A greener grass: Improving biofuel feedstock production of switchgrass (*Panicum virgatum* L.) by inoculation with endophytic rhizobacteria

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Dedication

This thesis is dedicated, with love, to my parents: my mother, Chanthalan Ker, and to my father, Tin Ker, who passed away before its completion. Without your love, courage, and hopes for a better future, this thesis would never have been possible.

Contents

Abstract

Switchgrass (SG, *Panicum virgatum* L.), a temperate perennial grass, was chosen by the US Department of Energy's Herbaceous Energy Crops Program as the 'model' bioenergy crop for further research in North America. Current research on SG for bioenergy feedstock production focuses on improving breeding selection, agronomy and crop physiology, energy potential, and its contribution to mitigating greenhouse gas emissions. However, there is a lack of knowledge regarding plant-microbe interactions with SG, how these associations play a role in its growth and productivity, and their function and potential role in agro-ecosystems. Moreover, as SG has been reported to produce high biomass yields with minimal to no synthetic nitrogen (N) fertilizer, this suggested to us that SG could be obtaining at least some of the N to meet its requirements from plant growth promoting rhizobacteria (PGPR) capable of biological N_2-fixation (BNF). The objectives of this research were to determine if: (1) SG associates with PGPR, (2) PGPR we isolated from SG can be used as inoculants capable of promoting SG growth under a controlled environment, and (3) inoculation with PGPR can increase the growth and productivity of SG for biofuel production under a low-N input system. Switchgrass rhizomes were collected in Québec, Canada, from a discontinued biomass trial of 11 varieties that had not received N fertilizer or any other management input since 2000. Isolates were chosen on N-free solidified media and screened for their ability to promote plant growth using plant assays conducted in growth chambers. Switchgrass seedlings were inoculated, or not, with batches of mixed isolates and fertilized with N-free Hoagland's solution. Molecular analyses of

16S rRNA gene sequences identified the mixed bacterial inoculum as *Paenibacillus polymyxa*, a N_2-fixing bacterium, and several other PGPR (*Pseudomonas, Serratia* and *Rahnella* spp.) capable of producing auxin and/or solubilizing phosphate. Field trials of inoculated SG seeds were conducted in 2010 on three sites comprising different soil types. The factors tested were the bacterial treatment, either uninoculated control or seed inoculated, and a fertilizer treatment, either 0 or 100 kg N ha^{-1}.

Establishment year results showed that inoculation of SG plants with a mixed PGPR culture produced higher tiller density and larger tillers than uninoculated plants, which was the probable cause of the 40% yield increase. This 40% yield increase persisted under N fertilization, at least at the 100 kg N ha^{-1} rate. Inoculated SG plants also had better N cycling than uninoculated plants, as they contained more N within tillers during anthesis but not after senescence, suggesting a greater amount of N was translocated to below-ground roots and rhizomes of inoculated than uninoculated plants. Greater N storage in roots and rhizomes could mean better early-season regrowth and provide an advantage over weeds. PGPR inoculation also affected the N balance of the harvested biomass by contributing additional non-fertilizer N (ANFN) to SG plants. Interestingly, this bacterial effect was not inhibited in the presence of N fertilizer. The combination of PGPR and N fertilizer provided a substantial N contribution to SG, although the exact amount will require additional research. This investigation showed that SG does associate with PGPR and that PGPR can be effectively utilized as inoculants to enhance SG yields in low-N input systems. This research will help in the development of an environmentally beneficial switchgrass-microbe system, reduces N requirements and has the potential to become a best N management practice.

Résumé

Le panic raide (PR, *Panicum virgatum* L.) est un graminées vivace qui pousse dans les climats tempéres. Le PR a été choisi dans le cadre du programme de recherche sur les agrocarburants, ≪ The Herbaceous Energy Crops Program ≫, du département de l'énergie américain comme étant le ≪ modèle ≫ de culture agroénergétique pour l'avancement de la recherche en Amérique du Nord. La recherche actuelle sur l'emploi du PR dans la production de biocarburant est principalement axée sur la sélection de cultivars plus productifs, l'agronomie et la physiologie de la plante, son potentiel énergétique ainsi que sa contribution à la réduction des gaz à effet de serre. Pourtant, il y a un manque de connaissance par rapport aux interactions plante-microbe chez le PR et le rôle que jouent ces associations sur la productivité de la culture ainsi que leur fonction et leur rôle dans l'agroécosystème. De plus, il a été démontré que PR donne de hauts rendements sans apport ou avec un apport minime d'azote (N) fertilisant synthétique. Ceci nous porte à croire que les besoins en N pourraient être comblés par l'interaction avec des rhizobactéries qui favorisent la croissance des plantes, connues sous le terme RFCP capable de fixation biologique de l'azote. L'objectif de l'étude ici présentée est de déterminer si : (1) le PR s'associe avec les RFCP, (2) les RFCP que nous avons isolés des rhizomes du PR sont capable de promouvoir la croissance de la plante dans un environnement contrôlé, et finalement (3) inoculation de RFCP peut augmenter la croissance et la productivité du PR pour de la production de biocarburant sous un apport minime de fertilisant N. Les rhizomes de PR ont été

recueillis sur le site d'une ancienne étude de biomasse de 11 variétés n'ayant pas reçu de traitement dazote fertilisant ou de tout autre soin depuis l'abandon du site en 2000. Les souches bactériennes cultivées dans milieu sans azote ont été choisi selon leur capacité de promouvoir la croissance de plantes en chambre d'incubation. Certaines boutures de PR ont été inocules avec des groupes de souches bactériennes et fertilisées avec une solution d'Hoagland sans azote. Des analyses moléculaires des gènes de type 16S rRNA nous ont permises d'identifier la nature de l'inoculant bactérien comme étant *Paenibacillus polymyxa*, une bactérie fixatrice d'azote, ainsi que plusieurs autres RFCP (espèces de *Pseudomonas, Serretia* et *Rahnella*) producteur d'auxine et/ou capable de dissoudre les phosphates. Des essais de champ ont été faits en 2010 dans trois types de sols différents. Les facteurs testés ont été les traitements bactériens, soit contrôle non inoculé ou semences inocules et un traitement fertilisé, soit de 0 ou de 100 kg N ha^{-1}.

Les résultats de la première année ont montré que les plants inoculés avec une mixture de RFCP ont produit une densité de talle une grosseur des talles supérieure aux plants non inoculés, ce qui explique probablement la différence de productivité de 40%. Cette augmentation de 40% a aussi été observée chez les plants fertilisés avec le taux de 100 kg N ha^{-1}. Les plants de PR inoculés ont montré un meilleur cycle de l'azote que les plants non inoculés puisqu'ils contenaient plus de N dans leurs talles durant l'anthèse et non âpres la sénescence ce qui suggère qu'une plus grande quantité de N a été stocké dans les racines et dans les rhizomes pourrait donner lieu à une meilleure repousse en début de saison et donner au PR une meilleure chance de compétitionner contre les mauvaises herbes. La présence de RFCP a affecté l'équilibre de N de la biomasse récoltée en contribuant un apport supplémentaire de N ne provenant pas de fertilisant aux PR. Il est intéressant de noter que cet effet bactérien n'a pas été inhibé par la présence de fertilisant N. La combinaison de RFCP

et de fertilisant N a contribué un apport important de N au PR, mais il faudra une étude plus approfondie afin de déterminer le montant exact. Cette étude a démontré que le PR s'associe avec les RFCP et que les RFCP peuvent être utilisé comme inoculent pour améliorer la productivité du PR en milieu pauvre en N. Cette étude contribue au développement d'un système panic raide-microbe, réduisant la quantité de N requis et ayant le potentiel de devenir un axe de bonne gestion en matière de fertilisation.

Contributions of the authors

This thesis consists of three manuscripts, of which the candidate was fully responsible for the design, development and execution of the laboratory, growth chamber and field-based research studies, collection and analysis of the data, discussion of the results, and writing of the thesis and manuscripts. However, this work could not have been achieved without the contribution of several colleagues, the thesis co-supervisors (Drs. Donald L. Smith and James W. Fyles), and in some instances, members of the candidate's supervisory thesis committee (Drs. Brian T. Driscoll and Philippe Seguin), who share authorship on the manuscripts and whose contributions are described below. In addition, the initial research idea was conceived by D. L. Smith, while the concept of the switchgrass-microbe system for temperate environments was largely developed by K. Ker and J. W. Fyles, with input from D. L. Smith.

Chapter 4 was co-authored by Drs. D. L. Smith from the Plant Science Department, J. W. Fyles and B. T. Driscoll from the Department of Natural Resource Sciences of McGill University, and by Andrea Jilling, an undergraduate research assistant. As the thesis co-supervisors, D. L. Smith and J. W. Fyles provided guidance, advice and support during the preparation, laboratory work, sample and data analysis, interpretation of findings, and writing of this manuscript. B. T. Driscoll provided advice and guidance in the planning and development of the laboratory work, inoculation and screening method used in the growth studies, and editing of the manuscript

before submission for publication. In addition, B. T. Driscoll provided laboratory facilities and expertise for the molecular work. A. Jilling provided invaluable assistance in all of the laboratory and growth chamber studies (preparation, data collection), as well as conducting some of the growth studies herself, under the close supervision of K. Ker.

Chapters 5 and 6 were co-authored by Drs. D. L. Smith, J. W. Fyles, B. T. Driscoll, and P. Seguin from the Plant Science Department of McGill University. The contributions of D. L. Smith and J. W. Fyles are as described above. B. T. Driscoll provided advice on the seed inoculation method used in the field studies, guidance during the field work, and editing of the manuscripts before submission for publication. P. Seguin provided advice on the design of the field experiment, data collection and analysis of the results, expertise regarding the agronomy of switchgrass, as well as editing of the manuscripts before submission for publication.

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List of Abbreviations

ACC	amino-cyclopropane-1-carboxylate
ADF	acid detergent fiber
ADL	acid detergent lignin
ANFN	additional non-fertilizer nitrogen
ANOVA	Analysis of Variance
ARA	acetylene reduction assay
ATCC	American Type Culture Collection
BLASTN	nucleotide Basic Local Alignment Search Tool
BNF	biological nitrogen fixation
cfu	colony forming unit
cm	centimetre
CO_2	carbon dioxide
CRD	completely randomized design
d	day
dNTP	deoxynucleotide triphosphate
DOE	Department of Energy
DW	dry weight
EDTA	ethylenediamine-tetra-acetic-acid
FW	fresh weight
FWD	forward

g	gram
GHG	greenhouse gas
ha	hectare
HECP	Herbaceous Energy Crops Program
HS	Hoagland's Solution
IAA	indole-3-acetic acid
K	potassium
kg	kilogram
km	kilometre
L	litre
LB	Luria Bertani
m	metre
Mg	megagram
mg	milligram
M	molar
μg	microgram
μm	micrometre
μM	micromolar
mL	millilitre
min	minute
MPN	most probable number
nm	nanometre
NDF	neutral detergent fiber
N	nitrogen
NFB	nitrogen-fixing bacteria
NUE	nitrogen use efficiency
N_2O	nitrous oxide

OD	optical density
P	phosphorus
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGP	plant growth promotion
PGPR	plant growth promoting rhizobacteria
PVK	Pikovskaya
RCBD	randomized complete block design
RDP	Ribosomal Database Project
REAP	Resource Efficient Agricultural Production
REV	reverse
s	second
SG	switchgrass
SE	standard error
TAE	tris acetate EDTA
TY	tryptone yeast
UV	ultraviolet
WUE	water use efficiency

Chapter 1

Introduction

Czech born agronomist Johanna Döbereiner wrote: *After I moved to Brazil ... in the early 1950s, I began my research by searching for diazotrophic bacteria which may occur in association with plant roots. I was motivated to study this topic by observing the continuously high-yielding sugarcane crops and other grasses of Brazil which required no fertilization* (Döbereiner and Pedrosa, 1987). Döbereiner went on to become a naturalized Brazilian citizen and is heralded as one of Brazil's most influential scientist (Baldani and Baldani, 2005). Her observations regarding the tropical grasses that grow alongside km 47 near the research centre, Embrapa Agrobiologia, in the state of Rio de Janeiro led to a series of discoveries, including: (1) isolation of several free-living, N_2-fixing bacteria (NFB) associated with graminaceous plants, (2) evidence showing the contribution of fixed nitrogen (N) from the bacteria to the host plant, and (3) the existence of beneficial plant-diazotroph interactions that enhance plant growth and soil fertility (Baldani and Baldani, 2005; Boddey et al., 1995; Döbereiner, 1996). The contributions made by Döbereiner on associative NFB with agronomically important crops expanded our understanding of plant-diazotroph interactions, and provided us with a framework from which to broaden this base of knowledge (Baldani and Baldani, 2005). The observations made by Döbereiner were the inspiration that led to the research undertaken here.

During the 1960s, Döbereiner and her team discovered *Beijerinckia fluminensis* and *Azotobacter paspali*, two new NFB associated with sugarcane and bahia grass, respectively (Baldani and Baldani, 2005; Döbereiner, 1961; Döbereiner et al., 1973). The 1970s marked the introduction of the acetylene reduction assay, which allowed the indirect measurement of nitrogenase activity, the enzyme responsible for catalyzing atmospheric N_2 to ammonia in diazotrophic bacteria (Hardy et al., 1968), and the discovery of spirillum-type NFB, namely *Azospirillum lipoferum* and *A. brasilense* by the Brazilian researchers (Baldani and Baldani, 2005; Döbereiner and Pedrosa, 1987; Tarrand et al., 1978). This time period also coincided with the 1970s oil crisis, which influenced the Brazilian government to seek energy independence from foreign fossil fuel consumption and to focus their attention on improving current sugarcane to bioethanol production (see Section 2.2.1). As a result, government funding on sugarcane research was plentiful. Efforts by the Brazilian research team expanded, allowing for the discovery of other NFB; *Herbaspirillum seropedicae* was isolated from plants of maize, sorghum, and rice (Baldani et al., 1986; Baldani and Baldani, 2005). Arguably, the most notable of diazotrophic bacterium discovered by the Brazilian researchers was *Gluconacetobacter diazotrophicus*, first isolated from sugarcane plants (Cavalcante and Döbereiner, 1988; Gillis et al., 1989). This bacterium has shown remarkable ability to enhance plant growth by providing fixed N to the host plant; as much as 50 to 80% of the plant N (equivalent to 150 to 170 kg N ha^{-1}) is derived from biological N_2-fixation (Baldani et al., 2002; Boddey, 1995; Döbereiner, 1996). The significant contribution of this bacterium to plant growth has enabled growers to reduce the amount of N fertilizer used for sugarcane production (Boddey, 1995; Boddey et al., 1995; Baldani et al., 2002). As synthetic N fertilizer is produced by the energy intensive Haber-Bosch process, incorporation of NFB into sugarcane production lowers the energy ratios (output/input) of bioethanol production (Boddey, 1995; Boddey et al., 1995; Baldani et al., 2002). By incorporating beneficial, and naturally-occurring mi-

crobes into sugarcane bioethanol production as a means of reducing N fertilizer use and enhancing yields, the energy ratio of sugarcane can effectively be increased from 4.53 to 5.79 (Boddey, 1995; Boddey et al., 1995; Baldani et al., 2002). Strategies, such as in this Brazilian example, could be modeled and potentially improved for other plant-diazotroph systems. Most importantly, could this 'model system' be implementted for temperate grown biofuels feedstock grasses?

In a manner similar to that of Döbereiner, we observed that switchgrass, a temperate perennial grass, has continued to produce reasonable yields with no fertilizer input. Our observations stem from a variety trial for biomass that was initiated in 1996 at the Lods Agronomy Research Centre (McGill University, Montreal, Canada) by the NGO, REAP-Canada (Resource Efficient Agricultural Production). Eleven upland and two lowland varieties were selected, and fertilized with 50 kg ha^{-1} of urea during the three years of the trial. After the trial ended, the switchgrass stands were allowed to remain on the field, but did not receive fertilizer or any other management input. Yet, a decade later, several varieties still produced reasonable biomass yields, as much as 5 Mg ha^{-1}. Further, several studies have reported switchgrass yields over 10 Mg ha^{-1} with minimal N fertilizer input (Fike et al., 2006a; Lemus et al., 2008; Parrish and Fike, 2005; Tilman et al., 2006). These observations led us to pose a question similar to that of Döbereiner and the Brazilian group: *Where does switchgrass obtain the needed N to support its growth?*

Chapter 2

Literature Review

2.1 Switchgrass (*Panicum virgatum* L.)

2.1.1 'Model' bioenergy crop: A brief history of its selection

It was not for nothing that switchgrass (SG) was chosen by the US Department of Energy (DOE) Herbaceous Energy Crops Program (HECP) as the 'model' bioenergy crop deserving of further research (Parrish and Fike, 2005; Wright and Turhollow, 2010). Initiated in 1984, the primary objective of the HECP was *to develop data and information that will lead to commercially viable systems for producing herbaceous biomass for fuels and energy feedstocks* (Wright and Turhollow, 2010). Other considerations that were included under the HECP were: (i) to achieve the primary goal while minimizing adverse environmental effects, (ii) to increase the production of biomass for energy without significantly reducing food production (crops screened were those that could be grown as winter crops and on marginal croplands), (iii) to produce fuels or energy feedstocks rather than chemicals, (iv) to have the greatest possible impact on total biomass energy use (emphasis was placed on lignocellulosic crops) (Wright and Turhollow, 2010).

In 1985, several universities and one private company were selected by the HECP to perform screening on both annual and perennial, legumes and other herbaceous crops selected for their bioenergy feedstock potential. After five years of trials, the general consensus among the investigators was that, because of its consistently high yields across a range of soil types, management regimes, and environmental conditions, coupled with reasonably good economic returns, SG was selected as the focal candidate for continued bioenergy research and development (Wright and Turhollow, 2010). Specifically, the HECP determined that SG' attributes included:

- adaptable to current production and cultivation practices
  - no-till management could be used to reduce soil erosion
  - ability to establish from seed
  - harvested and stored as hay
  - minimal fertilizer input requirements
  - reasonable production costs
- biomass potential and uses
  - used for both biomass and forage
  - high cellulose and hemicellulose contents; relatively low lignin content
  - high genetic variability within species indicates excellent breeding and selection potential
- environmental conditions
  - adaptable to wide environmental, climatic, and edaphic gradient
  - persistence and effectiveness in reducing soil erosion
  - deep root system suggests good soil carbon storage potential

Thus, was SG chosen. A 'model' bioenergy crop that was never at the top of any investigators list, but due to its adaptability, performance consistency, revenue potential, and general ease of cultivation, it was on every investigators' candidate list.

2.1.2 The biology of switchgrass

Biogeography

Switchgrass is a warm-season grass native to North America. Its habitat ranges from the Atlantic Coast to Nevada, USA, and latitudinally from Central America to the prairies of southern Canada, with a northern adaptation limit of about 51°N (Parrish and Fike, 2005; Porter, 1966). However, it can be cultivated in other regions of the world, such as Northern Europe (Elbersen et al., 2001).

Morphology

Switchgrass is a member of the Poaceae family, subfamily Panicoideae, and belongs to tribe Paniceae (Gould and Shaw, 1983). It is a C_4, perennial grass. A description of its morphology is provided below (Table 2.1, Figure 2.1). Two ecotypes of SG are recognized; lowland varieties which are common in wetter environments of lower latitudes, while upland varieties are predominantly found in drier more mesic regions of mid- to northern latitudes (Elbersen et al., 2001; Parrish and Fike, 2005; Porter, 1966). Switchgrass ploidy levels vary between ecotypes, with lowland types being generally tetraploid (2n = 4x = 36), while most upland types are octoploid (2n = 8x = 72), including cultivars such as 'Cave-In-Rock' and 'Pathfinder' (Church, 1940; Hopkins et al., 1996; Parrish and Fike, 2005). The high ploidy level of SG explains its wide adaptation to a variety of environments (Parrish and Fike, 2005). Consequently, SG displays cultivar x environment variability, such that disparity is often observed

for the same cultivars grown under different environmental conditions (Casler and Boe, 2003; Parrish and Fike, 2005).

Table 2.1 Morphological description of switchgrass (*Panicum virgatum* L.)

Character	Description
Growth habit	tall, erect; large scaly rhizomes
Height	0.5-3.0 m
Leaf sheath	round, glabrous and red-purplish
Leaf blade	flat, elongate, 6-12 mm wide, distinctly veined
Auricle	absent
Ligule	ciliate and membranous, 1.5-3.5 mm long, pilose
Inflorescence	open panicle, 15-45 cm long
Spikelet	2-flowered, with one fertile, floret and one sterile floret

References: Best et al. (1971); Gould and Shaw (1983)

2.1.3 Switchgrass: A better biofuel?

Perennial grasses, like SG, are desirable bioenergy feedstock for several reasons. They can be used as biomass feedstocks or combusted for direct heat and electricity (Lewandowski et al., 2003; Samson et al., 2005; Sanderson et al., 2006), or as lignocellulosic feedstocks for the production of bioethanol (Ragauskas et al., 2006; Schubert, 2006). Bioenergy production from perennial grasses does not necessarily displace food crop production, as with corn or soybean for bioethanol and biodiesel production (David and Ragauskas, 2010; Tilman et al., 2006). In comparison to annual biofuel crops (i.e. corn, wheat, soybean), the perennial nature of grasses results in greater energy return, as less intensive labour, equipment and fossil fuel energy are required each production year (Heaton et al., 2004; Lewandowski et al., 2003; Parrish and Fike, 2005). Moreover, perennial grasses such as SG utilize the C_4 pho-

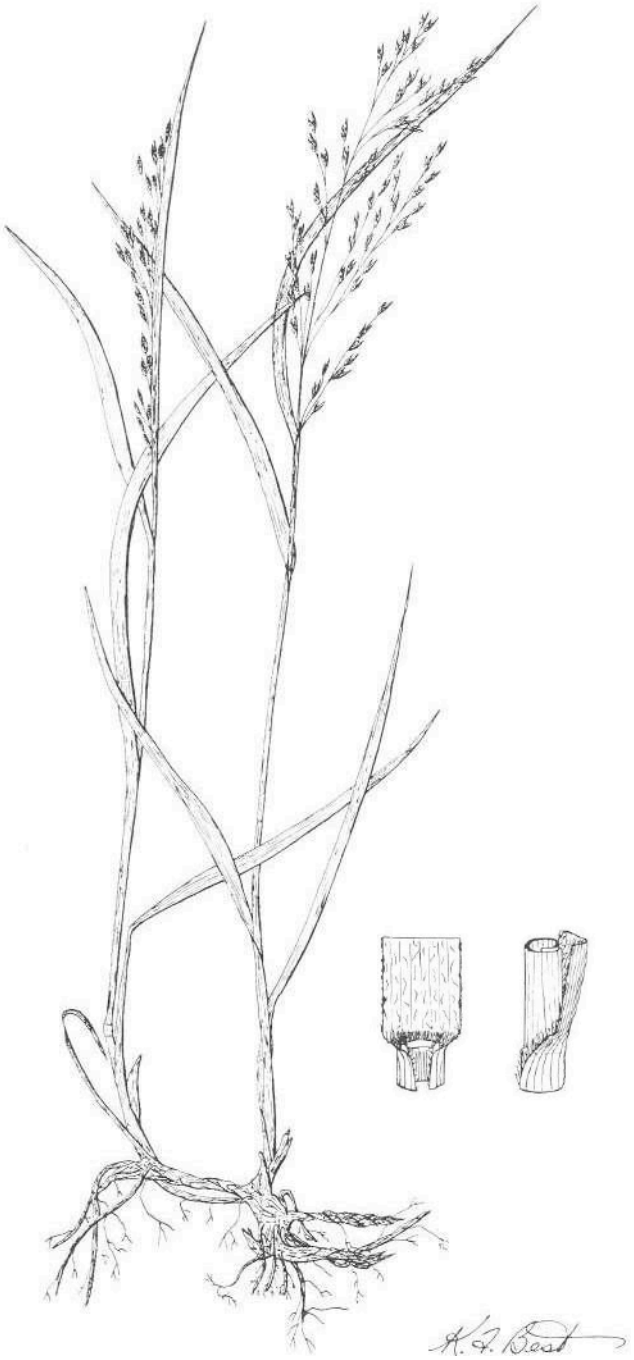

Figure 2.1 Diagram of *Panicum virgatum* L. (Best et al., 1971) Note: diagram is not to scale.

tosynthetic pathway, and these crops usually have higher nutrient, water and solar radiation efficiencies than C_3 plants (Heaton et al., 2004; Lewandowski et al., 2003; Parrish and Fike, 2005). Perennial crops generally have lower N fertilizer demands, in part, due to the internal cycling of N and other nutrients from the above ground biomass to the rhizomes in autumn where it is stored and reused for translocation to new emerging shoots in the following spring (Heaton et al., 2004; Lewandowski et al., 2003; Parrish and Fike, 2005). As such, crop harvest after senescence (either late autumn/early winter or early spring) usually results in a better quality biofuel as the feedstock will have a low ash (Si), mineral (mainly N, Cl, K) and water contents and therefore release less pollution when combusted (Heaton et al., 2004; Lewandowski et al., 2003; Parrish and Fike, 2005). Because of their perennial nature and extensive root systems, perennial grasses have the potential for soil carbon storage; as much as 64% of the plant can be comprised of lignin (Frank et al., 2004; Lemus and Lal, 2005; Parrish and Fike, 2005; Zan et al., 2001). Perennial grasses can also be adapted to marginal lands and are of potential use in phytoremediation strategies on contaminated soils (Lemus and Lal, 2005; Lewandowski et al., 2003; Parrish and Fike, 2005; Tilman et al., 2006).

2.1.4 Improving the sustainable production of switchgrass for bioenergy

While bioenergy production from SG offer desirable justifications for its cultivation, continued and future bioenergy production still need to be ameliorated. The long history of agronomic research by the HECP has provided agronomists and crop producers with considerable knowledge regarding how to manage SG as a biomass feedstock (Parrish and Fike, 2005; Wright and Turhollow, 2010). Moving forward, the next steps will be to improve on current management practices, so that improved SG cultivation can be achieved on abandoned, marginal, and/or contaminated lands

(Campbell et al., 2008; Schmer et al., 2008; Searchinger et al., 2008; Varvel et al., 2008). Lands not designated as agricultural are usually lacking nutrients such as N, P, and K, essential for maximum crop growth (Campbell et al., 2008; Field et al., 2008). However, conversion of marginal lands for biofuel production may require tillage of the land, as well as large inputs of nutrients and water, prior to its use. Thus, improving the N use efficiency of SG as well as other aspects of current fertilizer management (i.e. the amount applied, timing of application, and ratios of nutrients required) are considerations that should be addressed before exploring biofuel feedstock production on marginal lands. Switchgrass already has the added advantage of requiring low N inputs, and as a perennial grass, already has an efficient nutrient cycling system in place (Heaton et al., 2004; Lewandowski et al., 2003; Parrish and Fike, 2005). However, to maintain or even improve the yields of SG on marginal lands, will not only require better management but also an improved understanding of the natural interactions that exist between SG, the soil, and the surrounding microbiota. In this way marginal lands can be reclaimed and soil fertility improved.

Biofertilizers offer an attractive complement to fertilizer management by helping improve the nutritient requirements of crops through beneficial associations with microorganisms. Biofertilizers, as defined by Vessey (2003), are substances that contain living microorganisms which, when applied to seed, plant surfaces, or soil, colonize the rhizosphere or the interior of the plant and promote growth by increasing the supply or availability of primary nutrients to the host plant. The living microorganisms are usually plant growth promoting rhizobacteria (PGPR), either residing symbiotically or as free-living microorganisms within or surrounding the roots and rhizosphere of plants (Dobbelaere et al., 2003; Glick, 1995; Vessey, 2003). PGPR can improve plant growth either directly by providing nutrients to the host plant, or indirectly by enhancing root growth or by aiding other beneficial microorganisms

associations (Dobbelaere et al., 2003; Glick, 1995; Vessey, 2003). PGPR have been successfully utilized to enhance crop yields, including legumes, maize, rice, and wheat, by improving their nutrient status and consequently reducing the amount of fertilizer required (Dobbelaere et al., 2001; Lupwayi et al., 2005; Reis et al., 2001). However, the question remains: *Could inoculation of biofuel crops with PGPR contribute to improving its feedstock production and energy ratio?* More specifically, *can biofertilizers be part of a viable strategy for improving the N management of SG?*

Current bioethanol production from sugarcane in Brazil requires very little N fertilizer input due to the contribution of biological N_2-fixation (BNF) from diazotrophs and other forms of growth stimulation from other PGPR (Boddey et al., 1995; Döbereiner, 1996; Pessoa-Jr et al., 2005). Bioethanol from the diazotroph-sugarcane system has been argued to be the most cost effective and most energy efficient form of biofuels (Boddey, 1995; Nass et al., 2007; Pessoa-Jr et al., 2005; Walter, 2009). An examination of Brazilian sugarcane to bioethanol production as a 'model system' may be a useful starting point to answer these questions.

