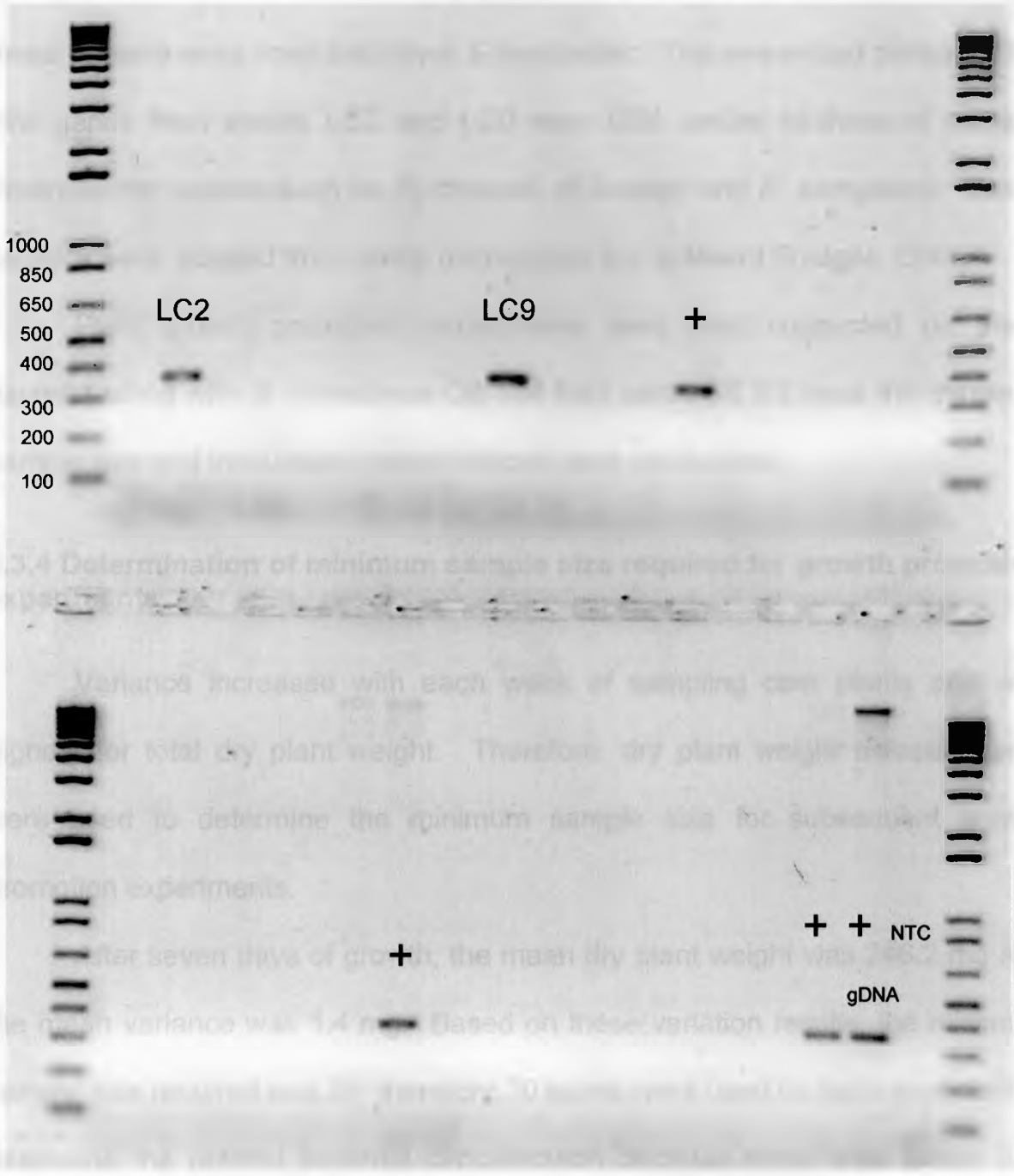

Figure 3.1 Amplification of 360 bp region of the *nifH* gene in *Enterobacter* sp. LC2 and LC9 isolated from surface-sterilized root tissue incubated in a semi-solid combined carbon medium (CCM).

+ - indicates positive controls from a bacterial colony (*A. zeae* N7)
gDNA - indicates positive control from extracted gDNA (*A. zeae* N7)
NTC - indicates the "no template control" to ensure no contamination occurred

16S rDNA fragments of strains LC2 and LC9 were sent for sequencing for further identification. Results from a comparative BLAST search determined that these bacteria were from the genus *Enterobacter*. The sequenced portion of the 16S genes from strains LC2 and LC9 were 95% similar to those of multiple *Enterobacter* species such as *E. cloacae*, *E. ludwigii* and *E. aerogenes*. These bacteria were isolated from sandy rhizosphere soil in Mount Brydges, Ontario.

Plant growth promotion experiments were then conducted on these bacteria along with *S. canadense* Q2-104 from section 3.3.2 once the minimum sample size and inoculation concentrations were established.

3.3.4 Determination of minimum sample size required for growth promotion experiments

Variance increased with each week of sampling corn plants and was highest for total dry plant weight. Therefore, dry plant weight measurements were used to determine the minimum sample size for subsequent growth promotion experiments.

After seven days of growth, the mean dry plant weight was 246.2 mg and the mean variance was 1.4 mg. Based on these variation results, the minimum sample size required was 25; therefore 30 seeds were used for each treatment to determine the optimal bacterial concentration because these trials lasted one week.

After three weeks, the mean dry plant weight was 608.7 mg and the mean variance was 11.1 mg. Based on week three variation results, the minimum sample size required was 46 seeds. Based on this result, 48 seeds were used

for each treatment in bacterial inoculation trials testing the growth promotion ability of *S. canadense* Q2-104, *Enterobacter* sp. LC2, and *Enterobacter* sp. LC9, as well as *G. azotocaptans* DS1, *A. zeae* N7, and *S. canadense* CR11. These trials were 15 days; therefore, to be conservative, week three variation results were used instead of week two variation results.

3.3.5 Determination of optimal bacterial treatments

The response of the corn plants to the bacterial inocula for each strain varied with respect to shoot height, fresh plant weight, and dry plant weight. For *S. canadense* Q2-104, the mean shoot height was 10% lower ($p = 0.02$) in the 10^8 CFU/mL treatment compared to the control group (Figure 3.2), the mean fresh plant weight of all the treatment groups did not significantly differ ($p = 0.47$) from that of the control group (Figure 3.3), although the dry plant weight of 10^7 CFU/mL treatment was 18% larger ($p < 0.001$) than that of the control group (Figure 3.4).

