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Associative Nitrogen-Fixing Bacteria and their Potential to Support the Growth of Bioenergy Grasses on Marginal Lands

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Associative nitrogen-fixing bacteria and their potential to support the growth of bioenergy grasses on marginal lands

By

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Rising demands for both food and energy have lifted up the idea of producing renewable biofuels from bioenergy grasses that can companion with associative N_2-fixing bacteria. Associative N_2-fixing bacteria can partially fulfill the N requirements of bioenergy grasses enabling successful application for marginal lands. The overall objective of this study was to measure the potential N_2-fixation of bacteria associated with three bioenergy grasses, giant miscanthus (*Miscanthus x giganteus*), switchgrass (*Panicum virgatum*; 'Alamo') and energycane (hybrid of *Saccharum spontaneum* and *S. officinarum*; HO 02-147) as well as identifying N_2-fixing bacteria associated with these energy grasses and applying these isolates to grasses to increase biomass yield. It was hypothesized that three different energy grasses, giant miscanthus, switchgrass, and energycane have different capabilities to support the growth of associative N_2-fixing bacteria, and thus exhibit different rate and quantities of N_2-fixation.

The dynamics of delta$^{15}N_{air}$ in the root-zones of three perennial bioenergy grasses, biomass yield and nitrogen derived from (%Ndfa) were obtained. Greater potential for N_2-fixation was identified in energycane associated plant system compared to switchgrass

and giant miscanthus. *Sorghum bicolor* (M81-E) was used to calculate the %Ndfa. Moreover, a $^{15}N_2$ enriched greenhouse study was carried out to estimate and compare the contribution of ANF to support field data. Greenhouse data further confirmed that energycane associated bacteria has greater potential N_2-fixation compared to giant miscanthus and switchgrass. Composition and diversity of N_2-fixing bacteria associated as endophytes and in the rhizosphere community of these three energy grasses were identified in marginal lands. Diverse N_2-fixing bacteria were observed to be associated with different grasses and *Azospirillum sp*. was identified in energycane. The effect of N_2-fixing bacteria on biomass was studied by inoculating three energy grasses with the bacteria isolated from energycane. Increased root lengths of giant miscanthus were observed upon inoculation. However, no other changes in biomass yield or shoot lengths were observed in three bioenergy grasses.

Further research is needed to quantify endophytic and rhizosphere N_2-fixing bacteria and to determine their role in association in an effort to increase the biomass yield of other grasses and reduce N fertilizer inputs.

DEDICATION

Every challenging work needs one's hard work as well as guidance from one's elders, especially those who are very close to our heart. It is my humble effort that I dedicate to my loving husband, father, mother, mother in-law, aunt, brother and sister in-law whose affection, love, encouragement and prayers of day and night help me to achieve such success.

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ACRONYMES

BNF	Biological Nitrogen Fixation
ANF	Associative Nitrogen Fixation
Ndfa	Nitrogen Derived from Air
LB Medium	Luria Bertani Medium
N	Nitrogen
%N	Nitrogen percentage
ANOVA	Analysis of Variance
DGGE	Denaturing Gradient Gel Electrophoresis
NFb	Nitrogen Free broth
IRMS	Isotopic Ratio Mass Spectrometry
GC-TCD	Gas Chromatography-Thermo Couple Detector
PCR	Polymerase Chain Reaction
NH_4NO_3	Ammonium Nitrate
kg N ha^{-1}	Kilogram Nitrogen per Hectare
kg ai ha^{-1}	Kilograms of Active Ingredients per Hectare

CHAPTER I

INTRODUCTION

Marginal lands supporting bioenergy grasses can play an important role in biofuel production due to their ability to associate with N_2-fixing bacteria. These bacteria fix atmospheric N_2 and can provide it as a usable form to the plants, and thus sustain high dry matter yield. As the global population increases, the resulting pressure on farmers to use fertile lands to increase the world food supply realized greater attention. Marginal lands have limited productivity, and therefore require additional management for usage. However, recent increases in prices of synthetic fertilizer have also made it difficult for small farmers to achieve profitable crop production in marginal lands with most food crops that are used for biofuel production. A potential alternative source for supplying N to these lands for improving their applicability is through the use of improving the richness of bacterial communities capable of N_2-fixation.

One type of N_2-fixing microbial population that has recently gained more attention is associate nitrogen fixing (ANF) bacterial populations. These bacteria are free-living in the soil, but tend to associate with certain plants through a non-endosymbiotic relationship to provide fixed N_2. This allows for growth in marginal lands with little to no additional N supplied in the form of fertilizers if the plant is able to associate with ANF bacteria. However, it is not known if other types of grasses, particularly those that are beneficial for bioenergy production, have the ability to associate with N_2-fixing bacteria.

In order for biological N_2-fixing by ANF bacteria to become a sustained and reliable source of N for biofuel crop production, certain questions have to be answered. For instance, does the efficiency of N_2-fixation differ between various feedstock crops and how much residual N supplied through these cropping systems? In order for these questions to be answered, measurements of biological N2-fixation are needed between various feedstock grass species. This dissertation is focused upon analyzing the N_2-fixation capability among three different feedstock grasses through various methods. The assessment of this capability is needed in order to efficiently utilize marginal lands and to increase arable land management.

1.1 Bioenergy grasses

Bioenergy grasses are also known as feedstock biofuel crops since they serve as a source for biofuel production. Energy grasses are composed of sugars and lignocellulose compounds in their cell wall and can be utilized to produce renewable biofuels (Bhalla et al., 2013; Zeng et al., 2014), which also holds the potential to decrease urban air pollution and CO_2 accumulation in the atmosphere (Farrell et al., 2006; Havlík et al., 2011). The Energy Independence and Security Act of 2007 set a target of 60.5 billion liters of bioethanol to be produced by 2022 (Rogers et al., 2012; Lima et al., 2014). Perennial grass species such as switchgrass (*Panicum virgatum*), giant miscanthus (*Miscanthus x giganteus*) sugarcane (*Saccharum sp*.) and energycane (hybrid of *Saccharum spontaneum* and *Saccharum officinarum*) have been proposed as key bioenergy crops in the United States and Europe based on their low input of fertilizers and ability to utilize marginal lands (Rogers et al., 2012; Lima et al., 2014). These bioenergy crops are currently being

explored as sustainable sources for bioenergy and each of these grasses possesses different characters that allow for their use as a source in biofuel industry.

1.1.1 Switchgrass

Switchgrass was selected as a model energy crop in 1991 and since then much research has been conducted to improve and estimate the ability of this grass for biofuel production (McLaughlin and Adams Kszos, 2005). Switchgrass belongs to the subfamily Panicoideae of the Gramineae family. This tall growing perennial C4 grass is native to North America and is widely adapted to a variety of different environment and soil conditions such as sands to clay loam soils in pH values ranging from 4.9 – 7.6 (Lewandowski et al., 2003). The shoots of lowland switchgrass can grow more than 3 m in height and the roots can extend to more than 3.5 m long (Weaver, 1968). Life span of switchgrass can be up to more than 10 years. Normally the annual yield of switchgrass is 16 – 22 Mg dry matter ha^{-1} and lowland varieties namely "Alamo" and "Kanlow" have higher yields compared to other varieties (Lewandowski et al., 2003).

1.1.2 Giant miscanthus

While the United States Department of Energy (DOE) considers switchgrass as a model energy crop, European researchers have focused on Miscanthus as their model energy crop since 1992 (Hodkinson et al., 2002). Giant miscanthus is another C4 perennial grass native to temperate regions of southern Asia and China. Giant Miscanthus (*Miscanthus x giganteus* or Mxg) is the most efficient yield producer, requiring low inputs of water and high temperature conditions (Beale and Long, 1995; Clifton-Brown and Lewandowski, 2000). *Miscanthus x giganteus* can grow up to 4 m height with a life

span of 20 - 25 years. Normally, it takes 3 - 5 years to reach for their maximum yield (25 Mg ha^{-1}) supplied for biofuel industry (Maughan, 2011).

1.1.3 Energycane

Energycane refers to high biomass sugarcane hybrids with a high-fiber content and a lower sugar content (Tew and Cobill, 2008). Energycane was introduced in order to overcome the lack of cold tolerance ability in sugarcane crops. Two sugarcane species, *Saccharum spontaneum* and *Saccharum officinarum* were hybridized in order to produce energycane. However, research is ongoing to improve the cold tolerance ability of energycane (Gupta et al., 1978). Energycane provides 53.6% of juice (wet basis) and 26.7% of fiber (dry basis), which consists of sucrose as major sugar and cellulose (43.3%), hemicellulose (23.8%) and lignin (21.7%) as the major fibers (Kim and Day, 2011). Energycane will yield 25 Mg ha^{-1} of dry biomass at their maximum within a year.

1.1.4 Annual and perennial grasses

Bioenergy grasses are included in the family Poaceae and are known as monocotyledon. According to the life cycle of different grasses, they are divided into 3 different categories; annual, perennial and biennial. Annual plants survive for one year only. Seeds produce within one year and all the aboveground and belowground materials die after a year. Most of the food crops, such as corn (*Zea mays*) and wheat (*Triticum sp.*), are annual crops and thus seeds must be replanted annually, increasing the expense for production. These crops are considered first-generation bioenergy crops described in section 1.2. However, the biomass yield is low compared to perennial bioenergy crops in terms of biofuel production.

The rhizomes of perennial crops, such as the bioenergy grasses energycane, sugarcane, switchgrass, and giant miscanthus, do not die annually, allowing for longer growth periods for these crops. Additionally, the life cycle expands for several years and the above ground materials can be collected as feedstock for biofuel production. This allows for greater yields with minimal input. Perennial grasses such as giant miscanthus (60 t DM ha^{-1} yr^{-1}) and switchgrass (22 t DM ha^{-1} yr^{-1}) have greater biomass yields compared to annual grasses such as corn and maize (5.5 t DM ha^{-1} yr^{-1}) (Weijde et al., 2013). Therefore, selecting perennial grasses to utilize as a source for biofuel production has an advantage over annual grasses as a source for biofuel production.

1.2 Generations of biofuels

The first generation of biofuels production began in the 20th century and produced ethanol from food crops, such as sugarcane and corn. Brazil primarily used sugarcane, US used corn, Germany used oilseed to produce bioethanol and Malaysia used palm oil to produce biodiesel. One major limitation in these first generation biofuel crops is the conflict for consumption (also known as the food versus fuel debate), as these are all food crops. Therefore the use for bioenergy production has affected the price of food and also had a negative impact on fertile lands.

The second generation of biofuel crops primarily uses non-food crops and forest residues for biofuel production, which addressed many of the limitations associated with first generation crops. Additionally, the energy yield (GJ/ha/year) was also found to be greater when using lignocellulosic biomass as a feedstock in comparison to the first generation food crops (Rahman et al., 2013). Arable lands could also be used for second-

generation energy grasses with low inputs of fertilizer, providing additional benefits to their use and applicability.

Third generation biofuels are mainly focused on utilization of algae cultures. Algae can be cultured with low-costs and could obtain high-energy as a renewable feedstock. Moreover it could produce more energy per acre compared to conventional crops. However, fourth generation bio-fuels are based on capturing and storing of CO_2 for fuel production. Metabolic engineered algae are considered as a fourth generation of biofuel (Dutta et al., 2014). Accordingly the biofuel technologies are evolving rapidly to answer the energy requirement with other mankind and environmental protection.

1.3 Insight to biofuel production

Biofuels are fuels that are produced from biological materials and are therefore made by renewable sources. Solar energy is fixed by plants via photosynthesis and stored as polymers in plant cells such as lignocellulose materials and sugars. There are three types of biofuels: bioethanol, biodiesel and biogas. The production of biofuels from lignocellulosic feedstocks can be accomplished in two processing routes such as biochemical and thermochemical pathways to produce bioethanol and biogas. However, plant oils are used to produce biodiesel. In biochemical pathways, enzymes such as cellulase and hemicellulase from microorganisms are used to convert cellulose and hemicellulose to simple sugars, which are then fermented to produce ethanol. Hence lignocellulose materials are converted into glucose, lactate, and acetate by acidogenesis, acetogenesis and methanogensis to form biogas (CH_4 and CO_2). Thermochemical pathways convert lignocellulosic compounds to synthesis gas (syn gas, $CO + H_2$) by pyrolysis/ gasification technologies, which result in the production of long carbon chain

biofuels such as synthetic diesel or aviation fuel that can be directly used in machineries and vehicles.

1.4 Processes of nitrogen cycle and their importance

Nitrogen (N_2) is the most abundant (79%) gas in the atmosphere. Nitrogen is one of the primary nutrients in all organisms; it is needed for production of biomolecules such as nucleic acids, proteins and chlorophyll. However, higher order organisms cannot directly utilize it in the inert N_2 form. Dinitrogen must be converted into ammonium or nitrate in order to be utilized by plants. The process by which this occurs is a very energy expensive process.

Nitrogen in the atmosphere and soil has several fates (Fig. 1.1) and can exist as gaseous form in atmosphere (atmospheric nitrogen N_2, N_2O, NO), inorganic ions in soil (ammonium and nitrate) and organic compound in soil (amino acids). Denitrification, volatilization, immobilization, leaching and crop uptake decreases the availability of N in the soil while nitrification, N_2 -fixation and mineralization are all geochemical processes that increase available N in the soil.

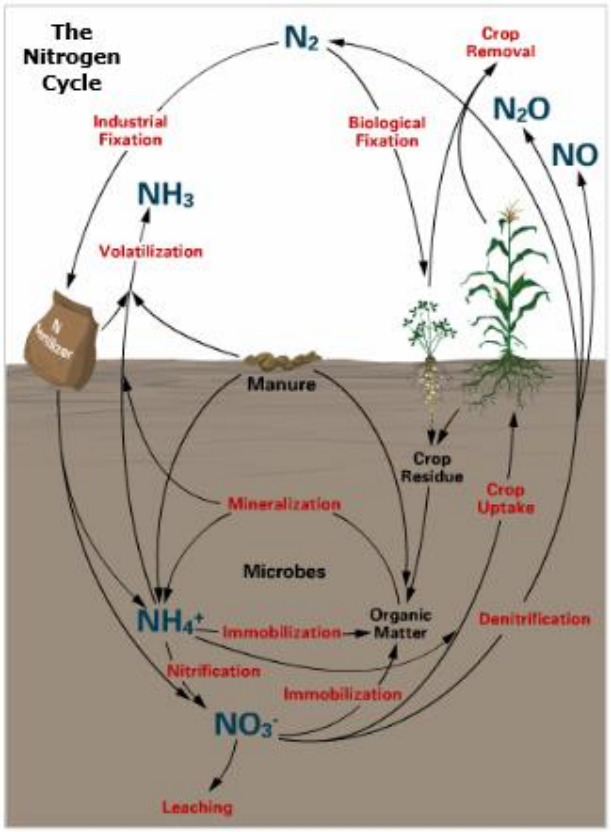

Figure 1.1 Nitrogen cycle on, above, and below the Earth's surface (Johnson et al., 2005)

1.4.1 Denitrification

Denitrification is the process that converts NO_3^- to N_2, hence removing bioavailable N (Fig. 1.2). Several intermediate products are produced during this process, including NO, N_2O and NO_2^-. Nitrous oxide (N_2O) is considered as a greenhouse gas that can react with ozone, thus contributing to air pollution. Denitrification is an anaerobic process performed by a diverse group of prokaryotes and certain eukaryotes. It primarily occurs in soils and water logged areas, anoxic zones in lakes and oceans (Risgaard-Petersen et al., 1998). Denitrifiers are chemoorganotrophs and include species within the

genera of *Bacillus*, *Paracoccus* and *Pseudomonas*. Denitrification is detrimental and costly in agricultural lands where the loss of fertilizer nitrate occurs.