2.2 Sustainable biofuel production from sugarcane: The Brazilian story

The story of Brazilian bioethanol from sugarcane can be viewed as an exemplary model with which to understand plant-microbe interactions for the purposes of improving the energetics of biofuel production. Similar to switchgrass, sugarcane is a C_4, graminaceous crop; however, it is native to tropical climates. Research on associative bacteria that colonize sugarcane has been ongoing since the late 1950s in Brazil (Baldani and Baldani, 2005; Boddey et al., 1978; De-Polli et al., 1977; Döbereiner, 1961; Döbereiner et al., 1973; Döbereiner and Pedrosa, 1987). By comparison, only a

handful of reports have been published with switchgrass and microbes (Brejda et al., 1998; Day et al., 1975; Miranda and Boddey, 1987; Tjepkema, 1975). An understanding of the process, successes, and setbacks of the Brazilian experience is fundamental to designing future strategies for improving the sustainable production of SG as a biofuel feedstock.

2.2.1 The catalysts for bioethanol research: Energy crisis and energy independence

Since the discovery of fire, humans have been using plant matter as a source for heat and fuel. Indeed, the first commercial automobiles were capable of running on 100% ethanol from vegetable matter (Erikson and Carr, 2009). However, fossil fuels are more energy dense, and with the advances in the production of gasoline, commercial production of fuel ethanol ceased by the end of the 1950s in the US (Erikson and Carr, 2009). However, a resurgence of interest in alternative energy production occurred in the 1970s, largely propelled by the oil crisis following OPEC's (Organization of Petroleum Exporting Countries) oil embargo to North America (Erikson and Carr, 2009). Petroleum prices in the US quadrupled from 1973 to 1974, then tripled from 1978 to 1979, following supply disruptions in the Middle East (Erikson and Carr, 2009). This also led to growing concerns regarding energy independence. Further, health and environmental concerns were mounting over the use of lead as a gasoline additive, which prompted the re-introduction of ethanol as an additive (Erikson and Carr, 2009). Coupled with this, the hydrocarbon-based solvent, methyl tertiary butyl ether (MTBE) added to gasoline to reduce carbon monoxide emissions, was discovered to posed a severe health risk as it was discovered to be a potential carcinogen (Erikson and Carr, 2009). This further propelled the use of ethanol as an additive to gasoline (Erikson and Carr, 2009).

2.2.2 Sugarcane and the Brazilian National Alcohol Program

As a response to the oil crisis, the Brazilian government launched the National Alcohol Program (PROALCOOL) on November 14, 1974, a nation-wide program intended to promote the use of ethanol as automobile fuel *in lieu* of gasoline (Nass et al., 2007; Pessoa-Jr et al., 2005; Walter, 2009). The main drivers of this initiative were: (i) the country's strong dependence on imported oil, (ii) the rising price of gasoline as a result of the OPEC oil embargo, and (iii) powerful sugarcane lobbyists who were looking for alternative markets to help sustain the industry during times of highly fluctuating sugar prices (Nass et al., 2007; Pessoa-Jr et al., 2005; Puppim de Oliveira, 2002; Walter, 2009). In addition, the country already had in place the method and the infrastructure necessary to produce ethanol from sugarcane (Nass et al., 2007; Puppim de Oliveira, 2002; Walter, 2009). Further, second only to India, the country was the major producer of sugarcane during this period (FAO, 1973). An increase in the production of sugarcane would also lead to more jobs, especially in rural areas, thereby reducing the number of impoverished people (Pessoa-Jr et al., 2005; Walter, 2009).

Sugarcane (*Saccharum officinarum* L.), was first cultivated in southeast and western India, and was imported to Brazil by the Portuguese in the 14th century (Baldani et al., 2002; Boddey et al., 2003; Nass et al., 2007). By the seventeenth century, Brazil had become the world's major source of sugar (Boddey et al., 2003; Nass et al., 2007), and is currently the leading global producer of sugarcane (FAO, 2009). As of 2009, about 3% of Brazil's total cultivated area, or about 8.5 million ha, is devoted to sugarcane production (FAO, 2009). Second only to the US, Brazil has become a major producer of ethanol (Walter, 2009). Through extensive breeding and selection aimed at increasing drought and disease resistance, the main 'commercial' sugarcane planted today is a hybrid of *S. officinarum* and other species of *Saccharum* (Baldani et al.,

2002). Because it now benefits from both technological improvements in cultivation, and the low amount of N fertilizer required for its production, ethanol from sugarcane has very high, positive energy balances; one unit of fossil fuel used can produce eight units of ethanol (Nass et al., 2007). Further, soil N reserves were not being depleted in spite of centuries of burning and removal of sugarcane during harvest (Baldani et al., 2002; Boddey et al., 1995; Döbereiner, 1996). This and the fact that sugarcane yields could be maintained with moderate and even no N fertilizer input suggested that the crop may be obtaining its N requirement through other means, such as from biological nitrogen fixation (Baldani et al., 2002; Boddey et al., 1995; Döbereiner, 1996).

2.3 Biological nitrogen fixation (BNF)

2.3.1 The N cycle: Mechanism of BNF

Biological nitrogen fixation (BNF) is an energy intensive enzymatic conversion of atmospheric dinitrogen (N_2) by nitrogenase to ammonia (NH_3), and is accomplished only by prokaryotic microorganisms (Burris and Robertson, 1993; Robertson and Vitousek, 2009; Saikia and Jain, 2007; Schubert, 1982). The overall stoichiometry of N_2-fixation is as follows (Dixon and Kahn, 2004; Mylona et al., 1995; Rees et al., 2005):

$$N_2 + 8\ e^- + 8\ H^+ + 16\ \text{MgATP} \rightarrow 2\ NH_3 + H_2 + 16\ \text{MgADP} + 16\ P_i$$

Although 79% of dry air is comprised of atmospheric N_2 (equivalent to 4 x 10^{21} g N), in this form it cannot be assimilated by plant and higher life forms (Burris and Robertson, 1993; Rees et al., 2005; Robertson and Vitousek, 2009; Saikia and Jain, 2007). As such, it is most often the limiting nutrient for plant and animal life and is only made available through the process of fixation. BNF is one of three mechanisms, which generate enough energy to break the strong, triple-bond of atmospheric N_2

(Burris and Robertson, 1993; Robertson and Vitousek, 2009; Saikia and Jain, 2007; Schubert, 1982). The other two mechanisms are through lightning, and industrial fixation by the Haber-Bosch process (Burris and Robertson, 1993; Robertson and Vitousek, 2009; Saikia and Jain, 2007; Schubert, 1982). In these ways atmospheric N_2 moves to other parts of the N cycle, a biogeochemical cycle whereby N is converted between various chemical forms through microbially mediated processes (Arp, 2000; Robertson and Vitousek, 2009; Saikia and Jain, 2007).

2.3.2 Fertilizer use: Impacts on agriculture and environment

Global N_2-fixation is estimated to be between 200 to 300 Tg N annually (Tg or teragram is equivalent to 10^{12} g, or million (10^6) metric tons), with 120 Tg N fixed through BNF (includes agriculture, grassland, and marine cyanobacteria BNF), and lightning providing < 10 Tg N (Arp, 2000; Herridge et al., 2008; Smil, 1999; Vitousek et al., 1997). Industrial fixation, through the Haber-Bosch process, contributes 83 Tg N to the system (Arp, 2000; Herridge et al., 2008; Smil, 1999; Vitousek et al., 1997). Originally developed in the early 20th century as a means to make the ammonia necessary for explosives, and now utilized for production of N fertilizer, the Haber-Bosch process has revolutionized agricultural production (Feldman and Tarver, 1983). The process utilizes natural gas (CH_4) to produce hydrogen, which is then reacted with N_2 under high temperature and very high pressure to form ammonia (NH_3) (Jensen and Hauggaard-Nielsen, 2003; Robertson and Vitousek, 2009). The current estimate of global N fertilizer use is about 139 million tonnes (FAO, 2008). However, as the global population continues to rise, becoming greater than 7 million in 2011, and estimated to be 7.50 million by 2020, food production will also need to increase to meet these demands (Tilman, 1999; UN, 1999). Consequently, fertilizer consumption will inevitably increase.

However, fertilizer use has negative impacts, both on the environment and on economics, due to the high cost associated with its production. Negative environmental impacts of fertilizer use include (Bohlool et al., 1992; Smil, 1999; Socolow, 1999; Vitousek et al., 1997):

- N_2O: nitrous oxide
  - a potent greenhouse gas (GHG); 310 times more effective at heat trapping than CO_2
  - emissions depletes stratospheric ozone
- NO_x: nitric oxide (NO) and nitrogen dioxide (NO_2)
  - reactive N gases in the atmosphere contribute to air pollution
  - contributes to acid precipitation (includes rain, fog, and snow)
  - contributes to respiratory illness
- NO_3^-: nitrate
  - leached into groundwater can contaminate drinking water
  - can cause methemoglobinemia in infants and ruminant livestock
  - excess NO_3^- in lakes and rivers can contribute to eutrophication

In addition, these effects can irreversibly impact flora and fauna biodiversity, as changes in vegetation composition due to inadvertent fertilization from nearby agricultural systems can impact surrounding plant and animal populations (Socolow, 1999; Vitousek et al., 1997). Further, continued and unabated use of N fertilizers will accelerate the depletion of fossil fuels, in the form of natural gas, used in its production (Bohlool et al., 1992). In addition to the cost of production, a great deal of energy is also used for transportation, storage, and application of N fertilizer, all

totaling ∼92,000 kJ of energy per kilogram of fertilizer-N processed, distributed, and applied (Bohlool et al., 1992). Therefore, changes in N fertilizer management for crop production are required in order to minimize the negative impacts of its use on the environment and economic profit.

2.3.3 N_2-fixing bacteria: Contribution to crop productivity and soil fertility

Even before the discovery of N_2-fixation by diazotrophic bacteria within the nodules of leguminous plants were reported, the benefits of leguminous plants to soil fertility was well recognized (Allen, 2008; Chorley, 1981; Lupwayi et al., 2005). Indeed, polycropping of maize, squash and bean, famously known as the "three sisters", was practiced by native Americans of North, South, and Central America, in an effort to maximize both agronomic and dietary benefits (Hart, 2008). Beans provided the native Americans with a rich source of protein and amino acids, while it was observed that polycropping of beans with maize and squash enhanced the growth and vigor of the latter two crops (Hart, 2008). Legume-cereal rotation studies have shown that cereal crops grown after a legume planting require less fertilizer N than the same crop grown after a non-leguminous plant (Lupwayi et al., 2005). In addition to providing soils with greater fixed N from root nodules after decay, legumes, such as soybean, produce less residue when harvested thereby shortening the N immobilization period following decomposition (Lupwayi et al., 2005). Further, enhanced yield and soil N values from non-symbiotic or associative N_2-fixation has been documented. Long term agricultural experiments on wheat by the Rothamsted group (data from 1852-1967) showed that plots receiving no fertilizer N, received about 30 kg N ha^{-1}, potentially from non-symbiotic N_2-fixing associations with the roots of wheat (Jenkinson, 1982; van Berkum and Bohlool, 1980). Pot experiments on sugarcane conducted in Brazil revealed that some varieties benefited from non-symbiotic BNF more so than others

(Boddey et al., 2003). This may explain why certain varieties are preferred by Brazilian farmers, especially by those whose fields have lower soil N (Boddey et al., 2003). In this way, plant associations with symbiotic and associative NFB can influence the N balance of the soil and the availability of N to accompanying or subsequent crops (Hardarson and Atkins, 2003; Lupwayi et al., 2005; van Berkum and Bohlool, 1980).

Other benefits of BNF to plant growth include enhanced soil N use efficiency, as inoculated plants have been documented to take up more soil N than non-inoculated plants (Jensen and Hauggaard-Nielsen, 2003). The greater N uptake may be attributed to the 'starter-N' effect of bacterial inoculation, whereby root growth is stimulated during the early growing stages (Jensen and Hauggaard-Nielsen, 2003). This could result in greater water and nutrient uptake and photosynthesis during later growth stages (Jensen and Hauggaard-Nielsen, 2003).

2.3.4 N_2-fixing bacteria: Rhizobia and diazotrophs

It was Boussingault, in 1838, who first suggested that leguminous plants utilize atmospheric N_2 (Aulie, 1970; Burris, 1988; Lupwayi et al., 2005; Postgate, 1982). However, at the time, it was generally believed that plants acquired their N from ammonia in the air that was released through decomposition (Aulie, 1970; Burris, 1988; Lupwayi et al., 2005; Postgate, 1982). The concept was highly debated until 1888, when Hellreigel and Wilfarth demonstrated that N_2-fixation was localized in the root nodules of pea plants and probably other leguminous plants (Aulie, 1970; Burris, 1988; Lupwayi et al., 2005; Postgate, 1982). The final component to this story came from Beijerinck, who in 1888, first isolated *Bacillus radicicola* (later renamed by Frank in 1889 to *Rhizobium leguminosarum*), a bacterium from legume root nodules that was capable of fixing N_2 from the air (Aulie, 1970; Burris, 1988; Lupwayi et al., 2005; Postgate, 1982). Beijerinck later complemented his finding with the discovery

of *Azotobacter chroococcum*, a free-living, aerobic bacterium capable of N_2-fixation outside of the root nodule (Burris, 2000). The discovery of BNF, its role in the N cycle, and its importance in agriculture and ecosystem functioning, may be one of the most profound achievements of the nineteenth century (Aulie, 1970).

Since then, several symbiotic and associative NFB have been isolated and characterized. Rhizobia comprise the dominant group of symbiotic NFB in agricultural systems; colonization of the host plant forms the characteristic nodules in leguminous plant roots where N_2-fixation occurs (Beringer et al., 1979; Burris and Robertson, 1993; van Rhijn and Vanderleyden, 1995). The root nodules provide a microaerophilic environment within which BNF occurs; nitrogenase is irreversibly poisoned by high levels of O_2 (Beringer et al., 1979; Burris and Robertson, 1993; van Rhijn and Vanderleyden, 1995). Leghaemoglobin (in the root nodule) functions to protect nitrogenase against O_2 inactivation by providing a high-affinity binding site for O_2 within the root nodules, thereby lowering the concentration of free O_2 (Beringer et al., 1979; Burris and Robertson, 1993; Mylona et al., 1995). In exchange, the bacteria receive photosynthates from the host plant, which in turn, provides the carbon (C) source required for BNF to occur (Beringer et al., 1979; Mylona et al., 1995; van Berkum and Bohlool, 1980). Thus, the root nodules function as both an N source and a C sink within the plant (Mylona et al., 1995). Direct transfer of the N fixed by rhizobia to the plant occurs in the root nodules and is usually in the form of ammonium or alanine, which is then assimilated by glutamine synthetase within plant cells (Beringer et al., 1979; Mylona et al., 1995). The more common rhizobial genera include *Rhizobium, Bradyrhizobium, Sinorhisobium, Mesorhizobium* and *Azorhixobium* (Beringer et al., 1979; van Rhijn and Vanderleyden, 1995). Their contribution to plant N requirement is sufficient to meet the needs of the host plants and, in the case of soybean, can be as much as 450 kg N ha^{-1} (Lupwayi et al., 2005).

Unlike the substantial BNF contributions from rhizobia, BNF transfers to plants from associative, diazotrophic interactions are usually smaller and take place without the production of nodules (Dobbelaere et al., 2003). It is hypothesized that diazotrophic bacteria have evolved under conditions of C-rich, N-poor soil environments, and their ability to fix N_2 have enabled them to become selectively enriched in these environments (Dobbelaere et al., 2003). The genera of known diazotrophs now include *Acetobacter, Azoarcus, Azospirillum, Azotobacter, Beijerinckia, Burkholderia, Enterobacter, Gluconacetobacter, Herbaspirillum, Klebsiella, Paenibacillus* and *Pseudomonas* (Döbereiner and Pedrosa, 1987; Dobbelaere et al., 2003). Associative diazotrophs are usually categorized as either *epiphytic* (residing in the soil rhizosphere or along the surface of the host plant) or *endophytic* (residing within the host plant, but can also survive along the root surface and in the soil rhizosphere) (Baldani et al., 1997; Dobbelaere et al., 2003). Infection by epiphytic diazotrophs may occur on the surface of the roots, such as the root hair and elongation zones, or the outer layers of the root cortex (James, 2000; McCully, 2001; Reinhold-Hurek and Hurek, 1998). Colonization by endophytic diazotrophs takes place at the root cortex through cracks formed during lateral root emergence (James, 2000; McCully, 2001; Reinhold-Hurek and Hurek, 1998). Penetration into the epidermis to the stele has also been observed (James, 2000; McCully, 2001; Reinhold-Hurek and Hurek, 1998). In these tissues, endophytic diazotrophs have been reported to colonize the intercellular spaces, the xylem vessels, and lignified xylem parenchyma of the apoplast (James, 2000; McCully, 2001; Reinhold-Hurek and Hurek, 1998).

Conclusive evidence showing direct transfer of N fixed by associative diazotrophs to the host plant is still debated (Dobbelaere et al., 2003; James, 2000). Several reasons for the lack thereof include: (i) no specific site of colonization, as in the root nodules of legume-rhizobia symbioses, which adds to the difficulty in determining

which bacterium is involved in N_2-fixation, (ii) N is released to the host plant upon bacterial cell death, unlike *Rhizobium* spp. which actively excrete N from their cells; this may explain why low levels of N_2-fixation have been recorded even using sensitive techniques such as ^{15}N isotope dilution and natural abundance methods, and (iii) colonization levels by associative diazotrophs are lower, between 10^3 to 10^5 cfu g^{-1} plant root, than with rhizobia (10^7 to 10^8 cfu g^{-1} plant root), even when inoculated with an initial cfu of 10^7 and higher (Dobbelaere et al., 2003; James, 2000; Mylona et al., 1995). While this adds to the difficulty in determining direct fixed N transfer, several studies have reported that inoculation by associative diazotrophs contributes in the range of 11 to 45 kg N ha^{-1} to plants of rice, bahia and elephant grasses, and *Brachiaria* sp., (Boddey et al., 1995; Reis et al., 2000, 2001; Rodrigues et al., 2008). *Herbaspirillum seropedicae* inoculated into rice seedlings increased N content by 30% (James et al., 2002), while *Azospirillum lipoferum* and *A. brasilense*, isolated from kallar grass, were inoculated into rice and provided nearly 70% of fixed nitrogen (Malik et al., 1997). Significantly greater plant growth was observed for agronomically important crops such as maize, wheat, sorghum, and oat, when inoculated with associative diazotrophs than to uninoculated plants (Dobbelaere et al., 2001; Hurek et al., 2002; Mehnaz and Lazarovits, 2006; Riggs et al., 2001). *Gluconacetobacter diazotrophicus* PA15 and *H. seropedicae* Z152 isolated from sugarcane, were inoculated onto maize and were shown to significantly increase maize yields in the greenhouse (32%) and field (11%) (Riggs et al., 2001). It has also been speculated that enhanced plant growth by associative diazotrophs could be a result of plant growth promoting mechanisms other than BNF (Dobbelaere et al., 2003; Glick, 1995).

2.3.5 Other mechanisms of plant growth promotion

Bacteria that increase plant growth when colonized onto the host plant are termed plant growth promoting rhizobacteria (PGPR) (Dobbelaere et al., 2003; Glick, 1995;

Lupwayi et al., 2005). Several mechanisms of growth promotion have been reported: (i) increased availability of nutrients in the rhizosphere, (ii) solubilization of nutrients such as phosphorus, (iii) enhanced phytohormone production, (iv) modulation of ethylene levels, (v) enhancement of other symbioses beneficial to the host, and (vi) combinations of modes of action (Dobbelaere et al., 2003; Glick, 1995). Enhanced root development in plants inoculated with NFB was attributed to greater meristemic growth due to increased production of auxins, such as indole-3-acetic acid (IAA), and cytokinins, by the bacteria (Dobbelaere et al., 2003; Glick, 1995; Lupwayi et al., 2005). Alternatively, root growth has also been attributed to lower levels of ethylene produced due to enhanced enzymatic activity of 1-amino-cyclopropane-1-carboxylate (ACC) deaminase (Penrose and Glick, 2003). *Pseudomonas* spp, containing ACC deaminase, were reported to increase the growth of maize (Shaharoona et al., 2006b) and peanut (Dey et al., 2004). Phosphate solubilization is another plant growth promoting mechanism common to many PGPR. *Gluconacetobacter azotocaptans*, co-inoculated with *A. lipoferum* and *A. brasilense*, were shown to increase maize shoot and root dry weights under greenhouse conditions through combined mechanisms of enhanced N nutrition from BNF, production of IAA and/or solubilization of phosphate (Mehnaz et al., 2006). Winter wheat inoculated with *Azospirillum brasilense* or *A. irakense*, produced more shoots (34 and 33%, respectively) resulting in higher plant dry weight (62 and 46%, respectively), than uninoculated plants (Dobbelaere et al., 2001). This was also attributed to increased root development in inoculated plants as a result of higher IAA production by the bacteria (Dobbelaere et al., 2001).

2.4 Beneficial microbes as part of a sustainable biofuels production system

2.4.1 Plant-microbe interactions with sugarcane: The 'model' system

Investigations of NFB in association with graminaceous plants of Brazil began in the late 1950s, but took off in the 1970s, with the introduction of the acetylene reduction assay to measure nitrogenase activity (Hardy et al., 1968) and with the discovery of several diazotrophic *Azospirillum* spp. (Baldani and Baldani, 2005; Döbereiner and Pedrosa, 1987; Tarrand et al., 1978). Since then, species of *Herbaspirillum* have been isolated from sorghum, miscanthus, wheat, and sugarcane by the Brazilian researchers and other groups (Baldani et al., 1986; James et al., 1997; Kirchhof et al., 2001; dos Reis Jr et al., 2000; Rothballer et al., 2006). Perhaps the most notable of plant-associated NFB discovered by the Brazilian group was the diazotrophic bacterium *Gluconacetobacter diazotrophicus* (synon. *Acetobacter diazotrophicus*), first isolated from sugarcane plants in the late 1980s (Cavalcante and Döbereiner, 1988; Gillis et al., 1989). Since then, it has been isolated from other plants, including cameroon grass, rice, wheat, coffee, and sugar beets (Baldani and Baldani, 2005; Madhaiyan et al., 2004; Muthukumarasamy et al., 2005; Saravanan et al., 2008; Youssef et al., 2004), and has also been isolated/detected from rhizosphere soils (Muthukumarasamy et al., 2002; Saravanan et al., 2008). This bacterium has shown remarkable ability to enhance plant growth by providing fixed N to the host plant; as much as 50 to 80% of the plant N (equivalent to 150 to 170 kg N ha^{-1}) is derived from BNF (Baldani et al., 2002; Boddey, 1995; Döbereiner, 1996). As a result, soil fertility could be improved in the form of soil N gains; as much as 38 kg N ha^{-1} yr^{-1} (averaged over 9 years) was detected in the unburned top 20 cm layer of soil from colonized sugarcane plants (Boddey et al., 2003). Other desirable characteristics of the bacterium include its plant growth promotion by mechanisms other than through BNF, such

as through the production of auxins, IAA and gibberellin, and by phosphate solubilization (Muthukumarasamy et al., 2006; Saravanan et al., 2008). The bacterium has also been reported to tolerate moderate levels of heat and salt concentrations, abiotic factors which may be of importance in drought affected soils (Tejera et al., 2003). Inoculation of maize with *G. diazotrophicus* has also been reported (Riggs et al., 2001). While maize yields were enhanced, N deficiency of plants was still evident, which the authors suggested plant growth promotion by *G. diazotrophicus* was probably due to mechanism(s) other than through BNF (Riggs et al., 2001). However, the capacity of *G. diazotrophicus* to colonize plants other than its natural host may prove beneficial to a wider range of crop plants if it is able to cause plant growth promotion.

2.4.2 Sugarcane-microbe 'model' system for temperate grown biofuels?

The Brazilian sugarcane-diazotroph system is an exemplary model that contains all the right components necessary for sustainably producing bioethanol:

(i) sugarcane cultivation in Brazil has undergone centuries of improvement

(ii) bioethanol from sugarcane is well understood and efficiently produced

(iii) government incentives that promote sugarcane cultivation and research

(iv) a naturally existing plant-microbe association that can be exploited

These key components illustrate the concentrated efforts of many Brazilian groups that have come together with a unifying goal in mind. However, could these same objectives be accomplished here, in northern North America?

The 1970s' oil crisis was one of the main motivators for Brazil's drive to energy independence. The crisis also prompted the United States to initiate government

incentives and research in alternative forms of energy. Yet, since the 1970s oil crisis, new drivers have emerged and existing concerns have intensified, creating an urgent need for immediate action (Duncan, 2004; Erikson and Carr, 2009; Faaij, 2006; Kerr, 1998; Stephanopoulos, 2007):

1. fossil fuel reserves are being depleted and will eventually be exhausted
2. oil and gas prices have increased
    - growing demand for oil as a result of global population growth and rising standard of living
    - instability in countries that make up OPEC
3. concerns over energy security and independence
4. increased public awareness and concerns regarding factors that contribute to climate change
5. increased need to strengthen the agricultural economy
6. advances in biological science and technology
    - plant breeding and genetic engineering
    - advances in biomass conversion technology help to improve the cost of bioethanol production

The HECP has selected switchgrass as its 'model' herbaceous energy crop (Parrish and Fike, 2005; Sanderson et al., 2006; Wright and Turhollow, 2010). Despite only decades of agronomic research, our understanding of SG is considerable and expanding (Parrish and Fike, 2005; Sanderson et al., 2006; Wright and Turhollow, 2010). While bioethanol production from lignocellulosic feedstocks is still in its infancy compared to

bioethanol from sugarcane, research is underway to advance current production methods (Sanderson et al., 2006; Wu et al., 2006). The US has implemented substantial government initiatives in SG research, production and conversion to fuel energy (Duncan, 2004; Erikson and Carr, 2009). A component that remains unexplored is: *Does SG naturally associate with beneficial microbes that could be isolated and exploited to improve its productivity?*

2.4.3 Are there beneficial switchgrass-microbe associations?

Unlike the extensive body of literature pertaining to beneficial microbe associations with sugarcane and other graminaceae, few studies have investigated diazotrophic associations with switchgrass (Brejda et al., 1998; Davis et al., 2010; Day et al., 1975; Miranda and Boddey, 1987; Tjepkema, 1975). Many exploratory examinations of N_2-fixation *in situ* use the acetylene reduction assay (ARA) to measure nitrogenase activity. However, the results of the ARA are notoriously error prone. Although the assay is simple and relatively inexpensive, it is an indirect measurement of nitrogenase activity, thus, extrapolation errors, and different incubation periods can lead to under- and over-estimation of N_2-fixation (Boddey, 1987; Döbereiner, 1980; Shah et al., 1975). In addition, quantification of fixed N transfer from the diazotroph to the plant is inherently difficult to measure. Unlike the active transfer of fixed N by *Rhizobium* spp. within the root nodules of leguminous plants, for associative diazotrophic interactions, N is usually released to the host plant upon death and decay of bacterial cells (see Section 2.3.4). Even very sensitive ^{15}N isotope dilution techniques may be unable to quantify fixed N transfer (Dobbelaere et al., 2003; James, 2000). Nonetheless, nitrogenase activity (measured by the ARA) has been detected in the roots and soil rhizosphere of *Panicum* spp. (Davis et al., 2010; Day et al., 1975; Miranda and Boddey, 1987; Tjepkema, 1975). However, it is possible that the low nitrogenase activities observed deterred further investigations into possible microbe interactions

with SG. Although, the low nitrogenase activities do not necessarily imply that SG does not associate with NFB. Further, it is quite likely that beneficial microbes that promote plant growth other than through BNF are associated with the roots and rhizosphere of SG. This may explain the low N input requirement and high biomass yields of SG (Parrish and Fike, 2005; Wright and Turhollow, 2010). Diazotrophic interactions with other graminaceous crops have been reported, including miscanthus (Eckert et al., 2001; Kirchhof et al., 2001), elephant grass (Reis et al., 2001), kallar grass (Malik et al., 1997), and bahia grass (Baldani and Baldani, 2005; De-Polli et al., 1977). Thus, it is quite possible that SG also associates with beneficial microbes. Their discovery may be only a question of time.