For *Enterobacter* sp. LC2, the mean shoot height of 10^7 CFU/mL treatment was 8% higher ($p = 0.04$) than that of the control group (Figure 3.5) and the 10^5 CFU/mL treatment produced a 14% smaller ($p < 0.001$) mean fresh plant weight compared to the control group (Figure 3.6). The mean dry plant weight of the 10^6 CFU/mL treatment was 9% larger ($p = 0.03$) than in the control group (Figure 3.7).

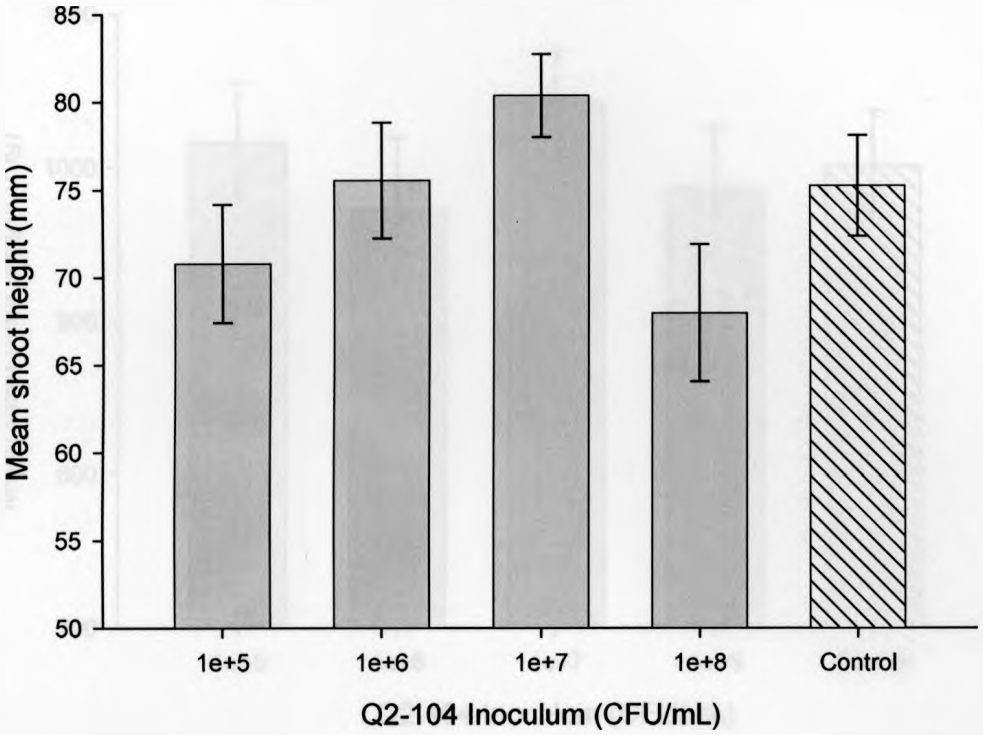

Figure 3.2 Mean (± standard error) shoot height (mm) of corn plants inoculated with *S. canadense* Q2-104 bacteria at four concentrations and grown for one week. Values are means of three trials consisting of 30 replicates per treatment.

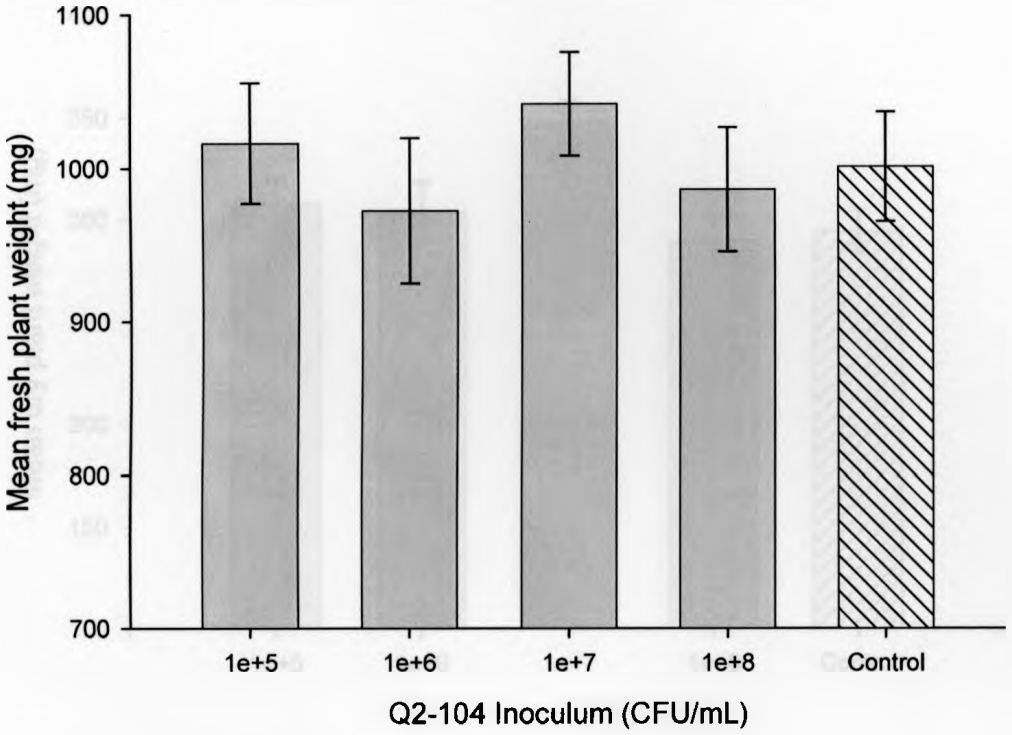

Figure 3.3 Mean (± standard error) fresh plant weight (mg) of corn plants inoculated with *S. canadense* Q2-104 bacteria at four concentrations and grown for one week. Values are means of three trials consisting of 30 replicates per treatment.