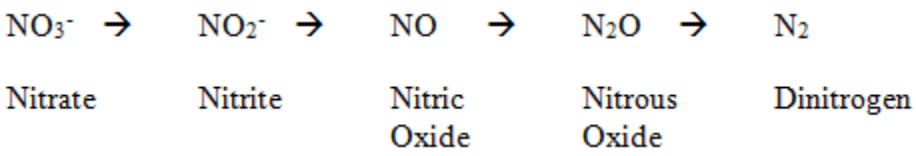

Figure 1.2 Denitrification process

1.4.2 Ammonia Volatilization

Ammonia volatilization is a process whereby urea is converted into NH_4^+ and NH_3 (Fig. 1.3). Nitrogen lost through the conversion of NH_4^+ to NH_3 mostly occurs in agricultural fields due to the addition of fertilizers. The volatilization losses increase at higher pH and hot and windy conditions.

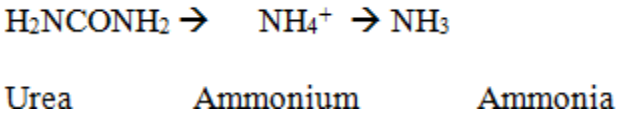

Figure 1.3 Volatilization process

1.4.3 Immobilization

All living organisms need N for their structural and metabolic activities. In soil ecosystems, microorganisms compete with plants to obtain their N requirement. These soil microorganisms are capable of utilizing the NH_4^+ and NO_3^- from the soil, thus minimizing the availability to crops. This process is called immobilization (Fig. 1.4). In

this process, N is stored in the form of amino acids in microorganism which can be re-entered into soil by mineralization after the microorganisms are died.

NH_4^+ and NO_3^- → RNH_2

Ammonium and Nitrate Organic Nitrogen

Figure 1.4 Immobilization process

1.4.4 Leaching

Leaching is the process where nutrients are lost from the soil through rain or drainage. Soil clay particles and NO_3^- nitrate are both negatively charged. Therefore the possibility of leaching NO_3^- from soils is high as they repel each other. Leaching can be significant in an agricultural soil where NO_3^- is applied as fertilizers. If a large amount of leached NO_3^- enters water bodies, the ecosystem of the water body can be subject to eutrophication and hypoxic regions can be formed (Riley et al., 2001). The application of slow release fertilizers and split applications could minimize the potential for leaching.

1.4.5 Crop uptake

Crop uptake is the principle goal of N management in an agricultural system. Plants actively utilize N during the growing season. Crops can obtain N from the soil in the form of NO_3^- and NH_4^+. However, the growth efficiency is dependent upon additional factors, such as temperature and soil moisture. Additionally, NO_3^- remaining after plant growth could be subjected to leaching or denitrification in moist soils and can decrease the crop yield in the next growing season.

1.4.6 Nitrification

Nitrification is the process that converts NH_3 to NO_2^- and to NO_3^- aerobically by prokaryotes (Fig. 1.5). Some plants cannot utilize N in the form of NH_4^+. Therefore by nitrification of NH_4^+ is needed to convert it into NO_3^-, which can be absorbed by plants. There are two steps in nitrification: 1) NH_3 oxidation and 2) NO_2^- oxidation. Certain members of the aerobic autotrophic genera of *Nitrosomonas*, *Nitrosospira*, and *Nitrosococcus* can oxidize NH_4^+ into NO_3^- using ammonia monooxygenase enzyme. Additionally, some archaea also can carry out this reaction (Koenneke et al., 2005). Once produced, the NO_2^- is oxidized into NO_3^- by the nitrite oxidizing bacteria, which include members of the genera of *Nitrospira*, *Nitrobacter*, *Nitrococcus*, and *Nitrospina.*

NH_4^+ → NO_2^- → NO_3^-

Ammonium Nitrite Nitrate

Figure 1.5 Nitrification process

1.4.7 Mineralization

Mineralization is the process by which microorganisms decompose organic N into available inorganic forms (Fig. 1.6). Organic N, which is composed into soil by debris, is converted into NH_3 and then to NH_4^+ by microbial and environmental processes. Microbial cell walls, nucleic acids in microbes and plant debris can added into soil and breakdown to small component and then into simple organic forms then to NH_3 and NH_4^+. Several environmental factors, such as temperature, moisture, and the nature and abundance of soil organic materials, affect the mineralization process.

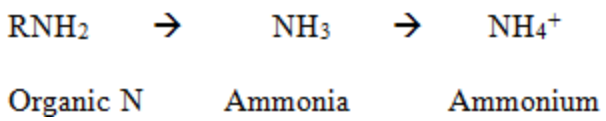

Figure 1.6 Mineralization process

1.4.8 Biological N_2-fixation

Atmospheric N_2 can be converted into NH_3through biological N_2 -fixation reactions (Fig. 1.7). Nitrogen gas is very stable as it has a triple bond between two N atoms. Therefore, N_2-fixation is energetically demanding. The process is performed by free living, symbiotic or associative microorganisms.

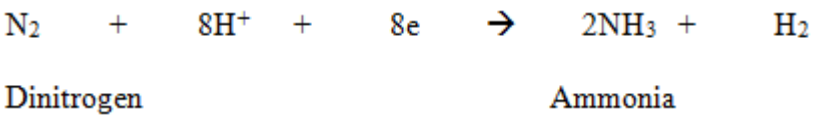

Figure 1.7 Biological N_2-fixation process

1.4.9 Free living N2 -fixation

Cyanobacteria (blue green algae) and certain other prokaryotes are capable of fixing N_2 through special internal structures called heterocysts. The nitroganase enzyme is the key enzyme for N_2 -fixation process and resides within the heterocyst primarily to protect this enzyme from O_2. However, certain other prokaryotes protect the nitrogenase from O_2 by performing this reaction inside vesicles in the cells. These include members of such genera as *Nostoc, Anabena*, *Azetobactor, Klebsilla,* and *Clostridium.*

1.4.10 Symbiotic N_2-fixating microorganisms

Microorganisms that are capable of fixing N_2 that are found in close association to the host plants are called symbiotic N_2 fixers. An example of this type of microorganism is *Rhizobium*. Rhizobia cannot fix atmospheric N_2 unless they have infected into roots of appropriate legumes. After colonizing the rhizobia near legume roots, these bacteria may form infection threads within the roots. Specificity of symbiotic fixation is due to the association of different "Nod" factors secreted from rhizobia and the interaction of these factors with different transmembrane receptors on the roots of specific legumes. Upon infection of *Rhizobium* within the legume roots, cortex cells begin to divide and form nodules, along with multiplication of the *Rhizobium* inside the nodules. Within these nodules, *Rhizobium* can fix atmospheric N_2 by utilizing C fixed by plants.

1.4.11 Associative N_2-fixing microorganisms

Associative N_2-fixers also have close associations with plants and can infect into plant roots to fix N_2. However, they do not form special nodule-like structures in roots. Associative N_2 fixers can be found as endophytes as well as in close association near the rhizosphere region.

1.5 Associative N_2-fixation in feed stock crops

A vast amount of research regarding ANF has been conducted in several non-leguminous grasses. However, little information regarding other biofuel crops such as energycane, switchgrass and miscanthus is available.

Increased application of N fertilizer onto biofuel feedstock grasses such as sugarcane systems has been shown to result in a decline in plant yields and depletion of

soil N pools (Wiedenfeld, 1995). This decrease in productivity in plants was found to be associated with the suppressed activity of associative N_2 fixers upon availability of N fertilizer.

Several studies have been conducted in sugarcane, rice, kallar grass and maize and have found more than six genera of associative N_2 fixers and nine species (Triplett, 1996; Baldani et al., 1997; James, 2000). Normally, in Brazil, the average recommended application rates of N fertilizers are around 80 kg N ha^{-1} for plant sugarcane and 120 kg ha^{-1} for the ratoon crop to get a biomass yield of 70 Mg ha^{-1} (Lima et al., 1987). However, the same yield has been observed without any applications of N fertilizers into the field, suggesting sugarcane may utilize N from biological N_2-fixation (Dobereiner, 1961; Ruschel et al., 1975).

Several genera of ANF bacteria have been identified within sugarcane, such as *Azotobacter*, *Enterobacter*, *Bacillus*, *Klebsiella*, *Azospirillum*, *Herbaspirillum*, *Gluconacetobacter*, *Burkholderia* and *Azoarcus* (Tarrand et al., 1978; Gillis et al., 1989; Reinhold-Hurek et al., 1993; Gillis et al., 1995; Baldani et al., 1997). It is known that *Gluconobacter diazotrophicus* (*Acetobactor diazotrophicus*) effectively fixes N_2 in the sugarcane plant system (Boddey and Dobereiner, 1995; Baldani et al., 1997). Some other associative N_2-fixers such as *Herbaspirillum rubrisubalbicans*, *H. seropedicae*, *Enterobacter cloacae* and *Klebsiella oxytoca* have also been found inside sugarcane plants (Baldani et al., 1997; James, 2000; Mirza et al., 2001).

The most common bacteria isolated from sugarcane tissues are *Gluconacetobacter diazotrphicus*; these are obligate endophytes as they cannot be isolated from root free soils (Gillis et al., 1989; James and Olivares, 1998). *Azospirillum*

has also been identified in rhizopsphere soils, in associations with roots of tropical forage grasses, and from cereal plants. Hence, most *Azospirillum* species have been found in superficial layers of root cortex (Bashan and Levanony, 1991). Another ANF microorganism, *Burkholderia sp*. has been isolated in quite diverse environments and rhizosphere soils (Paungfoo-Lonhienne et al., 2014).

Moreover, microbes belonging to the genera *Herbaspirillum* and *Azospirillum*, including *Azospirillum doebereinerae,* were isolated from miscanthus (Kirchhof et al., 2001, Eckert et al., 2001). *Burkholderia phytofirmans* was identified in switchgrass as a growth promoting and N_2 -fixation bacterium associated with the roots. However, there is limited information regarding ANF in the sugarcane hybrid energycane as well as ginat miscanthus, and switchgrass.

1.5.1 Rhizosphere and endophytic associative N_2-fixing microorganisms

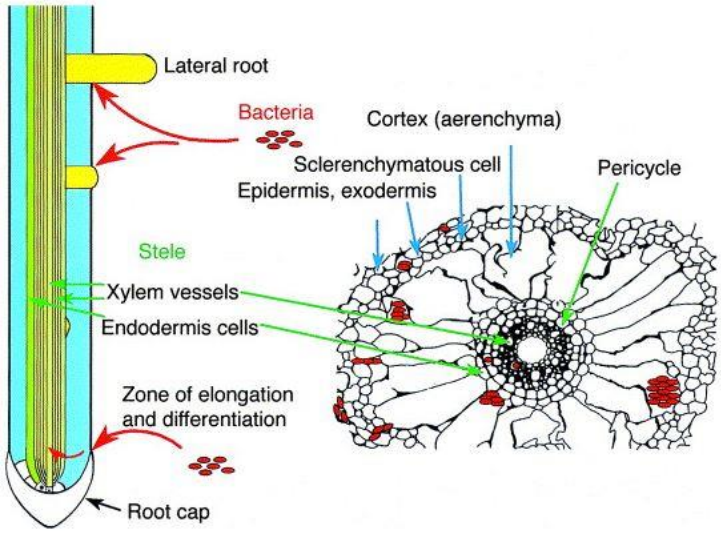

Figure 1.8 Site of infection and colonization of rice root (Reinhold-Hurek and Hurek, 1998)

The rhizosphere encompasses the soil directly surrounding a plant root. Thus the free-living ANF microorganisms can be found within the rhizosphere of the roots. Bacterial root colonization initiates with the chemical compounds called root exudates, such as ions, enzymes, mucilage, and diverse carbon-containing primary and secondary metabolites. Root exudates can make positive and negative interactions with plant and soil microbes (Bais et al., 2004). However, plants can communicate to specific microorganisms for their own ecological and evolutionary benefits (Compant et al., 2005). It has been identified that ANF can be mediated by highly specific flavonoid compounds ejected by plants (Webster et al., 1998; Balachandar et al., 2006). These chemical compounds can strongly contribute to competitiveness of microbes in colonization and initiate motility towards the plant through chemotaxis. However, very little is known about the role of this important bacterial communication tool in endophytes and ANF bacteria within grass systems (Bauer and Mathesius, 2004).

These signaling mechanisms are beneficial for bacterial colonization in rhizosphere region of roots. After the colonization, bacteria can fix N_2 by close attached to the roots in rhizosphere region and some can infect into the host plants (Fig. 1.8) through cracks formed in the lateral root junctions, wounds and root cracks, and the root elongation region (Reinhold-Hurek and Hurek, 1998). Certain species of the genera *Pseudomonas, Azospirillum,* and *Bacillus* are often found to colonize within rhizospheres and internal tissues. However, in general, the rhizospheric bacterial population density is greater than the endophytic bacterial population (Hallmann et al., 1997). Some N_2-fixing microorganisms found as endophytes are capable of stimulating drastic physiological changes that modulate the growth and development of plants (Conrath et al., 2006).

1.5.2 Synthetic inoculation of N_2-fixing microorganisms into bioenergy grasses

A study of inoculation of N_2-fixing *Rhizobium* into legumes demonstrated that the inoculation of these bacteria into soybean plants improved the yield obtained (Dobereiner, 1977). *Azospirillum, Bacillus,* and *Flavobacterium* have also been found to enhance non-legume plant growth (Kloepper et al., 1989). Cereal crops inoculated with *Azospirillum spp.* had a decrease in yield with an increase in the application of addition of N fertilizers due to available fertilizer N utilization by plants (Jagnow, 1987). An additional study conducted with sugarcane inoculated with five endophytic bacterial species found that the best treatment to increase the yield was the mixture of all five associative N_2 fixers: *Gluconacetobacter diazotrophicus*, *Herbaspirillum seropedicae*, *Herbaspirillum rubrisubalbicans*, *Azospirillum amazonense* and *Burkholder sp.* (Oliveira et al., 2002). Biological N_2-fixation (BNF) was found to contribute 30% of the total N accumulation of sugarcane when inoculated with these five ANF microorganisms (Oliveira et al., 2002).

Generally, upon the inoculation of bacteria, the bacterial population is decreased progressively and then increased gradually (Albrecht et al., 1981; Bashan and Levanony, 1988). The key obstacle for the introduced bacterial survival is the heterogeneity of native bacterial population in soil. However, these previous studies demonstrate that competition of indigenous microorganisms is occurring with synthetic inoculations with N_2-fixing bacteria and this competition can affect plant biomass yields.

1.6 Methods to measure N_2-fixation

It is very important to identify and quantify the rate of biological N_2-fixation associated with grass-microbe system in order to identify a suitable crop for certain areas

and to understand the fertilizer management in a particular field. There are several methods currently used to measure the capability of a plant to be associated with N_2-fixing microbes, such as acetylene reduction, isotopic measurements, enrichment culturing techniques, and molecular methods. Each of these methods measure the probability of N_2-fixation differently; therefore, each has its own advantages and disadvantages. These methods are further described in this section.

1.6.1 Acetylene reduction method

The acetylene reduction technique has been the most common method to assess BNF since 1960's (Hardy et al., 1968; Van Berkum and Bohlool, 1980). In this method, acetylene is introduced into the plant system and ethylene produced is measured using gas chromatography after a period of time. Both acetylene and N_2 have triple bonds and can be reduced by the nitrogenase enzyme, which is responsible for N_2-fixation. The reduction of acetylene needs two electrons whereas N_2 needs eight electrons. Therefore, the reduction of one molecule of N_2 is equivalent to the reduction of four molecules of acetylene.

This method is very easy to conduct, though the results are not necessarily an accurate quantitative measurement of N_2-fixation. Nitrogen, as well as acetylene, has triple bonds in between two molecules in chemical nature; therefore in theory, for every N_2 molecule that is fixed, three molecules of acetylene are reduced. There could be reduction of N_2 instead of acetylene and can produce H_2 from H_2O that affect the reduction stoichiometry. A nutrient limited environment can also result in an underestimation of the rate of N_2-fixation due to a disruption in the potential activity of the microbial community.