Chapter 3

Research Questions and Objectives

This doctoral research seeks to answer the question: *What is the N source that is providing SG with the N required to support biomass growth?* We hypothesized that SG could be obtaining part of its required N from biological N_2-fixation. In order to answer this principle question, three underlying questions need to be considered:

1. Does switchgrass associate with plant growth promoting rhizobacteria?

    - In particular, we were interested in determining whether SG associates with PGPR capable of biological N_2-fixation.
    - The specific objectives of this research question can be found in chapter 4. In this chapter, our objectives were to: 1) identify N_2-fixing bacteria that associate with SG, and 2) determine if these isolates can be used as inoculants capable of promoting SG growth.
    - The research reported in this chapter was exploratory; we screened several hundred potential isolates for plant growth promotion under N limiting, controlled-environment growth conditions.

2. Can inoculation with PGPR increase the growth and productivity of SG for biofuels under a low-N input field production system?

- Our objective (presented in chapters 5 and 6) was to test the chosen microbe(s) on SG in plot-scale field sites to determine whether the positive plant growth effects observed under controlled conditions could be replicated under field conditions.
- Chapter 5 presents the agronomic results of PGPR inoculation on yield and yield components of SG. This includes data on stand dynamics (i.e. tiller density, tiller size, tiller population and distribution), as changes within a population could greatly affect overall yield. The specific objective of chapter 5 was to determine if positive growth effects of the inoculation could be observed as early as the first year of seeding.
- The specific objective of chapter 6 was to determine the inoculum effects on the N dynamics of SG, in particular, the effects on N uptake and utilization efficiencies in the establishment year. Chapter 6 presents the N response of SG (i.e. soil N uptake, fertilizer N recovery, N concentration, and N cycling) to PGPR inoculation.

3. Can a plant-microbe production system, as in the example with sugarcane, be replicated with SG?
   - As SG is considered the 'model' bioenergy crop in N. America, the development of a low-N input, biofuel production system could greatly enhance its efficiency and thus its overall utility for biofuel production.
   - In chapter 7, we explore this question through a summary of the research and provide recommendations for future research.

Preface to Chapter 4

Chapter 4 describes the rationale behind the project hypotheses, and focuses on the isolation, screening and selection, and identification of PGPR that associate with SG. Emphasis was placed on selecting isolates that could potentially contribute to plant growth through BNF. This was accomplished using a combination of classical microbiological approaches (selection of isolates was performed on N-free solidified media), and plant bioassays in the absence of a N source, under controlled growth conditions. Chapter 4 was co-authored by the candidate's supervisors, Drs. Donald L. Smith and James W. Fyles, supervisory committee member, Dr. Brian T. Driscoll, and research assistant Andrea Jilling. Chapter 4 will be submitted to *Soil Biology and Biochemistry* for publication.

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Chapter 4

Isolation and identification of rhizosphere endophytes that promote the growth of switchgrass (*Panicum virgatum* L.)

4.1 Abstract

Switchgrass (SG, *Panicum virgatum* L.) is a preferred biofuel crop in North America because it is a perennial grass that can be grown on marginal lands and thus does not necessarily displace food production. Switchgrass produces high biomass yields with minimal to no synthetic nitrogen (N) fertilizer, which prompted us to hypothesize that SG is obtaining part of its N from an alternative source, perhaps through biological nitrogen fixation (BNF) by free-living, plant growth promoting rhizobacteria (PGPR). The objectives of this study are to (1) isolate and identify PGPR that associate with SG, and (2) determine which organisms contribute to plant growth enhancement. We focused our investigation on N_2-fixing, endophytes and their potential use as inoculants. Switchgrass rhizomes were collected in Québec, Canada, from a discontinued biomass trial of 11 varieties that had not received management or synthetic fertilizer input for ten years. Isolates were selected on N-free solidified media

and screened for the ability to promote plant growth using plant assays conducted in growth chambers. Switchgrass seedlings were inoculated, or not, with batches of mixed isolates, and fertilized with N-free Hoagland's solution. Strains capable of promoting SG growth were identified using 16S rRNA gene sequence analysis and evaluated for N_2-fixation capability by amplifying the *nifH* gene. The mixed inoculum that resulted in the greatest plant growth was comprised of *Paenibacillus polymyxa*, a N_2-fixing bacterium, and several other possible PGPR genera (*Pseudomonas, Serrtia,* and *Rahnella*) capable of producing auxin and/or solubilizing phosphate.

Key words: switchgrass, biological N_2-fixation, PGPR, *Paenibacillus polymyxa*, *Pseudomonas*, *Serratia*, *Rahnella*

4.2 Introduction

Sugarcane is considered a 'model' bioenergy crop for tropical areas. The high ratio of energy output to input seen in Brazilian sugarcane production demonstrates that efficiency in biofuel production is attainable. Sugarcane success has been attributed to lowered nitrogen (N) fertilizer input through management of beneficial plant-microbe associations (Baldani et al., 2002; Boddey, 1995; Döbereiner, 1996). The use of N fertilizers is energy expensive and contributes to greenhouse gas (GHG) emissions (Farrell et al., 2006), both through CO_2-production during fertilizer manufacture and because 1-2% of the applied N fertilizer is converted into N_2O via microbially driven processes such as denitrification (IPCC, 2007). Plant growth promoting rhizobacteria (PGPR), such as *Gluconacetobacter diazotrophicus*, that reside within sugarcane plant tissues (termed endophytes) contribute 50 - 80% (150 to 170 kg N ha^{-1} yr^{-1}) of plant N through biological N_2-fixation (BNF) (Baldani et al., 2002; Boddey, 1995; Boddey et al., 2003; Döbereiner, 1996). A reduction of N fertilizer input by this amount could increase energy ratios from 4.53 to as much as 5.79 (Boddey, 1995). As biofuels are

becoming increasingly important energy sources, it would be worthwhile to develop similar low-input and highly productive plant-microbe, biofuel systems applicable to temperate climates.

Many species of associative, free-living microbes capable of BNF have been isolated from a wide variety of plant species, including grasses such as miscanthus (Davis et al., 2010; Eckert et al., 2001; Miyamoto et al., 2004), rice (Boddey et al., 1995; Gyaneshwar et al., 2001; Muthukumarasamy et al., 2007; Ueda et al., 1995), and sorghum (Rout and Chrzanowski, 2009). *Gluconacetobacter diazotrophicus* was first isolated from sugarcane (Cavalcante and Döbereiner, 1988; Gillis et al., 1989; James et al., 1994; Stephan et al., 1991) and later on from other plants (Loganathan and Nair, 2003; Madhaiyan et al., 2004; Mehnaz et al., 2006; Muthukumarasamy et al., 2007). Its natural association with sugarcane and other biofuel grasses, such as elephant grass (*Pennisetum purpureum*), along with its capacity to contribute significant amounts of fixed N to the plant, make it an ideal model species with which to investigate strategies to increase crop yield while reducing N fertilizer inputs (Boddey et al., 1991, 2003; Reis et al., 2001). The use of PGPR as inoculants to improve the yield of other agronomically-important crops, through BNF or other means (i.e. phosphate solubilization, auxin production), has been extensively investigated (Dobbelaere et al., 2001; Riggs et al., 2001; Rodrigues et al., 2008; Shaharoona et al., 2006a,b).

In North America, switchgrass (SG, *Panicum virgatum* L.) is considered as the 'model' bioenergy crop because it is a cellulosic feedstock that can generate high biomass yields with minimal to no input of synthetic N fertilizer (Hill et al., 2006; Parrish and Fike, 2005; Schmer et al., 2008; Tilman et al., 2006). But where is the N coming from to support high SG production? We hypothesized that SG is meeting its N requirement from an association with N_2-fixing microorganisms. Few investigations into plant-microbe associations with species of *Panicum*, and in particular, *P.*

virgatum, have been reported. Most of these studies were conducted in the 1970s to late 1980s, and to our knowledge, no other studies have been reported since that time. Among *Panicum* sp., nitrogenase activity was first detected in the roots of *P. maximum* by Döbereiner et al. (1972). Tjepkema (1975) reported nitrogenase activity in root and rhizome fractions of *P. virgatum* collected from Wisconsin, U.S.A. Miranda and Boddey (1987) concluded that plant-associated BNF contributions were possible for several ecotypes of *P. maximum* grown in pots containing ^{15}N-enriched soils. The authors estimated the associated BNF contributions to be between 24 to 38 % of total N incorporated (5 - 10 kg N ha^{-1}). Although studies have been conducted to determine plant growth promoting effects of NFB inoculants in several other biofuel crops (Boddey et al., 2003; Davis et al., 2010; Mehnaz and Lazarovits, 2006), no study to date has identified the N_2-fixing microorganisms that contribute N for *P. virgatum* growth or if any contribution of BNF can increase the efficiency of *P. virgatum* for biofuel production.

The objectives of this work were to: 1) identify N_2-fixing bacteria that associate with SG, and 2) determine if these isolates can be used as inoculants capable of promoting SG growth. In this report, we present the findings of our screening of over 300 putative NFB isolated from eleven different varieties of SG. To our knowledge, this is the first report of N_2-fixing, SG endophytes shown to improve plant growth.

4.3 Materials and Methods

4.3.1 Rhizome sampling: location and site history

Endophytic bacteria were isolated from SG rhizomes obtained from a field site in Ste-Anne-de-Bellevue, Québec, Canada (45°28'N 73°45'W). The field was the site of a

SG variety trial in which the genotypes tested were evaluated for biomass productivity. Eleven upland ecotypes (Table 4.1) were established in a randomized complete block design, with four replications. Each plot measured 4 x 4 m. The soil type was a Chicot fine sandy loam. The trial was initiated in 1996, fertilized with 60 kg urea N ha^{-1} yr^{-1}, and managed until 2000 after which no further N fertilizer was applied.

Table 4.1 Switchgrass 2000 and 2008 fall harvest yields[1]

Label	Ecotype	Origin and latitude	Relative maturity	Yield (Mg DW ha^{-1})	
				2000	2008
V1	Cave-In-Rock	S. Illinois - 38'N 30'W	Medium-late (130 d)	9.2	5.1
V2	Carthage	N. Carolina - 36'N 00'W	Very late (155 d)	9.5	3.7
V3	Sunburst	S. Dakota - 43'N 80'W	Early-medium (120 d)	7.9	2.8
V4	REAP[2] 922	S. Dakota - 43'N 80'W	Early-medium (120 d)	9.5	3.5
V5	REAP 921	S. Illinois - 38'N 30'W	Early-medium (120 d)	11.8	4.8
V6	Shawnee	S. Illinois - 38'N 30'W	Medium-late (135 d)	8.3	3.6
V7	Late Synethic	Unknown	Late (145 d)	9.0	4.2
V8	NU[3] 95	Unknown	Medium (130 d)	10.4	3.5
V9	SU[4] 95	Unknown	Late (145 d)	7.7	3.7
V10	NU 94-2	Unknown	Medium (130 d)	9.7	3.8
V11	REAP 961[5]	S. Ontario - 42'N 00'W	Early (105 d)	8.5	1.3

[1]Jannasch et al. (2001), Technical Report: Development of bioenergy feedstocks: Agronomy data from Eastern Canada

[2] Resource Efficient Agriculture Production

[3] Northern Upland

[4] Southern Upland

[5] Also referred to as Long Point

4.3.2 Isolation of bacteria from *P. virgatum* rhizome tissue

Rhizomes were collected from plots of each variety in July 2008, and stored at 4°C until required. All root segments were cut from the rhizome pieces prior to washing in dH_2O and blotting dry with paper towels. Rhizome fragments of approximately 5

g (fresh weight) were asceptically transferred into separate sterile magenta containers, surface sterilized with 95% ethanol for 5 min, rinsed with sterile ddH_2O, and soaked in 3% sodium hypochlorite (bleach) for 20 min. The rhizomes were rinsed with sterile Luria Bertani broth (LB; per mL ddH_2O: 10.0 g tryptone, 5.0 g yeast extract, 5.0 g NaCl) for 5 min, and then rinsed with several changes of sterile ddH_2O. Each rhizome fragment was macerated in a sterile phosphate buffered saline solution (PBS; per mL ddH_2O: 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, 0.20 g KCl, 8.00 g NaCl) using a sterile mortar and pestle, and the extracts were filtered through four layers of sterile cheesecloth. The extracts were serially-diluted and plated on N-free solidified LG medium (modified *Azotobacter* medium; per L ddH_2O: 10.0 g sucrose, 0.5 g K_2HPO_4, 0.2 g $MgSO_4{\bullet}7H_2O$, 0.2 g NaCl, 0.001 g $MnSO_4{\bullet}H_2O$, 0.001 g $FeSO_4$, 0.001 g $NaMoO_4{\bullet}2H_2O$, 5.0 g $CaCO_3$, 15.0 g Bacto-agar; Döbereiner 1995) and incubated at 30°C for 4 d. Colonies were chosen based on different colony morphologies and restreaked (twice) on N-free LG medium to obtain pure isolates. Permanent preparations of the pure isolates were frozen at -80°C in tryptone yeast (TY) medium containing 7% dimethyl sulfoxide.

4.3.3 Bacterial screening bioassay

Plant growth assays were performed in growth chambers to screen for combinations of bacteria that promoted plant growth in the absence of exogenous N fertilization. The isolates were grouped into batches of eight to ten according to the plot/ variety from which the rhizomes were obtained. Positive controls, consisting of known associative N_2-fixers (*Azotobacter chroococcum* ATCC® 4412, *Burkholderia vietnamiensis* ATCC® BAA-248, *Azospirillum brasilense* ATCC® 29711 and *A. doebereinerae* DSM® 13400, *Herbaspirillum frisingense* DSM® 13128^T and *H. seropedicae* ATCC® 35894, and *Gluconacetobacter diazotrophicus* ATCC® 49037) purchased from the American Type Culture Collection (ATCC) or from the HelmholtzZentrum

münchen, German Research Center for Environmental Health, were also included in the screening bioassay. These isolates were grouped together to form the 'ATCC' batch treatment.

Initial screening: To ensure the colony forming units (cfu) per mL were approximately uniform, appropriate dilutions were performed for each isolate and the cfu per mL estimated spectrophotometrically at 600 nm, 0.5 optical density (O.D.) units. **Batch screening:** Appropriate dilutions were performed for each isolate to ensure the cfu mL^{-1} were in the range of 10^7 to 10^8. All individually cultured isolates were verified for purity by streak plating onto LB plates and culturing for 2 d at 30°C. All isolates tested in the growth experiments were verified to be pure cultures.

Switchgrass (*P. virgatum* var. 'Cave-In-Rock') plants were grown in a growth chamber in modified Leonard assemblies (Leonard, 1943), arranged in a completely randomized design (CRD). The Leonard assemblies contained vermiculite:sand (1:1, v/v) and were fertilized with a modified, N-free Hoagland's Solution (HS, Hoagland and Arnon 1950). The HS contained the macronutrients: 0.5 M K_2SO_4, 1 M $MgSO_4$, 0.05 M $Ca(H_2PO_4)_2{}^{\bullet}H_2O$, and FeEDTA (trace). A stock micronutrient solution was prepared separately (1.43 g H_3BO_3, 0.91 g $MnCl_2{}^{\bullet}4H_2O$, 0.11 g $ZnSO_4{}^{\bullet}7H_2O$, 0.04 g $CuSO_4{}^{\bullet}5H_2O$, and 0.01 g $Na_2MoO_4{}^{\bullet}2H_2O$), and 1 mL L^{-1} was added to the macronutrient solution. Once assembled, the entire unit was re-covered with aluminum foil and autoclaved for a minimum of 4 h. In a laminar flow hood, the plant seeds were surface sterilized in 25% sodium hypochlorite (bleach) for 20 minutes and thoroughly rinsed several times with sterile ddH_2O, sown into the assembly, recovered with aluminum foil and then placed in the growth chamber (25/20°C day/night, 16/8 h photoperiod, relative humidity 70%) until emergence (approximately 4-5 d). Seedlings were then inoculated with 10 mL of grouped isolates or treated with the non-inoculated control medium. Prior to inoculation, each isolate was separately cultured in LB medium with

shaking and incubated at 30°C for 2 d. Bacterial cells (1 mL of inoculant) were collected by centrifugation (14,500 rpm for 30 s), washed free of the growth medium, and re-suspended in sterile ddH_2O (performed twice). One mL of each isolate was added to the 'batch' (8 to 10 isolates per batch) and the resulting solution was brought to 10 mL with sterile ddH_2O. Uninoculated control treatments received equivalent amounts of sterile ddH_2O. Plants were watered with sterile ddH_2O bi-weekly and fertilized with N-free HS 15 days after inoculation. Plants were harvested 4 to 6 weeks later.

Plant growth promotion (PGP) by bacterial isolates was determined from measurements of fresh and dry weights (FW and DW) of shoots, roots and total per plant biomass. Leaf N concentrations (%) were measured using an Elemental Analyzer (NC2500 Elemental Analyzer, ThermoQuest Italic S.P.A., Italy) from a composite sample of three replicates pooled together in order to facilitate grinding and to provide enough biomass for the analysis. N content (mg g^{-1}) was determined by multiplying the N concentration by shoot DW. Batch isolates showing the greatest PGP were selected for further rounds of screening without N (Table 4.5, Bioassays 1 to 3) and with the addition of HS amended with 10% N in the form of 1 M NH_4NO_3 solution (Table 4.5, Bioassay 4). In addition, individual isolate inoculation (Table 4.6) were performed on SG plants receiving N-free HS to determine if and which isolates improved plant growth.

4.3.4 Statistical analyses

One-way analysis of variance (ANOVA) and contrast analyses were performed using the R statistical software package (R: A Language and Environment for Statistical Computing, 2010). Fisher F-tests were performed for pooled data to verify the assumption of equal variances among sample populations. Unless otherwise indicated,

all pooled data are verified to be from the same population. All the data were verified for the assumptions of normality and homoscedasticity. When the assumptions were not met, nonparametric Kruskal-Wallis analyses were performed. Differences were considered significant when their probability by chance alone was less than 0.05. Biologically interesting differences with probabilities between 0.05 and 0.1 are also presented. When this occurs, the P values are given.

4.3.5 Phosphate solubilization

Phosphate solubilization by selected strains was measured according to the quantitative methods of Johri et al. (1999) and Nautiyal (1999). Bacterial strains were tested by plate assay, in triplicate, using Pikovskaya (PVK) media (pH 7.0; 10 g glucose; 5 g $Ca_3(PO_4)_2$; 0.5 g $(NH_4)_2SO_4$; 0.2 g NaCl; 0.1 g $MgSO_4 \bullet 7H_2O$; 0.2 g KCl; 0.5 g yeast extract; 0.002 g $MnSO_4 \bullet H_2O$; and 0.002 g $FeSO_4 \bullet 7H_2O$) supplemented with 1.5% Bacto-agar (Nautiyal, 1999). The halo (area of medium cleared) and colony diameter were measured after 14 days of incubation at 30 ± 1 °C. Halo size was calculated by subtracting colony diameter from the total diameter.

4.3.6 Auxin production

The presence of indole-3-acetic acid (IAA) like substances were detected and quantified according to the combined methods of Dey et al. (2004) and Sarwar and Kremer (1995) in L-tryptophan agar. Isolates were cultured in LB media and incubated at 30°C for 48 h. One mL of culture was pour plated in L-tryptophan agar (0.204 g L-tryptophan; 4 g Difco nutrient broth, 15 g agar in 1L of ddH_2O) in triplicate and incubated at 30 ± 1 °C for 48 h in the dark. After incubation, one agar disk (∼ 0.12 cm^3) was removed from each plate and placed in freshly prepared Salkowsky reagent (2 mL of 0.5 M $FeCl_3$ in 98 mL of 35% perchloric acid; Sarwar and Kremer

1995) in triplicate, and incubated in the dark for 30 minutes until the appearance of pink colour. The amount of IAA produced, expressed as μg mL^{-1}, was measured spectrophotometrically at 535 nm and compared to a standard curve based on commercial IAA (Sigma-Aldrich, Canada).

4.3.7 Extraction of total genomic DNA

Genomic DNA was extracted using a phenol-chloroform extraction and purified using the method of Meade et al. (1982). The purity was assessed from the A260/A280 and A260/A230 extinction ratios using a NanoDrop (Thermo Scientific, Wilmington, Delaware, U.S.A.).

4.3.8 Molecular biology techniques

Full length 16S rRNAs (1465 bp) were amplified from isolates by polymerase chain reaction (PCR) using the universal forward (FWD) and reverse (REV) primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'), respectively (Eurofins MWG Operon, AL, USA). PCR reaction conditions included 1 μL template DNA, 1 μL FWD primer (10 μM), 1 μL REV primer (10 μM), 5 μL 10X PCR buffer, 1 μL deoxynucleotide triphosphates (dNTPs) mixture (10 mM each), 3 μL of 25 mM $MgCl_2$, 1 μL Taq DNA polymerase (5U) and ddH_2O to 50 μL. Unless otherwise specified, all PCR components were purchased from BioShop Canada Inc., Mississauga, ON. The following amplification program was run in a PTC-100^{TM} Programmable Thermal Controller (MJ Research, Inc., MN, USA): initial denaturation for 3 min. at 94°C; 30 s at 94°C, 30 s at 55°C, 1 min. for 72°C (40 cycles); and a final elongation at 72°C for 10 min. PCR products were analyzed by electrophoresis on 1% agarose (Bio-Rad, Mississauga, ON) gel run in 1X TAE (Tris Acetate EDTA) buffer with ethidium bromide staining and visualized using a UV

transilluminator.

Amplification of the *nifH* gene was performed using one of four sets of primers (19F/407R, MinF/MinR, PolF/PolR and ZehrF/ZehrR) (Table 4.2). PCR reaction conditions were as described for the 16S rRNA gene. Thermocycler amplification programs are given in Table 4.3. Target bands were excised and purified using QIAquick Gel Extraction Kit (Qiagen Inc. Mississauga, ON), and re-amplified with the same initial *nifH* primer sets.

Table 4.2 Sequences of various primers tested in this study for *nifH* gene amplification[a]

Name of primer	References	Forward primers
19F	Ueda et al. 1995	GCI WTY TAY GGI AAR GGI GG
MinF	Minerdi et al. 2001	GGC AAG GGC GGT ATC GGC AAG TC
PolF	Poly et al. 2001	TGC GAY CCS AAR GCB GAC TC
ZehrF	Zehr and McReynolds 1989	TGY GAY CCI AAR GCI GA
		Reverse primers
407R	Ueda et al. 1995	AAI CCR CCR CAI ACI ACR TC
MinR	Minerdi et al. 2001	CCA TCG TGA TCG GGT CGG GAT G
PolR	Poly et al. 2001	ATS GCC ATC ATY TCR CCG GA
ZehrR	Zehr and McReynolds 1989	ADN GCC ATC ATY TCN CC

[a] Table adapted from Poly et al. 2001
[b] Sequence position with reference to the *A. vinelandii nifH* coding sequence (Genbank accession number M20568). The International Union of Pure and Applied Chemistry Conventions was used to describe DNA sequence degeneracies: Y = C/T; S = G/C; R = A/G; B = C/G/T; D = G/A/T; H = T/C/A; N = A/G/C/T; W = A/T; I = inosine.

Table 4.3 Summary of PCR reaction conditions for amplification of *nifH* gene

PCR Reaction Conditions	19F/ 407R[a]	MinF/MinR[b]	PolF/ PolR[c]	ZehrF/ZehrR[d]
Initial denaturation	94°C for 3 min	94°C for 3 min	94°C for 3 min	94°C for 3 min
Number of cycles	40	30	30	30
Denaturation	94°C for 30 s	94°C for 1 min	94°C for 1 min	93°C for 1.2 min
Annealing	50°C for 1 min	62°C for 1 min	55°C for 1 min	50°C for 1 min
Elongation	72°C for 30 s	72°C for 2 min	72°C for 2 min	70°C for 1.5 min
Extension	72°C for 5 min	72°C for 10 min	72°C for 5 min	72°C for 5 min

Adapted from: [a] Poly et al. (2001); [b] Minerdi et al. (2001); [c] Poly et al. (2001), Ueda et al. (1995); [d] Poly et al. (2001), Zehr and McReynolds (1989)

4.3.9 DNA sequencing and analyses

DNA sequencing was done using the Genome Québec Innovation Centre (McGill University) service on a 3730XL DNA analyzer systems (Applied Biosystems). 16S rRNA gene sequences were compared against the GenBank database using the nucleotide Basic Local Alignment Search Tool (BLASTN) (Altscul et al., 1990) and the Ribosomal Database Project (RDP) using the Sequence Match software (Cole et al., 2005). Alignments were constructed using the CLUSTAL W program in the MACVECTOR 7.0 software package (Oxford Molecular Ltd). Phylogenetic trees were constructed using a neighbour-joining algorithm with the Jukes-Cantor model; bootstrap values were performed with 1000 replicates.

4.3.10 Nucleotide sequence accession numbers

16S rRNA gene sequences have been deposited in the Genbank database. Accession numbers for V1 isolates are JN887785 to JN887792, V5 isolates are JN887793 to JN887800, V7 isolates are JN887801 to JN887805, and V10 isolates are JN887806 to JN887808. Accession numbers for reference sequences using phylogenetic studies are indicated in the relevant tables.

4.4 Results

4.4.1 Bacterial screening bioassay

Initial screening

The initial bacterial screening bioassay revealed that batch inoculation of SG with bacteria isolated from treatments V1, V5, and V10 increased shoot, and total plant DW as compared to the uninoculated control plants (Table 4.4). These treatments correspond to bacteria isolated from SG varieties 'Cave-In-Rock', 'REAP 921', and 'NU 94-2', respectively (Table 4.1). Interestingly, the treatments that performed the best in the growth chamber (V1, V5 and V10), all correspond to varieties that also had the greatest yields under field conditions, as observed from the 2008 harvest results (Table 4.4, Figure 4.1). Also significant was the batch treatment from the ATCC group; these strains are known associative N_2-fixers.

Batch screening and selection

Further rounds of screening selection were performed on the treatments ATCC, V1, V5, and V10 batches (Table 4.5). Although not producing statistically significant effects during the initial screening bioassay, the V7 batch was also included in subsequent screening selection as it produced the fourth highest plant growth (Table 4.4). In the first round of screening, as compared with uninoculated control plants, treatment V1 increased shoot, root, and total plant DW; V7 increased shoot, root, and total plant DW; and V10 increased shoot, root, and total plant DW (Table 4.5). Treatment V5 only resulted in greater root growth, while the ATCC treatment did not produce significant differences. Based upon the results of the first round of screening, treatments V1, V7, and V10 were selected for further plant bioassays, while the ATCC and V5 treatments were not tested in the subsequent bioassays 2-4 (Table 4.5). In bioassay 2, shoot and total DWs of plants inoculated with the V1 batch were greater

than the uninoculated control. In bioassay 3, shoot and total DW (P = 0.08) of plants inoculated with the V10 batch were greater than the uninoculated control. A final round of screening bioassay was conducted; in this assay, shoot, root, and total plant DW were greater, if treated with batch V1 bacteria, than the uninoculated control. In addition, treatment V10 improved shoot and total plant DW as compared to the uninoculated control.

Single inoculant screening

Single isolate inoculation bioassays were also conducted to determine if and which isolates within the batch treatments V1, V5, V7, and V10 were responsible for PGP as observed in the previous bioassays (Table 4.6). Within the ATCC batch, shoot growth was enhanced due to inoculation with *A. chroococcum*, and *H. frisingense*, which resulted in greater plant DW for treatments inoculated with *A. chroococcum*, and only marginal plant growth enhancement for treatments inoculated with *H. frisingense* (P = 0.06). Inoculation with *H. seropedicae* marginally enhanced shoot (P = 0.07) and root (P = 0.06) growth, resulting in an overall greater total plant DW. Within the V1 batch, shoot growth was enhanced by isolates V1B1-13, V1B2-2, V1B2-11, and V1B2-12. Only isolate V1B1-13 enhanced root growth. Overall, total plant DW was increased by inoculation with isolates V1B1-13, V1B2-3, and V1B2-12. Within the V5 batch, shoot growth was enhanced with inoculation of V5B1-9, and marginally increased with treatment V5B1-7 (P = 0.08). Root and total plant DWs were not enhanced by any isolate from the V5 batch. Within the V10 batch, inoculation with isolate V10B2-1 marginally enhanced shoot DW (P = 0.07), while inoculation with V10B2-2 increased shoot DW, marginally enhanced root DW (P = 0.06), and resulted in overall greater total plant DW as compared to the uninoculated control.