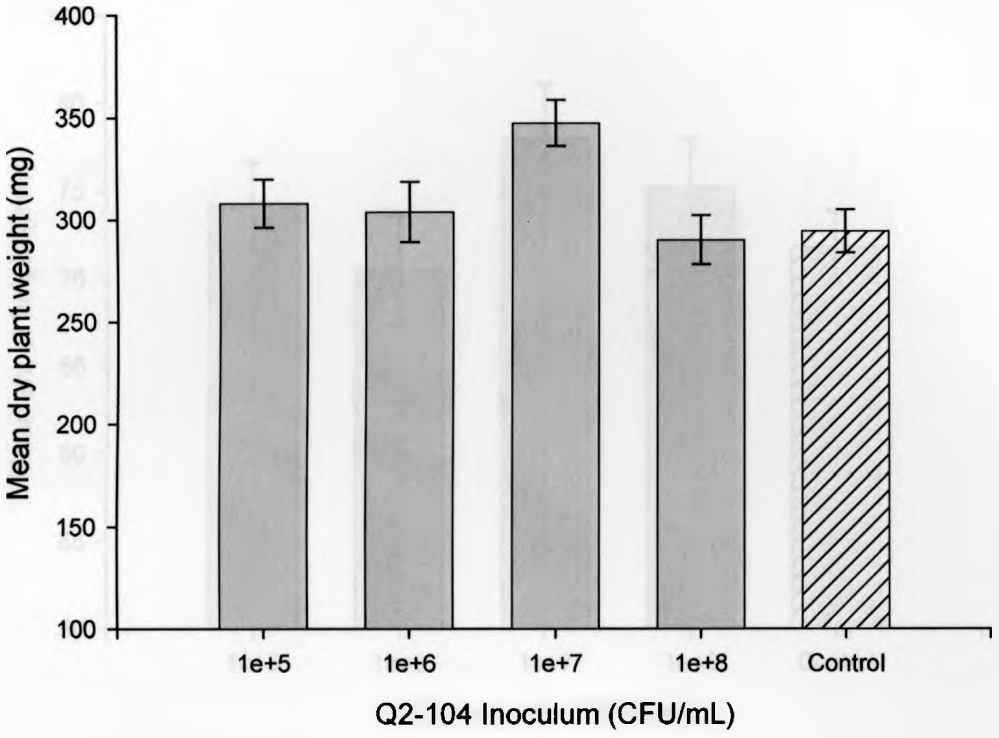

Figure 3.4 Mean (± standard error) dry plant weight (mg) of corn plants inoculated with *S. canadense* Q2-104 bacteria at four concentrations and grown for one week. Values are means of three trials consisting of 30 replicates per treatment.

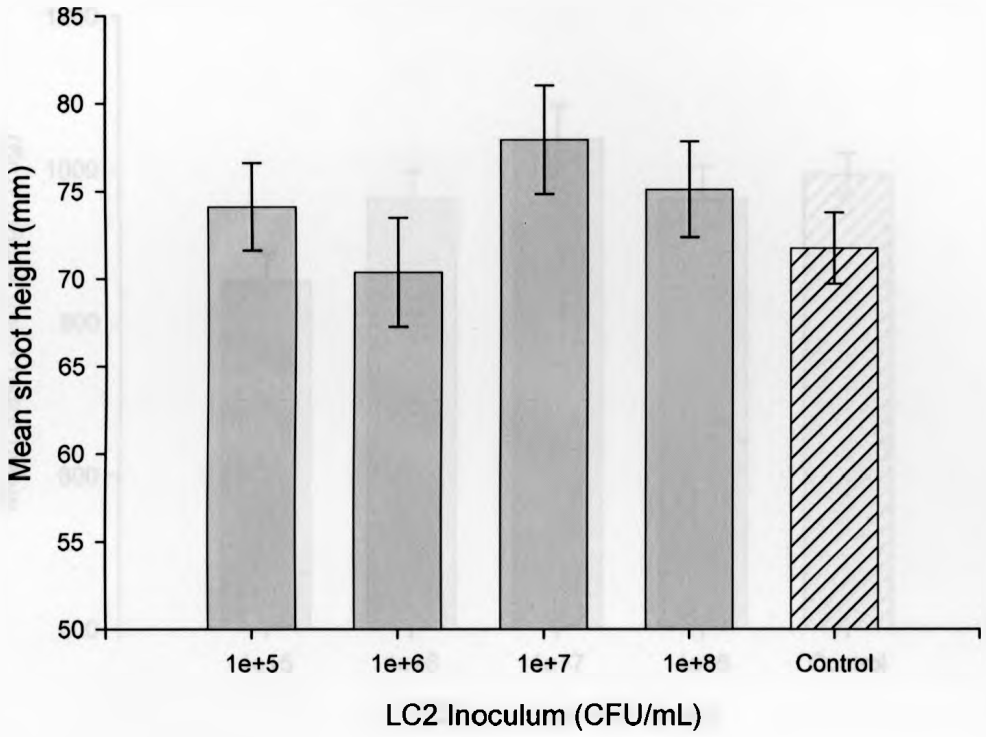

Figure 3.5 Mean (± standard error) shoot height (mm) of corn plants inoculated with *Enterobacter* sp. LC2 bacteria at four concentrations and grown for one week. Values are means of three trials consisting of 30 replicates per treatment.

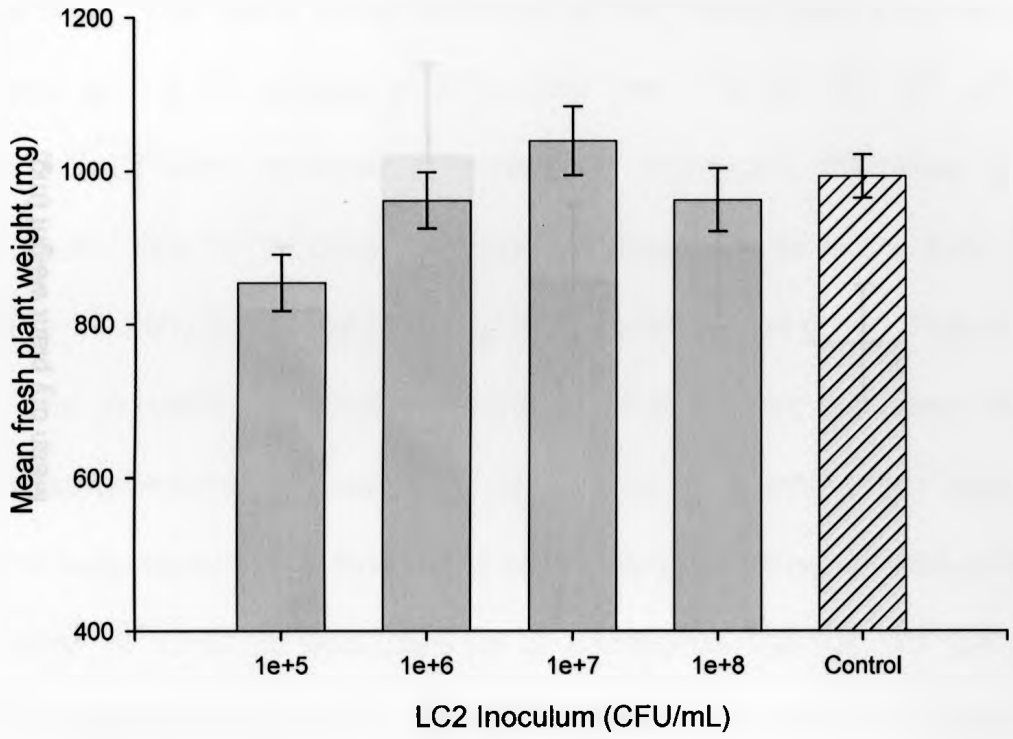

Figure 3.6 Mean (± standard error) fresh plant weight (mg) of corn plants inoculated with *Enterobacter* sp. LC2 bacteria at four concentrations and grown for one week. Values are means of three trials consisting of 30 replicates per treatment.