1.6.2 Isotope techniques

Isotope techniques were first applied to studying N_2-fixation in 1941 (Burris et al., 1943) and later non-symbiotic aerobic N_2-fixation was determined by injecting atmospheric labeled $^{15}N_2$ (Delwiche and Wijler, 1956). Identification of non-symbiotic ANF was significantly important to the N economy in soil when managing fertilizers in agricultural system. The isotope dilution method involves the addition of known amounts of isotopically enriched gas ($^{15}N_2$) or fertilizer (^{15}N labelled fertilizer) into an analyzed sample in order to detect the N_2-fixation. Recently, the $^{15}N_2$ tracer technique has been used to enhance the detection of N_2-fixation by stable isotope probing of N_2-fixing bacteria (Buckley et al., 2007). By this method ^{15}N labeled soil demonstrated that certain varieties of sugarcane (CB 45-3, sp 70-1143) can fix up to 70% of their N requirements and provide biomass yields in excess of 150 kg N ha^{-1} per year (Boddey and Dobereiner, 1995). Although most research has been conducted using sugarcane, a number of tropical forage grasses including *Brachiaria humidicola, B. decumbens, Paspalum notatum* and *Panicum maximum* have also shown relatively high (40% of their N-needs) N_2-fixation rates in ^{15}N isotope dilution studies (Boddey and Knowles, 1987).

The ^{15}N isotope dilution technique has also been applied to sugarcane plants grown in large (60 L) pots or a 120 m^2 concrete tank filled with ^{15}N-labeled soil (Lima et al., 1987; Urquiaga et al., 1992). These studies found that sugarcane varieties had the lowest ^{15}N enrichment. Higher N accumulation of soil/plant systems was powerful evidence for biological N_2-fixation. Other than the ^{15}N enriched soil and fertilizers, $^{15}N_2$ gas was incorporated into large chambers with control environments to measure the BNF (De-Polli et al., 1977).

The natural ^{15}N abundance method is commonly expressed in $\delta^{15}N$ value, denoting the relative deviation from the ratio ^{15}N:^{14}N in atmospheric N_2. The differences of ^{15}N abundance between atmospheric N_2 and other sources of N can be measured as particular soil processes of mineralization, nitrification, N_2-fixation, and denitrifiation (Boddey et al., 2000). A number of comparisons of BNF based on $\delta^{15}N$ method have been conducted with legumes (Virginia and Delwiche, 1982; Shearer and Kohl, 1986). However, very little work has been done to assess the applicability of $\delta^{15}N$ to non-legumes. Microbial screening studies have shown putative N_2-fixing bacteria are associated with non-legume species and ^{15}N studies can be applied into non-legumes along with several non-N_2-fixing reference plant species and also non cultivated soil microorganisms (Buckley et al., 2007). Additionally, ^{15}N natural abundance studies and $^{15}N_2$ dilution studies have shown potential N_2-fixation in *Azospirillum spp*. in grasses (Boddey and Knowles, 1987; James, 2000).

The simplest way to estimate the amount of fixed N_2 through accumulation of the crop. Isotopic measurement can be used to calculate the total N content of the non-fixing crop (derived solely from soil N) and then, subtracted from the total N content of the N_2-fixing non-legume crop, would give the accurate estimation of total N_2 fixed by the target N_2-fixing crop. Control plants must be utilized that exhibit similar patterns of soil N uptake and must be grown in close proximity to the test feedstock grasses to overcome the errors in estimation of diazotrophic derived N in plant soil systems (Shearer and Kohl, 1986).

The isotopic technique has been found to be more accurate than the acetylene reduction test in studies of BNF (Danso, 1995). However, isotopic labeling experiments

cannot be administered for long periods of time due to fluctuations in environmental factors and the high cost in materials (Maasdorp, 1987). Therefore, measurements of the natural abundance of N are more appropriate in field experiments and isotopic labeling studies are more reliable for green house experiments where factors can be more easily controlled.

1.6.3 Bacterial identification

Identification of ANF bacteria is one preliminary method of suspecting N_2-fixation. Though this information does not provide quantitation on the actual rates of N_2-fixation, enrichment culturing techniques and molecular techniques can provide valuable information on how to improve the microbial community to increase the rates of N_2-fixation.

1.6.3.1 Culturing techniques

In 1928 by Fred and Waksman and in 1949 by Winogadsky, first described the use of N-free agar and silica gel plates for culturing aerobic diazotrophs (Dobereiner, 1988). However, they could only isolate *Beijerinckia* and *Azotobacter* from the above culture medium. Later, NFb medium was used to identify *Azospirillum sp*. and then LGI medium was used to isolate associated diazotrophs from several grasses (Cavalcante and Dobereiner, 1988). Moreover, bromothymol blue is used as a pH indicator to characterize acid or alkali production after utilizing a carbon source in the medium.

1.6.3.2 Molecular analysis

Every living organism has DNA, RNA and protein, which provide useful tools for identification. Certain genes are highly conserved within species, allowing for their

identification. Therefore molecular analysis is one of the main portions in characterizing and identifying functions of potential N2 -fixing microorganisms.

1.6.3.2.1 *nif* gene

Biological N_2-fixation occurs in many different genera through the expression of different *nif* genes (Brill, 1983). The *nif* genes encode the N_2-fixation enzyme nitrogenase (Fig. 1.9). Nitrogenase enzyme consists of two protein components: dinitrogenase reductase (Fe protein) and dinitrogenase (FeMo-cofactor). The *nifH* gene is responsible for encoding the Fe-protein while *nifD, nifk* are responsible for encoding the Fe-Mo protein (Fig. 1.10). There are other genes (Table.1) that serve as regulatory genes (*nifL* and *nifA*), electron transport genes (*nifJ, nifF*), assemble of Fe-Mo cofactor encoding genes, and genes for processing Mo-Fe protein. However, *nifH* has several roles in N_2-fixation (Ludden, 1993).

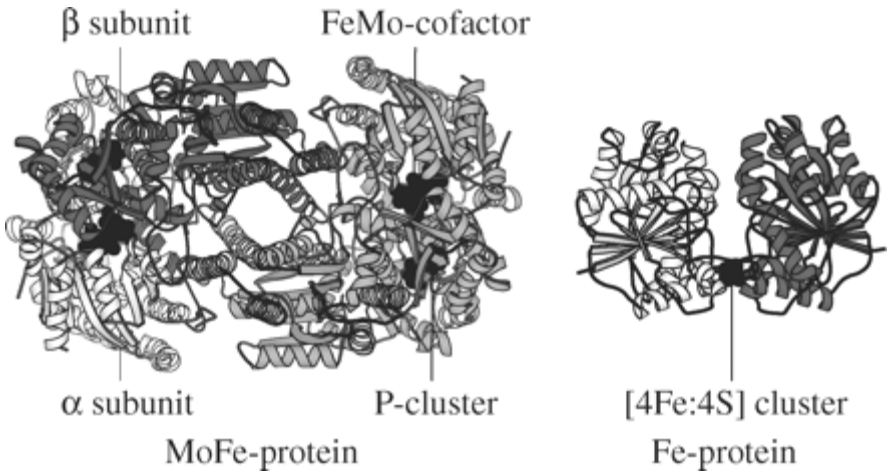

Figure 1.9 Nitrogenase enzyme

(Rees et al., 2005)

Table 1.1 *nif* gene and functions (Sofi and Wani, 2007)

Gene	Structure	Function
nifH	Fe-protein subunit	FeMo biosysnthesis
nifD	Mo-Fe α-subunit	Cofactor of holoprotein α 2β2 tetramer
nifK	Mo-Fe β-subunit	MoFe cofactor biosynthesis
nifB	Mo-Fe β-subunit	FeMo cofactor biosynthesis
nifE	Mo-Fe β-subunit	FeMO cofactore biosynthesis. Forms α 2β2 tetramer with *nifN* gene product
nifN	FeMo protein	Processing of Mo
	FeMo protein	Encodes a homocitrate
nifA	FeMo protein	Positively regulates the activity of *nif* transcription
nifL	FeMo protein	Negatively gergulates the activity of *nif* transcriptase
nifF	Flavodoxin	Physiological reduction of Fe-protein
nifJ	Pyruvate flavodoxin oxidoreductase	Reduction of glavodozin
nifM	Pyruvate flavodoxin oxidoreductase	Activity of Fe-protein
nifS	Pyruvate flavodoxin oxidoreductase	Processing of MoFe protein

Table 1.1 (Continued)

nifU	Pyruvate flavodoxin oxidoreductase	Processing of MoFe protein
nifY	Pyruvate flavodoxin oxidoreductase	Processing of MoFe protein but not required for diazotrophic growth
nifT	unknown	Not required for diazotrophic growth
nifW	unknown	Required for full activity of MoFe protein
nifZ	unknown	Required for full activity of MoFe protein
nifX	unknown	Involved in FeMo cofactor biosynthesis

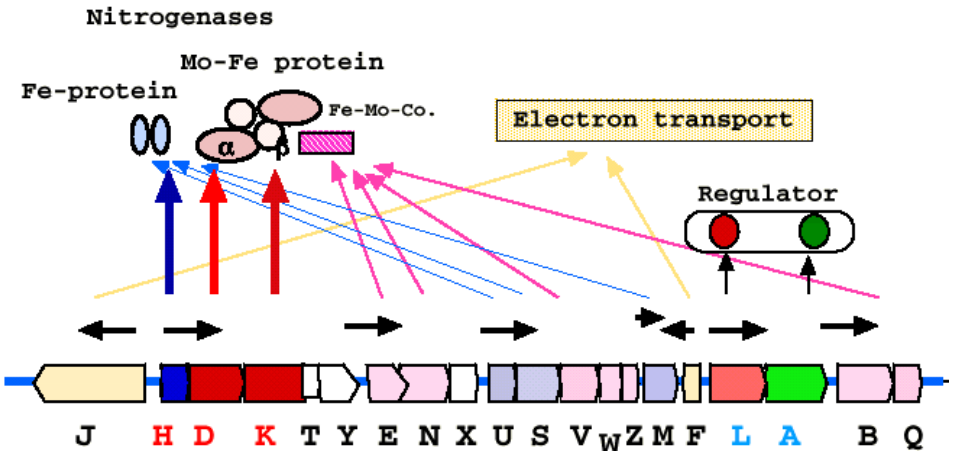

Figure 1.10 Physical association of *nif* gene map of *Klebsiella pneumonia* (Watanabe, 2000)

1.6.3.2.2 16S rRNA gene

Ribosomal RNA (16S rRNA) sequence analysis was first used by Carl Woese in order to identify various bacteria and for phylogenetic studies (Fox et al., 1977). Ribosomal RNA (16S rRNA) is a component of 30s subunit of ribosomes in prokaryotic

microorganisms. It is highly conserved within the same genus and species. Ribosomal RNA (16S rRNA) sequences are useful for classification of uncultivable bacteria, discovery and classification of novel bacterial species, and for identifying bacterial phylogenetic relationships. The most common primer used in 16s rRNA are 27f and 1492r primers, which reflect entire 16s rRNA of mostly identified bacteria (Hugenholtz et al., 1998; Rappe and Giovannoni, 2003). However, there are limitations associated with 16S rRNA gene sequences, such as no consensus quantitative definition of genus or species levels and microheterogeneity in 16S rRNA gene (Clarridge, 2004) by selecting as individual predictor. Therefore, 16S rRNA along with functional genes gives a more accurate prediction of specific genus or species level of a perticular organisms.

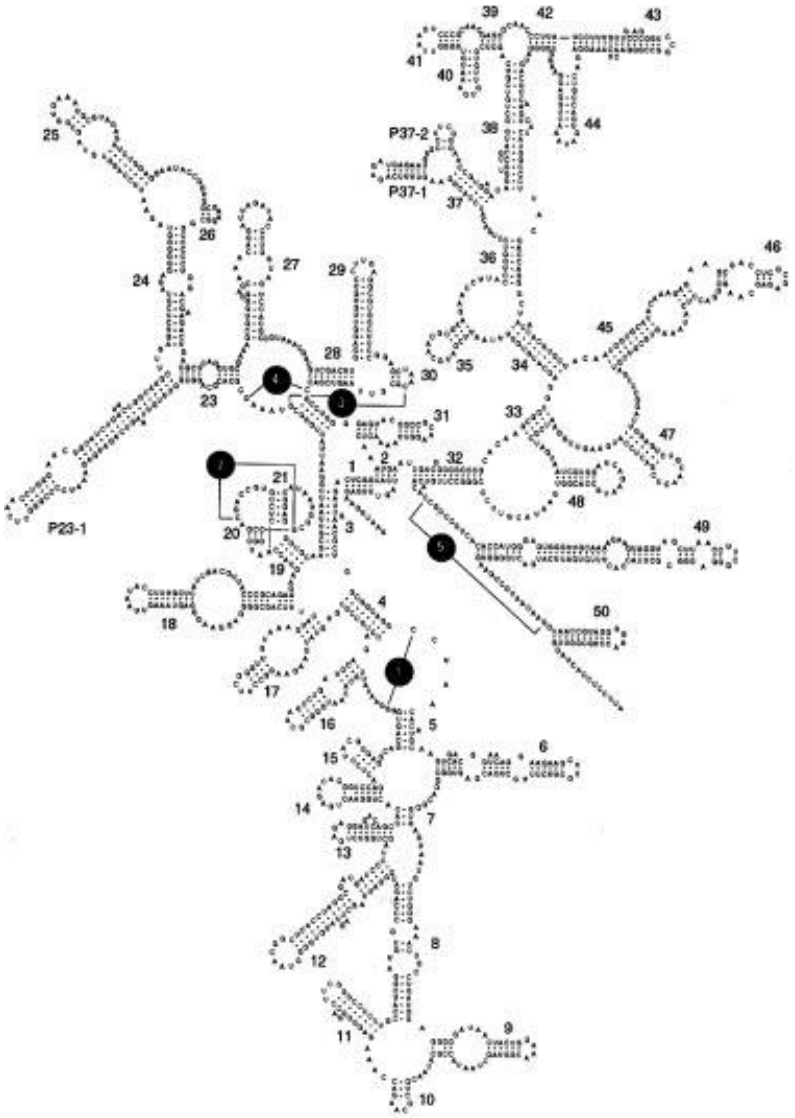

Figure 1.11 16S rRNA gene

(Van de Peer et al., 1996)

1.6.3.2.3 Microbial community structure

Soil can have 4–5 x 10^{30} microbial cells in 1 g (Singh et al., 2009) that have a key role in ecological processes in soil structure formation and recycling elements, such as C and N. Due to the complexity of the microbial composition in soil and other ecosystems, microbial ecology is used to identify the interactions and functions of microbes present in

soil and other environments. Moreover, as microbial community structures are complex in nature, fingerprinting techniques have been employed to identify the community structure in soil and plant ecosystems, such as denaturation gel electrophoresis (DGGE).

In DGGE the amplified PCR products with specific molecular marker (16S rRNA gene) from sample DNA are electrophoresed on a polyacrylamide gel containing a linear gradient of DNA denaturant such as urea and formamide (Muyzer et al., 1993). According to the different sizes of denatured DNA, bands can be seen in the gel. The taxonomic identification is then completed using computer assisted cluster analysis by software packages.

1.7 Significance of research

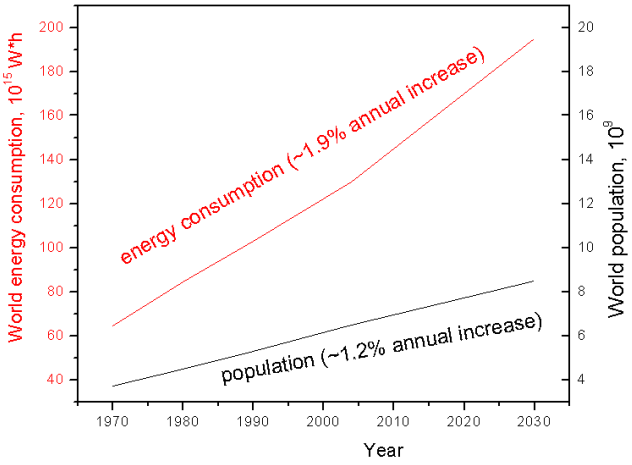

Figure 1.12 Comparison of the energy consumption and population growth in the world.