Singly inoculated treatments that enhanced plant growth the most from bioassay

5 were selected for final screening and selection in bioassay 6. As isolates from the V7 batch were not included in bioassay 5 screening due to lack of space in growth chambers, these isolates were included in this screening bioassay. Isolates from the ATCC group were not included in this screening process as we were more interested in selecting for endophytic SG isolates for further field assays rather than associative N_2-fixers isolated from other plant species. Within the V1 batch, root growth was enhanced by inoculation with isolates V1B1-13, V1B2-2, V1B2-3, V1B2-11, resulting in greater total per plant DW by inoculation with V1B2-2, V1B2-3, and V1B2-11. Within the V5 batch, root growth was enhanced by inoculation with V5B1-9, while total per plant DW was improved by inoculation with V5B1-7. Within the V7 batch, root growth enhancement was observed with isolates V7B3-2 and V7B3-6; while increased total per plant DW was observed with SG inoculated with V7B3-7. Within the V10 batch, marginal increases in root ($P = 0.06$) and total per plant ($P = 0.09$) DW were observed with SG plants inoculated with V10B2-2.

4.4.2 Nitrogen concentration and content

Initial screening

The corresponding shoot N concentration and content of the initial batch screening indicated that while there were no differences in total N concentration, the N content was greater as compared to the control, mostly as a result of the larger shoot biomass (Table 4.4). Thus, treatments that showed the greatest PGP were also the treatments that resulted in greater N content in shoots. These treatments correspond to batches ATCC, V1, and V5.

Batch screening and selection

The results of further screening of these batches/ strains, along with the other top two treatments, V7 and V10, are shown in Table 4.5, bioassays 1-4. For cost purposes, shoot replicates were pooled before grinding to form a composite sample; hence statistical analyses were not performed for bioassay 1. In this assay, no differences were observed for shoot N concentration of batch treatments, but differences were observed for the N content in that, batch treatments ATCC, V1 and V7 resulted in greater N contents than the control. Bioassays 2 and 3 represent data from two, growth chamber experiments pooled together. In bioassay 2, no significant differences for the N concentration were observed among treatments, however, the N content of batch treatment V1 was greater than the control. In bioassay 3, no differences were observed among treatments for either the shoot N concentration or content. In bioassay 4, only the N content of batch V10 was greater than the control.

4.4.3 Phosphate solubilization

Several isolates from the V1 batch exhibited substantial ability to solubilize phosphate (Table 4.7). These isolates included V1B1-12, V1B1-13, V1B2-2 and V1B2-13. Isolates V1B2-3 and V1B2-12 exhibited limited ability to solubilize phosphate, while isolates V1B1-11 and V1B2-11 were unable to solubilize phosphate on PVK plates. With the exception of isolate V5B1-6, all other isolates that comprised the V5 batch were able to solubilize phosphate. Only the isolate V7B3-1 within the V7 batch exhibited phosphate solubilization ability, however, this capacity was slight. Within the V10 batch, only isolate V10B2-1 was able to solubilize phosphate.

4.4.4 Production of IAA-like substances

All isolates screened and selected for further testing produced varying amounts of IAA-like substances (Table 4.7). Of the eight isolates comprising the V1 batch, four isolates (V1B2-13, V1B1-12, V1B2-2, and V1B1-13) produced the greatest amounts of IAA-like substances, ranging from 31 to 37 μg mL^{-1}. The other four isolates (V1B2-12, V1B2-11, V1B2-3, and V1B1-11) produced about 3 to 26 μg mL^{-1} of auxin-like compounds. Isolates comprising the V5 batch produced moderate to high amounts of IAA-like substances, ranging from 14 to 32 μg mL^{-1}. Within the V7 batch, three isolates (V7B3-2, V7B3-3, and V7B3-6) showed moderate amounts of auxin production, 22 to 30 μg mL^{-1}, while two isolates (V7B3-1 and V7B3-7) showed lower amounts of auxin production, 9 to 13 μg mL^{-1}. Isolate V10B2-1 showed high IAA-like substance production, 32 μg mL^{-1}, while the other two isolates, V10B2-2 and V10B3-1, exhibited low production of IAA-like substances, 14 to 18 μg mL^{-1}.

4.4.5 Isolate identification

Analyses of 16S rRNA gene sequences

Over 300 putative NFB were isolated from rhizomes of the eleven SG varieties sampled. Of these, four sets of isolates from varieties 'Cave-In-Rock' (V1), 'REAP 921' (V5), 'Late Synthetic' (V7), and 'REAP 961' (V10), showed significant PGP as observed in the growth chamber screening. The majority of isolates sequenced were from the phylum *Proteobacteria*, many of which were from the families *Enterobacteriaceae*, *Pseudomonadaceae*, and *Sphingomonadaceae* (Tables 4.8 and 4.9). Isolate V1B1-11 was identified as *Paenbacillus polymyxa* (99 % similarity), a species belonging to the phylum *Firmicutes*, and isolate V10B3-1 was identified as *Chryseobacerium* sp. (97 % similarity), which belongs to the phylum *Bacteroidetes*. The V1 batch was composed of eight isolates, but it is likely that only four bacterial species comprised

this grouping. Isolates V1B1-13 and V1B2-11 are closely related or identical (100 % rRNA gene sequence identity) to a species of *Serratia*, most likely *S. plymuthica* or *S. proteamaculans*. Three isolates, V1B1-12, V1B2-3 and V1B2-12, were identified as a species of *Rahnella*, with V1B1-12 and V1B2-12 being most likely the same species of *Rahnella*, while isolates V1B2-13 and V1B2-2 are similar species of *Pseudomonas*. The V5 batch was also composed of eight isolates, seven of which were identified as a species of *Rahnella*, and V5B1-7 was identified as a species belonging to the genus *Serratia*. The V7 batch consisted of five isolates, three of which belong to the genus *Sphingomonas* or the species *Novosphingobium capsulatum*; the other two isolates belong to the genus *Serratia*. Three different isolates were included in the V10 batch, and they were identified as a species of *Rahnella*, a species of *Pseudomonas*, and a species of *Chryseobacerium*.

Analyses of *nifH* gene sequences

Four *nifH* primer sets were used to amplify various sized fragments of the *nifH* gene. While this process was not successful for the majority of isolates analyzed (Tables 4.8 and 4.9), several isolates were observed to be *nifH* positive, including isolate V1B1-11 (identified as *P. polymyxa*), as well as all of the V7 batch isolates. The *nifH* gene amplified from V1B1-11 had a BLAST match closest to *P. polymyxa nifH*, while all five isolates from the V7 batch had BLAST matches closest to the *Rhizobium* sp. *nifH* gene.

4.5 Discussion

Switchgrass is a high biomass cellulosic feedstock requiring minimal N fertilization. This observation prompted us to hypothesize that SG may be meeting its N requirement by its association with free-living PGPR capable of fixing their own N and

supplying N to SG. Here we report several PGPR isolated from the rhizomes of SG that exhibit significant PGP through N_2-fixation, and that possess other important plant growth promoting mechanisms, which include P solubilization and production of IAA-like substances. To our knowledge, this is the first report of beneficial plant-microbe interactions involving bacteria isolated from SG and their effect on plant growth. Such bacteria could be advantageous when applied to field trials of SG fertilized with little to no N fertilization, and/or grown on marginal and underutilized land.

We sampled SG rhizomes from a variety trial abandoned in 2000 when the trial ended (Section 4.3.1, Table 4.1). Several varieties did poorly without management or fertilization (i.e. V11, 'REAP 961') and eventually succumbed to weed competition, while other varieties, such as V1 ('Cave-In-Rock'), V5 ('REAP 921'), V7 ('Late Synthetic'), and V10 ('NU 94-2'), fared better, managing to produce between 3.8 to 5.1 Mg ha^{-1} and compete very effectively with weed species. By comparison, a fertilized and well-managed field in southeastern Canada would produce over 10 Mg ha^{-1} of biomass (Madakadze et al., 1998a). We selected bacteria from rhizomes, and not roots or rhizosphere, because of the larger structure of rhizomes (i.e. bigger diameter), which would provide areas of niches for potential NFB due to a greater range of O_2 tension across the rhizome, provide more carbohydrate reserves and is a site of new tissue/tiller growth (Reinhold-Hurek and Hurek, 1998; Rout and Chrzanowski, 2009; Steen and Larsson, 1986). We were able to isolate over 300 putative NFB on N-free solidified media. From this sample population, the strains that performed the best in the growth chamber experiments conducted without N fertilizer (V1, V5, and V10) were all isolated from varieties that also had the greatest yields under field conditions, as observed from the 2008 harvest results (Table 4.4, Figure 4.1). Therefore, the results suggest that variety type may not be the sole determining factor of SG

plant productivity. It does, however, support our hypothesis that other factors, such as plant-microbe associations, may be involved in influencing plant productivity. It also suggests that plant genotype can have an important influence over the nature of these associations.

Also significant was the batch treatment from the ATCC group; the strains included in this batch are known associative N_2-fixers. It was predicted that the ATCC group would have positive effects on shoot and total plant growth, as several of the bacteria that comprised this treatment have been reported to increase productivity of sugarcane (Boddey et al., 1995, 2003; Govindarajan et al., 2006), rice (James et al., 2002; Muthukumarasamy et al., 2007; Rodrigues et al., 2008), and corn (Dobbelaere et al., 2001; Mehnaz and Lazarovits, 2006). However, none of the ATCC bacteria had been previously tested with SG. This is also the first report of PGP of SG by bacteria that made up the ATCC batch, namely species of *Azospirillum*, *Herbaspirillum*, *Azotobacter*, *Burkholderia*, and *Gluconacetobacter*. Further experiments to test plant growth effects by the bacteria that make up the ATCC batch are being pursued by our laboratory. An in-depth investigation into these plant-microbe interactions will be of agronomic and economic interest, in particular, the use of these well characterized and often tropically isolated bacteria to enhance SG growth under field conditions. However, the use of locally isolated bacteria may be the best approach to improving SG growth under field conditions in N. America, as it may be difficult to obtain rights and permits to use exotic bacteria for field trials or commercial field production. Further, locally isolated strains are certain to be well adapted to local conditions, whereas exotic strains may perform well under growth chamber conditions, but not in the field. All of the species of bacteria tested in the ATCC batch were bacteria isolated from other plant species sampled from areas such as Brazil, Germany, and Vietnam (Baldani et al., 1986; Eckert et al., 2001; Gillis et al., 1989, 1995; Kirchhof

et al., 2001; Tarrand et al., 1978). The bacteria isolated from SG and tested in the growth chamber studies, were all isolated from rhizomes of SG sampled from Québec, Canada. These isolates are also generally benign bacteria ubiquitous to agricultural and other types of soils.

Further growth chamber screening of the best isolate batches (V1, V5, V7, and V10 bacteria) showed that the V1 batch (bacteria isolated from 'Cave-In-Rock') consistently outperformed the other batches of bacteria (Table 4.5). We observed that the V1 batch tended to also result in greater SG shoot N concentration and content than the uninoculated control treatment (Table 4.5). Due to the small sample size, the results of the N analyses were not significant in most cases, but we frequently observed higher numerical values due to inoculation. In addition, the N analyses of two pooled bioassays showed that inoculation with the V1 batch improved N concentration and content as compared to the uninoculated control treatment (data not shown). Screening of the individual isolates that comprised each of the batches showed that the majority of isolates comprising the V1 batch were able to increase plant growth, while only a few individuals of the V5 or V7 batches showed PGP (Table 4.6). Further, batches V1 and V10 were comprised of four and three different genera, respectively, while batches V5 and V7 were comprised of essentially the same species of bacteria, i.e. only two species for each of these two batches (Tables 4.8 and 4.9). The apparent diversity within batches V1 and V10, as compared to the uniformities within the V5 and V7 batches, may explain the magnitude of PGP observed by each of the batches. Generally, and most consistently, batches V1 and V10 increased plant growth the most, while PGP by either the V5 or V7 batches were minor, and not consistent throughout the various screening bioassays (Table 4.5). These results support our hypothesis that endogenous N_2-fixing bacteria helped to improve growth and productivity of SG, with isolates from the V1 batch showing the greatest effects. In

general, inoculation with batch V1 resulted in the greatest plant growth promotion, with batches V10, then V7, and finally V5 following suite.

Sequencing of 16S rRNA genes revealed that the majority of isolates tested belong to the phylum *Proteobacteria*, with isolate V1B1-11, identified as *Paenibacillus polymyxa*, belonging to the phylum *Firmicutes*. Among the various batches of isolates, the V1 batch was comprised of the most diverse group of bacteria with isolates belonging to the genera *Rahnella*, *Serratia*, *Pseudomonas*, and *Paenibacillus* (Table 4.8), many of which have already been shown to promote plant growth through BNF and other mechanisms (Lal and Tabacchioni, 2009; Mehnaz and Lazarovits, 2006; Pratibha et al., 2010; Shaharoona et al., 2006b). In this study, several isolates within the V1 batch were able to solubilize phosphate, with varying levels of high to marginal P solubilizing ability (Table 4.7). All isolates tested showed some ability to produce IAA-like substances. A bacterium's capacity to solubilize P and produce auxin are properties that could prove beneficial under field conditions.

Species of the genus *Pseudomonas* are well known for their plant growth promoting abilities, often through such properties as P solubilization (Mehnaz and Lazarovits, 2006), IAA production (Mehnaz and Lazarovits, 2006; Patten and Glick, 2002), and ACC deaminase activity (Shaharoona et al., 2006b). Recently, a strain of *P. stutzeri* A1501 was reported by Yan et al. (2008) to be capable of N_2-fixation. *Serratia* sp., most notably, *S. plymuthica* has been reported to be an effective biocontrol agent against weeds and phytopathogenic fungi (Berg, 2000; Dandurishvili et al., 2010; Liu et al., 2010), while *R. aquatilis* is also known for its ability to promote plant growth through its biocontrol and P solubilizing properties (El-Hendawy et al., 2005; Kim et al., 1997; Pratibha et al., 2010; Samina et al., 2010). Species from both genera have been shown to be N_2-fixers (Berge et al., 1991; Gyaneshwar et al., 2001; Islam et al., 2009, 2010).

However, with the exception for isolate V1B1-11, identified as *Paenibacillus polymyxa*, attempts to amplify the *nifH* gene of all other V1 isolates were unsuccessful. *P. polymyxa* has been reported to promote plant growth (Çakmakçi et al., 2006; Lal and Tabacchioni, 2009) through cytokinin and auxin production (Lebuhn et al., 1997; Timmusk et al., 1999), antifungal activity (Kim et al., 2010; Son et al., 2009), and has been shown to invade plant roots by the formation of biofilms (Timmusk et al., 2005). The bacterium has also been reported to be a N_2-fixer (Achouak et al., 1999; Coelho et al., 2003). In this study, isolate V1B1-11 was not able to solubilize P and produced only low amounts of IAA-like substances (Table 4.7). However, it is probable that the inclusion of isolate V1B1-11 in the V1 batch resulted in an additive advantage when compared to the other batches, as the V1 batch consistently increased plant growth. Further, as N was the only limiting nutrient, and water was in constant supply, it is clear that while some combination of BNF, P solubilization and auxin producing ability are favourable for maximizing plant growth, the isolates we tested were able to promote plant growth without the added benefits of these other mechanisms. It is apparent that other factors, such as BNF, may be the predominant bacterial trait required for plant growth, since inoculated plants grown in the controlled studies were larger than the uninoculated controls. Furthermore, although the plants were clearly stressed and N-limited, as exhibited by the small biomass and light green to yellow leaves, inoculation with the bacterial treatments increased plant growth without lowering N concentrations within shoot tissues. Plant growth promotion beyond N uptake capabilities would result in a dilution effect, resulting in lower N concentration within the tissues. Therefore, the results of this study suggest that plants inoculated with the bacterial treatments were able to grow beyond their N-limited capacity by acquiring N from another source, probably through BNF.

4.6 Conclusions

In this investigation, we report the first findings of beneficial plant-microbe associations between SG and several PGPR, in particular *P. polymyxa*, an N_2-fixing bacterium isolated from SG rhizomes. Switchgrass is an agronomic and economically important crop, grown for its large biomass potential and high lignocellulosic content, traits desirable for biofuel production. Furthermore, SG is known for its low N requirement, which prompted us to hypothesize that it may be getting at least some of its N through BNF. Indeed, this may be the case as inoculation of SG with isolates grouped into the V1 batch, was demonstrated to consistently improve plant growth in the absence of N fertilization. This suggest that the plants were obtaining their N from somewhere, possibly through BNF, as the plants were N-limited but still maintained similar, and in the case of the V1 treatment, greater N concentrations than the uninoculated control. Furthermore, the isolates possessed other plant growth promoting mechanisms, such as P solubilization and production of IAA-like substances, properties that could prove beneficial under field conditions. These findings were identified in growth chamber studies, but future investigations will focus on the inoculation effects of the V1 batch in field trials with SG. An increase in SG biomass without the application of N fertilization in the field would have tremendous implications agronomically and economically. Less need for N fertilizer use would improve energy ratios, increase economic gain, and reduce environmental impacts of biofuel production by mitigating GHG emissions, particularly N_2O, and CO_2 emissions associated with fertilizer production and its use.

4.7 Tables and Figures

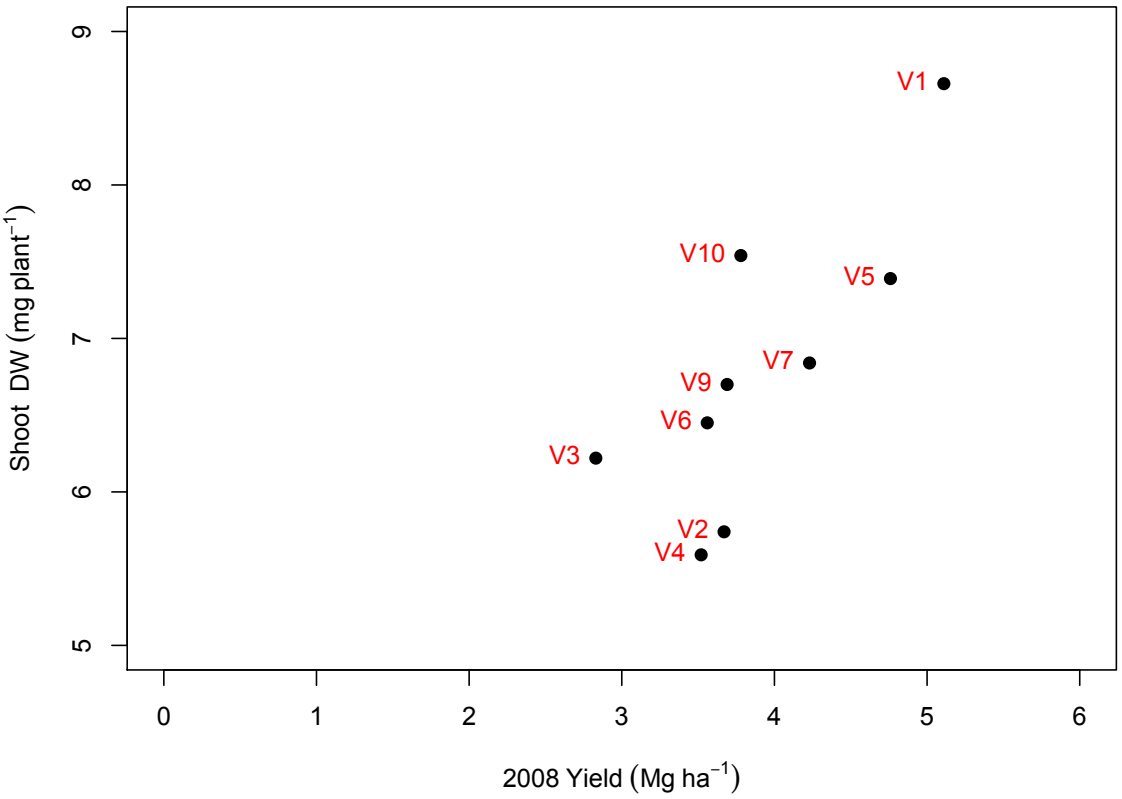

Figure 4.1 Plot showing shoot dry weight (DW; mg plant^{-1}) by yield (Mg ha^{-1}) relationships of the initial bioassay isolate screenings. Values shown represent the means of shoot DW (n = 3) by yield (n = 4). Varieties V8 and V11 are not shown as the isolates from these varieties were not tested in this growth chamber screen.

Table 4.4 Summary of results of initial bacterial screening bioassay and comparison to 2008 switchgrass (SG) harvest yield data, along with the mean number of bacterial strains isolated from each variety and most probable number (MPN) of N_2-fixing bacteria (NFB) per variety. Plant DW (n = 3) means and standard errors (SEs) of SG inoculated or not (control) with bacterial strains isolated from SG rhizomes are shown. Treatments represent 'batches' of 8 to 10 isolates grouped according to location of the plot sampled. Treatments within each chamber were isolated with a different set of 'batch' inoculants. Within a column, values with asterisks indicate significant differences from the control at $P < 0.05$ according to the R statistical software package (R: A Language and Environment for Statistical Computing, 2010).

Treatment	Plant DW[1] (mg)			Total Shoot N		2008 Yield (Mg DW ha^{-1})	Total number of isolates	MPN of NFB (cfu mL^{-1})
	Shoot	Root	Total	%N	Content			
Control	5.59 (0.45)	5.93 (0.19)	11.50 (0.53)	0.50 (0.03)	2.81 (0.28)	NA	NA	NA
ATCC	8.32** (0.40)	6.40 (0.45)	14.73* (0.64)	0.52 (0.03)	4.33** (0.32)	NA	NA	NA
V1	8.66*** (1.00)	6.84 (0.10)	15.50* (1.04)	0.51 (0.01)	4.38** (0.38)	5.11 (0.39)	39	1.3 x 10^5
V2	5.74 (0.39)	4.59 (0.11)	10.33 (0.33)	0.51 (0.03)	2.94 (0.19)	3.67 (0.65)	39	1.6 x 10^6
V3	6.22 (0.58)	5.72 (0.77)	11.93 (1.00)	0.49 (0.03)	3.11 (0.48)	2.83 (0.63)	49	4.9 x 10^6
V4	5.59 (0.76)	5.67 (0.42)	11.27 (1.13)	0.48 (0.01)	2.68 (0.32)	3.52 (0.63)	44	3.0 x 10^6
V5	7.39* (0.82)	5.11 (0.84)	12.50 (1.44)	0.56 (0.03)	4.16* (0.60)	4.76 (0.35)	42	1.5 x 10^6
V6	6.45 (0.47)	5.50 (0.65)	11.93 (1.05)	0.48 (0.04)	3.11 (0.48)	3.56 (0.62)	38	2.4 x 10^6
V7	6.84 (0.32)	5.91 (0.71)	12.73 (1.01)	0.49 (0.03)	3.41 (0.38)	4.23 (0.64)	47	1.6 x 10^6
V8	NA[2]	NA	NA	NA	NA	3.51 (0.72)	50	2.8 x 10^5
V9	6.70 (0.77)	5.25 (1.55)	11.93 (2.27)	0.48 (0.03)	3.19 (0.33)	3.69 (0.77)	61	1.6 x 10^5
V10	7.54* (0.20)	5.38 (0.94)	12.90 (1.00)	0.45 (0.03)	3.40 (0.11)	3.78 (0.72)	45	1.6 x 10^6
V11	NA[3]	NA	NA	NA	NA	1.27 (0.11)	57	1.2 x 10^5

*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$

[1] Growth chamber result from bacterial screening.

[2] *Not applicable:* V8 treatment was not tested in this chamber due to lack of space.

[3] *Not applicable:* V11 treatment was not tested in any chamber due to lack of space as well as poor harvest yield.

Table 4.5 Mean plant dry weights (DWs) and shoot nitrogen (N), given with standard errors (SEs) of switchgrass inoculated or not (control) with bacterial strains isolated from switchgrass rhizomes. Plant DWs (mg) are presented for shoot, root, and total biomass separately. Biomass results represent data from chamber experiments with n=3, n=6, and n=6 pots per treatment for bioassays 1, 3, and 4, respectively, while bioassay 2 represent pooled data from two growth chamber experiments with n= 12 pots per treatment. Shoot N data are presented as %N and N content (mg N plant^{-1}) and are the means of composite sample[1] (n = 1 for bioassay 1, and n = 3 for bioassays 2, 3, and 4). Treatments selected showed the greatest plant growth promotion based upon the results of the screening bioassay shown in Table 4.4. Treatments represent plants inoculated with 'batches' V1, V5, V7, and V10 (8 to 10 isolates per batch; V10 was comprised of 3 isolates) grouped according to location of the plot sampled. Within a row, values with asterisks indicate differences significant from the control at $P < 0.05$ according to the R statistical software package (R: A Language and Environment for Statistical Computing, 2010).

Bioassay	Treatments				
	Control	V1	V5	V7	V10
Bioassay 1					
Plant DWs (mg)					
Shoot	5.14 (0.19)	7.38*** (0.41)	5.55 (0.46)	7.02** (0.23)	5.97* (0.36)
Root	3.67 (0.52)	4.88 (0.76)	4.76 (0.80)	5.20^{+} (0.26)	4.84 (0.59)
Total	8.81 (0.53)	12.26** (0.53)	10.31 (1.07)	12.23** (0.47)	10.81* (0.95)
Shoot N					
% N	0.49[2]	0.54	0.46	0.50	0.44
N content	2.47[2]	4.00	2.55	3.48	2.65
Bioassay 2					
Plant DWs (mg)					
Shoot	4.00 (0.27)	4.91* (0.28)	NA[3]	3.97 (0.18)	3.71 (0.20)
Root	3.95 (0.20)	4.20 (0.25)	NA	3.36^{+} (0.21)	4.05 (0.19)
Total	7.94 (0.33)	9.11* (0.43)	NA	7.33 (0.33)	7.76 (0.25)
Shoot N					
% N	0.64 (0.02)	0.69 (0.02)	NA	0.72 (0.08)	0.73 (0.07)
N content	2.57 (0.20)	3.40* (0.19)	NA	2.70 (0.30)	2.69 (0.23)
Bioassay 3					
Plant DWs (mg)					
Shoot	3.76 (0.12)	4.08 (0.20)	NA	3.79 (0.11)	4.45** (0.21)
Root	4.20 (0.13)	4.07 (0.16)	NA	4.24 (0.24)	4.22 (0.18)
Total	7.96 (0.21)	8.15 (0.25)	NA	8.03 (0.33)	8.67^{+} (0.29)
Shoot N					
% N	0.61 (0.03)	0.68 (0.01)	NA	0.63 (0.12)	0.65 (0.10)
N content	2.29 (0.09)	2.79 (0.01)	NA	2.39 (0.48)	2.93 (0.60)
Bioassay 4					
Plant DWs (mg)					
Shoot	3.92 (0.48)	5.31* (0.24)	NA	4.48 (0.47)	5.76** (0.44)
Root	2.01 (0.28)	2.68* (0.09)	NA	2.24 (0.25)	2.18 (0.07)
Total	5.92 (0.62)	7.99* (0.32)	NA	6.72 (0.71)	7.95* (0.42)
Shoot N					
% N	3.73 (0.46)	4.05 (0.11)	NA	4.49 (0.05)	4.36 (0.29)
N content	14.63 (2.22)	21.56 (1.88)	NA	20.18 (3.54)	25.12* (1.86)

*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; $^{+}$ $P < 0.1$

[1] Samples (n = 3) were pooled and ground into a composite sample (n = 1) for total N analysis.

[2] Standard errors not shown as samples (n = 3) were pooled into a composite sample (n = 1).

[3] *Not applicable:* V1, V7, and V10 batch treatments showed the greatest plant growth promotion and were selected for further plant bioassays. The V5 treatment was not tested in the subsequent bioassays.

Table 4.6 Plant dry weight (DW) means and standard errors (SEs) of switchgrass inoculated or not (control) with bacterial strains isolated from switchgrass rhizomes. Biomass results represent data from chamber experiments with n=3 and n=6 pots per treatment for bioassays 5 and 6, respectively. Treatments selected showed the greatest plant growth promotion based upon the results of the screening bioassay shown in Table 4.4. Treatments represent plants inoculated with single strains that comprised the batches ATCC, V1, V5, V7, and V10. Within a column, values with asterisks indicate significant differences from the control at $P < 0.05$ according to the R statistical software package (R: A Language and Environment for Statistical Computing, 2010).