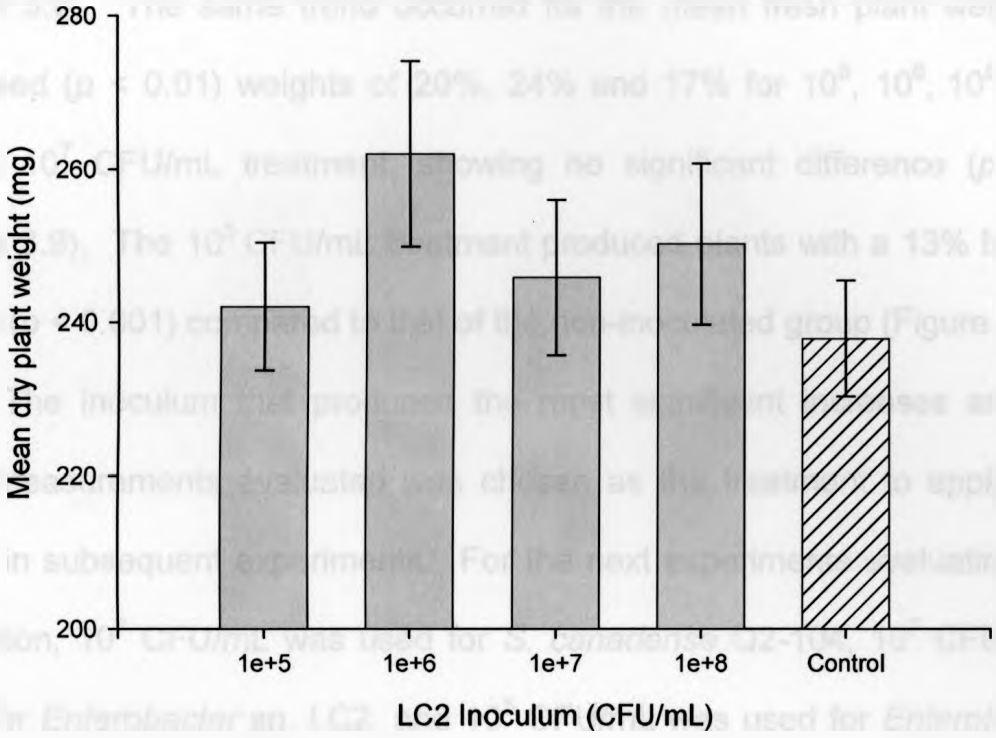

Figure 3.7 Mean (± standard error) dry plant weight (mg) of corn plants inoculated with *Enterobacter* sp. LC2 bacteria at four concentrations and grown for one week. Values are means of three trials consisting of 30 replicates per treatment.

For *Enterobacter* sp. LC9, significant increases ($p < 0.01$) in the mean shoot height compared to the control group were observed in all treatment concentrations: 16%, 18%, 12% and 18% for 10^5 – 10^8 CFU/mL, respectively (Figure 3.8). The same trend occurred for the mean fresh plant weights with increased ($p < 0.01$) weights of 20%, 24% and 17% for 10^5, 10^6, 10^8 CFU/mL except 10^7 CFU/mL treatment, showing no significant difference ($p = 0.10$) (Figure 3.9). The 10^5 CFU/mL treatment produced plants with a 13% higher dry weight ($p < 0.001$) compared to that of the non-inoculated group (Figure 3.10).

The inoculum that produced the most significant increases among the three measurements evaluated was chosen as the treatment to apply to corn seeds in subsequent experiments. For the next experiments evaluating growth promotion, 10^7 CFU/mL was used for *S. canadense* Q2-104, 10^7 CFU/mL was used for *Enterobacter* sp. LC2, and 10^5 CFU/mL was used for *Enterobacter* sp. LC9.

The optimal treatments of *G. azotocaptans* DS1 and *A. zeae* N7 were not evaluated because of previous successful sand experiments completed by Mehnaz and Lazarovits (2006). Positive results for two corn varieties with respect to increased shoot and root dry weight were observed when seeds were inoculated with 10^8 CFU/mL. Also, *S. canadense* CR11 was previously evaluated for growth promotion (Mehnaz *et al.*, 2010) using the same inoculum density with positive results for increased shoot and root dry weight therefore 10^8 CFU/mL was used for experimentation.