The curves have been calculated based on the data of the International Energy Agency Report 2004

According to the National Agricultural Statistics Service of the United States Department of Agriculture (NASSUSDA), N fertilizer prices have continued to increase

rapidly since 1960 (USDA, 2010). Nitrogen is one of the major nutrients needed to increase plant growth and therefore the biomass yield. Moreover, the world energy consumption is increasing at high rates as the world's population increases (Fig.1.12). Therefore the goal is to replace 30% of petroleum-based oil imports with alternative energy sources by 2025 (Ragauskas et al., 2006) and decrease N fertilizer utilization to improve the cost yield in the biofuel industry. The second generation of biofuels grasses are able to grow in low N environments and can produce high biomass yields. Studies that address the N utilization dynamics of these grasses and their associated N_2-fixing bacteria is needed to further improve the yield produced from these grasses. This dissertation uses both isotopic and molecular techniques on three bioenergy grasses to identify the ANF microorganisms and to define the potential N dynamics over period of time.

1.7.1 Aims of research

Studies over the past 50 years have shown the occurrence of ANF bacteria and N_2-fixation in grasses. However, it is not known if ANF occurs with biofuel feedstock grasses. Therefore, the central hypothesis of this study is that different biofuel feedstock grasses will each have unique capabilities to associate with N_2-fixing bacteria, which will affect the biomass yield. There is an urgent need to identify N_2-fixation and estimate the rates of N_2-fixation of bioenergy grasses to accelerate research on grass N_2-fixation in the biofuel industry. This hypothesis was tested through the following four objectives:

1. Characterize the dynamics of $\delta^{15}N$ in soil and plant pools and to estimate N_2-fixation in soil and plants of three perennial bioenergy grasses over three year time. Hypothesis for this aim is that N in plant shoot and root of

bioenergy grasses and root zone soil would be partially derived from recently fixed atmospheric N_2 and have lower $\delta^{15}N$ than sorghum-a confirmed non fixing plant and energycane would decline $\delta^{15}N$ throughout time.

2. Identify the potential for N_2-fixation associated with three feedstock grasses using the isotopic reduction technique. The hypothesis for this aim is that N_2-fixation by bacteria associated with roots of intact switchgrass, giant miscanthus and energycane will be different when exposed to $^{15}N_2$ under greenhouse conditions.
3. Isolate, identify and characterize root-associated and endophytic bacteria in three different feedstock grasses. The hypothesis for this aim is that diversity will be observed in ANF with three different bioenergy grasses under field conditions.
4. Evaluate the growth of three feedstock grasses by co-inoculation of ANF bacteria isolated from the field. The hypothesis for this aim is that inoculation of *Azospirillum lipoferum* isolated from energycane will increase the growth of giant miscanthus and switchgrass under greenhouse conditions.

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CHAPTER II

ISOTOPIC $\delta^{15}N$ EVIDENCE CONSISTENT WITH HIGH RATES OF ASSOCIATIVE NITROGEN-FIXATION IN THE ROOT-ZONE OF THREE PERRENIAL BIOENERGY GRASSES GROWING ON MARGINAL LANDS

2.1 Abstract

Associative N_2-fixation by bacteria associated with perennial bioenergy grasses has the potential to replace or supplement N fertilizer and support sustainable production of energy. Bioenergy grasses can yield large quantities of biomass needed for the production of biofuels, however, the capacity for these grasses to sustain growth in N-limited marginal ecosystems is one prerequisite to their economical viability. Three grasses considered as candidate feedstocks included in our experiment were; *Miscanthus x ginganteus* (giant miscanthus, Freedom), *Pancium virgatum* (switchgrass, Alamo) and *Saccharum sp.* (energycane, HO 02-147). *Sorghum bicolor* (sweet sorghum, M81-E), which had no detectable *nif* genes amplicons, and $\delta^{15}N$ (relative to air) was used as a plant reference of bioavailable N. Our objective was to measure isotopic natural abundance in perennial grasses and soil N pools over three years. The $\delta^{15}N$ in roots, shoots and soil of perennial grasses were expected to decline each year, and become significantly less relative to the sorghum reference plant. It was also expected that energycane would have lower $\delta^{15}N$ than the other plants due to its shared genetic heritage with sugarcane, which is well-known to support high rates of ANF in its root-zone. At the

beginning of the experiment, soil $\delta^{15}N$ was positive, ranging from +6.7 to +7.7. Roots and shoot $\delta^{15}N$ reflected the soil enrichment of ^{15}N during early stages of growth, especially in switchgrass. Shoot $\delta^{15}N$ declined significantly over the three years of growth from ~ +7.9 to +3.0, while roots $\delta^{15}N$ also declined but less dramatically. As expected, energycane $\delta^{15}N$ tended to be significantly lower than the other two grasses, especially compared to giant miscanthus. Energycane, giant miscanthus, and switchgrass were estimated to contain ~38, 21, and 31% of the N derived from the atmosphere (%Ndfa) via associative N_2-fixers. Soil $\delta^{15}N$ of the root-zone soils were dynamic across the three year period, but only root-zone soil associated with energycane showed significant quantities of plant Ndfa. These results suggest that perennial bioenergy grasses can support relatively high rates of ANF when grown on N poor soil. Efforts to understand plant-diazotroph interactions and the breeding of plants that support high amounts of ANF are likely to benefit the production of grass feedstocks and other graminaceous crops.

2.2 Introduction

Perennial grasses have the potential to produce biofuels that could help replace the demand for petroleum (Dhugga, 2007; Heaton et al., 2008; Yat et al., 2008; Kumar et al., 2009). For perennial bioenergy grasses to be economical and supportive of the overall agricultural economy, they should require little management (e.g. fertilizer), grow productively on marginal lands, and not compete for land used for food production (Farrell et al., 2006).

Diazotrophic bacteria associated with bioenergy grasses can fix atmospheric N_2, and provide a source of available N to benefit plant growth to increase biomass yield in

low N environment. The highest rates of ANF have been measured in sugarcane (e.g. CB 45-3, sp 70-1143), incorporating up to 70% of the N requirement and 150 kg N ha^{-1} $year^{-1}$ (Boddey and Dobereiner, 1995; Boddey et al., 1998). The bulk of research related to ANF has centered on sugarcane, but a few tropical forage grasses including *Brachiaria humidicola*, *B. decumbens*, *Paspalum notatum* and *Panicum maximum* have also been shown, in a several cases, to incorporate up to 40% of tissue N derived from N_2-fixation (Boddey and Knowles, 1987). Perennial temperate grasses have also been shown to support ANF and nitrogenase activity, but estimates of their contribution to plant Ndfa tend to be low and variable, with most rates ~10-20 kg N ha^{-1} $year^{-1}$. These rate estimates are overwhelmingly reliant on extrapolation from nitrogenase activity assays and were overwhelmingly measured on disturbed plant and soil samples. These studies nevertheless indicate that a number of different grasses can support root-zone diazotrophs (Lewandowski et al., 2003) yet the significance of ANF to support the bulk of N needs for these grasses is still in question (Jessup, 2009).

Both ^{15}N enrichment and ^{15}N natural abundance are valid methods to provide quantitative estimates of N_2-fixation (Bai et al., 2012; Munroe and Isaac, 2013). Enrichment methods can be used to confirm ANF and to provide estimates of N_2-fixation over relatively short periods of hours, and days, but longer time frames are difficult to conduct, and can suffer from isotope saturation following numerous exposures. Therefore, it is difficult to continuously utilize ^{15}N enrichment for long-term studies of N_2-fixation associated with plants across several years. The ^{15}N natural abundance method is relatively simple, inexpensive, and can be successfully used to determine ANF under natural conditions (Bai et al., 2012). However, the natural abundance method is

less sensitive in the short-term. As with any isotope study, turnover and gas transformations of the isotope may contribute to the underestimation of N derived from the atmosphere (%Ndfa). However, when N_2-fixation is expected to occur, natural isotopic change can provide useful indications of shifts or estimates of N_2-fixation over several years (Shearer and Kohl, 1986; Hogberg, 1997; Williams et al., 2006; Urquiaga et al., 2012).

Natural abundance isotopic studies were first implemented 70 years ago to identify symbiotic N_2 -fixation (Burris and Miller, 1941). Non-symbiotic aerobic N_2-fixation, determined by labeled $^{15}N_2$ later provided evidence of "non-symbiotic" fixation (Delwiche and Wijler, 1956). Since then, published ^{15}N natural abundance studies and ^{15}N-labeled N_2 experiments to investigate N_2-fixation by bacteria (e.g. *Azospirillum sp.)* in the root-zone of gramineae (Boddey and Knowles, 1987; James, 2000; Buckley et al., 2007) are relatively rare. Research is critically needed to investigate the full potential that N_2-fixation may play in grass feedstocks, crops, and the global N cycle.

The objective of this study was to characterize the dynamics of $\delta^{15}N$ in soil and plant pools and to estimate %Ndfa fixation in soil and plants in three perennial bioenergy grasses over three growing seasons. It was hypothesized that N in plant roots and shoots of perennial bioenergy grasses and root-zone soil would be partly derived from recently fixed atmospheric N_2 ($\delta^{15}N=0$), and have lower $\delta^{15}N$ than annual sweet sorghum, a confirmed non-diazotroph associated plant. It was also hypothesized that $\delta^{15}N$ in energy grass would decline with time among the perennial grasses, especially in energycane, a close relative of sugarcane.

2.3 Material and methods

2.3.1 Site description

The study was conducted at the Agronomy Unit 1 of the Leveck Animal Research Center located at Mississippi State University, Mississippi, USA (33° 28' N and 88° 47' W) and arranged as a randomized block design. Soils were not treated with N fertilizer after initial application. Initially low amount of N fertilizer were applied and plants subjected to grow in minimal N conditions. The soil at this site is mapped as a Catalpa (fine, smectitic, thermic fluvaquentic hapludolls) and was used extensively to grow pasture, hay and row crops such as cotton, corn and soybeans. The soils are dries quickly due to high evapotranspiration between March and September. These soils are subjected to flooding for a brief duration in late winter and early in spring and no agricultural management were applied. These traits support the designation of marginal (Larson et al., 1985; Barbier, 1997).

2.3.2 Bioenergy grasses

Three perennial bioenergy crops and one annual reference plant were established in a randomized block design with four replicated plots (0.75 x 0.52 m) from May 2010 to December 2012. Energycane (HO 02-147) was planted in two row plots with 3 stalks per row switchgrass ('Alamo'), giant miscanthus (*Miscanthus* x *giganteus*) and *Sorghum* (M81-E) seeds were planted, similarly. For simplicity, these plants will from now on be referred to as switchgrass, giant miscanthus, energycane, and sorghum, respectively.

Plantings of annual sorghum were planned following the perennial grass establishment year but were most successful in May 2012. Following planting of all species, a low but typical application of NH_4NO_3 was applied (54 kg ha^{-1}/19 kg N ha^{-1}) as

a quick release fertilizer to foster plant establishment. No fertilizer application was done after the initial stage. Typically, less than 30% of a fertilizer application is available and utilized by the plant (Staley et al., 1991). It has been shown that starter levels of N have little effect on early N_2-fixing activity, which tends to be relatively low during early growth establishment (Wada et al., 1986; Hogh-Jensen and Schjoerring, 1994). Herbicide (glyphosate) was applied at as aspot treatment 1.54 kg ai. ha^{-1} each year. Hand hoeing and weeding was also conducted in the second year. Trifluralin (0.84 kg ai. ha^{-1}) was applied in early spring in the third year but not in plots used to grow sorghum. Hand weeding was used to supplant the use of the Trifluralin in these plots.

2.3.3 Soil sampling

Soil samples were collected using a Hoeffer soil probe (2 cm diameter) at two depths: 0-10 cm and 10-30 cm. Six to ten cores, for the surface and subsurface, respectively, were placed randomly around a fixed circular growing base of the plant near the root-zone (Batten et al., 2006). These soil samples (~ 500 g) were stored on ice in sealed Whirl-Pak bags (Ocala, Florida) during transport and then subsequently stored at -80ºC. Samples were collected by replicate plot prior to planting in May 2010, followed by collections every 5-7 months at the beginning and end of the main growing season.

2.3.4 Preparation of soil for analysis

Roots were separated from soil samples and soil was sieved using sieve number 4 (4.75 mm). Subsamples were dried for 24 h, ground in a pestle and mortar for homogenization and finally pass through a 100 mesh sieve (150 µm). Soil was weighed (40 mg) into tin cups (5 x 9 mm, Costech #041077) that were then folded, sealed and

analyzed for N and $\delta^{15}N$ using a PDZ-Europa 20/20 Isotope Ratio Mass Spectrometer (Agilent, Oregon State University, Department of Crop and Soil Sciences).

2.3.5 Total biomass collection

At the end of the 2nd and 3rd growing season (January), total aboveground biomass was collected from each plot and a subsample was dried at 65°C for 4 days and used to determine annual yield (dry weight) in the field (Mg ha^{-1}).

2.3.6 Preparation of plant material for analysis

Roots were washed several times to remove soil particles and dried for 24 h at 65°C, ground with liquid N_2 and passed through a 60 mesh sieve (150 μm). When total aboveground biomass was collected, the subsample was used to estimate the $\delta^{15}N$ of shoots. When total biomass was not collected, similar subsamples of 6-8 maturing leaves (Ramos et al., 2001) of above ground material were taken from the field and were oven dried and ground as described. Both root and shoot materials were weighed (5-6 mg) and analyzed using isotope mass ratio mass spectrometry (IRMS).

2.3.7 Selection of reference plant

In May 2012, roots were surface sterilized and DNA (ZR Soil Microbe DNA Minikit, Zymo Research) was isolated from 0.5 g of crushed roots and 1.0 g of ground rhizosphere soil obtained from switchgrass, giant miscanthus, energycane and sorghum. PCR amplification was performed to amplify the 600-750 bp fragment (cluster I) in functional N_2-fixing gene *nifH,* (Kumari and Kumar, 2009) which is used to identify aerobic and anaerobic proteobacteria and N_2-fixing microorganisms in different environments (Ueda et al., 1995; Bergmann et al., 2009; Gaby and Buckley, 2014). After

PCR amplification of the *nifH* gene with an initial denaturation 5 min at 94°C then 30 cycles of 1 min at 94°C, 45 sec at 55°C, 1 min at 72°C, and a final extension of 5 min at 72°C. The amplified DNA was visualized in 0.8% agarose to identify the presence of N_2-fixing bacteria in our treatment and reference plants.

2.3.8 Calculations

The natural abundance of atmospheric N_2 was taken as 0.3663 atom% ($\delta^{15}N_{air}$ =0).

The following equations were used to determine $\delta^{15}N$ (‰).

$$\delta^{15}\mathrm{N}(‰) = 1000 \times \frac{\text{atom\% } (^{15}N \text{ sample}) - 0.3663}{0.3663} \quad 2.1$$

N derived from atmospheric N_2 in soils and plants was calculated using the following equation:

$$\%\mathrm{Ndfa} = 100\,(\delta^{15}N\,ref - \delta^{15}N\,fix)/(\delta^{15}N\,ref - \mathrm{B}) \quad 2.2$$

Where:

Ndfa- Nitrogen derived from air

$\delta^{15}N_{ref}$ - $\delta^{15}N$ of reference plant

$\delta^{15}N_{fix}$ - $\delta^{15}N$ of ANF plant

B - $\delta^{15}N$ of plant receiving all of its N through fixation

N derived from associative N_2-fixation (Ndfa) was calculated using sorghum as a reference plant for comparison to the three perennial bioenergy grasses (Hogberg, 1997). The value of B varies from species to species and with growth, but is close to $\delta^{15}N$ of 0 in legumes (Denton et al., 2013; Frankow-Lindberg and Dahlin, 2013). Therefore the B value was taken as $\delta^{15}N$ =0.