Treatment	Bioassay 5 DW (mg)			Bioassay 6 DW (mg)		
	Shoot	Root	Total	Shoot	Root	Total
Control$_{Ch1}$	**5.27 (0.09)**	**3.20 (0.31)**	**8.48 (0.24)**	**4.80 (0.22)**	**3.44 (0.63)**	**8.24 (0.72)**
ATCC*batch*						
Ac	6.28* (0.56)	4.16 (0.60)	10.44* (1.16)	NA	NA	NA
Ab	5.50 (0.22)	3.80 (0.42)	9.29 (0.63)	NA	NA	NA
Ad	6.06 (0.49)	2.89 (0.11)	8.95 (0.40)	NA	NA	NA
Hf	6.38* (0.32)	3.94 (0.55)	10.31^{+} (0.80)	NA	NA	NA
Hs	6.18^{+} (0.23)	4.36^{+} (0.18)	10.54* (0.05)	NA	NA	NA
Bv	5.97 (0.31)	4.00 (0.45)	9.97 (0.32)	NA	NA	NA
Gd	5.61 (0.40)	3.58 (0.13)	9.19 (0.26)	NA	NA	NA
V1*batch*						
V1B1-11	5.75 (0.01)	4.26^{+} (0.37)	10.01 (0.38)	NA	NA	NA
V1B1-12	6.21^{+} (0.49)	3.33 (0.48)	9.54 (0.90)	5.63 (0.35)	3.87 (0.50)	9.50 (0.24)
V1B1-13	6.59* (0.25)	4.72* (0.18)	11.31** (0.19)	4.90 (0.37)	4.82* (0.35)	9.72^{+} (0.54)
V1B2-2	6.66** (0.34)	3.64 (0.79)	10.30^{+} (1.12)	5.19 (0.46)	5.40** (0.15)	10.59** (0.59)
V1B2-3	6.23^{+} (0.32)	4.30^{+} (0.58)	10.53* (0.83)	5.24 (0.55)	5.01* (0.66)	10.25* (1.20)
V1B2-11	6.30* (0.32)	3.68 (0.16)	9.98 (0.35)	5.41 (0.63)	5.21** (0.16)	10.62** (0.53)
V1B2-12	6.45* (0.36)	3.97 (0.43)	10.42* (0.65)	4.52 (0.25)	4.14 (0.34)	8.66 (0.58)
V1B2-13	5.67 (0.43)	4.12 (0.29)	9.79 (0.71)	4.68 (0.46)	4.41 (0.28)	9.10 (0.74)
Control$_{Ch2}$	5.00 (0.39)	4.13 (1.02)	9.13 (1.10)	NA	NA	NA
V5*batch*						
V5B1-1	5.46 (0.21)	5.00 (0.46)	10.46 (0.66)	NA	NA	NA
V5B1-2	5.59 (0.02)	3.91 (0.34)	9.51 (0.34)	NA	NA	NA
V5B1-3	5.46 (0.31)	5.01 (0.12)	10.47 (0.32)	NA	NA	NA
V5B1-4	5.49 (0.39)	4.82 (0.51)	10.31 (0.57)	NA	NA	NA
V5B1-5	4.79 (0.26)	4.26 (0.38)	9.04 (0.42)	NA	NA	NA
V5B1-6	5.86 (0.42)	4.07 (0.48)	9.92 (0.90)	NA	NA	NA
V5B1-7	6.04^{+} (0.29)	5.42 (0.47)	11.46 (0.18)	5.73 (0.46)	4.46 (0.69)	10.19* (1.14)
V5B1-9	6.21* (0.26)	4.91 (0.58)	11.11 (0.82)	4.62 (0.27)	5.06* (0.10)	9.67 (0.37)
V7*batch*						
V7B3-1	NA[1]	NA	NA	5.20 (0.15)	4.54^{+} (0.54)	9.74^{+} (0.61)
V7B3-2	NA	NA	NA	4.66 (0.26)	4.70* (0.26)	9.36 (0.51)
V7B3-3	NA	NA	NA	5.07 (0.52)	2.88 (0.65)	7.95 (0.13)
V7B3-6	NA	NA	NA	4.91 (0.18)	4.76* (0.24)	9.68 (0.22)
V7B3-7	NA	NA	NA	5.44 (0.35)	4.55^{+} (0.14)	9.99* (0.25)
V10*batch*						
V10B2-1	6.07^{+} (0.19)	5.24 (0.43)	11.30 (0.61)	5.21 (0.66)	2.80 (0.50)	8.01 (0.21)
V10B2-2	8.00*** (0.82)	6.18^{+} (1.75)	14.18** (2.57)	5.09 (0.16)	4.64^{+} (0.35)	9.73^{+} (0.29)
V10B3-1	5.11 (0.63)	4.73 (0.70)	9.84 (1.24)	NA	NA	NA

*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; $^{+}$ $P < 0.1$

[1] *Not applicable:* V7 individual isolates were not tested in this bioassay due to lack of space.

Table 4.7 Phosphate solubilization and indole-3-acetic acid (IAA) like substance production by plant growth promoting rhizobacteria isolated from switchgrass (SG) rhizomes. All assays were performed in triplicate.

Isolate	P solubilization[1]	IAA-like substance (μg mL^{-1})
Control	—	—
V1 *batch*		
V1B1-11	—	2.83 ± 0.09
V1B1-12	+++	36.15 ± 2.10
V1B1-13	++	31.49 ± 1.34
V1B2-2	++	33.15 ± 2.35
V1B2-3	+/-	11.76 ± 2.26
V1B2-11	—	20.69 ± 2.60
V1B2-12	+/-	25.51 ± 1.59
V1B2-13	+++	37.33 ± 1.46
V5 *batch*		
V5B1-1	++	31.82 ± 2.44
V5B1-2	+++	30.86 ± 0.47
V5B1-3	+	29.13 ± 0.84
V5B1-4	++	28.27 ± 0.83
V5B1-5	++	31.89 ± 1.08
V5B1-6	—	14.16 ± 1.22
V5B1-7	++	28.97 ± 1.31
V5B1-9	++	29.91 ± 1.06
V7 *batch*		
V7B3-1	+/-	12.83 ± 0.70
V7B3-2	—	30.04 ± 2.83
V7B3-3	—	29.55 ± 1.31
V7B3-6	—	22.62 ± 4.43
V7B3-7	—	9.74 ± 0.55
V10 *batch*		
V10B2-1	+++	31.95 ± 2.06
V10B2-2	—	14.02 ± 1.69
V10B3-1	—	18.04 ± 0.35

[1] Zone of inhibition: — 0 cm^2; +/ – < 0.05 cm^2; + 0.05 - 0.50 cm^2; ++ 0.50 - 1.00 cm^2; +++ > 1.00 cm^2

Table 4.8 Summary of Variety 1 ('Cave-In-Rock') isolate identification according to the 16S rRNA gene sequences in the GenBank database based on BLAST search results. Isolates selected for identification showed the greatest plant growth promotion based from the results of the screening bioassay.

Isolate	Accession number	Highest scoring 16S BLAST match	Phylum	Class	Family	Max identity (%)	Gram reaction	*nifH*	Highest scoring *nifH* BLAST match
V1 *batch*									
V1B1-11	JN887785	*Paenibacillus polymyxa*	Fir	Bacilli	Paen	99	positive	present	*P. polymyxa*
V1B1-12, V1B2-3 V1B2-12	JN887786 JN887789 JN887791	*Rahnella* sp. *R. aquatilis*	Pro	Gam	Ent	96-98	negative	Inconclusive[1]	Inconclusive
V1B1-13, V1B2-11	JN887787 JN887790	*Serratia* sp. *S. plymuthica* *S. proteamaculans* *S. liquefaciens*	Pro	Gam	Ent	96-98	negative	Inconclusive	Inconclusive
V1B2-2, V1B2-13	JN887788 JN887792	*Pseudomonas* sp. *P. brenneri* *P. collierea*	Pro	Gam	Pseu	97-100	negative	Inconclusive	Inconclusive

Abbreviations: Fir = *Firmicutes*; Pro = *Proteobacteria*; Gam = *Gammaproteobacteria*; Paen = *Paenibacillaeae*; Ent = *Enterobacteriaceae*; Pseu = *Pseudomonadaceae*

[1] Repeated attempts to amplify the *nifH* gene were unsuccessful.

Table 4.9 Summary of Varieties 5 ('REAP 921'), 7 ('Late Synthetic'), and 10 ('REAP 961') isolate identification according to the 16S rRNA gene sequences in the GenBank database based on BLAST search results. Isolates selected for identification showed the greatest plant growth promotion based from the results of the screening bioassays.

Isolate	Accession number	Highest scoring 16S BLAST match	Phylum	Class	Family	Max identity (%)	Gram reaction	*nifH*	Highest scoring *nifH* BLAST match
V5 *batch*									
V5B1-1 to V5B1-5	JN887793 to JN887797	*Rahnella* sp.	Pro	Gam	Ent	96-98	negative	Inconclusive[1]	Inconclusive
V5B1-7, V5B1-9	JN887799, JN887800	*R. aquatilis*							
V5B1-6	JN887798	*Serratia* sp. *S. plymuthica* *S. liquefaciens* *S. proteamaculans* *S. grimesii*	Pro	Gam	Ent	98	negative	Inconclusive	Inconclusive
V7 *batch*									
V7B3-1, V7B3-7	JN887801 JN887805	*Serratia* sp. *S. plymuthica* *S. liquefaciens* *S. proteamaculans* *S. grimesii*	Pro	Gam	Ent	98	negative	present	*Rhizobium* sp.
V7B3-2, V7B3-3, V7B3-6	JN887802 JN887803 JN887804	*Sphingomonas* sp. *Novosphingobium capsulatum*	Pro	Alpha	Sph	96	negative	present	*Rhizobium* sp.
V10 *batch*									
V10B2-1	JN887806	*Rahnella* sp. *R. aquatilis*	Pro	Gam	Ent	98	negative	Inconclusive	Inconclusive
V10B2-2	JN887807	*Pseudomonas* sp. *P. brenneri* *P. collierea*	Pro	Gam	Pseu	98	negative	Inconclusive	Inconclusive
V10B3-1	JN887808	*Chryseobacerium* sp.	Bac	Fla	Flav	97	negative	Inconclusive	Inconclusive

Abbreviations: Pro = *Proteobacteria*; Bac = *Bacteroidetes*; Gam = *Gammaproteobacteria*; Alpha = *Alphaproteobacteria*; Fla = *Flavobacteria*; Ent = *Enterobacteriaceae*; Sph = *Sphingomonadaceae*; Pseu = *Pseudomonadaceae*; Flav = *Flavobacteriaceae*

[1] Repeated attempts to amplify the *nifH* gene were unsuccessful.

Preface to Chapter 5

In Chapter 4, we identified free-living, beneficial PGPR that associate with SG rhizomes. Based on the plant assay screening process, we determined four sets of bacteria (batch V1, V5, V7, and V10) that enhanced plant growth in the absence of exogenous N input. It was concluded that strains from batch V1, comprising of *Paenibacillus polymyxa* and species of *Pseudomonas, Serratia,* and *Rahnella*, consistently outperformed the other batches of inoculants tested. For this reason, it was concluded that the V1 batch should be tested under field conditions.

Chapters 5 and 6 present the establishment year results of a field experiment conducted in Sainte-Anne-de-Bellevue, replicated over three sites. These results are further subdivided into yield and yield component responses of bacterial seed inoculation on SG growth and productivity (presented in Chapter 5), and into SG N dynamics, as affected by bacterial seed inoculation (presented in Chapter 6), respectively. Chapter 5 was co-authored by the candidate's supervisors, Drs. Donald L. Smith and James W. Fyles, and supervisory committee members, Drs. Philippe Seguin and Brian T. Driscoll. We plan to submit the material from Chapter 5 (under a different title) to *Biomass and Bioenergy* for publication.

Ker, K., Seguin, P., Driscoll, B. T., Fyles, J. W., and Smith, D. L. (2011). Switchgrass establishment and seeding year production can be improved by inoculation with rhizosphere endophytes. To be submitted to *Biomass and Bioenergy.*

Chapter 5

Inoculation of switchgrass (*Panicum virgatum* L.) by rhizosphere endophytes improves establishment year crop productivity

5.1 Abstract

Nitrogen-fixing bacteria (NFB) and other plant growth promoting rhizobacteria (PGPR) have been used to increase growth and final yield of corn and sugarcane, two graminaceous crops utilized as bioenergy feedstocks. Switchgrass (SG, *Panicum virgatum* L.), a native perennial grass of North America, is also a desirable bioenergy crop because it can be grown on marginal lands and thus does not necessarily displace food production. Yet, no investigations have been conducted on the use of NFB and/or PGPR as inoculants to increase SG growth and productivity. It has been noted that SG produces high biomass yields with minimal to no fertilizer nitrogen (N) input, which prompted us to hypothesize that SG is obtaining part of its

N from an alternate source, perhaps through biological nitrogen fixation (BNF) from root/rhizome associated PGPR. We isolated bacteria from SG rhizomes that had not received fertilizer N input for over ten years and which have increased plant growth in the absence of fertilizer N under growth chamber conditions. The bacteria were identified as a strain of *Paenibacillus polymyxa*, a N_2-fixing bacterium, and several other PGPR capable of solubilizing phosphate and/or producing auxins. Our objective was to test this PGPR inoculum under field conditions with low-N inputs. Here, we present the findings regarding SG productivity in the establishment year. Switchgrass seeds inoculated with the PGPR culture had greater tiller density, and larger tillers resulting in a yield increase of approximately 40% as compared to uninoculated plants. PGPR can be effectively utilized as inoculants to enhance SG yields in a low-N input production system.

Key words: switchgrass, biofuel, biological N_2-fixation, PGPR, *Paenibacillus polymyxa*, establishment year

5.2 Introduction

Since its selection by the US Department of Energy (DOE) Herbaceous Energy Crops Program (HECP) as the most promising energy crop for biofuel development, switchgrass (SG, *Panicum virgatum* L.) has garnered considerable attention (Parrish and Fike, 2005; Sanderson et al., 2006; Wright and Turhollow, 2010). Consequently, research on this crop has increased in the past several decades (Sanderson et al., 2006). Originally cultivated as a forage crop, there was very little agronomic development of SG until the last couple of decades of the 20th century (Parrish and Fike, 2005; Sanderson et al., 2006). In the comparatively short time frame since its recognition as a crop worthy of extensive breeding, our agronomic knowledge of SG has

grown considerably (David and Ragauskas, 2010; Parrish and Fike, 2005; Wang et al., 2010; Wright and Turhollow, 2010; Wullschleger et al., 2010). However, while a good amount is known about cultivating SG, very little is known about the plant-microbe interactions of SG, and their possible influence on plant productivity.

Beneficial microbes, either living in symbiosis or free-living micro-organisms co-existing with plants, have been well documented to enhance plant growth (Glick, 1995; Gray and Smith, 2005; Dobbelaere et al., 2003; Vessey, 2003). These plant growth promoting rhizobacteria (PGPR) have been shown to enhance growth through several mechanisms: (1) biological N_2-fixation (BNF), (2) solubilization of nutrients such as phosphorus, (3) producing phytohormones such as indole-3-acetic acid (IAA) and related compounds, (4) modulating production of ethylene through increased enzymatic activities (i.e. ACC-deaminase), (5) production of siderophores or antibiotics that decrease the growth of phytopathogens, and (6) enhancing other symbioses beneficial to the host (Glick, 1995; Gray and Smith, 2005; Dobbelaere et al., 2003; Vessey, 2003). The use of PGPR as inoculants to increase yield has been studied in several agronomically important crops, including sugarcane, corn, wheat, and rice (Boddey et al., 2003; Dobbelaere et al., 2001; Riggs et al., 2001; Rodrigues et al., 2008; Shaharoona et al., 2006a,b). In particular, PGPR such as *Gluconacetobacter diazotrophicus*, that are capable of BNF, have been shown to enhance sugarcane yield by providing 50 to 80% of its plant nitrogen (N) from fixed N (Boddey, 1995; Döbereiner, 1996; Pessoa-Jr et al., 2005). This contributes to the high energy ratio output reported for sugarcane to bioethanol, as greater yield per N input means less energy used for crop production (Boddey et al., 2003; Döbereiner, 1996; Pessoa-Jr et al., 2005).

The sugarcane-*G. diazotrophicus* system, although specific to the tropical grass, raises interesting questions as to whether this type of plant-microbe interaction could be replicated with a temperate bioenergy crop. *Miscanthus* x *giganteus*, a native

tropical grass that has been bred to produce high biomass yields under temperate conditions (Heaton et al., 2009; Lewandowski et al., 2003; Pyter et al., 2010), has been shown to associate with several N_2-fixing bacteria (NFB) including *Azospirillum doebereinerae* (Eckert et al., 2001) and *Herbaspirillum frisingense* (Kirchhof et al., 2001). Davis et al. (2010) recently reported significant N_2-fixation activity of *M.* x *giganteus in situ*, which the authors concluded supplied an important portion of miscanthus' high N demand. Sorghum, another bioenergy crop, has also been reported to associate with NFB (Rout and Chrzanowski, 2009). Plant growth promotion (PGP) of sorghum by NFB has been reported under controlled conditions when inoculated with *G. diazotrophicus* and the arbuscular mycorrhizal fungi *Glomus intraradices* (Adriano-Anaya et al., 2006), and under field conditions when inoculated with a combination of *Azospirillum brasilense*, or *Rhizobium* sp., and *Glomus fasciculatum* (Saini et al., 2004). By comparison, little research has been conducted on plant-microbe interactions with SG. However, some studies have reported nitrogenase activity within the roots, rhizomes, and soil rhizosphere of SG and other species of *Panicum*, suggesting that some level of BNF is occurring in association with SG (Davis et al., 2010; Day et al., 1975; Döbereiner et al., 1972; Miranda and Boddey, 1987; Tjepkema, 1975).

Recently, we have isolated several strains of PGPR from the rhizomes of SG (Chapter 4, Section 4.4.5). Switchgrass has been shown to produce consistently high yields even with minimal to no fertilizer N inputs (Hill et al., 2006; Parrish and Fike, 2005; Schmer et al., 2008; Tilman et al., 2006). This observation led us to hypothesize that SG could also be obtaining its N from associative BNF. We then sampled from a field site that was once a variety trial for biomass production, but was abandoned when the trial ended in 2000 (Mehdi et al., 2000). Since then, the field has not been managed and has not received any fertilizer inputs. The eight bacterial strains that we isolated from 'Cave-In-Rock' rhizomes, were identified from 16S rRNA gene sequences to be

a strain of *Paenibacillus polymyxa*, two strains of *Pseudomonas* sp., two strains of *Rahnella* sp., and three strains of *Serratia* sp. All strains showed moderate to high capacity to produce indole-3-acetic acid (IAA) like substances, four of the eight isolates were able to solubilize phosphate, and *P. polymyxa* was confirmed to possess the *nifH* gene, a marker for N_2-fixation capabilities. Moreover, following inoculation of SG seedlings with the grouped isolates, SG biomass yield was consistently enhanced, compared to the uninoculated control plants under growth chamber conditions and in the absence of exogenous N fertilization (Chapter 4, Section 4.5).

Our objective, therefore, was to test these eight isolates in a mixed bacterial inoculation on SG seeds to determine whether the positive plant growth effects observed under controlled environment conditions could be replicated under field conditions. Further, effects on stand dynamics were also considered, as changes within a population could greatly affect overall yield. In this report, we present the findings of a mixed bacterial inoculation on SG productivity in its first year of establishment. A poorly established field could force a crop producer to reseed the following year, resulting in lost revenues and time (Perrin et al., 2008). Factors that contribute to successful establishment include, but are not limited to, seed quality, seeding rates, seed dormancy, date of planting, planting methods (e.g. row spacing, depth of planting), soil type and temperature, weed and pest control, number of cuttings per season, and fertilizer amendments (Aiken and Springer, 1995; Parrish and Fike, 2005; Sanderson and Reed, 2000; Schmer et al., 2006). As this time period is critical for ensuring continued SG growth and success, we were interested in determining whether positive growth effects following inoculation could be observed as early as the first year of seeding.

5.3 Materials and Methods

5.3.1 Field design

Field experiments were conducted in Ste-Anne-de-Bellevue, Québec, Canada (45°28'N 73°45'W) in 2010 on three soil types, a Chateauguay clay loam, a Bearbrook clay and a Chicot fine sandy loam. Field soil characteristics, macronutrients, previous crops and N fertilization history, and seeding, fertilization, and harvest dates are summarized in Table 5.1. Sites were selected to provide a range of soil types, with different physical and chemical properties, to test the hypothesis that seed inoculation with a mixed culture of bacterial isolates increased SG production across a range of soils and environmental conditions. The factors tested in field trials were the bacterial treatment, either an uninoculated control or the mixed bacterial culture, and N fertilizer level, either 0 or 100 kg N ha^{-1}. The SG cultivar, 'Cave-In-Rock', was seeded at a rate of 10 kg ha^{-1} (Mehdi et al., 2000; Parrish and Fike, 2005) using a disk drill (Fabro, Swift Current, SK, Canada). The experiment was organized following a randomized complete block design with four replicates, with each plot being 5 m (length) x 1.3 m (width), and containing 7 rows at a spacing of 18 cm. Each plot was separated by 1.5 m spaces to minimize the possibility of inoculants spreading to uninoculated plots. Prior to seeding, soil samples (20 per field site) were taken using an auger (15-20 cm depth, 10 cm diameter), combined to form one composite sample, air dried, ground and sifted through a 10 μm sieve and used for the determination of soil characteristics (Table 5.1). Fields were plowed and N fertilizer (27:0:0%, N:P:K) was added to fertilized plots at a rate of 33.3 kg N ha^{-1} (1/3 of the total N amount) prior to seeding; the remaining N (66.6 kg N ha^{-1}) was hand broadcasted approximately ten weeks later (Table 5.1). Unfertilized plots did not receive any fertilizer application. All sites were hand weeded; herbicide was not applied on any of the field sites. The mean monthly precipitation level and temperature during the interval May 1st to October 31st in

2010 was 117 mm and 17.1°C, respectively (Pierre Elliot Trudeau Airport weather station, 19 km from the field site; Environment Canada, National Climate Data and Information Archive).

5.3.2 Seed inoculation

Eight bacterial strains were isolated in July 2008 from the rhizomes of SG, cultivar 'Cave-In-Rock', from a SG for biomass variety trial that had been abandoned after the trial ended, but was allowed to grow unmanaged and unfertilized for over ten years (Chapter 4, Section 4.3.1). Serial dilutions of the macerated juice from surface sterilized rhizomes were streaked on N-free LG medium (Döbereiner, 1995) to generate individual colonies that were selected based on differences in morphology (Chapter 4, Section 4.3.2). Permanent preparations of the pure isolates were obtained from a second round of streaking and colony selection, prior to freezing at -80°C in tryptone yeast medium containing 7% dimethyl sulfoxide (Chapter 4, Section 4.3.2).

Inoculation was performed by seed coating with peat (sieved through 500 μm, rate of 8 g peat per kg of seed) containing the mixed bacterial culture suspension 24 h before seeding in the field (Dobbelaere et al., 2001; Lupwayi et al., 2005). Each isolate was individually cultured in sterile Luria Bertani broth (LB; per mL ddH_2O: 10.0 g tryptone, 5.0 g yeast extract, 5.0 g NaCl) at 30°C for 48 h with shaking. One mL of each strain (diluted to ensure the colony forming units per mL was in the range of 10^8 to 10^9) was added together and brought to 10 mL with sterile ddH_2O, to form one culture of mixed bacterial inoculant (Chapter 4, Section 4.3.3). All individually cultured isolates had been verified for purity by streak plating onto LB plates and culturing for 48 h at 30°C. The mixed inoculant was added to SG seeds (rate of 140 mL inoculant per kg seed), vortex mixed, and allowed to sit at room temperature for 24 h, then air dried (approximately 1 h) in a laminar flow hood, prior to seeding in the

field. Control plots were inoculated in the same manner with an equivalent amount of sterile LB media and were seeded first in the field to ensure that cross-contamination did not occur. All isolates tested in the field experiments were verified to be pure cultures. To minimize the possibility of inoculants spreading to adjacent plots in the field, buffer zones of 1.5 m between plots were seeded with turf grass (60 % perennial ryegrass, 25 % creeping red fescue, 15 % Kentucky blue grass).

5.3.3 Harvest, data collection, and analyses

Prior to harvest in the fall, six height measurements per plot were randomly taken and averaged to generate one height value per plot. Tillers were manually cut by one of two persons, at a 5 cm stubble height in two, one-meter row-lengths randomly selected from each plot on October 26, 2010 for all field sites (Table 5.1). The tillers were separated by length into small ($<$ 0.5 m), medium (0.5 to 1.0 m), and large ($>$ 1.0 m), and counted to determine stand density, before fresh weights by size group were taken. The subsample was dried to a constant weight at 65°C (at least 72 h), at which time, dry weight (DW) measurements for the total and small-, medium-, and large-sized tiller counts were taken. Yield was estimated from this subsample, and per tiller biomass was determined as the total tiller DW divided by the total number of tillers. Tiller distribution was determined as the percentage of either small-, medium- or large-sized tillers over the total number of tillers per plot. Hemicellulose, cellulose, and lignin concentrations were determined from plant dry material (ground to 0.5 mm particle size) using the acid detergent fiber (ADF), neutral detergent fiber (NDF), and acid detergent lignin (ADL) procedures on an Ankom 2000 Fiber Analyzer (ANKOM Technology Corp., Fairport, NY) (ANKOM, 2005a,b,c). Cellulose concentration was estimated as the difference between ADL and ADF, while hemicellulose concentration was estimated by subtracting ADF from NDF.

5.3.4 Statistical analyses

Two-way analyses of variance (ANOVA) were performed using the R statistical software package (R: A Language and Environment for Statistical Computing, 2010). Fisher F-tests were performed for pooled data to verify the assumption of equal variances among the two sample populations. All the data were verified for the assumptions of normality and homoscedasticity. When the assumptions were not met, nonparametric Kruskal-Wallis analyses were performed. Differences were considered significant when their probability by chance alone was less than 0.05. Biologically interesting numerical differences with probabilities between 0.05 and 0.1 are also presented. When this occurs, the P values are given. Simple linear regression was used to determine the relationship of biomass yield to the dependent variable, stand density.

5.4 Results and Discussion

5.4.1 Yield and yield components

Across fields, inoculation of SG seeds with a mixed PGPR culture resulted in overall greater yield ($P < 0.01$; Figure 5.1C) than uninoculated plots, due to increased stand density ($P < 0.01$; Figure 5.1B) in both unfertilized and fertilized plots. Positive relationships between increasing stand density and increasing yield (Figure 5.2) has been reported (Boe, 2007; Boe and Beck, 2008). In this study, inoculation with the mixed PGPR culture increased the number of tillers per area, leading to higher yields of inoculated than uninoculated SG plants. Per tiller biomass, however, was not affected by inoculation (Figure 5.1A). The fertilizer treatment also mirrored the bacterial treatment in that stand density ($P < 0.001$), and yield ($P < 0.001$) were greater in fertilized than unfertilized plots, but per tiller biomass was not affected by the fertilization treatment (Figure 5.1). There was no interaction between the

fertilizer and bacterial factors, but field effects were observed for variables measured. Overall, inoculation with PGPR resulted in a yield increase of 43%, as opposed to the fertilizer treatment alone, which increased yield by 83%. Further, a combination of fertilizer input and inoculation with PGPR increased yield by 123%. Therefore, in comparison to an unfertilized, uninoculated SG field, a potential yield increase of approximately 40% is anticipated with the PGPR inoculum alone, with a further 40% increase (above fertilizer input alone) when in combination with a fertilizer input of 100 kg N ha^{-1}.

The components that contribute to yield within a perennial grass system, such as plants per unit area and tillers per plant, can greatly affect overall yield (Boe and Beck, 2008; Parrish and Fike, 2005). While PGPR studies have been conducted on various grasses (Dobbelaere et al., 2003; Lucy et al., 2004; Malik et al., 1997), field investigations into their effects on stand dynamics have not been reported. We, therefore, examined each variable: stand density, tiller biomass, and tiller size distribution for each field. To our knowledge, this is the first field investigation of plant growth promotion by PGPR on SG productivity. Inoculation with the mixed PGPR culture increased the densities of medium- and large-sized tillers as compared to uninoculated plots, which led to greater stand density (Table 5.2, Figure 5.1). Thus, more tillers per area, as well as more medium- and large-sized tillers were observed in the inoculated plots. As expected, fertilizer treatment increased medium- and large-sized tiller densities (Table 5.2).