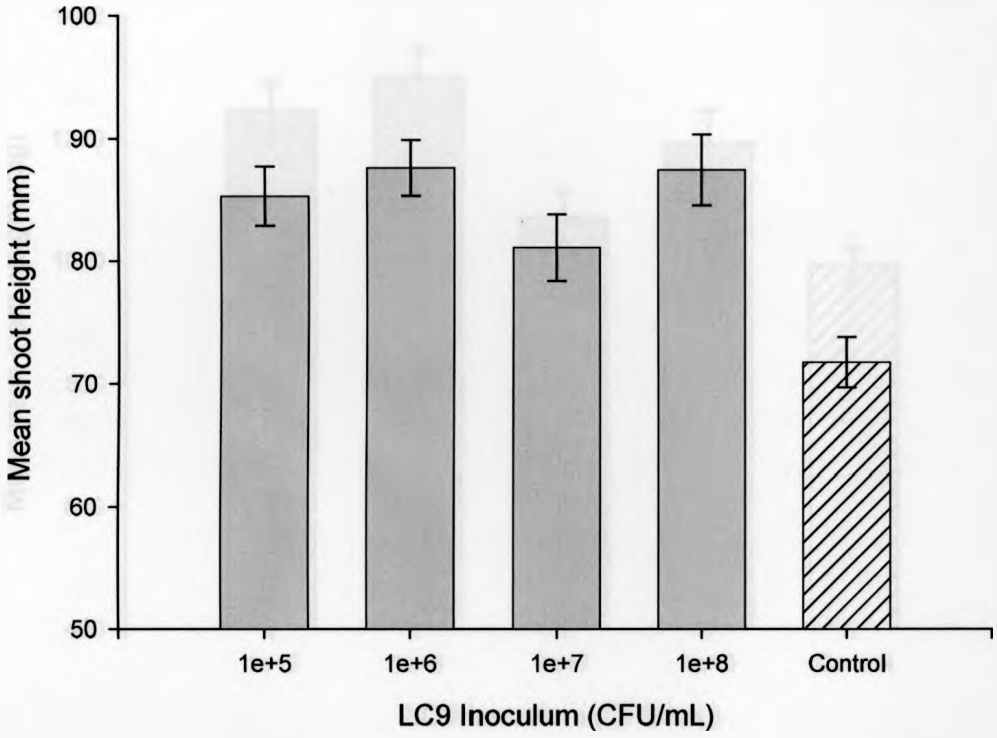

Figure 3.8 Mean (± standard error) shoot height (mm) of corn plants inoculated with *Enterobacter* sp. LC9 bacteria at four concentrations and grown for one week. Values are means of three trials consisting of 30 replicates per treatment.

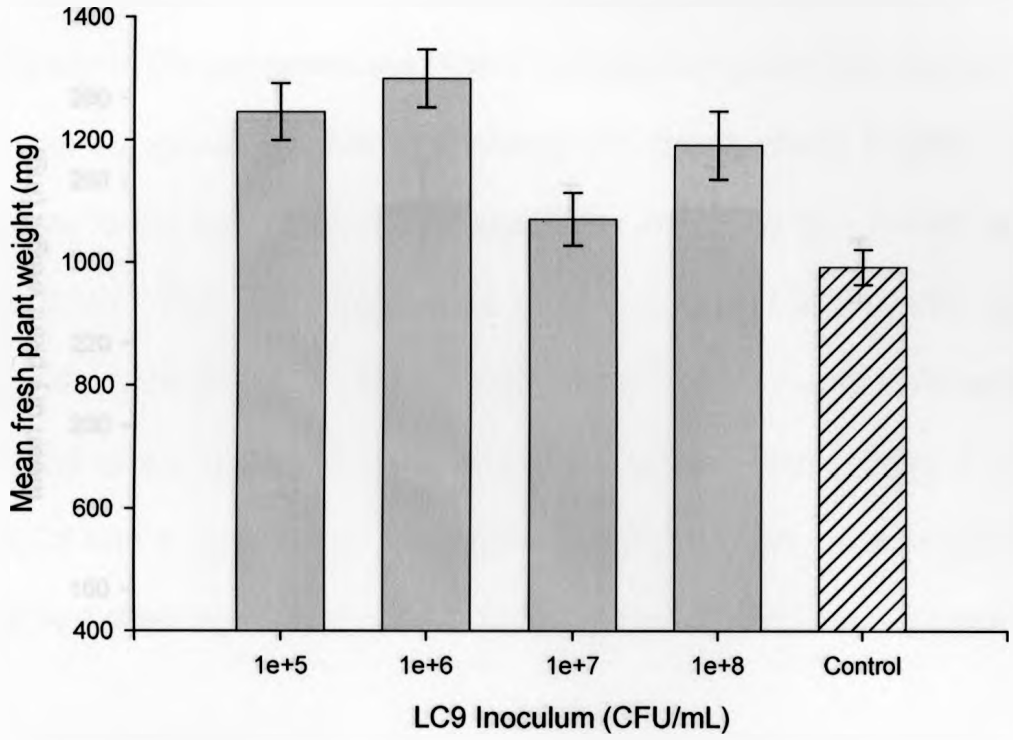

Figure 3.9 Mean (± standard error) fresh plant weight (mg) of corn plants inoculated with *Enterobacter* sp. LC9 bacteria at four concentrations and grown for one week. Values are means of three trials consisting of 30 replicates per treatment.

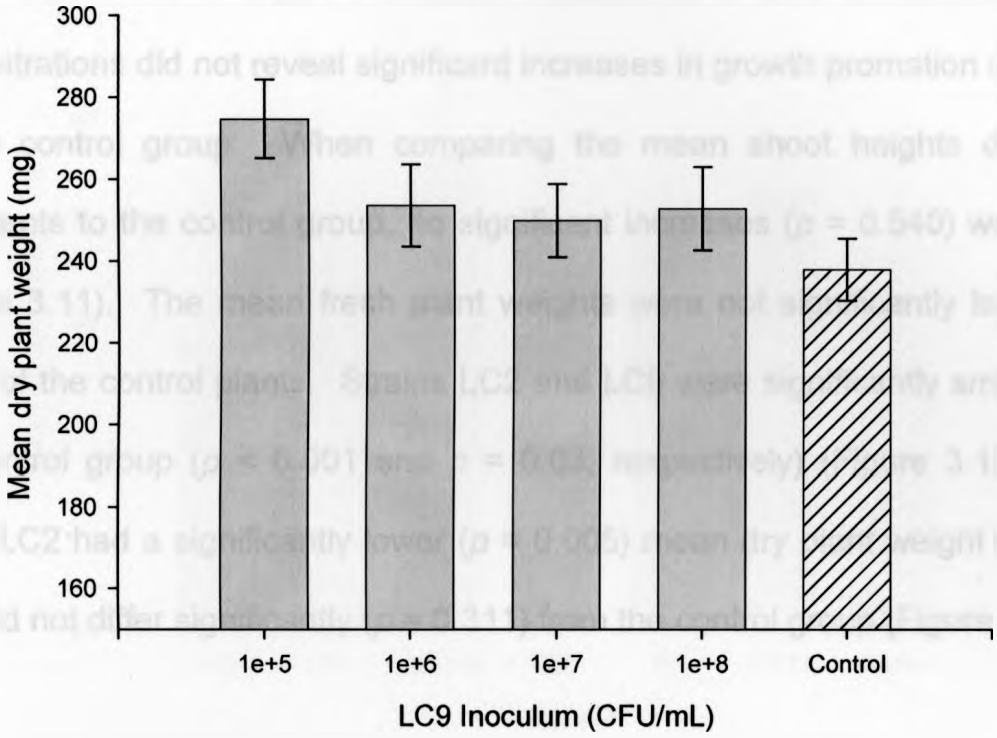

Figure 3.10 Mean (± standard error) dry plant weight (mg) of corn plants inoculated with *Enterobacter* sp. LC9 bacteria at four concentrations and grown for one week. Values are means of three trials consisting of 30 replicates per treatment.