Accordingly, the equation can be simplified and written as follows (Boddey et al., 2001):

$$Ndfa = (\delta^{15}N_{ref} - \delta^{15}N_{fix})/ \delta^{15}N_{ref} \quad 2.3$$

2.3.9 Statistical analysis

Two-way analysis of variance (ANOVA) of plant species, time and their interaction on response variables was used to determine the statistical significance of differences in the $\delta^{15}N$, %N, %Ndfa and biomass for roots, shoots and soil associated with the growth of three feedstock grasses. The least significant difference (LSD) test at $p<0.05$ was used to assess the significance of statistical differences among treatment means. Statistical analysis was carried out using PROC MIXED and the repeated measures function in SAS Version 9.3 (SAS Institute, Cary, NC, 2010).

2.4 Results

2.4.1 Root-zone soil $\delta^{15}N$

Soil $\delta^{15}N$ before planting mean averaged +6.6 in the surface (0-10 cm) with low variation between plots (Fig. 2.1a). Overall, $\delta^{15}N$ in the root-zone soil following plant growth was significantly lower in energycane compared to switchgrass and giant miscanthus ($p<0.05$), suggesting greater flow of atmospheric N_2 to the energycane plant-soil system. The $\delta^{15}N$ value in the root-zone soil of energycane was also significantly lower ($p<0.05$) following the last sample collected in December 2012 in comparison to May 2012, also suggesting greater flow of atmospheric bacterial fixed N_2 in energycane compared to switchgrass and giant miscanthus soils. Overall, the tendency for greater variability in $\delta^{15}N$ compared to the initial pre-plant sampling (May 2010) may be

indicative of the heterogeneity of N_2-fixation, which would occur directly adjacent to a root.

In the subsurface (10 to 30 cm; Fig. 2.1b), there was considerably more variation in soil $\delta^{15}N$ than the surface, and plots randomly selected to receive switchgrass had significantly greater values compared to giant miscanthus and energycane ($\delta^{15}N$, +7.7 vs +7.2). The subsurface was more difficult to sample. However, five to six less number of sample cores of sub surface help to explain the greater variability in $\delta^{15}N$. These results make it difficult to arrive at conclusions about the overall lower $\delta^{15}N$ in the subsurface which also tended to decline in energycane planted soil compared to other perennial bioenergy grasses during the three years of growth. However, results are consistent with changes in $\delta^{15}N$ observed in the surface soil.

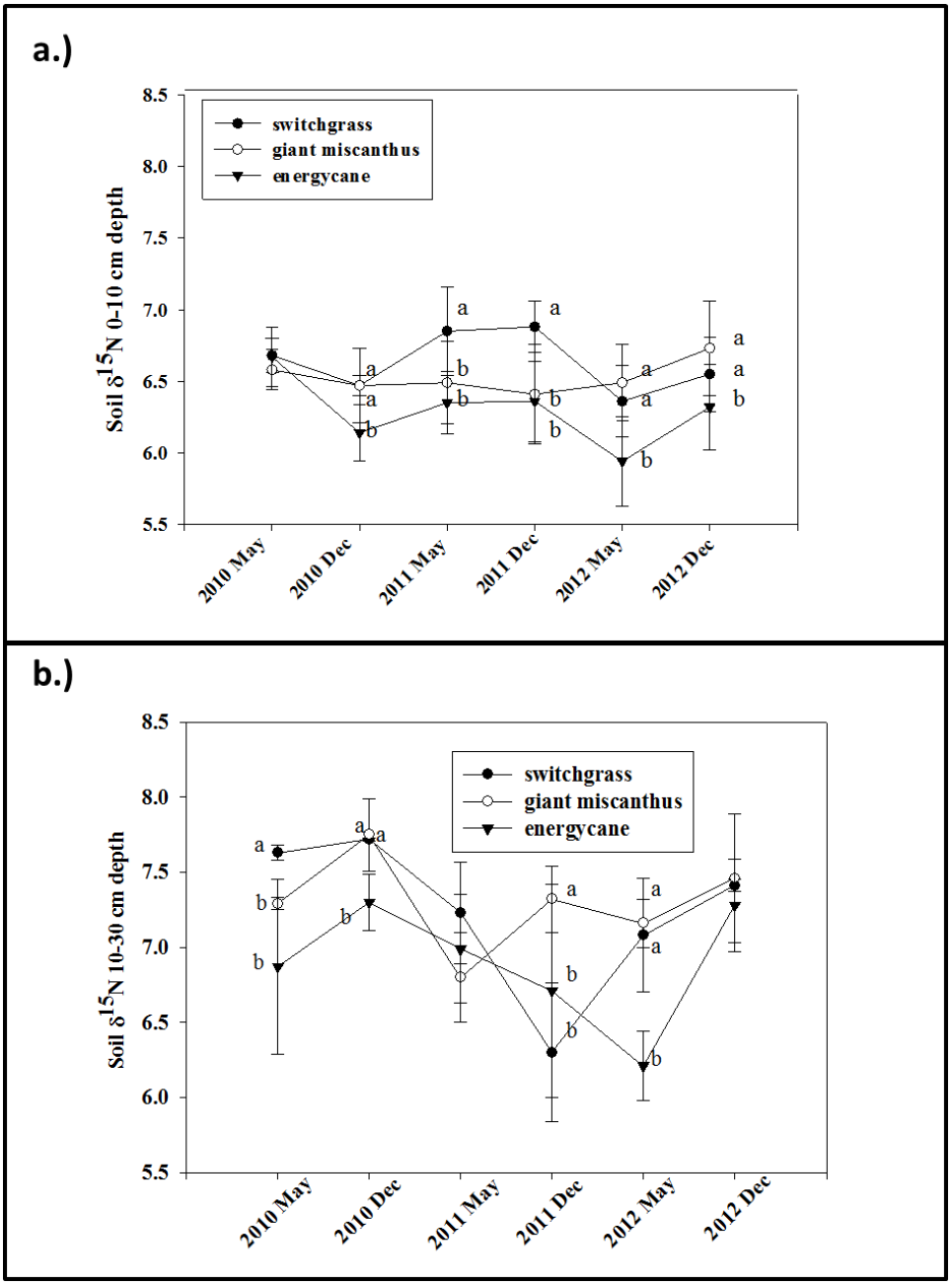

Figure 2.1 Mean soil δ15N in switchgrass, giant miscanthus and energycane grown over three years in field plots at different time points.

a) 0-10 cm depth, and b) 10-30 cm depth Letters (a,b) denote significant difference between the species and error bars represent standard error (n=4; $p<0.05$)

2.4.2 Root zone soil %N

Overall, variability in the root-zone soil %N was fairly low during the first two years of plant growth, but became more variable and also significantly greater in energycane, switchgrass and giant miscanthus plots in May 2012 at the surface, but not the subsurface soils, compared to other sampling periods ($p<0.05$, Fig.2.2a). Subsurface soil had a lower %N compared to surface soil (Fig.2.2b). Changes in pools of total N are often difficult to detect in only a few years of field experimentation; however, the significantly greater concentrations detected may be indicative of the process of N accrual. An increase from 1.4 to 1.5 mg N g^{-1} soil between May 2010 and Dec 2012 in the top 10 cm (bulk density, D_b, ~1.3 to 1.4 g cm^{-3}) is equal to approximately 130 kg N ha^{-1}.

This amount of N would be a relatively large increase based on typical annual estimates of ANF. However, amounts of soil N can vary significantly throughout a growing season, and are further complicated by variations in D_b. We could not continuously track D_b without compromising the integrity of the plots. However, estimates of D_b based on similar planted and bare soil plots suggest, they are not significantly different, though averages are 5 to 7% greater in unplanted plots (data not shown).

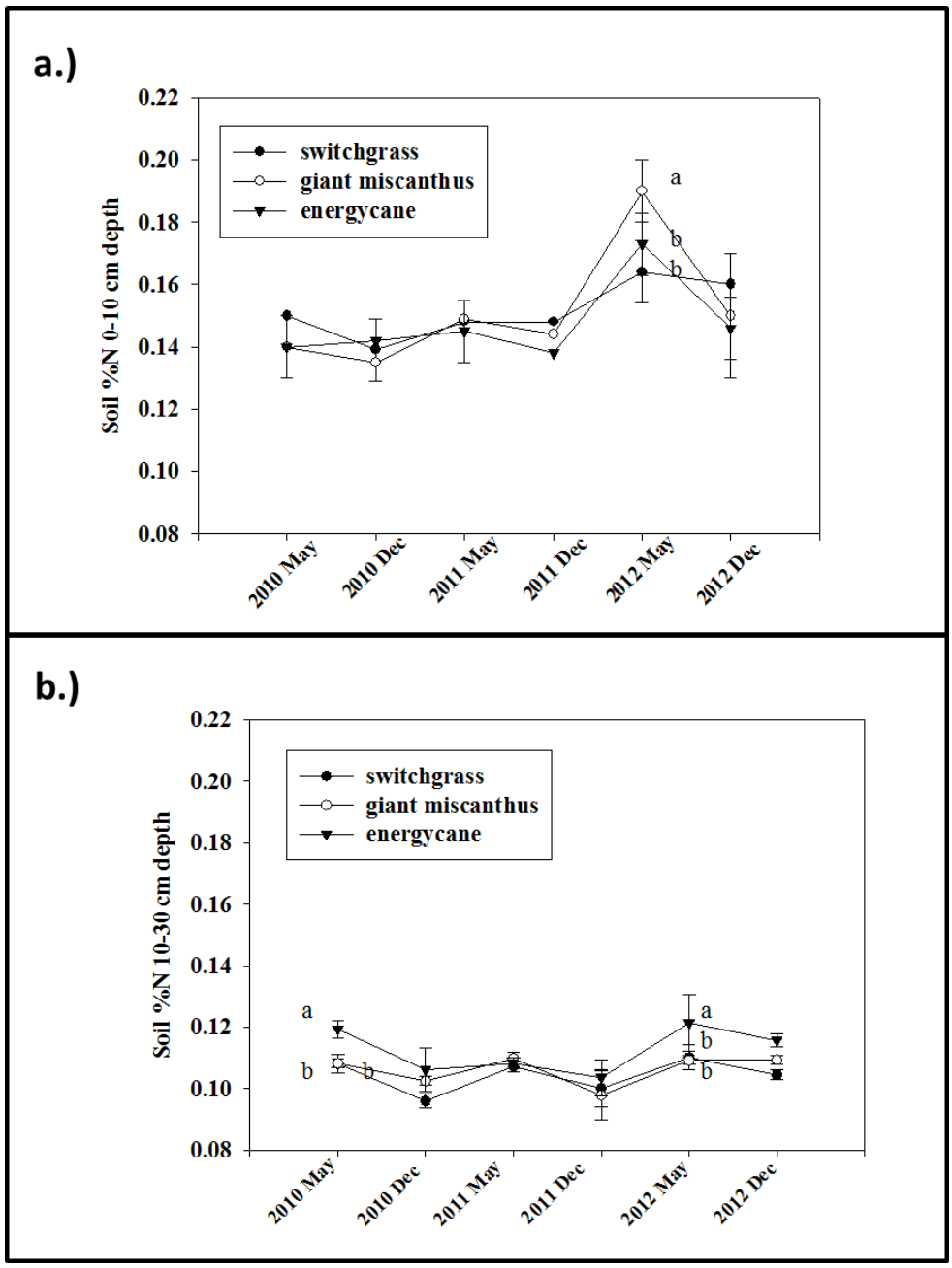

Figure 2.2 Mean soil %N in switchgrass, giant miscanthus and energycane grown over three years in field plots at each time point

a) 0-10 cm depth, and b) 10-30 cm depth. Letters (a,b) denote significant difference between the species and error bars represent standard error (n=4; $p<0.05$)

2.4.3 Temporal variation in shoot and root $\delta^{15}N$

Roots and shoots could not be sampled during the first year of plant establishment without compromising the future growth and success of the perennial feedstock grasses (Fig.2.3). The $\delta^{15}N$ of new shoots during the first growing season immediately following the establishment year (May 2011) reflect those of whole soil with little evidence for significant amounts of ANF. Similar but slightly lower $\delta^{15}N$ values were observed for roots at the first sampling in December 2010. However, $\delta^{15}N$ of energycane was significantly lower than switchgrass and giant miscanthus, and was significantly lower than whole-soil values. The $\delta^{15}N$ of shoots were dynamic and significantly greater in spring (May) compared to winter (December) for both years. Overall, the values tended to demonstrate a pattern of decline, with root and shoot $\delta^{15}N$ declining with time in all perennial bioenergy grasses ($p<0.05$). When considered over the entire study period, root $\delta^{15}N$ varied significantly ($p<0.05$) between the three grass species (Fig.2.3b), with energycane having the lowest values. Overall, the results were consistent with patterns of decline in $\delta^{15}N$ indicative of the process of N_2-fixation.

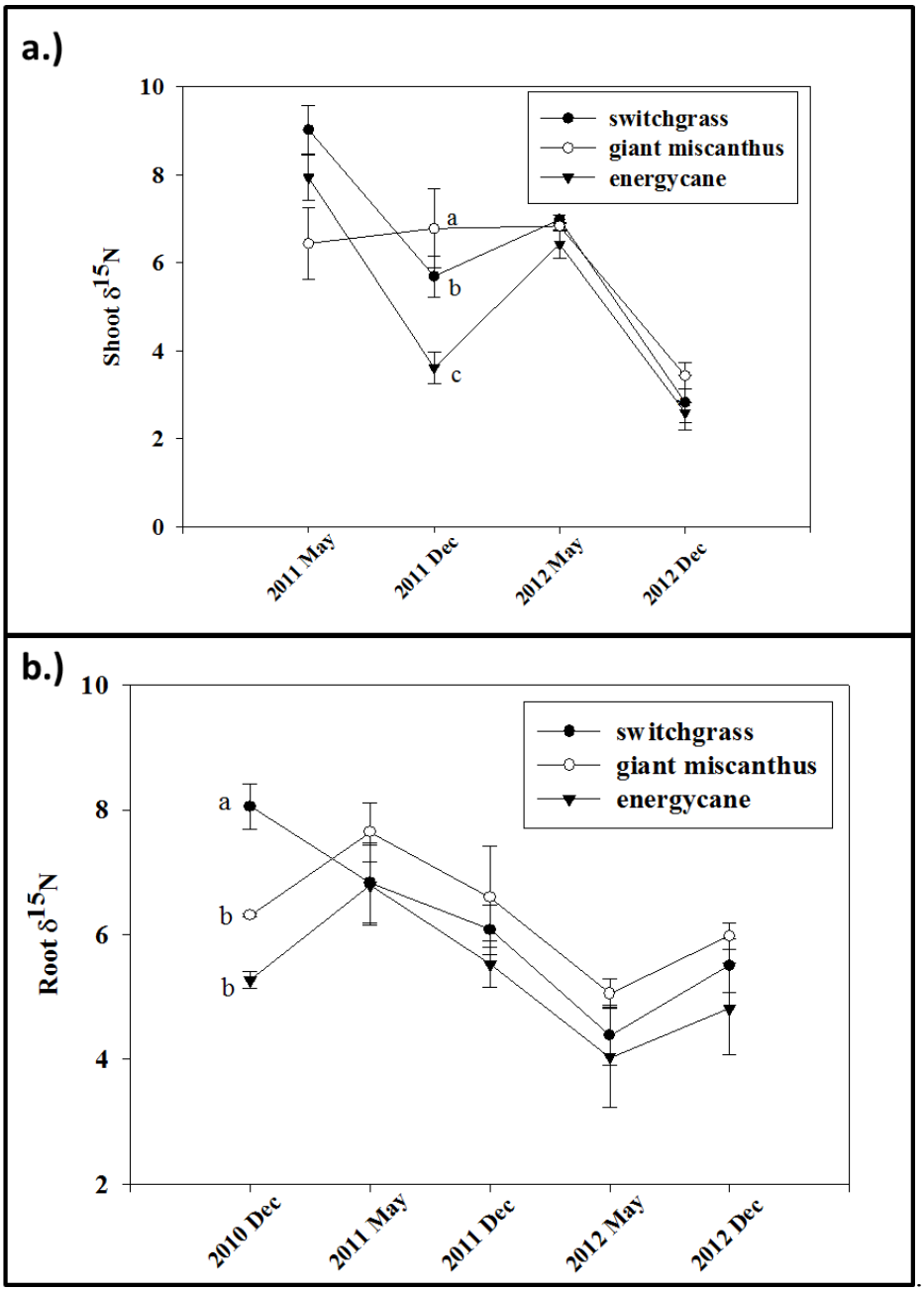

Figure 2.3 Mean δ15N of a) shoot and b) root in switchgrass, giant miscanthus and energycane grown over three years.