Although no differences were observed between inoculated and uninoculated per tiller biomass for small- and medium-sized tillers, a marginally significant ($P = 0.08$) increase was observed for large-sized tillers as a result of inoculant treatments, as tillers were larger for both fertilized and unfertilized treatments than the uninoculated plants. This suggests that whether through earlier germination and growth,

leading to a longer growing season, or through better N acquisition, or possibly both, inoculated plants tended to have greater biomass, when fully developed, than uninoculated plants. While the densities of inoculated plots were greater than uninoculated plots, this did not change the overall proportional distribution of small-, medium-, and large-sized tillers, as no differences were observed due to inoculation. However, a marginally significant effect was observed for large-sized tillers ($P = 0.09$) in that inoculated plots tended to have a greater proportion of large-sized tillers within the sites. Furthermore, inoculated plants were also taller than the uninoculated plants (Table 5.2), which could also contribute to the greater per tiller biomass observed for large-sized tillers.

The greater number of tillers we observed as a result of inoculation may have arisen either from earlier plant emergence in the spring allowing for a head start on the season, or a more efficient N-acquiring root and rhizome system by inoculated SG plants, which could then allocate additional nutrients to new shoot development. The latter explanation may be the case, as PGPR have been documented to increase nutrient acquisition mainly through BNF, solubilizing immobile nutrients such as P, and stimulating root growth which would expand the rhizosphere (Glick, 1995; Dobbelaere et al., 2003; Vessey, 2003). While evidence for associative N_2-fixation with SG *in situ* has been reported (Brejda et al., 1994; Davis et al., 2010; Tjepkema, 1975), few studies have explored plant growth promoting bacterial effects on perennial grass growth, let alone on SG growth. As stated earlier, the inoculum used in this study was comprised of a mixture of PGPR, which have been shown to be capable of solubilizing P, producing IAA-like substances, and, in the case of *Paenibacillus polymyxa*, to possess the *nifH* gene and therefore possibly be an N_2-fixing bacterium (Chapter 4, Section 4.5). Therefore, it is possible that inoculation with the PGPR culture enhanced plant growth through a combination of these bacterial mechanisms.

The production of IAA-like substances may have resulted in greater root growth, which would expand the rhizosphere and allow for more access to nutrients and water. The abilities of the mixed culture to solubilize P and to fix atmospheric N would result in better nutrient acquisition, which in turn, could produce larger rhizomes and root systems. This may have led to a greater number of new shoots, while concomitantly augmenting leaf development.

5.4.2 Fiber analyses: lignin, cellulose, and hemicellulose

Fertilizer treatment resulted in greater cellulose concentration ($P < 0.05$), but did not affect hemicellulose or lignin concentration (Table 5.3). There was no effect of inoculation, nor was there an interaction between fertilizer and the bacterial treatment for any of the fiber components measured. These results are not entirely surprising given that we did not observe any bacterial effects (and minimal fertilizer effects) on per tiller biomass, indicating that increases in yield and consequently cellulose concentration would be a result of increased stand density and not necessarily from larger tillers.

5.5 Conclusions

The dynamics of a SG population can be altered by factors such as fertilizer input (George and Reigh, 1987; Muir et al., 2001; Stroup et al., 2003), cultivar (Beaty et al., 1978), environment (Casler and Boe, 2003; Casler et al., 2004), soil water availability (Berdahl et al., 2005; Evers and Parsons, 2003; Schmer et al., 2010), temperature and the resulting growing degree days (Madakadze et al., 1998b), row spacing (Muir et al., 2001; Sanderson and Reed, 2000), and previous harvest frequency and method (Schmer et al., 2006). The results of this field study indicate that a mixed PGPR

inoculation affected SG productivity in the establishment year. More tillers per area within a stand, as well as a greater population of medium- and large-sized tillers, were produced with PGPR treated stands. This resulted in a greater proportion of taller and larger plants, and led to a subsequent increase in yield. Overall, a potential yield increase of approximately 40% is anticipated with the PGPR inoculum alone. This 40% increase persists in the presence of N fertilizer, at least at the 100 kg N ha^{-1} rate. By contrast, fertilization alone at this rate produced an 83% yield increase as compared to unfertilized fields. Thus, the enhanced plant population and stand dynamics observed in the inoculated plots demonstrate that plant-microbe associations do play a role in altering the dynamics of SG population.

Future work should be directed at investigating plant and soil N dynamics, which may provide further indices on the microbial effects on plant development. *In situ* measurements of N_2-fixation, including nitrogenase activity and ^{15}N natural abundance, would substantiate the hypothesis of plant growth promotion through associative BNF. Additionally, improvements in the agronomy of this system, such as testing inoculation methods, differing inoculation dates, varying cultivars and N fertilizer rates, should be considered. Research into these areas would provide a better understanding of plant-microbe interactions with SG, a clear sense of the potential agricultural applications of these interactions and promote a more sustainable approach to its cultivation.

5.6 Tables and Figures

Table 5.1 Soil characteristics of fields 1 to 3.

Parameters	Field 1	Field 2	Field 3
Characteristics			
Type	Chateauguay clay loam	Bearbrook clay	Chicot fine sandy loam
pH	6.16 ± 0.10[1]	4.80 ± 0.02	5.52 ± 0.03
Organic matter	3.42 ± 0.15	3.56 ± 0.08	3.31 ± 0.05
Soil Nutrients			
N (mg g^{-1})	1.22 ± 0.02	1.13 ± 0.04	1.16 ± 0.02
P (mg g^{-1})	1.02 ± 0.01	0.99 ± 0.02	0.96 ± 0.02
K (mg g^{-1})	8.97 ± 0.13	10.60 ± 0.17	5.87 ± 0.28
Ca (mg g^{-1})	11.41 ± 0.40	9.98 ± 0.07	9.47 ± 0.15
Mg (mg g^{-1})	6.39 ± 0.12	7.18 ± 0.16	5.30 ± 0.00
Previous crops/ N fertilization rate (kg ha^{-1})			
2009	sweet corn/ 0	fallow/ 0	beans/ 57
2008	soybean/ 20	oats/ 0	fallow/ 0
2007	bean/ 57	corn/ 150	peas/ 0
2006	sweet corn/ 0	fallow/ 0	sweet corn/ 0
2005	soybean/ 20	oats/ 0	oats/ 0
Experiment			
Seeding date	May 20, 2010	May 27, 2010	June 4, 2010
Fertilizer input *33.3 kg N ha^{-1}*	May 19, 2010	May 26, 2010	June 4, 2010
Fertilizer input *66.6 kg N ha^{-1}*	July 20, 2010	August 4, 2010	August 10, 2010
Harvest date	October 26, 2010	October 26, 2010	October 26, 2010

[1] Standard error (n = 3)

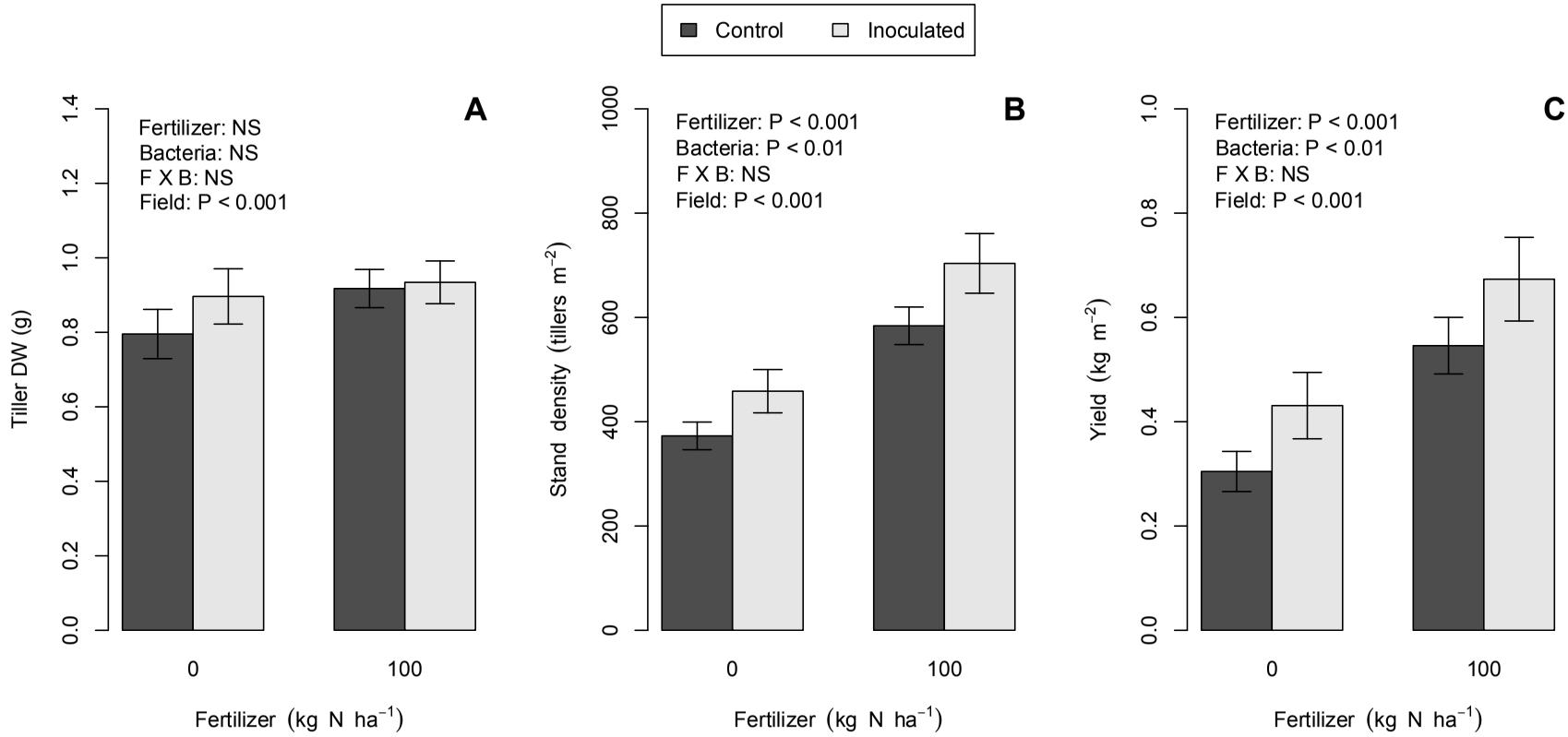

Figure 5.1 **A to C:** Mean (n = 12) (**A**) tiller dry weight (DW; g), (**B**) stand density (tillers m^{-2}), and (**C**) yield (kg m^{-2}) with standard error (SE) bars of unfertilized (0 kg N ha^{-1}) and fertilized (100 kg N ha^{-1}) switchgrass plants inoculated or not (control) with a mixed rhizobacterial culture. ANOVA results are also presented. F x B refers to 'Fertilizer x Bacteria' interaction. Means represent mean pooled data from fields 1, 2, and 3.

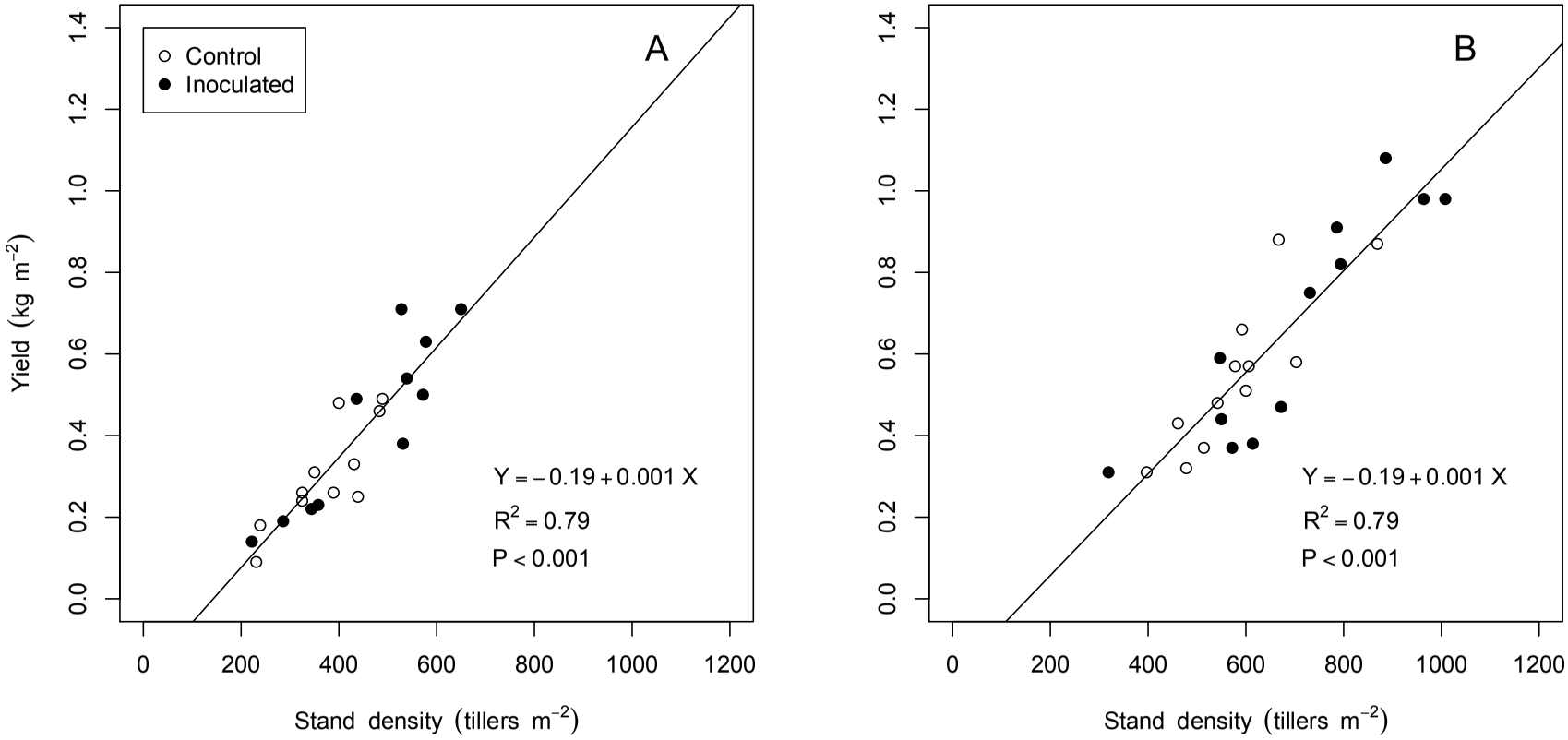

Figure 5.2 **A and B:** Response of mean (n = 24) yield (kg m^{-2}) to stand density (tillers m^{-2}) for unfertilized (0 kg N ha^{-1}; **Figure A**) and fertilized (100 kg N ha^{-1}; **Figure B**) switchgrass plants inoculated or not (control) with a mixed rhizobacterial culture harvested during senescence. Means represent pooled data from fields 1, 2, and 3.

Table 5.2 Harvest summary showing the mean (n = 12) stand density (tillers m^{-2}), tiller dry weight (DWs; g), tiller distribution among size classes (%), and height (cm), with SEs of unfertilized (0 kg N ha^{-1}) and fertilized (100 kg N ha^{-1}) switchgrass plants inoculated or not (control) with a mixed rhizobacterial culture. 'Sm', 'med', and 'lg' refer to length size of small (< 0.5 m), medium (0.5 to 1.0 m) and large (> 1.0 m) tillers. Means represent pooled data from fields 1, 2, and 3. ANOVA results are also presented. Numerical differences with probabilities between 0.05 and 0.1 are presented for their biological meaning.

Fertilizer	Bacteria	Stand density			Tiller DWs			Tiller Distribution			Height
		Sm	Med	Lg	Sm	Med	Lg	Sm	Med	Lg	
0 N	Control	108	229	36	0.19	0.83	2.02	30.5	60.4	9.1	103.9
		(17)	(23)	(9)	(0.02)	(0.05)	(0.26)	(5.0)	(3.5)	(2.0)	(3.3)
	Inoculated	110	274	74	0.20	0.83	2.42	26.9	58.4	14.7	110.8
		(12)	(32)	(18)	(0.01)	(0.04)	(0.14)	(4.5)	(2.9)	(3.0)	(2.3)
100 N	Control	120	369	95	0.18	0.83	2.18	21.8	63.0	15.3	112.9
		(15)	(26)	(21)	(0.01)	(0.03)	(0.08)	(3.1)	(1.8)	(2.7)	(2.9)
	Inoculated	130	441	134	0.18	0.78	2.36	20.5	62.3	17.2	116.2
		(21)	(43)	(28)	(0.01)	(0.04)	(0.08)	(3.5)	(1.8)	(2.8)	(3.1)
ANOVA											
Fertilizer		NS	***	***	0.1	NS	NS	**	NS	*	**
Bacteria		NS	*	*	NS	NS	0.08	NS	NS	0.09	*
F X B[1]		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Field		***	***	***	NS	0.07	NS	***	***	***	***
Block		*	NS	NS	*	NS	NS	*	*	NS	NS
Harvester		*	NS	NS	NS	NS	NS	*	0.1	NS	**

$***$ P < 0.001; $**$ P < 0.01; $*$ P < 0.05; NS, not significant

[1] F x B refers to 'Fertilizer x Bacteria' interaction

Table 5.3 Hemicellulose, cellulose, and lignin concentrations (mg g^{-1}), with SEs of unfertilized (0 kg N ha^{-1}) and fertilized (100 kg N ha^{-1}) switchgrass plants inoculated or not (control) with a mixed rhizobacterial culture. All values are expressed as the mean (n = 12) %, dry basis. Means represent pooled data from fields 1, 2, and 3. ANOVA results are also presented.

Fertilizer	Bacteria	Hemicellulose	Cellulose	Lignin
0 N	Control	24.8	27.8	7.5
		(0.9)	(1.1)	(0.3)
	Inoculated	25.8	26.9	7.5
		(0.7)	(0.6)	(0.3)
100 N	Control	25.0	29.1	7.4
		(1.1)	(1.1)	(0.3)
	Inoculated	24.6	29.5	7.6
		(0.8)	(0.9)	(0.4)
ANOVA				
Fertilizer		NS	*	NS
Bacteria		NS	NS	NS
F X B[1]		NS	NS	NS
Block		***	NS	NS
Field		***	***	***

$***$ P < 0.001; $**$ P < 0.01; $*$ P < 0.05; NS, not significant

[1] F x B refers to 'Fertilizer x Bacteria' interaction

Preface to Chapter 6

In Chapter 5, the effect of a mixed PGPR inoculum on establishment year SG productivity was evaluated for effects on yield and yield components. It was concluded that seed inoculation with a mixed PGPR solution increases tiller density and the proportion of larger-sized tillers. This resulted in greater yields of inoculated than uninoculated plants.

Chapter 6 presents the results regarding the N dynamics of bacterial seed inoculation on SG productivity. As the mixed PGPR inoculum consisted of the N_2-fixing bacterium, *Paenibacillus polymyxa*, we focused our investigation on the effects of inoculation on N concentration and cycling of SG in its establishment year. Chapter 6 was co-authored by the candidate's supervisors, Drs. Donald L. Smith and James W. Fyles, and supervisory committee members, Drs. Philippe Seguin and Brian T. Driscoll. We plan to submit the material from chapter 6 to *Biomass and Bioenergy* for publication.

Ker, K., Seguin, P., Driscoll, B. T., Fyles, J. W., and Smith, D. L. (2011). N dynamics of switchgrass: Effects of inoculation by endophytic plant growth promoting rhizobacteria on establishment year N use efficiency. To be submitted to *Biomass and Bioenergy.*

Chapter 6

N dynamics of switchgrass: Effects of inoculation by endophytic plant growth promoting rhizobacteria on establishment year N use efficiency

6.1 Abstract

Biofuel agro-ecosystems that incorporate nitrogen (N) efficient and low-N requiring crops, such as switchgrass (SG, *Panicum virgatum* L.), may improve sustainable production of feedstocks and the resulting fuels. Improvement in sustainable production of SG, as a purpose-grown biomass feedstock crop, could be realized through investigation of plant-microbe interactions associated with plant growth promoting rhizobacteria (PGPR), capable of biological nitrogen fixation (BNF). The objective of this study is to increase production of SG biofuels by developing a low-input switchgrass-microbe system. We collected SG rhizomes from sites that have not received N fertilization for ten years and isolated pure strains of N_2-fixing, and other plant growth promoting bacteria (PGPR), from these below-ground tissues. The bacteria were identified as

Paenibacillus polymyxa, an N_2-fixing bacterium, and several other PGPR capable of solubilizing phosphate and/or producing auxins. Field trials utilizing these strains in a mixed PGPR inoculum resulted in greater biomass yields and recovery of fertilizer N, and improved N cycling of inoculated than uninoculated plants, in the establishment year. Inoculated plants also contained more N in tillers during anthesis but not at senescence, suggesting a greater amount of N was translocated to rhizomes for overwintering. Greater N storage in rhizomes could mean better early-season regrowth and provide an advantage over weeds. The amount of N removal of biomass at harvest was also greater for inoculated than uninoculated plants. PGPR inoculation also resulted in positive N balances, suggesting improved access to N from non-fertilizer N sources, possibly through BNF and improved soil N uptake due to enhanced root and rhizome growth.

Key words: switchgrass, biomass, nitrogen use efficiency, PGPR, biological nitrogen fixation, establishment year

6.2 Introduction

Perennial rhizomatous grasses (PRGs), such as switchgrass (SG, *Panicum virgatum* L.), are high yielding, lignocellulosic bioenergy crops that produce greater net energy returns than first generation biofuels, such as corn and wheat (Fike et al., 2006a; Heaton et al., 2004; Hill et al., 2006; Lewandowski et al., 2003; Varvel et al., 2008). Heaton et al. (2004) list several characteristics that exemplify the positive attributes of PRGs, including SG, as bioenergy crops, among which are their ability to recycle nutrients annually, winter standing, long canopy duration, low-input requirement, and high water and nitrogen (N) use efficiencies (WUE, NUE). Indeed, SG utilizes nutrients like N more effectively than annual crops. This may explain why

several studies have reported that SG requires much less fertilizer input to generate high yields than many other crops (Fike et al., 2006a; Lemus et al., 2008; Schmer et al., 2008; Tilman et al., 2006). Fike et al. (2006a) compared low- and high-input SG production of two upland and two lowland cultivars across the upper southeastern USA over ten years. Although high-input systems produced greater SG yields, the biomass yield per kg N was greater in the low-input system. Tilman et al. (2006) reported that low-input, high-diversity grassland systems that included SG had 238% greater yields than monoculture grassland biofuel production systems and had a total CO_2 sequestration rate of 4.4 Mg ha^{-1} $year^{-1}$ in soil and roots after ten years. In contrast, Schmer et al. (2008) argued that monoculture production of SG on marginal land with moderate inputs can be as net energy efficient as low-input systems and can produce greater quantities of energy per unit of land. It is clear that N management systems can greatly affect the productivity and level of greenhouse gas (GHG) mitigation.

Strategies in place to efficiently and sustainably produce SG with minimal N inputs must consider how N is utilized and cycled by the plant (Lemus et al., 2008; Parrish and Fike, 2005; Sanderson et al., 2006). Switchgrass plants undergo cycles of new and continuous vegetative growth, asexual and sexual reproduction, and physiological decline or senescence (Clark, 1977; Heaton et al., 2009; Jones and Lazenby, 1988; Lewandowski et al., 2003). During senescence, nutrients are cycled to rhizomes for winter storage, and then cycled back for regrowth the following spring (Clark, 1977; Heaton et al., 2009; Jones and Lazenby, 1988; Lewandowski et al., 2003). This process allows the plant to use the same unit of N to build new leaves, tillers, and other plant parts year after year (Vitousek, 1982). Thus, NUE, defined by Moll et al. (1982) as the yield of grain per unit of available N in the soil, has become a challenging focus for agronomists and breeders for improving SG productivity. In the case of

SG production for bioenergy, an increase in total biomass and not grain yield *per se* is the desired end product. An improvement in NUE can mean better N uptake efficiency (the ability of the plant to remove N, including residual and fertilizer N, from the soil) and/or utilization efficiency (the ability to use N to produce yield) (Moll et al., 1982). An enhancement in either or both of these components, may improve SG's already efficient nutrient cycling process, which may potentially further decrease the requirements of any low-input management system developed for SG production. Thus, for crops such as SG, optimization of N fertilizer use will inevitably lead to greater GHG mitigation, improve the economics and decrease energy costs associated with fertilizer production and application (Sanderson et al., 2006; Schmer et al., 2008; Tilman et al., 2006).

For several decades, N fertilizer use for sugarcane bioethanol production in Brazil has been optimized through the application of plant growth promoting rhizobacteria (PGPR) capable of N_2-fixation (Baldani and Baldani, 2005; Boddey et al., 2003). Beneficial soil and plant microbes have long been known to reside near the roots in the rhizosphere, and within above-ground plant tissues (Dobbelaere et al., 2003; Glick, 1995). Indigenous populations of N_2-fixing bacteria (NFB) have been isolated from sugarcane tissue, and have been successfully re-inoculated into sugarcane setts and micro-propagated plantlets prior to planting in the field (Boddey et al., 2003; Cavalcante and Döbereiner, 1988; Gillis et al., 1989; Reis et al., 1999). These endophytes (bacteria that reside within plant tissues) including *Gluconacetobacter diazotrophicus* and *Azospirillum* sp., have been reported to contribute 50 to 80% of the crop's N requirement as fixed N, equivalent to 150 to 170 kg N ha^{-1} yr^{-1} (Boddey et al., 1995, 2003; Döbereiner, 1996). A reduction of N fertilizer input by this amount can increase energy ratios (output/input) from 4.53 to as much as 5.79 (Boddey, 1995). Implementation and improvement of plant-microbe systems such as the sugarcane-endophyte

system has substantially improved the energetics and economics of the sugarcane-to-ethanol production system in Brazil (Boddey, 1995). A similar grass-microbe system, requiring minimal N fertilizer use, and ideal for growth under temperate climates would be very desirable.

Recently, we have isolated several strains of PGPR from the rhizomes of SG (Chapter 4, Section 4.3.2). We hypothesized that because SG produces consistently high yields with minimal to no N fertilizer input, SG could be obtaining part of its required N from associative NFB. We sampled from a field site that was once a variety-for-biomass trial, but was abandoned when the trial ended in 2000 (Mehdi et al., 2000). Since then, the field has not received any fertilizer or other managment inputs. The eight bacterial strains that we isolated from 'Cave-In-Rock' rhizomes were identified, from 16S rRNA gene sequences, to be a strain of *Paenibacillus polymyxa*, two strains of *Pseudomonas* sp., two strains of *Rahnella* sp., and three strains of *Serratia* sp. (Chapter 4, Section 4.4.5). All strains showed moderate to high capacities for producing indole-3-acetic acid (IAA) like substances, four of the eight isolates were able to solubilize phosphate, and *P. polymyxa* was confirmed to possess the *nifH* gene, a marker for N_2-fixation capabilities (Chapter 4, Section 4.4.5). Moreover, significant plant growth promotion was observed as inoculation of SG seedlings with the grouped isolates yielded consistently enhanced plant biomass as compared to the uninoculated control plants under growth chamber conditions (Chapter 4, Section 4.5). Improved plant growth by NFB that also possess P solubilization and IAA production capabilities, such as *P. polymyxa* and species of *Pseudomonas* and *Herbaspirillum*, has also been reported for other plant species (Çakmakçi et al., 2006; Islam et al., 2009; Lal and Tabacchioni, 2009). However, to our knowledge, no study has been undertaken regarding PGPR inoculation effects on SG under field conditions.

Our objective, therefore, was to test these eight isolates in a mixed PGPR inoc-

ulation on SG seeds to determine whether the positive plant growth effects observed under controlled conditions could be replicated under field conditions. Further, we were interested in determining the effects of PGPR inoculation on the N dynamics of SG and whether a response to the inoculation could be observed as early as the year of seeding, as this time period is critical for ensuring the long-term productivity and success of a SG stand. The effect of a mixed PGPR inoculum on yield and yield components in the establishment year was discussed in Chapter 5, Section 5.4.1. In this report, we summarize the inoculum effects on the N dynamics of SG, in particular the effects on N concentration, content, translocation, cycling and fertilizer recovery. An estimate of total N contributed through PGPR inoculation was also calculated through N balance comparisons. The effect of PGPR inoculation on shoot nutrient concentrations and contents were also investigated.