3.3.6 Testing growth promotion ability of strains Q2-104, LC2, and LC9 in sand under greenhouse conditions

Evaluation of strains Q2-104, LC2, LC9, *G. azotocaptans* DS1, *A. zeae* N7, and *S. canadense* CR11 bacterial inoculations at their determined optimal concentrations did not reveal significant increases in growth promotion compared to the control group. When comparing the mean shoot heights of the six treatments to the control group, no significant increases ($p = 0.540$) were found (Figure 3.11). The mean fresh plant weights were not significantly larger than those of the control plants. Strains LC2 and LC9 were significantly smaller than the control group ($p < 0.001$ and $p = 0.03$, respectively) (Figure 3.12). Also, strain LC2 had a significantly lower ($p = 0.005$) mean dry plant weight but strain LC9 did not differ significantly ($p = 0.311$) from the control group (Figure 3.13).

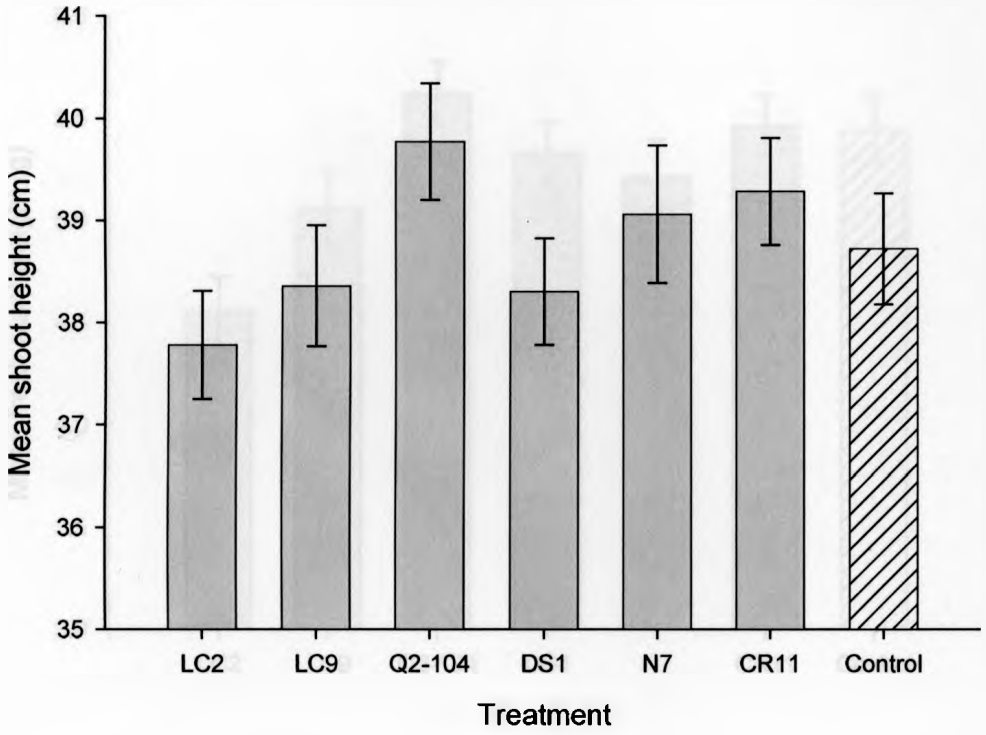

Figure 3.11 Mean (± standard error) shoot height (cm) of corn plants inoculated with bacteria and grown for 15 days. Values are means of three trials consisting of 48 replicates per treatment.

Bacterial treatments:
Enterobacter sp. LC2
Enterobacter sp. LC9
Sphingobacterium canadense Q2-104
Gluconacetobacter azotocaptans DS1
Azospirillum zeae N7
Sphingobacterium canadense CR11

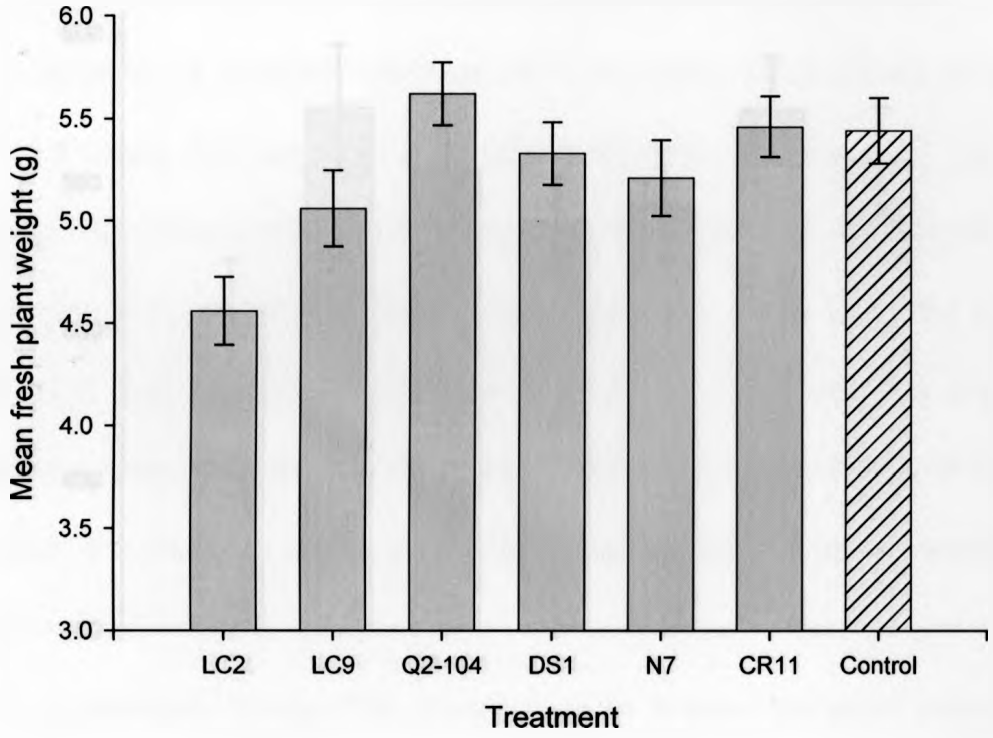

Figure 3.12 Mean (± standard error) fresh plant weight (g) of corn plants inoculated with bacteria and grown for 15 days. Values are means of three trials consisting of 48 replicates per treatment.