Letters (a,b) denote significant difference between the species and error bars represent standard error (n=4; $p<0.05$)

2.4.4 Temporal variation in root and shoot N

Shoot %N had similar temporal dynamics to that of $\delta^{15}N$ (Fig. 2.4a). There were discernable valleys and troughs in %N in shoots with the greatest values during early growing season and lowest during the late growing season. This pattern reflects the demand of N associated with plant growth during the growing season. Root dynamics were less variable (Fig. 2.4b), but shifts along with shoot dynamics were likely the result of translocation between shoot and belowground rhizomes and roots between seasons. There were, however, no persistently different %N content of the plants and considering these are relatively low fertilized perennial grass system, consistent with low bioavailable N soil conditions than would be a tendency to support N_2-fixation (Boddey, 1995).

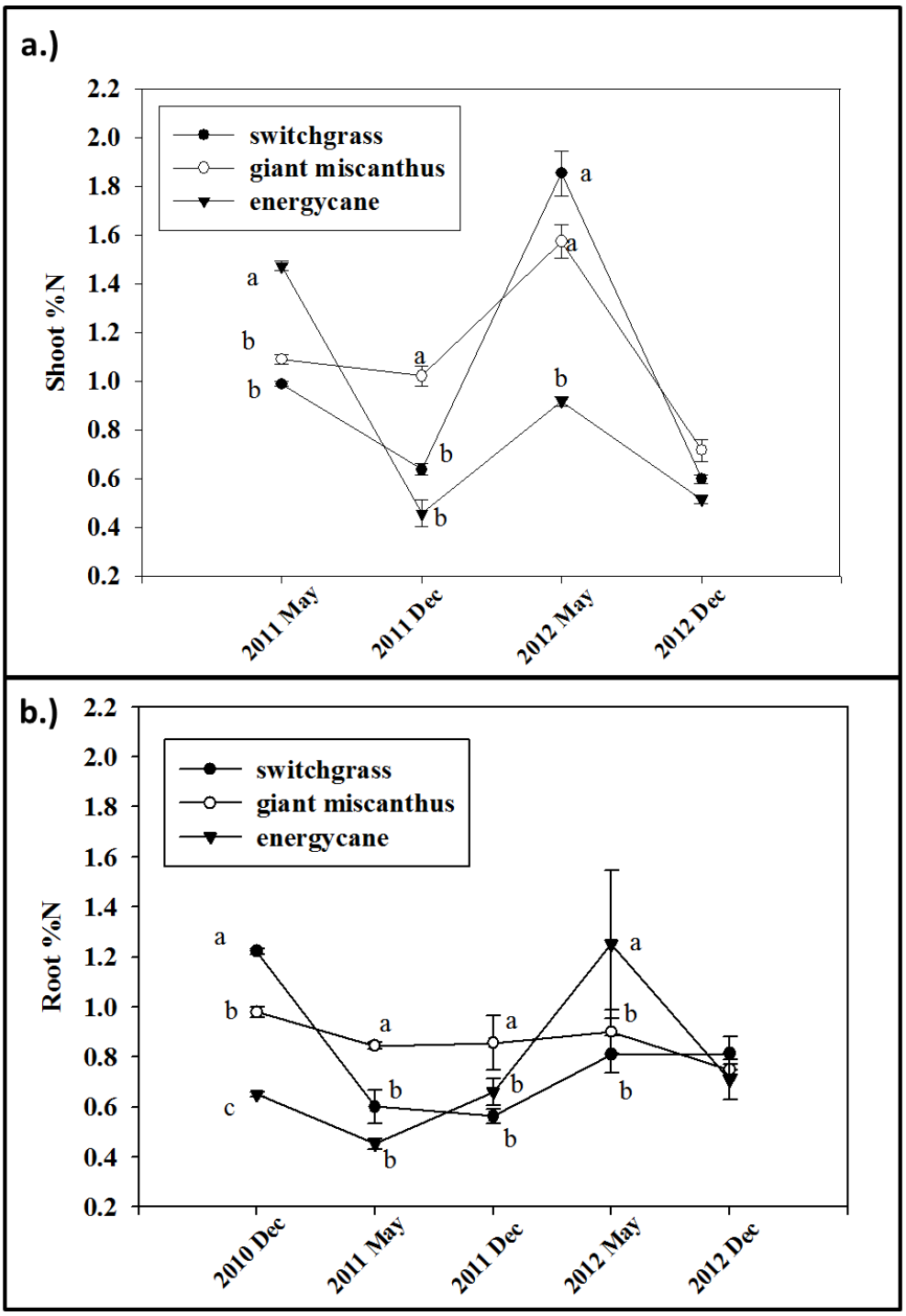

Figure 2.4 Mean %N of a) shoot and b) root in switchgrass, giant miscanthus and energycane grown over three years

Letters (a,b,c) denote significant difference between the species and error bars represent standard error (n=4; $p<0.05$)

2.4.5 $\delta^{15}N$ of sorghum reference, bare soils, and %Ndfa of grasses

Reference plots containing the annual, sorghum, and bare soil plots were used to comparison and to calculate %Ndfa of the three treatment grasses energycane, switchgrass and giant miscanthus (Table. 2.1). Overall, the greatest %Ndfa was found in shoots followed by roots and soils (Table.2. 2). Both switchgrass and energycane roots had greater %Ndfa compared to giant miscanthus. Shoot %Ndfa was greater in all three grasses in December 2012 compared to May 2012. Reference plant and soils, and soils in plots without any vegetation had similar $\delta^{15}N$ and were significantly greater than those of the perennial bioenergy grasses (Table.2.1). While *nifH* genes were detected and sequenced in the roots and rhizosphere of bioenergy grasses, *nifH* amplicons from root and root-zone soils of sorghum were below detection (Sarig et al., 1990), confirming the reliability of this sorghum variety as a non-fixing reference plant (Fig. 2.5).

Table 2.1 Isotopic $\delta^{15}N$ of shoot and root of reference plant (sorghum) and the $\delta^{15}N$ of soil which these plants were grown as well as bare soil plots in two different time points

$\delta^{15}N$	Roots	Shoots	soil 0-10 cm	soil 10-30 cm
Sorghum				
2012 May	5.3±0.1	7.5±0.2	7.0±0.1	7.3±0.1
2012 December	6.4±0.1	5.5±0.1	7.0±0.2	7.6±0.2
Bare soil plots				
2012 May	NA	NA	6.8±0.2	8.0±0.3
2012 December	NA	NA	6.8±0.1	7.9±0.1

mean n ± SE, n=4 (NA= not applicable)

Table 2.2 Nitrogen derived from air (%Ndfa) in Roots, Shoots, Soil in 0-10 cm and 10-30 cm depth of three perennial perennial bioenergy grasses in two different years

	2012 May			2012 December		
	Switchgrass	Giant miscanthus	Energycane	Switchgrass	Giant miscanthus	Energycane
Roots	17	4.8	24	14	6.7	24
Shoots	7.1	9.0	14	48	37	52
Shoot + Root	12[b]	6.9[c]	19[a]	31[b]	21[c]	38[a]
Soil 0-10 cm depth	6.8	4.5	12	4.4	1.6	7.6
Soil 10-30 cm depth	4.3	3.2	16	2.8	2.1	4.5

[a,b] Means followed by the same letter are not significantly different between different species in 2012 May and 2012 December, n=4, $p<0.05$ (LSD test)

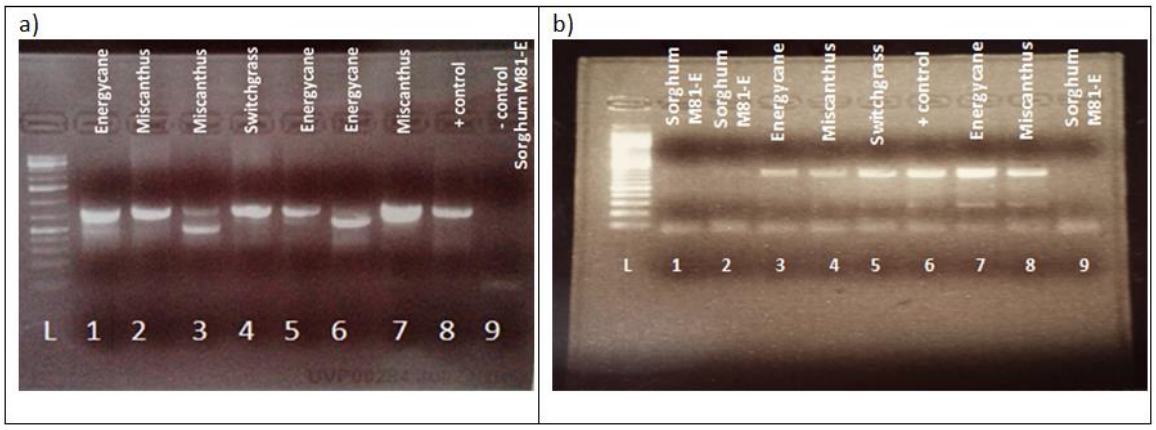

Figure 2.5 Electrophoretic analysis of the products of PCR (amplify with *nifH* primers) on the DNA preparations from soil and root of energycane, giant miscanthus, switchgrass and sorghum.

Lane-L, 100 bp ladder; a.) lane 1 (rhizosphere), 5 (root), 6 (root) DNA isolated from energycane of 2012 May; Lane 2 (rhizosphere), 3 (root), 7 (root) DNA isolated from giant miscanthus of 2012 May; Lane 4 (root) DNA isolated from switchgrass of 2012 May; Lane 8 positive control *Gluconoceobacter diazotrophicus* (cultures perches from ATCC® 49037 ™) ; Lane 9 (root) sorghum of 2012 May (negative control) b.) lane 1 (rhizosphere), 2 (root), 9 (root) DNA isolated from sorghum of 2012 December; Lane 2 (rhizosphere),7 (root) DNA isolated from energycane of 2012 December; Lane 4 (rhizosphere), 8 (root) DNA isolated from giant miscanthus of 2012 December; Lane 5 (root) switchgrass; Lane 6 positive control *Gluconoceobacter diazotrophicus* in 0.8% agarose gel stained with ethidium bromide (bands were present in 600-750 bp region)

2.4.6 Total biomass

There was no significant difference in dry matter yield among perennial grasses in the establishment year (Fig. 2.6). However, a higher dry matter yield was observed in energycane (15.9 Mg h^{-1}) compared to giant miscanthus (13.4 Mg h^{-1}) and switchgrass (14.2 Mg h^{-1}) in 2012. In 2013, energycane (23.1 Mg h^{-1}) and switchgrass (15.1 Mg h^{-1}) had significantly greater dry matter yield relative to 2012, and energycane had the highest yield compared to the other grasses. All perennial grasses, energycane, giant miscanthus and switchgrass had higher aboveground productivity compared to annual *Mg h* (8.5 Mg h^{-1}).

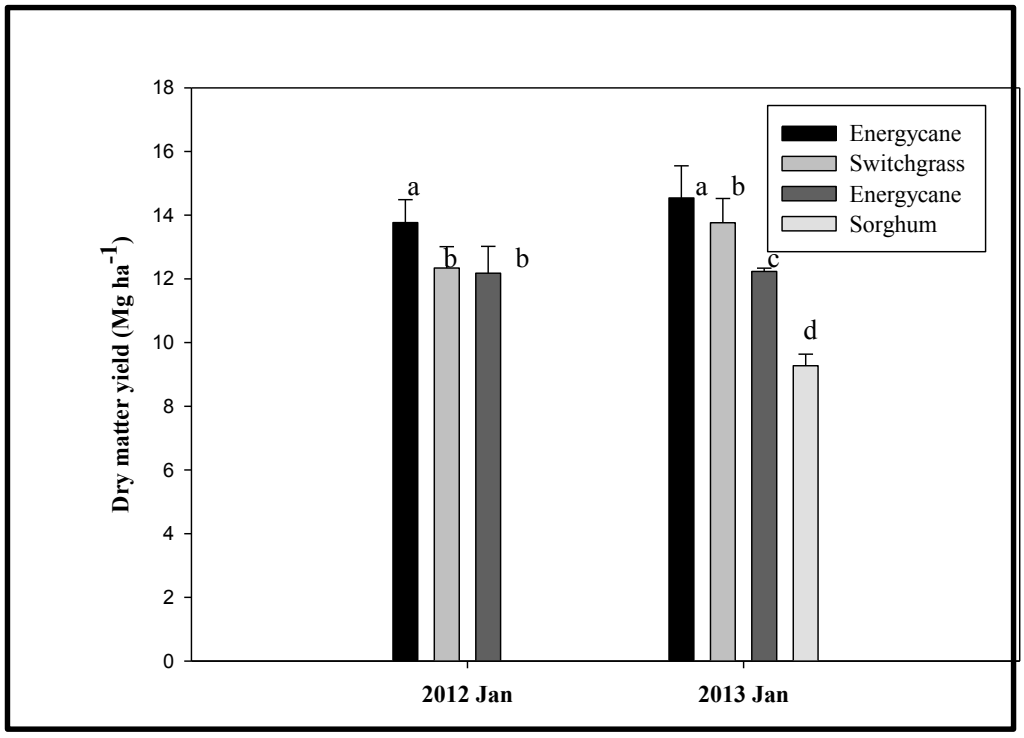

Figure 2.6 Total above ground biomass yield in switchgrass, giant miscanthus and energycane of two years

Letters (^{a,b,c,d}) denote significant difference between the species of 2012 and 2013 January and error bars represent standard error (n=4; $p<0.05$)

2.5 Discussion

The objective of this study was to assess the natural abundance dynamics of $\delta^{15}N$ in the root-zones of three perennial bioenergy grasses; and to use these changes to estimate the potential contribution of ANF as a source of plant N. It was hypothesized that ANF would result in a decrease in the $\delta^{15}N$ and greater plant %Ndfa in the root-zone and shoots of perennial bioenergy grasses giant miscanthus, switchgrass, especially in energycane, a hybrid of *Saccharum officinarum* (commercial sugarcane) and *Saccharum sponteneum* (wild cold hardy sugarcane). In support of this hypothesis, in the first and second years following perennial grass establishment, there was significantly lower $\delta^{15}N$ and significant quantities of Ndfa in perennial bioenergy grasses was observed compared to the non-fixing annual sorghum.