6.3 Materials and Methods

6.3.1 Field design

Field experiments were conducted in Ste-Anne-de-Bellevue, Québec, Canada (45°28'N 73°45'W) in 2010 on three soil types, a Chateauguay clay loam (Field 1), a Bearbrook clay (Field 2) and a Chicot fine sandy loam (Field 3). Detailed site descriptions and establishment as well as management methods have been described previously (Chapter 5, Table 5.1). Switchgrass cultivar, 'Cave-In-Rock', was seeded at a rate of 10 kg ha^{-1} (Mehdi et al., 2000; Parrish and Fike, 2005). The factors tested in the field trials were the bacterial treatment, either uninoculated control or the mixed bacterial culture, and an N fertilizer treatment, either 0 or 100 kg N ha^{-1} (27:0:0%, N:P:K). The experiment was organized following a completely randomized design with four replicate plots for each field.

6.3.2 Seed inoculation

Detailed culture protocol, and inoculation methods were described previously (Chapter 5, Section 5.3.2). Eight bacterial strains, isolated from the rhizomes of SG (cv. 'Cave-In-Rock'), were inoculated by seed coating with peat (sieved through 500 μm, rate of 8 g peat per kg of seed), 24 h before seeding in the field (Dobbelaere et al., 2001; Lupwayi et al., 2005). Each isolate was individually cultured in sterile Luria Bertani (LB) broth at 30°C for 48 h with shaking. One mL of each strain (diluted to ensure the colony forming units per mL was in the range of 10^8 to 10^9) was added together and brought to 10 mL with sterile ddH_2O, to form one culture of mixed bacterial inoculant. The mixed inoculant was added to SG seeds (rate of 140 mL inoculant per kg seed), vortex mixed, and allowed to sit at room temperature for 24 h, then air dried (approximately 1 h) in a laminar flow hood, prior to seeding in the field. Control plots were inoculated in the same manner with an equivalent amount of sterile LB media and were seeded first in the field to minimize any risk of contamination with bacterial inoculants. All isolates tested in the field experiments were verified to be pure cultures.

6.3.3 Harvest, data collection, and analyses

Tillers were sampled twice during the growing season, once during anthesis (August, 2010) and a second sampling at senescence (October, 2010), in order to determine plant N content during these two developmental stages. Fifty tillers were randomly sampled per plot, per field site by one of two persons/harvesters on August 24th and 26th, 2010. The fresh and dry weights were taken before and after oven drying at 65°C to a constant weight (at least 72 h), then ground. Approximately 0.5 to 2 mg of the ground plant samples were used for continuous-flow isotope ratio mass spectrometry analysis at the Stable Isotopes in Nature Laboratory (SINLAB), University of New

Brunswick, using a Carlo Erba NC 2500 interfaced with a Thermo-Finnigan Delta Plus Mass Spectrometer, for N analyses. During the fall harvest, tillers were cut to a 5 cm stubble height in two, one-metre lengths of row randomly selected from each plot on October 26, 2010 for all field sites (Chapter 5, Section 5.3.3). The tillers were counted to determine stand density. The subsamples were dried to a constant weight at 65°C (at least 72 h) then weighed. Yield was estimated from this subsample. Soil samples (five per plot) were taken using an auger (15-20 cm depth, 10 cm diameter), combined to form one composite sample, air dried, ground and sifted through a 10 μm sieve. Plant (mg N g^{-1} tiller) (dry material ground to 0.5 mm particle size) and soil N concentrations (mg N g^{-1} soil) were determined by wet oxidation in sulphuric acid and hydrogen peroxide (Parkinson and Allen, 1975), and measured using a Lachat QuickChem autoanalyzer (FIA+8000, Lachat Instruments, Milwaukee, Wisconsin). Plant P, K, Mg, and Ca concentrations were also determined by wet oxidation in sulphuric acid and hydrogen peroxide, and determined with a flame atomic absorption spectrophotometer (2380, Perkin-Elmer, Waltham, Massachusetts).

Nitrogen content/removal (g N m^{-2}) of harvested biomass was calculated by multiplying the percent N concentration by the dry biomass yield (kg m^{-2}). Per tiller N content (mg N $tiller^{-1}$) was calculated by multiplying the percent N concentration by the per tiller DW (g $tiller^{-1}$) sampled at anthesis or senescence. Fertilizer recovery, expressed as a percentage, was calculated as the difference between N removal from the uninoculated, unfertilized treatment and N removal from either the uninoculated or inoculated fertilized treatments, divided by the amount of fertilizer applied. The amount of N translocated below-ground was estimated by determining the difference between shoot N concentration at anthesis and senescence, and represents the amount of N translocated (mg N g^{-1} tiller) from shoots to rhizomes and roots upon senescence. Shoot P, K, Mg, and Ca content (g m^{-2}) was calculated by multiplying the

percent of each nutrient with the dry biomass yield.

6.3.4 Statistical analyses

Two-way analyses of variance (ANOVA) were performed using the R statistical software package (R: A Language and Environment for Statistical Computing, 2010). Fisher F-tests were performed for pooled data to verify the assumption of equal variances among the sample populations. T-tests were performed for the comparison of means. All the data were verified for the assumptions of normality and homoscedasticity. When the assumptions were not met, nonparametric Kruskal-Wallis analyses were performed. Differences were considered significant when their probability by chance alone was less than 0.05. Biologically interesting numerical differences with probabilities between 0.05 and 0.1 are also presented. When this occurs, the P values are given.

6.4 Results and Discussion

6.4.1 Nitrogen concentration, translocation, and cycling

In this report, we present establishment year results related to the effects of inoculation of a mixed PGPR on SG growth and productivity under field conditions. The agronomic responses of bacterial inoculation on yield and yield components were discussed in Chapter 5, Section 5.4.1. Here, we provide results pertaining to the effects of bacterial inoculation on the N dynamics of SG growth, including concentration and content, translocation, cycling, and fertilizer recovery of inoculated SG plants. The effect of PGPR on shoot nutrient concentrations and contents were also examined.

We evaluated the effect of bacterial inoculation on the N concentration in SG at

various growth stages (Figure 6.1). Switchgrass tillers were sampled twice during the growing season, once during anthesis (August 2010) and a second time at harvest (October 2010) when plants were senescing. During anthesis, N accumulation is at its maximum, in part, due to the large N requirement by plants for panicle and seed development (Clark, 1977; Heaton et al., 2009; Parrish and Fike, 2005). As day length decreases and temperature declines, SG plants undergo senescence, and the dying above-ground tissues cycle available N reserves to below-ground structures (roots/rhizomes) for winter storage (Clark, 1977; Heaton et al., 2009; Parrish and Fike, 2005). Anthesis, therefore, is the time when above-ground N concentration is likely to be highest, while senescence is the time when it is generally lowest. Senescence is also the time when above-ground biomass is largest.

The ANOVA results indicated that the tissue N concentrations were greater in the inoculated than uninoculated plants during anthesis ($P < 0.01$; Figure 6.1A), but not at senescence (Figure 6.1B). Fertilizer addition affected N concentration during anthesis ($P < 0.001$; Figure 6.1A), but interestingly, this effect was not observed at senescence, as unfertilized and fertilized plants had comparable tissue N concentrations (10 - 11 mg N g^{-1}; Figure 6.1B). Although N translocation was not directly measured, a comparison of the N concentration between the two sampling times suggest that inoculated plants translocated more N ($P < 0.01$) to the rhizomes than uninoculated plants, for both unfertilized and fertilized SG plants (Figure 6.1C).

By anthesis, SG plants inoculated with the mixed PGPR culture had accumulated more N in above-ground tissue than the uninoculated plants. However, by senescence no differences were observed between either uninoculated and inoculated plants, or between unfertilized versus fertilized plants. This would explain the disparity between N concentrations observed at different harvest times and account for the greater amount of N translocated. In preparation for winter, it is likely that senescing above-ground

tissues cycled whatever N was available to rhizomes, regardless of the initial amount. Therefore, inoculated SG plants were able to re-mobilize greater amounts of N to rhizomes and roots than uninoculated plants, resulting in the similar concentrations of N observed in the inoculated and uninoculated plant tissues at senescence. In this way, more of the N in inoculated plants was mobilized out of above-ground tissues, presumably to below-ground tissues, thereby establishing larger N reserves for the following spring. As discussed previously, these results are consistent with nutrient cycling studies in SG that also reported differences in N concentrations during different growth stages (Clark, 1977; Heaton et al., 2009; Lemus et al., 2008). The resorption physiology of biofuel crops has practical significance because a lower amount of N would make a cleaner feedstock for combustion or for chemical conversion and would require less N fertilization the following spring (Heaton et al., 2009; Lemus et al., 2008; Lewandowski et al., 2003).

In our study, a mixed PGPR culture, containing eight bacterial isolates capable of N_2-fixation, P solubilization, and IAA production (Chapter 4, Section 4.4.5), was inoculated onto SG seeds. While the mixed inoculum was composed of bacteria with several different plant growth promoting mechanisms, we concluded that under controlled-environment conditions, the positive growth effects that we observed were largely due to N_2-fixation, as the only limiting resource was N and not a lack of other nutrients or water (Chapter 4, Section 4.5). However, under the more complex conditions of the field environment, it is more likely that a combination of BNF, P solubilization and production of IAA-like substances contributed to the positive growth effects, either by providing the plants with access to more N, other nutrients and water, and/or by stimulating phytohormone production within the plant itself as a response to colonization (Chapter 4, Section 4.5). All of the isolates were able to produce IAA-like substances, which could have stimulated root elongation and

therefore, increased the root plus rhizosphere below-ground system of inoculated SG plants (Dobbelaere et al., 2003; Glick, 1995). Further, the ongoing periods of nutrient cycling within SG may mean more root exudates are released into the rhizosphere. An increase in root exudates may signal and promote bacterial colonization by the mixed PGPR inoculum, which in turn, would increase their proliferation and plant growth effects (Bais et al., 2006; Walker et al., 2003). Indeed, colonization of legumes with rhizobia is initiated via signals, such as flavonoids, exuded from plant roots into the rhizosphere and Nod factors secreted by the rhizobia response (Broughton et al., 2003; Gray and Smith, 2005). A similar response by PGPR, initiated by an increase in root exudates, could have occurred.

6.4.2 Nitrogen removal at harvest and fertilizer recovery

Inoculation of SG seeds with a mixed PGPR culture resulted in greater N content in harvested biomass, or essentially the amount of N removed ($P < 0.01$) at harvest, and more fertilizer N recovered ($P < 0.05$) by inoculated than uninoculated plants (Table 6.1). The amount of N removed in biomass in the inoculated SG plants were 1.6 and 1.4 times (approximately 60 and 36%) more than the amount removed in the uninoculated, unfertilized and fertilized plants, respectively (Table 6.1). The greater amount of N removal we observed in our study was due to the greater yields ($P < 0.01$; Table 6.1) of inoculated than uninoculated plants, rather than differences in N concentrations (Figure 6.1B), which did not differ among treatments by the end of the growing season. The results described in Figure 6.1 suggest a re-mobilization of N as the SG plants matured and is a reflection of the concentration of N and not necessarily total N, as is the case regarding the amount of N removal in harvested biomass. While the N concentrations were not different among treatments, both PGPR inoculation and N fertilizer addition increased the N content at harvest by increasing the biomass yield (by over 40%), in the case of PGPR (Chapter 5, Section 5.4.1). This 40%

increase persisted in the presence of N fertilizer, at least at the 100 kg N ha^{-1} rate.

In addition, the mixed PGPR culture included the N_2-fixing bacterium *Paenibacillus polymyxa*. It is possible that the higher amount of N within inoculated SG tissues was, in part, provided by *P. polymyxa* to the roots upon its death. It has been speculated that, contrary to symbiotic N_2-fixation between legumes and *Rhizobia* sp., where fixed N produced by living bacteria are actively released in the nodules, associative NFB release fixed N to the host plant after bacterial cell death and decay of its biomass (Dobbelaere et al., 2003; James, 2000; Mylona et al., 1995), however, we have no data related to this. *P. polymyxa* has also been reported to increase the aggregation of root-adhering soil, enhance production of the phytohormone cytokinin, and act as a biocontrol against other deleterious soil micro-organisms (Dobbelaere et al., 2003; Lal and Tabacchioni, 2009; Timmusk et al., 1999, 2005). An improvement in soil aggregation may help to regulate the water concentration of the rhizosphere, thus not only improving water acquisition but also enhancing microbial interactions by maintaining soil moisture concentration (Dobbelaere et al., 2003; Lupwayi et al., 2005).

Fertilizer recovery was lower than anticipated at 8 to 17% (Table 6.1). Fertilizer recovery for annual crops generally ranges from 30 to 70% during the year of application, with 10 to 40% incorporated into organic matter, 5 to 10% lost by leaching, and 10 to 30% lost in gaseous form (Bransby et al., 1998). For perennial crops like SG, fertilizer recovery rates can vary depending on the amount of N fertilizer used, cultivar and environment. Stout and Jung (1995) reported fertilizer recovery ranging from 19 to 53 % for first year SG growth on four different soil types fertilized with 84 kg N ha^{-1}. Lemus et al. (2008) also reported fertilizer recovery for SG at 90 and 180 kg N ha^{-1} to be low (10 to 25% per year) in the first year. The authors speculated that the amount of fertilizer N not recovered might represent N losses through leaching or volatilization, or that some portion of it may have become sequestered

in below-ground pools (roots, microbial biomass, and/or soil organic matter) (Lemus et al., 2008). It is probable that the majority of N lost in our study was due to these various transformations. As well, root to shoot ratios are generally much larger for perennial than annual crops (often 50% for perennials and sometimes as much as 10% for annuals), which could account for part of the loss (Bolinder et al., 2002; Gastal and Lemaire, 2002; Wilsey and Polley, 2006). Further, in our study, N application was split, with one third applied prior to seeding and the remainder applied as much as ten weeks later. Generally, N is applied in full prior to seeding (Lemus et al., 2008; Muir et al., 2001; Stout and Jung, 1995; Vogel et al., 2002), or applied as a split application under a two-cut management system (Fike et al., 2006a,b). It is well known that within *Rhizobium* - legume systems, the application of N fertilizer reduces or even eliminates colonization (Hardarson and Atkins, 2003; Lupwayi et al., 2005). For this reason, we chose to wait and apply some of the fertilizer N at a later time in order to allow the mixed PGPR inoculum the opportunity to colonize with the plant roots and rhizomes. Thus, SG plants did not have the full growing season within which to utilize all of the available N fertilizer. However, in our study, the percentage of fertilizer recovered ($P < 0.05$) was almost 1.6 times greater for inoculated (13%) than uninoculated (8%) plants (Table 6.1).

The results suggest that inoculation of SG seeds with the mixed PGPR culture produced greater biomass yields at fall harvest due to more efficient provision of N by below-ground to above-ground tissues during the growing season. Rhizome, root and soil N reserves would still be maintained for the following spring by inoculated SG plants by re-mobilizing the N to below-ground tissues prior to a killing frost. Fertilizer recovery is expected to be greater for inoculated than uninoculated SG plants, thus ensuring the 40% yield increase observed under N-limited conditions persists with N fertilization, at least at the 100 kg N ha^{-1} rate evaluated in this work.

6.4.3 Nitrogen balance of switchgrass-microbe systems: Improved access to non-fertilizer N

We evaluated the effect of PGPR inoculation and fertilizer N addition on total N balance, calculated as the difference between total N content of SG tillers sampled at anthesis and senescence, and also through the N content of biomass on a per unit area basis, making a comparison between SG that received PGPR inoculation or not (Tables 6.2 and 6.3). At anthesis, total N content per tiller was affected by both the inoculation and N fertilizer addition (Table 6.2). As expected, the N contribution of fertilizer N alone led to a positive N balance of 7.6 mg N tiller^{-1} ($P < 0.001$) in the uninoculated treatment (comparison of uninoculated, fertilized versus uninoculated, unfertilized plants). A positive N balance was also observed when SG was inoculated with PGPR ($P < 0.05$); about 4.0 mg N tiller^{-1} more N (a 33% increase), from additional non-fertilizer N (ANFN), was present in inoculated, unfertilized than uninoculated, unfertilized SG plants. Interestingly, when fertilizer N was added, inoculation with PGPR resulted in a numerical N balance increase of 3.4 mg N tiller^{-1} between inoculated and uninoculated SG plants; however, this N balance increase was not statistically significant ($P = 0.17$). A comparison between fertilizer treatments for inoculated plants showed an effect of PGPR inoculation ($P < 0.01$) on N balance; a N contribution of 7.0 mg N tiller^{-1}, likely due to a combination of fertilizer N and ANFN. The total N balance, due to both fertilizer application and inoculation with PGPR was large; inoculated, fertilized SG plants had 11.0 mg N tiller^{-1} more N ($P < 0.001$) than unfertilized plants. This additional N contribution most likely resulted from a combination of ANFN and fertilizer N. By senescence, a significant effect of fertilizer addition ($P < 0.05$) on N content per tiller was observed (Table 6.2). However, no effect on N content per tiller was observed by PGPR inoculation, at senescence, regardless of the presence or absence of fertilizer addition.

The total N content of harvested biomass, on a per unit area basis, at senescence, was also affected by both the inoculation and fertilizer N addition (Table 6.3). An effect of fertilizer N addition ($P < 0.001$) on N balance of the harvested biomass of uninoculated plants was observed, with a N contribution of 7.6 mg N m^{-2} more N (similar to the results described for per tiller biomass at anthesis; Table 6.2) detected in fertilized than unfertilized SG plants. By comparison, the N balance of PGPR inoculated plants in the presence of fertilizer N was larger, at 9.2 mg N m^{-2} (comparison of inoculated and fertilized plant versus inoculated and unfertilized plants; $P < 0.001$). It may be that enhancement of the below-ground system (roots plus rhizomes) increased uptake of both soil N and fertilizer N. Alternatively, increases in below-ground SG structures due to the combined effects of both PGPR inoculation and N fertilizer addition could have resulted in a greater increase in uptake of ANFN than the addition of PGPR alone.

PGPR inoculation increased the N balance of inoculated versus uninoculated SG plants, both in the presence and absence of added fertilizer N (Table 6.3). Without fertilizer N input, PGPR inoculation resulted in a positive N balance ($P = 0.055$) of 2.7 mg N m^{-2} (comparison of inoculated, unfertilized plants versus uninoculated, unfertilized plants); the ANFN could be due to increased uptake of soil N, as a result of enhanced root and rhizome systems, but BNF is also possible. Surprisingly, an increase in N balance also occurred as a result of PGPR inoculation ($P < 0.001$) in the presence of fertilizer N. The N contribution, probably from a combination of ANFN and fertilizer N, was 11.9 mg N m^{-2}. It is remarkable that the increase due to inoculation occurred in both the absence and presence of N fertilizer as BNF is generally inhibited by the presence of mineral nitrogen, which could suggest that ANFN was due to improved soil N uptake rather than BNF (Hardarson and Atkins, 2003; Lupwayi et al., 2005). However, there have been reports indicating that the nitrogen

fixing activities of sugarcane associated NFB are not inhibited by the presence of mineral N (Baldani et al., 2002; Baldani and Baldani, 2005; Boddey, 1995; Boddey et al., 2003). In this study, the increase due to inoculation was larger when fertilizer N was added (4.3 mg N m^{-2}; P = 0.054) than in the absence of fertilizer N (2.7 mg N m^{-2}; P = 0.055). The difference between the two may have been due to improved access to fertilizer and ANFN, although increased soil N uptake as a result of enhanced root and rhizome growth (perhaps due to production of auxin-like compounds by the inoculated PGPR) may also have increased uptake of fertilizer N. This will have to be resolved through future research.

The comparison of N content in tillers at two sampling times (anthesis and senescence) and in harvested yield biomass suggests that the differences indicated through final harvest of above-ground biomass are probably underestimates of the total effect of PGPR inoculation. As expected, the N obtained from fertilizer addition increased the N content and improved plant growth at anthesis but not at senescence. Similarly, at senescence, the bacterial treatment did not affect the N content of individual tillers. This is almost certainly due to translocation of N from above- to below-ground tissues as the plants senesced. These conclusions stemmed from observations that the N concentration in tillers at anthesis was greater for PGPR inoculated than uninoculated SG plants, but there was no difference at senescence. Further, the N concentrations were lower at senescence than anthesis (16.4 versus 10.3 mg N g^{-1} tiller for anthesis and senescence, respectively, without fertilizer input, and 24.9 versus 11.1 mg N g^{-1} tiller for anthesis and senescence, respectively, with fertilizer input). In fact, by senescence there was no difference in tissue N concentration between fertilized and unfertilized plants for PGPR inoculated (11.1 versus 10.3 mg N g^{-1} tiller for fertilized and unfertilized, respectively) and uninoculated (11.2 versus 10.6 mg N g^{-1} tiller for fertilized and unfertilized, respectively) treatments. These observations strongly suggest that

N was moved into below-ground tissues in the run-up to senescence. Therefore, the total estimated amount of ANFN made available by PGPR inoculation was most certainly greater overall than the amount estimated for harvested above-ground biomass at senescence. Given the differences in the N concentration of PGPR inoculated and uninoculated plants at anthesis and the greater N concentrations and contents per tiller at anthesis it seems very probable that the total improvement in access to ANFN due to PGPR inoculation is greater than the value estimated at senescence. The total amount of ANFN at anthesis will have to be determined through future research activities; this will allow an estimate of the full enhancement of SG access to ANFN due to inoculation with the PGPR tested.

Inoculation with the PGPR culture evaluated here contributes N to SG plants thereby meeting the N required to support its growth. The results suggest that the bacteria included in the PGPR inoculant provided plants with ANFN possibly from BNF and, in some cases, may also have improved access to fertilizer N. Inoculation may also have improved SG to other soil nutrients, due to a larger root and rhizome system or better nutrient acquisition due to greater availability of nutrients, such as through improved solubilization of soil P.

6.4.4 Other nutrients

Shoot concentrations of nutrients other than N (P, K, Mg, and Ca) were not affected by inoculation with the PGPR culture, but were lower for P ($P < 0.01$) and Ca ($P = 0.06$), and greater for K ($P < 0.001$) and Mg ($P < 0.05$) when N fertilizer was applied (Table 6.4). In contrast, the contents of P ($P = 0.06$), K ($P < 0.05$), and Mg ($P = 0.06$), but not Ca, were affected by the bacterial treatment, as inoculated SG plants had greater amounts of P, K, and Mg at harvest than uninoculated plants. The Ca content values tended to be higher in inoculated than uninoculated plants;

the lack of statistical differences observed was most likely due to high standard errors. The fertilizer treatment also affected the contents of nutrients, as greater amounts of each nutrient were observed in the fertilized SG plants.

Similar to N concentrations within tissues at fall harvest (senescence), differences in other nutrient concentrations were not detected among inoculated and uninoculated plants. However, the greater observed contents of other nutrients were largely a reflection of the greater biomass production at harvest, as was the case with the amount of N removal in biomass. Switchgrass plants likely sought out the extra nutrients in response to the higher growth demand. However, it is possible that inoculation by the mixed PGPR culture aided plants in acquiring these nutrients, as the inoculum was comprised of PGPR capable of BNF, solubilizing P, and producing auxins. In particular, the P content was 1.5 times greater in the inoculated than uninoculated plants, suggesting either greater P content as a function of plant need and/or greater P content because the inoculum helped provide more P through a greater solubilization of P in soil near the root/rhizome system. The results indicate that inoculation of SG plants by the mixed PGPR culture resulted in greater yields, which may have helped to increase the below-ground root and rhizome system. This enhanced below-ground system would allow plants to acquire greater amounts of N and other nutrients from the soil, thereby providing for greater above-ground growth. It is clear that the PGPR inoculum affected SG plant growth and the response of SG to PGPR warrants further investigation into these specific mechanisms.

6.5 Conclusions

Switchgrass is a perennial grass often reported to produce high yields with little fertilizer N input. This observation led us to hypothesize that SG is obtaining part of

its N requirement through BNF by associative N_2-fixing bacteria and other PGPR. We investigated the effects of a mixed PGPR culture, isolated from SG rhizomes, on the N dynamics and productivity of SG under field conditions within the establishment year. To our knowledge, this is the first field investigation of bacterial inoculation on SG.

The results suggest that inoculation with a mixed PGPR provided roots and rhizomes access to ANFN, possibly through a combination of BNF, P solubilization and auxin production with the latter two allowing improved growth (including root growth) and therefore better access to soil N and other nutrients (such as P, K, and Mg). PGPR inoculation affected the N balance of SG tillers sampled at anthesis and senescence, demonstrating the movement of N out of above-ground biomass at the end of the growing season. Greater concentrations of N were observed in the inoculated plants during the growing period, allowing for higher tiller density (Chapter 5, Section 5.4.1), which led to greater biomass yields at fall harvest. Yet, harvest results indicated that N concentration did not differ between inoculated and uninoculated plants, suggesting that inoculated plants were able to translocate more N to roots and rhizomes during senescence. Therefore, our estimate of ANFN contribution in the harvested biomass (per unit area) due to PGPR inoculation, at senescence, is likely an under-estimate of the actual contribution of ANFN due to PGPR inoculation, as biomass yield measurements at anthesis were not investigated in this study. This contribution to ANFN due to PGPR inoculation at anthesis, and how much of this N contribution is from BNF contributions, remains to be determined. Interestingly, the growth stimulation and increased access to ANFN following PGPR inoculation was not inhibited in the presence of N fertilizer. This suggests that crop yields could be improved by inoculation with this PGPR mixture even in the presence of fertilizer N. Our hypothesis of a combined positive PGPR effect on plant growth is supported

by these findings, and it seems that at least part of this growth response is related to improved access to N. Overall, bacterial inoculation of SG with PGPR enhanced NUE and could be an effective strategy to increase the establishment of this crop, especially under a low-input system. A well established field would reduce the need for re-seeding the following year, and keep establishment costs at a minimum.

6.6 Tables and Figures

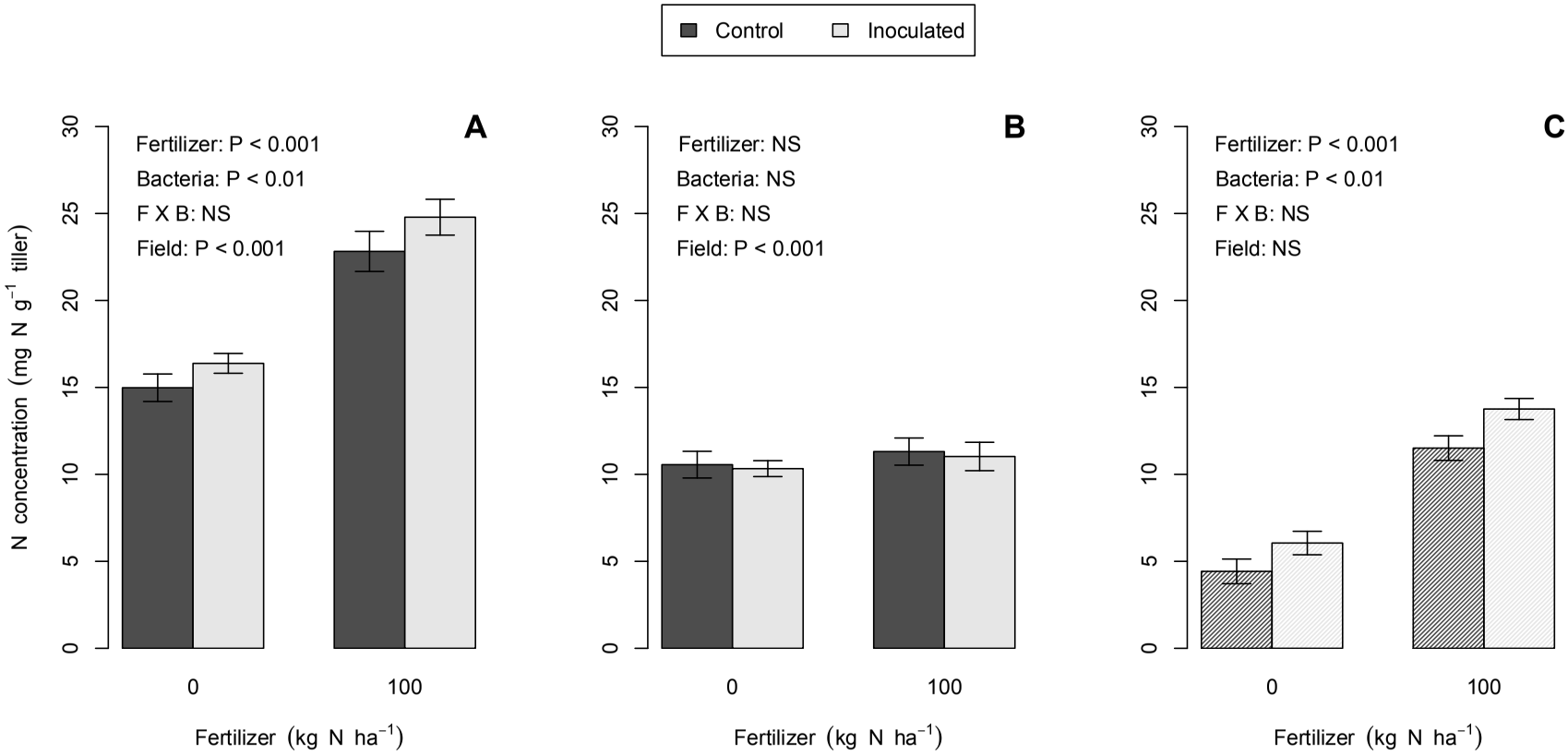

Figure 6.1 Mean (n = 12) N concentration (mg N g^{-1} tiller) with standard error (SE) bars of unfertilized (0 kg N ha^{-1}) and fertilized (100 kg N ha^{-1}) switchgrass plants inoculated, or not (control), with a mixed rhizobacterial culture sampled during (**A**) anthesis (August, 2010), and (**B**) senescence (October, 2010). The amount of N translocated (**C**) refers to the difference between shoot N concentration at anthesis and senescence, and represents the amount of N translocated from shoots to below-ground structures (rhizomes and roots) upon senescence. Means represent pooled data from fields 1, 2, and 3. ANOVA results are also presented. F x B refers to 'Fertilizer x Bacteria' interaction.