Bacterial treatments:
Enterobacter sp. LC2
Enterobacter sp. LC9
Sphingobacterium canadense Q2-104
Gluconacetobacter azotocaptans DS1
Azospirillum zeae N7
Sphingobacterium canadense CR11

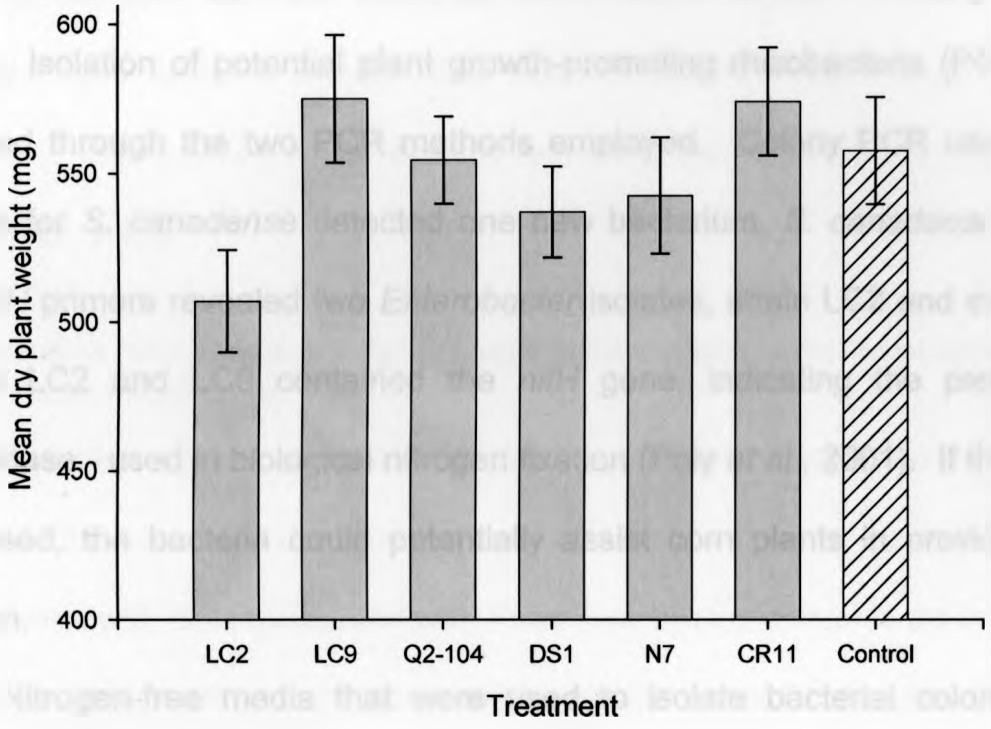

Figure 3.13 Mean (± standard error) dry plant weight (mg) of corn plants inoculated with bacteria and grown for 15 days. Values are means of three trials consisting of 48 replicates per treatment.

Bacterial treatments:
Enterobacter sp. LC2
Enterobacter sp. LC9
Sphingobacterium canadense Q2-104
Gluconacetobacter azotocaptans DS1
Azospirillum zeae N7
Sphingobacterium canadense CR11

3.4 Discussion

3.4.1 Isolation of potential PGPR

In this study, new potential plant growth-promoting bacteria were isolated from the corn rhizosphere soil and surface-sterilized corn roots using selective media. Isolation of potential plant growth-promoting rhizobacteria (PGPR) was achieved through the two PCR methods employed. Colony PCR using *cpn*60 primers for *S. canadense* detected one new bacterium, *S. canadense* Q2-104, and *nifH* primers revealed two *Enterobacter* isolates, strain LC2 and strain LC9. Strains LC2 and LC9 contained the *nifH* gene, indicating the presence of nitrogenase - used in biological nitrogen fixation (Poly *et al.*, 2001). If the gene is expressed, the bacteria could potentially assist corn plants in providing fixed nitrogen.

Nitrogen-free media that were used to isolate bacterial colonies were ideally expected to eliminate all non-nitrogen fixing bacteria. Theoretically, all the bacteria screened should have had gene detection with *nifH* primers because they require nitrogen to survive. This was not found with the colonies tested as most of them did not result in PCR detection. However, when randomly selected *nifH*-negative isolates were sequenced, the majority came from genera that have the ability to reduce nitrate, except for *Xanthomonas* (Palleroni and Bradbury, 1993; Willems *et al.*, 1991; Vauterin *et al.*, 1995; Wolf *et al.*, 2002). This finding is likely due to limitations of the *nifH* primers as they did not cover a wide enough range of bacteria to encompass all the species found and are not universal (Poly *et al.*, 2001).

Colony PCR gave unpredictable results in detection of bacterial species containing *nifH* genes. Positive controls often failed due to inconsistencies with using colonies as the DNA template. This problem occurred after repeated freezing/thawing of bacterial colonies suspended in water. When gDNA was extracted from bacteria, amplification was consistent. This problem was not reported in the literature where researchers used the same colony PCR method (Woodman, 2008).

3.4.2 Growth promotion ability of bacteria-inoculated corn in sand under greenhouse conditions

A preliminary experiment evaluated the optimal bacterial concentration for each inoculum and the minimum sample size required. The greenhouse sand experiments with corn inoculated with three new bacterial strains did not indicate growth promotion when comparing plant weights and shoot heights to those of the control group. Concurrently, corn plants inoculated with *S. canadense, G. azotocaptans,* and *A. zeae* did not have significant increases in corn plant growth despite other studies finding results to the contrary (Mehnaz *et al.*, 2006b; Mehnaz *et al.*, 2010; Marques *et al.*, 2010). My hypothesis was rejected as corn plant colonization by new potential plant growth-promoting bacteria as well as *S. canadense*, *A. zeae*, and *G. azotocaptans*, did not increase the rate of plant development.

A possible explanation for this outcome could be related to the corn variety chosen and it not being well suited to support bacterial growth. Bacteria can have variable success in different corn cultivars. More than one corn variety were tested in past greenhouse sand studies with only some varieties showing

increased plant growth for *S. canadense*, *A. zeae*, and *G. azotocaptans* (Mehnaz *et al.*, 2006b; Mehnaz *et al.*, 2010). In another study by de Salamone and Döbereiner (1996), only six of fifteen corn genotypes showed a consistently positive response to inoculation with a mixture of *A. brasilense* strains isolated from corn.

3.5 References

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Chapter 4

4.1 General Discussion and Conclusions

Plant growth-promoting rhizobacteria (PGPR) represent a wide variety of soil bacteria that, when grown in association with a host plant, result in stimulation of growth of their host. Many bacterial species and strains belonging to genera such as *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Gluconacetobacter*, *Herbaspirillum*, and *Pseudomonas* have already been established as PGPR (Probanza *et al.*, 1996; Glick, 1995; Boddey *et al.*, 1986). The search for PGPR and investigation of their modes of action are increasing at a rapid pace as efforts are made to exploit them commercially as biofertilizers.