To accurately determine Ndfa, the choice of a reference plant is a major experimental consideration (Shearer and Kohl, 1986). Some varieties of sorghum such as BRA 308 have been shown to support root-zone N_2 fixers (Coelho et al., 2009) and the plant acquisition of Ndfa. In our study, it was confirmed at two stages of plant growth, that there were no detectable amounts of the target *nifH* amplicon in the roots and rhizosphere soil of sweet *Sorghum* variety M81-E. These results support the first criteria for a suitable reference plant, which is to derive its N from soil and not from the atmosphere via root associations with diazotrophs (Sarig et al., 1990; Lee et al., 1994). Second, the source of soil N utilized by the reference plant should be representative of bioavailable soil $\delta^{15}N$ utilized by target plants. In the second case, plant $\delta^{15}N$ was consistent with expectations of a soil source of N and also consistent with values derived from other varieties of non-fixing sorghum (Lee et al., 1994; Urquiaga et al., 2012). The

plant pool used to estimate bioavailable N resembles, and on average significantly more depleted than whole soil ($\delta^{15}N$=0.7). This level of isotope differentiation between plant and soil isotope ^{15}N is also consistent with other studies in N-limited systems, whereby the $\delta^{15}N$ of the soils tend to be enriched relative to plants (Hogberg, 1997; Williams et al., 2006; Kahmen et al., 2008). The depletion of plants, as expected, is even greater than in soils observed or predicted to have plants growing symbiotically with N2-fixing bacteria (Shearer and Kohl, 1986; Hogberg, 1997; Robinson, 2001). Sorghum shows evidence supporting its use as a viable reference plant to calculate Ndfa for perennial diazotroph associated grasses.

Following the establishment year, it was likely that root productivity and depth of root exploration were greater in perennial than annual grasses (Tufekcioglu et al., 1998; Neukirchen et al., 1999; Koteen et al., 2011). Greater rooting biomass and depth of perennial grasses compared to sorghum (Myers, 1980) thus may have supported the uptake of heavier N (^{15}N) in the subsoil which would reduce estimates of fixation in perennial grasses (Urquiaga et al., 2012). The results thus may be conservative estimates of N_2-fixation across the three perennial bioenergy grasses.

Estimating rates of N_2-fixation in field experiments over several years is still a challenging, but a highly important research goal. Short-term experiments using enriched or depleted (labeled) ^{15}N could provide, perhaps, more accurate instantaneous rate estimates of N_2-fixation, but the labeling process can also create artifacts. Rate measurements, of a few hours or a day cannot be used to extrapolate to longer time periods of weeks, seasons, and multiple years. To our knowledge, long-term (across several years) labeling experiments have not been conducted, likely because of the

expense, experimental constraints on the application of label to the plant-soil system, and difficulty in the interpretation of fixation rates as more ^{15}N is added and cycled through the plant-soil system. Natural abundance experiments suffer from similar problems related to the movement of fixed N into the ecosystem, whereby, N losses to soil and the atmosphere, and interaction of other processes in the N cycle can affect $\delta^{15}N$. The atmosphere, however, has the advantage of being a reliable and stable source of N_2 ($\delta^{15}N=0$), and thus serves as a good proxy for the movement of fixed N_2 into plants.

Soil N has several fates, including denitrification, immobilization, leaching and plant uptake, and each of these have different and sometimes opposing effects on the $\delta^{15}N$ observed in soil and bioavailable plant N pools. Plants do utilize sources of NO_3^--N and NH_4^+-N in soil that can be both more or less depleted compared to bulk soil pools (Shearer and Kohl, 1989). Denitrification of depleted pools of NO_3^- could have played a role in shaping $\delta^{15}N$ in our samples, however, denitrification would have tended to increase soil $\delta^{15}N$; and therefore, reduce our estimates of N derived from ANF. Cool temperatures of 4-6°C (Ryden, 1986; Jordan, 1989; Ruz-Jerez et al., 1994) likely reduced the activity of denitrification in the winter. In the summer, plants were actively growing without fertilization. Plant N content was also indicative of low N availability, and so it was expected that plant demand for available N with low soil supply helped to reduce NO_3^- concentrations and the potential for losses via denitrification.

Lower $\delta^{15}N$ in shoots were observed in spring compared to those in winter. This dynamic could be explained by the normal transfer of N from aboveground to belowground roots and rhizomes (Gathumbi et al., 2002). This trend in belowground allocation is also consistent with the decline in %N of the shoots in winter, however, this

sampling also follows the period of high summer plant productivity and C flow, which would also be expected to decrease %N in shoots. When aboveground plant biomass was harvested in January, the plants were senescent and so translocation from the aboveground to the belowground had already taken place. However, the $\delta^{15}N$ of the shoots tended to be lower than that of the roots and so removal of plant shoots in the second year of the study would have resulted in the preferential loss of ^{14}N relative to ^{15}N and thus lowered estimates of diazotrophic derived N in the plant-soil system.

In the early periods of this experiment, the relatively high $\delta^{15}N$ in the roots and shoots could be interpreted as the result of low colonization and activity of N_2 fixers in the root-zone and greater N acquisition from soil sources. Plant and root $\delta^{15}N$ values from our study were generally similar to those seen in other systems dominated grasses, where the range of values between -2 and +8 are common, but can still vary considerably both positively and negatively (Handley and Scrimgeour, 1997; Tsialtas and Maslaris, 2005; Williams et al., 2006; Kahmen et al., 2008; Klaus et al., 2013). Growth of grasses in monoculture have also been observed to have $\delta^{15}N \sim 6.5$, but when grown in competition with other grasses, there is substantial depletion by 2 to 4 units (Tsialtas and Maslaris, 2005), consistent with the idea that competition for N and overall lower N availability helps to stimulate ANF (Williams et al., 2006).

Shoots and roots of perennial bioenergy grasses accumulated N consistent with an atmospheric source with a low $\delta^{15}N$, and by the end of the experiment it was estimated that ~1/3 of the plant N would have been derived from the process of ANF. Previous studies of sugarcane have demonstrated that up to 60-80% (> 150 kg N ha^{-1} $year^{-1}$) of plant N is derived from N_2-fixation (Boddey et al., 1995; Baptista et al., 2014). Perennial

grasses may not have shown the potential for these high rates, but nevertheless demonstrate that relatively large amounts of N are moving into plant tissues following N_2-fixation by diazotrophs. These results support the need for more research into the potential of feedstock grasses and other plants to associate with diazotrophs and increase Ndfa in agro-ecosystems.

In addition to evidence for the movement of fixed N into roots and shoots, the $\delta^{15}N$ of surface root-zone soil planted with energycane declined by the end compared to the beginning of the experiment. The decline in soil $\delta^{15}N$ further supports that N is moving from depleted ^{15}N-atmospheric sources to soil via ANF. Similarly, the lower soil $\delta^{15}N$ in subsurface soil in energycane compared to the other grasses further support the greater potential for N_2-fixation associated with energycane. Biomass yield was also greater for energycane, which provides the catalyst for greater biomass C and energy supply to diazotrophs, and greater demand for N that would help to support ANF in nutrient limited soils.

Energycane is a F_1 hybrid of *Saccahrum sp*. and *Saccahrum spontaneum*. The interbreeding of these two plants has increased the cold tolerance and survivability of energycane compared to sugarcane (Wang et al., 2008). Energycane produces relatively high fiber and low sugar compared to that of conventional cane (Jessup, 2009). It has retained the ability to resprout from stolons following winters that have killed several sugarcane varieties on the same field plots and had 30% more aboveground biomass growth compared to switchgrass and giant miscanthus following the third year of the experiment. These abilities to survive cold winters, outgrow other perennial grasses, and to associate with N_2-fixing bacteria to meet much of its N needs suggest it may be a

strong alternative and sustainable feedstock for biofuel production for some regions. Switchgrass is a highly adapted and genetically diverse native grass that can grow productively in many regions of the U.S., and along with giant miscanthus also shows strong potential to associate with N_2 fixers that can supply N for plant growth in temperate zones. Likely, high amounts of belowground C flow help to explain high rates of N_2-fixation. Furthermore, as C flow is increased and N is fixed, soil organic matter may accumulate and result in the sequestration of soil carbon. Across several years to decades, the growth of these grasses could feedback positively to support soil organic matter accumulation and thus the long-term sustainability of the biofuel feedstock system.

2.6 Conclusions

This study provided evidence that three perennial energy grasses derive N from atmospheric fixation by associative diazotrophs. Energycane, as expected, showed evidence for the greatest amounts of N derived from the atmosphere, followed closely by switchgrass, and to a lesser extent giant miscanthus. The high biomass yields of perennial bioenergy grasses suggest the potential for these grasses to grow sustainably in N-limited marginal lands with the support of diazotrophic N_2-fixation. Long-term studies spanning several years to a decade are needed to provide information on the sustainability of the biomass production of these perennial energy grasses. Monitoring growth and $\delta^{15}N$ over several more years, and the accumulation of N with a low isotopic signature should be complemented with further studies that utilize isotopically enriched $^{15}N_2$ and acetylene reduction techniques to confirm rates of N_2-fixation. Breeding of ANF-supportive plant

traits and a better understanding of the mechanisms of plant-diazotroph interaction will support the development of long-term sustainable feedstock and other crop systems.

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CHAPTER III

QUANYIFICATION OF BIOLOGICAL N2 -FIXATION ASSOCIATED WITH THREE BIOENERGY GRASSES $^{15}N_2$ ENRICHED SOIL

3.1 Abstract

Nitrogen isotope studies provide strong evidence that certain tropical grasses can obtain at least part of their N needs from biological N_2-fixation. However, no studies have been performed to directly analyze N_2-fixation in bioenergy grasses using isotopic measurements. In this study, soil collected from marginal land was mixed with sand and used to grow the bioenergy grasses such as energycane, giant miscanthus and switchgrass. Soil was enriched with 5% $^{15}N_2$ gas, traced by 5% Ne gas with each pot enclosed in a polyethylene bag. The $^{15}N_2$ recovery was then calculated. For controls, atmospheric N_2 and Ne gas were injected in same the manner. *Sorghum* was used as a negative control. After 24 h incubation with the N_2 (atmospheric N_2 and $^{15}N_2$), samples were analyzed by gas chromatography coupled with thermal conductive detector (GC-TCD) to quantify Ne. Observed recovery for Ne was assumed to equal to $^{15}N_2$ and was ~ 84% after 24 h incubation. Moreover, isotope ratio mass spectrometry (IRMS) was used to detect $\delta^{15}N$ values in soil, root and plants of three bioenergy grasses. Higher $\delta^{15}N$ values (+7.20 to +4.01 in roots, ~ +5.00 in soil and shoots) were obtained in labeled pots compared to controls, with %Ndfa values of 35%, 44% and 33% in energycane, switchgrass and giant miscanthus, respectively. Control pots were showed 60%, 58% and

38% in Ndfa energycane, switchgrass and giant miscanthus, respectively. These data suggest that energycane has a greater potential for N_2-fixation than switchgrass and giant miscanthus. This data, in combination with previous studies that indicated energycane produce the greatest biomass and suggest that energycane is suitable as a biofuel feedstock requiring low to no fertilizer input on marginal soil.

3.2 Introduction

Research related to optimizing the biomass yield of bioenergy crops capable of growing in low N environments has expanded due to a continual increase in the price of N fertilizer and disparity of fuel demands and supply. Switchgrass, miscanthus and energycane (hybrid of *Saccharum spontaneum* and *S. officinarum*) have extensively been researched in second generation biofuel industry. Therefore, within the past decade, considerable attention has been focused on the potential of N2 -fixation by bacteria associated with non-legume crops, especially with the bioenergy crops switchgrass, giant miscanthus, and sugarcane which partially satisfy their N requirement through N_2-fixation (Döbereiner, 1997; Davis et al., 2010).

A number of studies have been conducted to analyze the capability of N_2-fixation associated with sugarcane (Ohyama et al., 2014; Paungfoo-Lonhienne et al., 2014). Several N_2-fixing bacteria have been isolated from sugarcane, including predominately *Gluconacetobacter diazotrophicus* and *Herbaspirillum* (Baldani et al., 1997; James, 2000; Baldani et al., 2002; Boddey et al., 2003); switchgrass including (*Paenibacillus polymyxa* (Ker et al., 2014)) and miscanthus including *Azospirillum sp.* (Eckert et al., 2001), *Herbaspirillum spp.* (Kirchhof et al., 2001), *Clostridium spp.* (Saito and Minamisawa, 2006)). However, it is not known whether the presence of these microbes

corresponds to an increase in N_2-fixation rates, and therefore contributes to biomass yield.

Previous studies from our group (Chapter II) have indicated that variations exist in the potential biomass yield of bioenergy crops. We have identified greater potential for bacterial associative N_2-fixation in energycane compared with switchgrass and giant miscanthus. Moreover, no N_2-fixing bacteria were identified with the annual grass sorghum. Though many studies have investigated the N accumulation by ANF microorganisms associated with grasses, these studies have not been able to quantify the rates due to limitations associated with low rates of N_2-fixation (Trivelin et al., 1994).

Reduction of $^{15}N_2$ is a reliable direct method to measure rates of N_2-fixation (De-Polli et al., 1977; McNeill et al., 1994). The use of $^{15}N_2$ has been mostly applied in small scale greenhouse studies to obtain accurate results and confirm field experiments (Ruschel et al., 1979; Giller et al., 1984). This study was aimed at utilizing a $^{15}N_2$ labelling to confirm field data collected (Chapter II). It is important to define the rates of N_2 fixation and to trace the fate of the fixed N_2 by ANF bacteria with these grasses. Therefore, the main objective of this study was to quantify rates of N_2-fixation in a short period of time for the energy grasses giant miscanthus, switchgrass, and energycane using $^{15}N_2$ reduction method in controlled, greenhouse conditions. We hypothesized that different rates of N_2-fixation by bacteria associated with roots of intact switchgrass, giant miscanthus and energycane would be evident using the $^{15}N_2$ reduction method. Isotopic $\delta^{15}N$ and %Ndfa are key findings to predict reduction of $^{15}N_2$ in ANF associated with bioenergy grasses. Our previous findings support that energycane has the greatest potential to associate with ANF bacteria to obtain greater biomass yield.

3.3 Materials and methods

3.3.1 Soil enrichment with $^{15}N_2$

Soil was collected from the field edges of Agronomy Unit 1 of the Leveck Animal Research Center located at Mississippi State University, Mississippi, USA (33° 28' N and 88° 47' W) and sieved using sieve number 4 (4.75 mm) in order to homogenize the soils and remove large particles. Soil was mixed with sand in 1:1 ratio and approximately 2500 g soils were placed into pots. Soil properties, such as soil moisture content, bulk density and pH, were recorded prior to planting. The three bioenergy grasses used were: energycane (HO 02-147), giant miscanthus (*Miscanthus* x *giganteus*) and switchgrass ('Alamo'); *Sorghum* grass was used as a negative control. Rhizomes were collected from the field where soil was collected (Chapter II) and germinated in an incubator (Fig.3.1a). Six pre-weighed rhizomes with one shoot were planted into separate pots; Total weight of pots and rhizomes were recorded. Plants were watered every 2 other dys throughout the duration of the study. Pots were spaced in a greenhouse in randomized block design.

Isotopic gas $^{15}N_2$ was injected at the elongation phase of grasses (Fig.3.1b), which was represented by the formation of six leaves (Moore et al., 1991).

Figure 3.1 Germination of rhizomes and visual representation of plants (a.) elongation stage (b.) growth phase (logarithmic)

Neon gas was used as a tracer because it is an inert gas that has a similar interaction with water or air as N_2 does (Hamme and Emerson, 2004). Pots were covered with polyethylene bags and three rubber stoppers were embedded in the sides at 10.5 cm from top (middle of the pot) of pot to allow for injection of gas (Fig.3.3). Neon (99.9%, Sigma Aldrich 601691) and isotopic $^{15}N_2$ (98%, Sigma Aldrich 364584) were diluted to 5% by using 1 L gas sampling bags (SKC, Inc) (Fig. 3.2). Sixty ml of 5% Ne gas and 5% $^{15}N_2$ were injected through rubber seals using a 60 ml syringe. Control pots sealed with polyethylene bags and embedded with rubber stoppers were injected with $^{14}N_2$ (atmospheric gas) and Ne gas. Air volume in pots was determined by displacing the air with water. Gas was collected after 24 h post injection using a 10 ml syringe into 10 ml vacuum tubes to detect Ne gas. Soils were separated form whole plants and shoots and roots lengths were measured, and total dry matter yield was obtained by drying plants at 60°C for 3 days (Fig.3.4).