Table 6.1 Mean (n = 12) yield (kg m^{-2}), N removal (g N m^{-2}), and fertilizer recovery (%) with standard errors (SEs) of unfertilized (0 kg N ha^{-1}) and fertilized (100 kg N ha^{-1}) switchgrass plants inoculated, or not (control), with a mixed rhizobacterial culture harvested at senescence (October, 2010). Means represent pooled data from fields 1, 2, and 3. ANOVA results are also presented.

Fertilizer	Bacteria	Yield[1]	N removal	Fertilizer recovery
0 N	Control	0.30 (0.04)	4.5 (0.6)	NA[2]
	Inoculated	0.43 (0.06)	7.2 (1.1)	NA
100 N	Control	0.55 (0.05)	12.1 (1.0)	8.0 (0.8)
	Inoculated	0.67 (0.08)	16.4 (1.8)	12.7 (1.6)
ANOVA				
Fertilizer		***	***	NA
Bacteria		**	**	*
F X B[3]		NS	NS	NA
Block		NS	NS	NS
Harvester		NS	NS	NS
Field		***	**	*

$***$ $P < 0.001$; $**$ $P < 0.01$; $*$ $P < 0.05$; NS, not significant

[1] Data from Chapter 5, Section 5.6, Figure 5.1

[2] NA = not applicable

[3] F x B refers to 'Fertilizer x Bacteria' interaction

Table 6.2 Effect of a mixed rhizobacterial inoculum and fertilizer N addition on the total N balance (mg N tiller^{-1}) of switchgrass tillers harvested at anthesis (August, 2010) or senescence (October, 2010). Means (n = 12), with standard errors (SEs), represent pooled data from fields 1, 2, and 3. T-Test results are also presented.

Fertilizer	Bacteria	Mean (SE)	Difference	df[1]	T-Test	N contribution
Anthesis						
0 N	Inoculated	16.0 (1.4)	4.0	20.297	$P < 0.05$	ANFN[2]
0 N	Control	12.0 (1.1)				
100 N	Control	19.6 (1.4)	7.6	20.764	$P < 0.001$	Fertilizer N
0N	Control	12.0 (1.1)				
100 N	Inoculated	23.0 (2.0)	11.0	16.878	$P < 0.001$	ANFN
0 N	Control	12.0 (1.1)				+ Fertilizer N
100 N	Inoculated	23.0 (2.0)	3.4	19.544	NS	ANFN under
100 N	Control	19.6 (1.4)				N Fertilization
100 N	Inoculated	23.0 (2.0)	7.0	20.062	$P < 0.01$	ANFN
0 N	Inoculated	16.0 (1.4)				+ Fertilizer N
Senescence						
0 N	Inoculated	9.0 (0.6)	1.2	19.965	NS	ANFN
0 N	Control	7.8 (0.6)				
100 N	Control	10.1 (0.6)	2.3	20.826	$P < 0.05$	Fertilizer N
0N	Control	7.8 (0.6)				
100 N	Inoculated	10.0 (0.7)	2.2	20.595	$P < 0.05$	ANFN
0 N	Control	7.8 (0.6)				+ Fertilizer N
100 N	Inoculated	10.0 (0.7)	0.1	20.898	NS	ANFN under
100 N	Control	10.1 (0.6)				N Fertilization
100 N	Inoculated	10.0 (0.7)	1.0	20.339	NS	ANFN
0 N	Inoculated	9.0 (0.6)				+ Fertilizer N

$***$ $P < 0.001$; $**$ $P < 0.01$; $*$ $P < 0.05$; NS, not significant

[1] df = degrees of freedom

[2] ANFN = additional non-fertilizer N

Table 6.3 Effect of a mixed rhizobacterial inoculum and fertilizer N addition on the total N balance (g N m^{-2}) of switchgrass harvested biomass per unit area at senescence (October, 2010). Means (n = 12), with standard errors (SEs), represent pooled data from fields 1, 2, and 3. T-Test results are also presented.

Fertilizer	Bacteria	Mean (SE)	Difference	df[1]	T-Test	N contribution
0 N	Inoculated	7.2 (1.1)	2.7	15.661	P = 0.055	ANFN[2]
0 N	Control	4.5 (0.6)				
100 N	Control	12.1 (1.0)	7.6	18.161	P < 0.001	Fertilizer N
0N	Control	4.5 (0.6)				
100 N	Inoculated	16.4 (1.8)	11.9	13.565	P < 0.001	ANFN
0 N	Control	4.5 (0.6)				+ Fertilizer N
100 N	Inoculated	16.4 (1.8)	4.3	17.193	P = 0.054	ANFN under
100 N	Control	12.1 (1.0)				N Fertilization
100 N	Inoculated	16.4 (1.8)	9.2	18.128	P < 0.001	ANFN
0 N	Inoculated	7.2 (1.1)				+ Fertilizer N

$***$ P < 0.001; $**$ P < 0.01; $*$ P < 0.05; NS, not significant

[1] df = degrees of freedom

[2] ANFN = additional non-fertilizer N

Table 6.4 Mean (n = 12) shoot nutrient concentration (mg g^{-1}) and content (g m^{-2}) with SEs of unfertilized (0 kg N ha^{-1}) and fertilized (100 kg N ha^{-1}) switchgrass plants inoculated or not (control) with a mixed rhizobacterial culture harvested at senescence (October, 2010). Means represent pooled data from fields 1, 2, and 3. ANOVA results are also presented. Numerical differences with probability between 0.05 and 0.1 are presented for their biological meaning.

Fertilizer	Bacteria	P		K		Mg		Ca	
		Conc[1]	Content	Conc	Content	Conc	Content	Conc	Content
O N	Control	1.96	0.56	12.50	3.86	4.11	1.21	7.15	2.12
		(0.11)	(0.06)	(0.67)	(0.59)	(0.30)	(0.19)	(0.35)	(0.27)
	Inoculated	2.04	0.85	12.58	5.47	4.29	1.79	7.49	3.24
		(0.10)	(0.18)	(0.35)	(0.78)	(0.14)	(0.26)	(0.29)	(0.49)
100 N	Control	1.76	0.99	14.46	8.17	4.79	2.63	6.94	3.90
		(0.09)	(0.13)	(0.60)	(0.90)	(0.26)	(0.26)	(0.47)	(0.53)
	Inoculated	1.64	1.06	14.86	9.95	4.66	2.98	6.28	4.07
		(0.08)	(0.14)	(0.42)	(1.37)	(0.25)	(0.37)	(0.31)	(0.53)
ANOVA									
Fertilizer		**	***	***	***	*	***	0.06	**
Bacteria		NS	0.06	NS	*	NS	0.06	NS	NS
B X F		NS	NS	NS	NS	NS	NS	NS	NS
Block		NS	NS	NS	NS	NS	NS	NS	NS
Harvester		0.07	NS	*	*	NS	NS	NS	NS
Field		NS	***	NS	***	NS	***	NS	***

$***$ $P < 0.001$; $**$ $P < 0.01$; $*$ $P < 0.05$; NS, not significant

[1] Abbreviations: Conc = Concentration

Preface to Chapters 7 and 8

In the previous chapters I reviewed literature on switchgrass as a purpose-grown, bioenergy feedstock, described the lack of information regarding plant-microbe interactions with SG, and illustrated how bridging this knowledge gap could improve current SG feedstock production. This was followed by chapters presenting the rationale for my study, along with the research questions and overall hypothesis, and chapters now being submitted for publication and therefore disseminating the results of my efforts to address the existing knowledge gaps (4 to 6). In this next chapter (7), I revisit the rationale for the study by describing key findings and other elements that highlight the interest and novelty of the research. I also provide explanation regarding some of the decision processes underlying the research progression, that for reasons of brevity, continuity, and relevance were not discussed in the early parts of the thesis. I summarize key findings, present final conclusions, and identify priorities for future research. In the last chapter (8), I describe the contributions of knowledge of my work.

Chapter 7

Summary, Conclusion and Future Research

In the late 1950s, Brazilian researchers began investigations on diazotrophic interactions with native tropical grasses growing alongside the highway of Km 47 near the agriculture research station, Embrapa Agrobiologia, in Rio de Janeiro state. The foundation for these investigations stemmed from observations made by scientists, such as J. Döbereiner, that these tropical grasses produced high biomasses without any N fertilizer input. This observation, and the curiosity to know more, initiated decades-long research into beneficial plant-microbe interactions with agronomically important bioenergy crops, like sugarcane. Global events, such as the 1970s oil crisis, which sparked concerns over energy independence and the inevitability that fossil fuels would someday be depleted, along with strong government and industrial funding propelled the research by these scientists. Plant growth promoting rhizobacteria, such as *Gluconacetobacter diazotrophicus*, capable of biological N_2-fixation and of contributing substantial amounts of fixed N to the host plant, were isolated along with several other diazotrophic species of the genera *Azospirillum* and *Herbaspirillum*. Over 60 years of research has been conducted by Brazilian and other scientists around the

world on these particular microbes and their effects on plant growth.

This doctoral thesis evolved in a manner similar to the Brazilian sugarcane-microbe example. Research on SG as a bioenergy feedstock by the HECP had been ongoing since the 1980s. However, many people had never heard of SG until United States President George Bush's State of the Union Address in January 2006. The release of the IPCC's Fourth Assessment Report by the United Nations and award-winning documentaries such as *An Inconvenient Truth* and *The 11th Hour* propelled the issues of global warming into the public eye. This renewed concerns about the impact of fossil fuel consumption on climate change. At the same time, the price of fossil fuel had risen substantially and the inevitability of fossil fuel depletion seemed more imminent than in the 1970s. Government and industrial funding for improving bioenergy feedstock production and conversion to ethanol was plentiful. In January 2007 I began my PhD in this environment. Similar to the observations made by Döbereiner about tropical grasses, we had observed that an abandoned SG variety-for-biomass trial still continued to produce reasonable yields many years after the cessation of N fertilizer or other management inputs. While the parallels between the sugarcane-microbe and switchgrass-microbe models had not yet been made, we arrived at a question and hypothesis that resembled that of Döbereiner et al. for sugarcane: *Could SG be getting N to support its biomass growth from biological N_2-fixation?*

To answer this principle question, three underlying questions were considered:

1. Does switchgrass associate with plant growth promoting rhizobacteria?

2. Can inoculation with PGPR increase the growth and productivity of SG for biofuel production under a low-N input system?

3. Can a plant-microbe production system, as in the example with sugarcane, be replicated with SG?

Thus, this doctoral research was organized in such a way that each set of experiments was successive to the other. Experiments, each with specific objectives, were conducted to answer the first question, before the second and third research questions could be addressed.

7.1 Summary of results

In chapter 4, we set out to isolate potential PGPR from the rhizomes and rhizosphere of SG from the abandoned SG variety-for-biomass field site. Eleven upland varieties had been planted in a randomized complete block design with four replicates. I sampled (in duplicate) the rhizomes and rhizosphere of each replicate of each variety. Therefore, 88 samples of each of rhizome and soil rhizosphere were taken. From these, over 300 and 500 rhizome and rhizosphere isolates, respectively, were selected from N-free solidified plates. The choice was then made to focus on the isolates from the rhizomes rather than the rhizosphere, as we hypothesized that if SG was obtaining its N from BNF, endophytic bacteria were more likely able to contribute substantial amounts to the host plant than free-living bacteria residing in the rhizosphere. Bacterial screening assays conducted on SG seedlings under growth chamber conditions yielded several sets of isolates that consistently enhanced plant growth as compared to the uninoculated control plants. Enhanced plant growth was also observed with single isolate inoculated SG seedlings. Therefore, several options presented themselves with regards to which isolates(s) to choose from for further field testing. Initially we were interested in choosing one or two strains of PGPR, however, we felt it prudent to choose the isolates comprising the V1 batch (isolated from 'Cave-In-Rock') because of its consistent performance in the various bioassays. Identification of these isolates based on 16S rRNA gene sequences revealed that the V1 batch comprised a strain of *Paenibacillus polymyxa*, a bacterium capable of N_2-fixation, two strains of

Pseudomonas, two strains of *Serratia*, and three strains of *Rahnella* spp. Many of these isolates were also shown to solubilize P and all isolates produced IAA-like substances, properties that could prove beneficial under field conditions. It was concluded in chapter 4 that plant growth promotion was probably a result of BNF, as plants were grown without exogenous N fertilization and inoculation resulted in substantial increases in plant growth in comparison with uninoculated control plants. Further strengthening this conclusion, no dilution effect (reduction in N concentration) was observed between inoculated and uninoculated plants, in spite of the larger biomass of inoculated plants. This result suggested strongly that inoculated plants were able to grow better under N limitation conditions because of additional fixed N from the bacterial symbiont(s).

Field experiments were set up at the Emile A. Lods Agronomy Research Centre in Sainte-Anne-de-Bellevue. The results of this field investigation were presented in chapters 5 and 6, with the inoculation effects on yield and yield components presented in chapter 5, and the inoculation effects on N dynamics presented in chapter 6. Once again, several decision processes were followed, many of which were not discussed in the earlier sections of this thesis, in order to maintain flow and unity. As stated earlier, prior to this research, no other study had been reported on PGPR effects on SG under controlled or field conditions. Thus, questions arose regarding how best to inoculate SG for field experimentation, how much inoculant to use, whether follow-up inoculation should be performed, etc. Originally, we had intended to perform a spray inoculation onto existing plots of SG. These plots were established in the summer of 2007, and spray inoculation was scheduled to take place during the summer of 2009, after the field was firmly established. Sampling and isolation of bacteria from the rhizomes and rhizosphere occurred during the summer of 2008. However, it was suggested by a committee member (B. Driscoll) that I also consider other avenues of

inoculation, as this would minimize the chances of a *'negative result'* field experiment. Dr. Driscoll suggested that I look into seed inoculation and compare the effectiveness of seed versus spray inoculation. We therefore established a small field experiment in 2009 with seed inoculated SG, using the same protocol as described in Section 5.3.2. This proved to be wise counsel, as the spray inoculation yielded no significant findings, nor any effects on plant growth. Results from the seed inoculated treatment were also not statistically significant, however, several positive plant responses as a result of this inoculation technique were observed. We concluded that beneficial bacterial effects had occurred but statistical significance was not detected due to the small sample size and problems with weed control, which confounded the results. We decided to repeat the seed inoculation experiment the following year, 2010, on three fields comprising different soil types.

In chapter 5, we set out to determine if the positive growth effects of the PGPR inoculum on biomass growth that we observed under controlled-environment conditions could be replicated under field-environment conditions, and be observed as early as the establishment year. The results presented in this chapter show that seed inoculation of the mixed PGPR inoculum positively affected the growth and production of SG in the seeding year. Inoculated SG plants had greater tiller density, with a greater proportion of larger-sized tillers than uninoculated plants. This higher number of tillers per area resulted in a greater overall yield; approximately 40% greater yield is predicted with inoculation as compared to unininoculated, unfertilized plants. In addition, when fertilized with the recommended rate of 100 kg N ha^{-1}, this 40% yield increase is still observed for inoculated as compared to uninoculated SG plants. We concluded that the positive growth effects observed under field-environment conditions resulted from the combined plant growth promoting effects of the mixed inoculum, as the bacteria possessed capabilities of N_2-fixation, P solubilization, and auxin pro-

duction. We surmised that the mixed PGPR inoculum produced a more efficient N-acquiring root and rhizome system, which could allocate additional nutrients and water to new shoot development.

In chapter 6, the effects of the mixed PGPR inoculum on N dynamics of SG were examined. The results presented in this chapter suggest that inoculation with a mixed PGPR provided roots and rhizomes access to ANFN, possibly through a combination of BNF, P solubilization and auxin production with the latter two allowing improved growth (including root and rhizome growth) and therefore better access to soil N and other nutrients (such as P, K, and Mg). We also observed that inoculated SG plants had greater N concentrations within the tissues of tillers during anthesis when N concentration would be at its maximum, and concentrations that were not different during senescence when N would be at its lowest, as compared to uninoculated plants in both cases. The difference in N concentration between the two growth stages, which we referred to as the amount of N translocated, indicated that inoculated plants had probably translocated greater amounts of N to the below-ground roots and rhizomes for winter storage. Even during the establishment year, inoculated SG plants would be able to produce greater biomass yields at fall harvest due to more efficient provision of soil N, and possibly biologically fixed N, to above-ground tissues during the growing season. Fertilizer recovery can also be greater for inoculated than uninoculated SG plants, thus allowing the 40% yield increase observed under N-limited conditions to persist with N fertilization, at least at the 100 kg N ha^{-1} rate evaluated in this work. The conclusions we presented in this chapter were that inoculation of SG seeds with the mixed PGPR culture enhanced NUE and contributed N to SG plants, thereby helping to meet the N requirement to support its growth. The use of this SG-microbe system could be an effective strategy to improve the establishment of this crop, especially under a low-input management system.

7.2 Conclusion

The results of this thesis show that SG does associate with PGPR, including those capable of N_2-fixation, P solubilization and auxin production. The isolates showing the greatest potential for plant growth promotion were identified to be a strain of N_2-fixing bacterium, *Paenibacillus polymyxa*, and species of *Pseudomonas, Serratia*, and *Rahnella*. Inoculation of SG seedlings with a mixed PGPR culture containing these strains resulted in enhanced biomass yield and N concentration under controlled-environment growth conditions. It is likely that plant growth promotion in this instance was largely due to contributions from BNF as plants were grown without N fertilization, and N concentrations within plant tissues were not diluted in spite of the larger biomass, so that the total N in the plant tissues was increased. Seed inoculation with this mixed PGPR culture during field trials indicated that during the growing season, inoculated SG plants took up and utilized more N possibly due to greater access to ANFN, producing more tillers per area and consequently had greater yields than uninoculated plants. Fall harvest results showed that this N was cycled to rhizomes for overwintering, which could result in a greater competitive establishment advantage of inoculated plants over weeds in the spring. Further, while more N was taken up by inoculated plants during the growing season, amounts not different than those of the uninoculated plants were removed at harvest, which results in a cleaner feedstock for combustion and/or chemical conversion, and would result in a crop that will require less N fertilization the following year, as more N is returned to the soil with crop residues. In this instance, under field-environment conditions, it is likely that plant growth promotion was largely due to the combined contributions of plant growth mechanisms exhibited by the bacteria, as nutrient acquisition would be increased by the combined effects of enhanced root systems resulting from auxins produced by the bacteria, and increased capacity to solubilize immobile phosphate and greater contributions of fixed N by associated N_2-fixing bacterial cells.

In conclusion, a switchgrass-microbe low-N input system could effectively be utilized for improving current feedstock production systems under temperate-zone conditions. While this system may be different than the tropical sugarcane-microbe system discussed earlier, many lessons can be learned from the 60 plus years of research invested by Brazilian and other scientists around the world. The switchgrass-microbe system discussed here in this thesis can be improved and effectively utilized under different climates and on different soils. This switchgrass-microbe system will lead to a more sustainable and productive management system, which will result in a direct cost benefit, as less N fertilizer will be used for crop production thereby increasing the energy output of biofuel crops and providing a broad environmental benefit in the form of alternatives to fossil fuels. In addition, this system may potentially be used on marginal lands where nutrients such as N are more limiting.

7.3 Recommendations for future research

The general objectives of this research were to determine if SG associates with PGPR, if the PGPR could be used to enhance SG growth, and if a switchgrass-microbe low-N input system could be developed to enhance bioenergy feedstock production. While all of these objectives were met, several potential avenues for further research surfaced from this investigation, many of which, are based on examples of research progress made by the Brazilian scientists. At the Brazilian agricultural research facility, Embrapa Agrobiologia, the research is divided by discipline such that all aspects of the plant-microbe interaction are studied. Thus, my recommendations for future research include:

1. Molecular microbiology

    - Further characterization of the bacteria isolated from this study, including analyses of the whole genome shotgun sequences to identify the isolates to

species level and to determine if the isolates are able to fix N_2. Identification of other plant growth promoting properties, such as enhanced ACC deaminase activity, Fe-siderophore production, production of other plant hormones or their analogues (cytokinins, gibberellins, etc.) would provide a greater understanding of the mechanisms by which PGPR can enhance SG growth.

- Further isolation, identification, and characterization of the rhizosphere bacteria, including screening for isolates that promote plant growth using plant assays as described for endophytic bacteria.

2. Molecular plant-microbe interactions

   - Determination of the level of and sites of infection or colonization of SG plants (for both growth chamber and field inoculated plants) by the bacteria using a combination of staining (β-Glucuronidase or GUS stain) and microscopy (light, transmission electron microscopy, laser scanning microscopy) techniques, or fluorescence *in situ* hybridization (FISH) analyses (Fuentes-Ramírez et al., 1999; Gyaneshwar et al., 2001; James et al., 2002; Loganathan and Nair, 2003; Oliveira et al., 2009).

3. Plant physiology and biochemistry

   - Investigate the effects of the PGPR inoculum on plant development variables: root length, leaf area, tiller and rhizome development, etc.
   - Investigate the effects of the PGPR inoculum on cellular and biochemical variables: changes in meristemic cell growth, changes in enzymatic activity, etc.
   - Determine if the plant-microbial effects differ with different SG varieties. In the growth chamber plant bioassay, only the variety 'Cave-In-Rock' was

used during the different batch and singly inoculated trials. Cave-In-Rock was chosen because we had already selected this variety for use in the field trials based upon previous variety trial field experiments conducted within the southwestern Québec area (Madakadze et al., 1998a; Mehdi et al., 2000). However, it is possible that the plant response to bacterial inoculation is variety specific, and warrants re-examination of the screening bioassay using the specific SG varieties from which the bacteria were obtained.

4. Agronomy

- Test seed inoculation: formulation, inoculant amount, efficaciousness when seeding date is varied.
- Re-visit spray inoculation: It is possible that the timing and/or amount of inoculant used in the 2009 trial were wrong. The level of initial investigation was slight, thus, this method of inoculation warrants another investigation. Spray inoculation of established SG plants at the seedling stage (early to mid-May) may improve colonization levels and result in positive growth effects.
- Provided the plant response to the bacterial (PGPR) inoculation is indeed plant variety and bacterial species/strain related (as discussed above), field investigations using different SG varieties is another avenue of research to pursue.
- Longer term studies are required to address such questions as how long the effects of inoculation will last, whether re-inoculation of existing stands is effective and whether the growth promotion observed in the short-term studies results in an inherently high-production, low input system that can be maintained in the long term.

5. Plant and soil ecology

   - Determine BNF contribution of the bacteria to the SG plant using a combination of N balance study and $^{15}N/^{14}N$ isotopic ratio analyses: ^{15}N natural abundance technique, ^{15}N isotope dilution method, ^{15}N fertilizer labeling techniques.
   - Many research avenues exist to increase understanding of the feedbacks between PGPR, plant growth and nutrition, soil organic matter derived from residues of PGPR-supporting plant and soil nutrient dynamics. Such knowledge will be valuable in understanding the longer term function of SG-PGPR systems.

Chapter 8

Contributions to Knowledge

Research on SG, both as a forage and bioenergy crop, has focused on breeding selection, agronomy and crop physiology, energy potential, and its contribution to mitigating GHG emissions. However, there is a lack of knowledge regarding plant-microbe interactions with SG, how these associations play a role in its growth and productivity, and their functioning as part of agro-ecosystems and natural grasslands. The few studies that have investigated plant-microbe associations with SG were conducted in the 1970s and 80s, and only examined *in situ* microbial activity without the identification of the microbe(s) involved. To our knowledge, no study has been reported that have identified microbe(s) that interact with SG, and their effects on plant growth and productivity. The research conducted here strives to bridge some of these knowledge gaps, allowing for several avenues from which future research could stem. Thus, the experiments conducted to answer the questions and objectives of this work provide the following contributions to knowledge.

The study was the first to:

1. Demonstrate that strains of N_2-fixing bacteria associated with the rhizomes of SG. In this case we have shown that an N_2-fixing strain of *Paenibacillus*

polymyxa associates with the rhizomes of SG. This is the first study to isolate this bacterium from SG. In addition, this bacterium was part of a mixed inoculum that enhanced SG plant growth.

2. Isolate and identify pure strains of bacteria from the rhizomes of switchgrass. The results presented in Chapter 4 show that these bacteria are free-living, beneficial microbes that are able to enhance plant growth when re-inoculated into SG seeds and seedlings. These strains were shown to exhibit capabilities that include biological N_2-fixation, P solubilization and production of IAA-like substances.

3. Examine plant growth promotion of SG by endophytic and exogenous microbes. In chapter 4, we focused on selecting for endophytic rhizosphere bacteria of SG that enhanced plant growth, but we also investigated plant growth promotion of several free-living, N_2-fixing bacteria, obtained from the ATCC, and that were isolated from other plant species. The results of this chapter demonstrated that, under controlled environment conditions, colonization and plant growth promotion of SG is not exclusive to endophytic and/or naturally colonized microbes.

4. Demonstrate positive plant-microbe effects that resulted in enhanced SG growth and productivity under controlled, growth chamber and field environment conditions. In chapters 5 and 6, we have shown that seed inoculation of SG with PGPR I isolated can improve the yield and N cycling (N uptake, fertilizer recovery, N concentration, and N translocation) of SG under optimal management and N-limited (no N fertilizer applied) conditions in the field.

Advancements in knowledge:

1. We have developed a switchgrass-microbe low-N input production system. This system may be part of a viable strategy for reducing N fertilizer use, improving

SG energetics, and mitigating GHG emissions. The development of this system was explored in chapters 5 and 6, and discussed in chapter 7.

2. We have developed controlled, growth chamber and field inoculation techniques. With regard to investigations on plant-microbe interactions, several inoculation methods have been performed in other studies. However, not all methods allow for reproducible results, which adds to the difficulty in studying these interactions. Prior to this study, no information was available regarding how to inoculate SG with bacteria. Thus, this research allowed for the advancement of plant-microbe investigations with SG by providing a working method with which to study the effects of the interaction. In chapter 4 we have shown that inoculation of 4 to 5 day old SG seedlings does allow for measureable results in a controlled, laboratory environment. For field studies, seed inoculation with peat-based material has been the traditional way of inoculating legumes with rhizobia. As shown in chapters 5 and 6, this inoculation method is also viable for field studies with SG.

3. This study advances knowledge of physiological responses by SG, under controlled and field conditions, to PGPR inoculation. Prior to this research there have been no reports of SG-microbe interactions and the effects of PGPR on SG plant and/or crop physiology. Thus, this research provides a baseline for other research with which to compare SG physiological responses to bacterial colonization.

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