In this study, three PGPR bacterial species, namely, *S. canadense*, *A. zeae*, and *G. azotocaptans*, were detected and quantified from corn rhizosphere soil and surface-sterilized root tissue using real-time qPCR. The abundance of these bacteria in Ontario and Québec was previously unknown. Identifying bacterial quantities close to 10^6 CFU/g in more than one field site indicates that the bacteria flourish in their given environments and are potentially involved in multiple forms of nutrient exchange with the plant host. Determining how widespread the bacteria naturally exist in association with corn can give an indication of their ability to thrive in different environments with a range of corn varieties. This study provided a glimpse into the presence of a group of bacterial species associated with rhizosphere soil and roots. However, bacterial populations are dynamic, changing in response to climate, soil characteristics,

and plant secondary metabolite availability (Döbereiner and Day, 1975). Sampling multiple times over a growing season could reveal different results as to which bacteria persist and therefore may be better suited for use in broad-based commercial biofertilizers.

Three additional bacterial isolates, *S. canadense* Q2-104, *Enterobacter* sp. LC2, and *Enterobacter* sp. LC9, were identified as potential PGPR by means of colony PCR. *S. canadense* Q2-104 was anticipated to have similar growth-promoting effects as *S. canadense* CR11 reported by Mehnaz *et al.* (2007; 2010). *Enterobacter* species have been found previously in rhizosphere soils in Chile, among other regions (Shoebitz *et al.*, 2008). Hinton and Bacon (1995) found one isolate of *Enterobacter cloacae*, endophytically associated with corn that enhanced the biological control of systemic pathogens.

The capability of native bacterial species isolated from Ontario and Québec rhizosphere soil and corn roots to behave as PGPR was investigated. *S. canadense* CR11, *A. zeae* N7, and *G. azotocaptans* DS1 along with *S. canadense* Q2-104, *Enterobacter* sp. LC2, and *Enterobacter* sp. LC9 were evaluated under greenhouse conditions for corn growth promotion but no statistically significant increases in shoot height, dry plant weight, or fresh plant weight were observed. In general, shortly after bacteria are introduced into the soil, bacterial populations can decline progressively (Bashan and Levanony, 1988; van Elsas *et al.*, 1986). This phenomenon may prevent the build-up of a sufficiently large PGPR population in the rhizosphere soil to obtain the intended plant response. One of the goals of inoculating corn seeds with PGPR is to

establish high levels of biological nitrogen fixation (BNF). Nitrogen fixation with cereal crops is often considered negligible in comparison to rates of symbiotic nitrogen fixation attained by root nodulated plants. Therefore, the possibility of increasing nitrogen fixation with cereal crops by inoculation with wild type bacteria is a challenging task.

The development of molecular techniques has allowed microbial ecologists to explore microbial diversity and abundance far beyond that exposed by analysis of culturable microbes, as shown in Chapter 2. In contrast, in Chapter 3, despite finding bacterial isolates that are very closely related to PGPR, *S. canadense* Q2-104, and *Enterobacter* species containing *nifH* genes, they did not exhibit any growth-promoting effects in the greenhouse trials. The link between tracking PGPR and determining how to exploit their beneficial traits, whether it is biological nitrogen fixation, solubilization of phosphate, or facilitating the adsorption of iron, is where more research is needed (Vessey, 2003).

As research continues towards alternative fertilizers to provide nutrients to non-leguminous crops of agricultural significance, PGPR are promising candidates as they appear to be relatively widespread and diverse in their functional characteristics. Discovering a method to determine quickly if a bacterial species is able to assist in plant growth-promotion would ultimately narrow down the search. Also, studying the associative interactions between bacteria and non-leguminous plant species would aid in better understanding of the mechanisms involved, leading to advantageous PGPR.

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Appendix

Common media used throughout study.

LB Medium (Lysogeny Broth Medium)

10.0 g/L	tryptone
5.0 g/L	yeast extract
5.0 g/L	NaCl
15.0 g/L	agar (for solid medium)

LGI Medium

0.2 g/L	K_2HPO_4
0.6 g/L	KH_2PO_4
0.2 g/L	$MgSO_4 \cdot 7H_2O$
0.002 g/L	$CaCl_2$
0.1 g/L	$Na_2MoO_4 \cdot 2H_2O$
5.0 g/L	$FeCl_3$
5.0 mL	Bromothymol Blue 0.25% in 0.2M KOH
6	pH (adjusted with 5 M KOH)
1.8 or 15 g/L	agar (semi-solid or solid medium)

Malate Medium

0.1 g/L	K_2HPO_4
0.4 g/L	KH_2PO_4
0.2 g/L	$MgSO_4 \cdot 7H_2O$
0.02 g/L	$CaCl_2$
5.0 g/L	DL-malic acid
0.1 g/L	NaCl
0.02 g/L	biotin
6.8	pH (adjusted with 5 M KOH)
15.0 g/L	agar (for solid medium)

Modified Combined Carbon Medium

5.0 g/L	sucrose
5.0 g/L	mannitol
5.0 g/L	DL-malic acid
0.5 mL/L	sodium lactate (60% v/v)
0.8 g/L	K_2HPO_4
0.2 g/L	KH_2PO_4
0.2 g/L	$MgSO_4 \cdot 7H_2O$
0.06 g/L	$CaCl_2$
0.1 g/L	NaCl
0.1 g/L	yeast extract
25 mg/L	$Na_2MoO_4 \cdot 2H_2O$
28 mg/L	Na_2Fe EDTA
5 µg/L	Biotin
7	pH (adjusted with 5 M KOH)
15.0 g/L	agar (for solid medium)

NFM (Nitrogen-free Medium)

0.4 g/L	KH_2PO_4
0.1 g/L	K_2HPO_4
0.2 g/L	$MgSO_4 \cdot 7H_2O$
0.1 g/L	NaCl
0.02 g/L	$CaCl_2$
0.01 g/L	$FeCl_3$
0.002 g/L	$Na_2MoO_4 \cdot 2H_2O$
5.0 g/L	DL malic acid
7.2-7.4	pH (adjusted with 5 M KOH)
15.0 g/L	agar (for solid medium)

Rich Medium

2.0 g/L	dextrose
1.5 g/L	glutamic acid
1.5 g/L	peptone
0.5 g/L	K_2HPO_4
0.5 g/L	$MgSO_4 \cdot 7H_2O$
2.0 g/L	yeast extract
6	pH (adjusted with 5 M KOH)
15.0 g/L	agar (for solid medium)