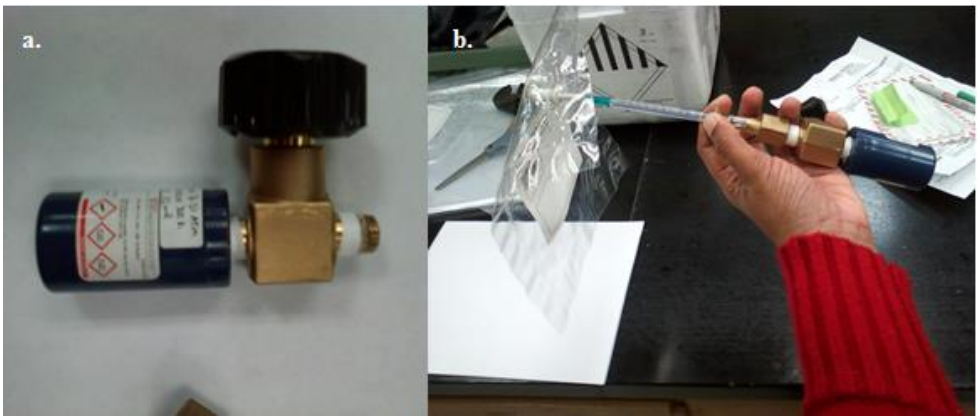

Figure 3.2 $^{15}N_2$ isotopic gas at 98 atom% and dilution into 5% using gas sampling bags (a.) $^{15}N_2$ isotopic gas (b.) dilution procedure

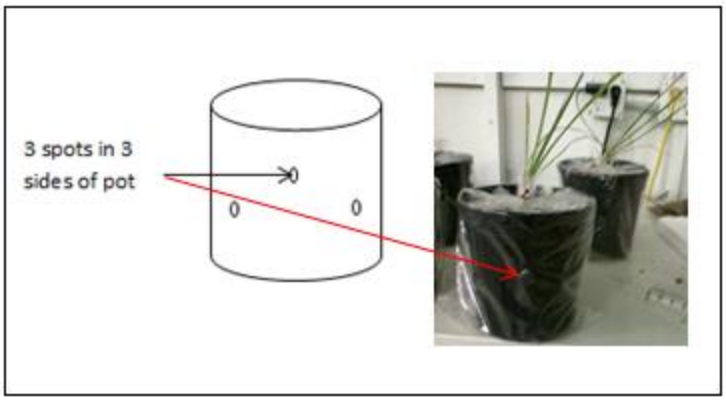

Figure 3.3 Diagram and visual representation of placement of rubber stoppers

Figure 3.4 Representative image of each grass harvested 24 h post inoculation

3.3.2 Analysis of soil for $\delta^{15}N$

Roots were separated from soil and soil was sieved using sieve number 4 (4.75 mm). Subsamples were dried for 24 h at 60°C, grounded in a pestle and mortar for homogenization and finally pass through a 100 mesh sieve (150 µm). Soil was weighed (10 mg) into tin cups (5x9 mm, Costech #041077) that were then folded, sealed and analyzed using dry combustion analyzer (Carlo Erba, Milan, Italy) and Isoprime mass spectrometer (Micromass, Beverly, MA).

3.3.3 Analysis of plant material for $\delta^{15}N$

Roots were washed with distilled water for several times to remove soil particles rinsed with ethanol and then distilled water 3 times. After washing, roots were dried for 24 h at 65°C, ground with liquid N_2 and passed through a 60 mesh sieve (150 µm). Total aboveground biomass was collected and was used to estimate the $\delta^{15}N$ of shoots. Subsamples of above ground material were oven dried at 65°C for 24 h, frozen with liquid N_2 and ground to pass through 60 mesh sieve. Both root and shoot materials were weighed (5-6 mg) into tin cups (5 x 9 mm, Costech #041077) that were then folded, sealed and analyzed for $\delta^{15}N$ by CFIRMS.

3.3.4 Calculations

Nitrogen concentrations (mg g^{-1}) were calculated from the %N in each grass and total N was obtained using dry matter yields of plants and N concentration. Total N in $^{15}N_2$ labeled plants and unlabeled plants were used to acquire the amount of N_2 fixed following the $^{15}N_2$ injection.

The natural abundance of atmospheric N_2 was taken as 0.3663 atom% ($\delta^{15}N_{air}$ =0).

The following equations were used to determine $\delta^{15}N$ (‰).

$$\delta^{15}\mathrm{N}(‰) = 1000 \times \frac{\text{atom\% } (^{15}N \text{ sample}) - 0.3663}{0.3663} \qquad 3.1$$

Nitrogen derived from atmospheric N_2 in soils and plants was calculated using the following equation:

$$\%\mathrm{Ndfa} = 100\,(\delta^{15}N\,ref - \delta^{15}N\,fix)/(\delta^{15}N\,ref - \mathrm{B}) \qquad 3.2$$

Where:

Ndfa- Nitrogen derived from air by ANF

$\delta^{15}N_{ref}$ - $\delta^{15}N$ of reference plant

$\delta^{15}N_{fix}$ - $\delta^{15}N$ of ANF plant

B - $\delta^{15}N$ of plant receiving all of its N through fixation

Nitrogen derived from air by ANF was calculated using sorghum as a reference plant for comparison to the three perennial bioenergy grasses (Hogberg, 1997). The value of B varies from species to species and with growth, but is close to $\delta^{15}N$ of 0 in legumes (Denton et al., 2013; Frankow-Lindberg and Dahlin, 2013). Therefore the B value was taken as $\delta^{15}N$ =0.

Accordingly, the equation can be simplified and written as follows (Boddey et al., 2001):

$$Ndfa = (\delta^{15}N_{ref} - \delta^{15}N_{fix})/ \delta^{15}N_{ref} \quad 3.3$$

3.3.5 Statistical analysis

One-way analysis of variance (ANOVA) was conducted with F- test to detect the effect of $\delta^{15}N$, N concentration (mg g^{-1}), %Ndfa and biomass for different grasses in SAS Version 9.3 (SAS Institute, Cary, NC, 2010). The least significant difference (LSD) test was used to determine a comparison among treatment means, with significance declared at $p \leq 0.05$.

3.4 Results

3.4.1 Comparisons of the $\delta^{15}N$ in soil, roots and shoots for miscanthus, energycane, and switchgrass

Soil $\delta^{15}N$ values and N concentration of labeled pots were not significantly different compared to unlabeled pots for the three energy grasses and the negative control

sorghum (n=3; Table 3.1). However, there was a trend for each grass to have an increase in $\delta^{15}N$ and N concentration in labeled plants comparison to the unlabeled samples, indicating that the isotopic labeling was successful.

Table 3.1 Soil $\delta^{15}N$ and N concentration for $^{15}N_2$ inoculation and non-inoculated energycane, giant miscanthus, switchgrass and negative control sorghum

Grass	$\delta^{15}N$		N concentration (mg g^{-1})	
	Labeled	Unlabeled	Labeled	Unlabeled
Energycane	5.16	4.57	1.27	1.14
Switchgrass	5.60	4.89	1.13	1.12
Giant miscanthus	5.34	4.30	1.07	1.08
Sorghum	5.64	4.58	1.01	1.09

Roots were highly indicative of labeling and unlabeling of $^{15}N_2$ in our experiment. As expected in root systems, increased $\delta^{15}N$ values were observed after labeling with $^{15}N_2$ in bioenergy grasses. The $\delta^{15}N$ of labeled roots of energycane were significantly different ($p>0.05$) than that of unlabeled roots of energycane. The $\delta^{15}N$ of the sorghum plants was greater than that of all energy grasses tested and significantly different from energycane, switchgrass and giant miscanthus (Table 3.2).The lower $\delta^{15}N$ values obtained in unlabeled plants indicated the potential for ANF in bacteria associated (Table 3.2). Though, there was no significant difference in N concentration of unlabeled plants, labeled energycane had a significantly greater N concentration compared to switchgrass and giant miscanthus. Therefore, the $^{15}N_2$ injection accounted for the increased N_2-fixation associated with energycane, and showed a greater concentration of N in plant-root system.

Table 3.2 Root $\delta^{15}N$ and N concentration for $^{15}N_2$ inoculation and non-inoculated samples of energycane, giant miscanthus, switchgrass and negative control sorghum

Grass	$\delta^{15}N$		N concentration (mg g^{-1})	
	Labeled	Unlabeled	Labeled	Unlabeled
Energycane	5.62^{bc*}	2.83^{bc}	12.6^{a}	13.21^{a}
Switchgrass	4.55^{c*}	2.39^{c}	9.23^{b}	11.95^{a}
Giant miscanthus	6.3^{b*}	4.45^{b}	10.83^{ab}	10.70^{a}
Sorghum	7.45^{a}	7.27^{a}	5.8^{c}	5.75^{b}

Mean root $\delta^{15}N$ values in switchgrass, giant miscanthus, energycane and sorghum of $^{15}N_2$ labeled and unlabeled pots.
$^{(a,b,c,d)}$ Letters denote significant difference between the different species (n=3; $p<0.05$).
$^{(*)}$ Asterisk following the values indicate significant difference between labeled and unlabeled pots

Shoots of energycane, switchgrass and giant miscanthus had significantly decreased $\delta^{15}N$ values compared to the negative control sorghum. Nitrogen concentration of the shoots was greater in switchgrass compared to energycane. However the N concentration of the three bioenergy grasses was significantly increased when compared with the negative control (Table 3.3). The $\delta^{15}N$ of the unlabeled and labeled plants was not significantly different. Since a lack of incorporation of $^{15}N_2$ into shoots through roots may indicate lower concentrations of N of shoots.

Table 3.3 Shoot $\delta^{15}N$ and N concentration for $^{15}N_2$ inoculation and non-inoculated samples of energycane, giant miscanthus, switchgrass and negative control sorghum

Grass	$\delta^{15}N$		N concentration (mg g^{-1})	
	Labeled	Unlabeled	Labeled	Unlabeled
Energycane	3.83^{b}	2.66^{c}	10.91^{b}	10.46^{b}
Switchgrass	3.58^{b}	3.43^{ab}	19.99^{a}	18.99^{a}
Giant miscanthus	4.39^{b}	4.23^{b}	11.74^{b}	10.27^{b}
Sorghum M81-E	7.24^{a}	6.79^{a}	7.88^{c}	6.63^{c}

Mean shoot $\delta^{15}N$ values in switchgrass, giant miscanthus, energycane and sorghum of $^{15}N_2$ labeled and unlabeled pots.
(^{a,b,c,d}) Letters denote significant difference between different species (n=3; $p<0.05$).

Total biomass yield was not a useful indicator of characterizing treatment effect due to the short period of incubation with $^{15}N_2$. However, the total N in labeled pots indicated increased values and, interestingly, the negative controls gave values equal to zero (-0.27), indicating no N_2-fixation occurred for sorghum (Table 3.4.). The amount of N_2 fixed after 24 h incubation for the three bioenergy grasses has considerable and indicate the potential for association with N_2-fixing bacteria (Table 3.4.). Root to shoot ratios was also not indicative of identifying treatment effects. However, annual grasses had a lower root to shoot ratio (~1.4) in comparison to the perennial bioenergy grasses (~2.1). Total N accumulation after 24 h incubation period of three bioenergy grasses were calculated for three replicates of each species.

The %Ndfa of the three bioenergy grasses was calculated (Table 3.5.) and a significantly greater %Ndfa was observed for energycane and switchgrass for unlabeled plants. Associative N_2-fixing bacteria discriminate ^{14}N vs ^{15}N; thus this could be

indicative of the lowering of the %Ndfa of labeled plants in this experiment. However, no significant difference was observed in giant miscanthus.

Table 3.4 Estimated fixation following a 24 h incubation period for three bioenergy grasses

Plant		Dry matter (g)	Total N (mg)	Amount of N_2 fixed after 24 h (mg)
Energycane $^{15}N_2$ labeled	Root	10.01	126.13	9.88
	Shoot	11.20	122.19	17.60
	Total	21.21		27.47[a]
Energycane $^{15}N_2$ unlabeled	Root	8.80	116.25	
	Shoot	10.00	104.60	
	Total	18.80		
Switchgrass $^{15}N_2$ labeled	Root	10.10	93.22	5.99
	Shoot	11.11	222.09	22.70
	Total	21.21		28.69[a]
Switchgrass $^{15}N_2$ unlabeled	Root	7.30	87.24	
	Shoot	10.50	199.40	
	Total	17.80		
Giant miscanthus $^{15}N_2$ labeled	Root	9.53	103.21	4.77
	Shoot	9.42	110.60	18.16
	Total	18.95		22.93[a]
Giant miscanthus $^{15}N_2$ unlabeled	Root	9.20	98.44	
	Shoot	9.00	92.43	
	Total	18.20		
Sorghum $^{15}N_2$ labeled	Root	6.12	35.50	-0.21
	Shoot	6.37	50.20	-0.06
	Total	12.49		-0.27[b]
Sorghum $^{15}N_2$ unlabeled	Root	6.21	35.71	
	Shoot	7.58	50.26	
	Total	13.79		

Mean amount of fixed N_2 after 24 h in switchgrass, giant miscanthus, energycane and sorghum in labeled pots.
([a,b]) Letters denote significant difference between the different grass species (n=3; $p<0.05$).

Table 3.5 The %Ndfa of three bioenergy grasses grown in green house with $^{15}N_2$ labeled and unlabeled pots

Bioenergy grasses	**%Ndfa in Unlabeled plants**	**%Ndfa in labeled plants**
Energycane	60.9[a]*	43.3[b]
Switchgrass	58.6[a]*	51.3[b]
Giant miscanthus	38.2[a]	35.9[a]

[a,b] Means followed by the same letter are not significantly different between species. *Following the values indicate significant difference within same species at different treatment. $p<0.05$ (LSD test)

3.5 Discussion

Several studies have been conducted using $^{15}N_2$ incorporation into legumes, rice and sugarcane (Eskew et al., 1981; Molero et al., 2014; Ohyama et al., 2014). Yet no such experiments have been performed with $^{15}N_2$ incorporation into bioenergy grasses energycane, switchgrass, and giant miscanthus to directly measure the N_2-fixation. Here, the effect of the incorporation of $^{15}N_2$ was analyzed in these three bioenergy grasses. It was hypothesized that energycane has a greater propensity to associate with N_2 fixers and that this would be indicated by an increase in plant N, $\delta^{15}N$ and %Ndfa in the root-zone and shoots in comparison to giant miscanthus, switchgrass and the negative control sorghum. Moreover, switchgrass and giant miscanthus showed greater potential to associate with N_2 fixers compared to sorghum.

Most $^{15}N_2$ incorporation studies have been conducted in chambers inside controlled environments (Ito et al., 1980; Chalk et al., 2014). However, these studies are limited in the fact that they are expensive, time sensitive, and may lead to

misinterpretations of data due to leaks (Ito et al., 1980). In this study, a more cost effective mechanism of directly measuring N_2-fixation was tested by measuring the reduction of isotopic $^{15}N_2$ and included the leakage by using trace gas as Ne. The degree of enrichment of ^{15}N is dependent on the rate of N_2 fixation of diverse N_2-fixation bacteria associated with different bioenergy grasses and the quantity of N contained in the different grasses. Therefore, it is possible to gauge potential rates of N_2-fixation by associated bacteria using this method.

In this experiment, the results indicated that the soil alone is not a good indicator to measure short term direct N_2-fixation. However, roots were a good indicator to measure the N_2-fixation. This is because ANF bacteria colonized within the rhizosphere and roots. The $^{15}N_2$ incorporated into the soil may also diffuse into the plant root system, where the activity of N_2-fixing bacteria are greater and in abundance and able to fix N_2 into NH_4^+. Therefore the roots would show greater N concentrations as well as lower $\delta^{15}N$ values compared to the soil and shoots. This is supported by previous findings that have indicated N_2 fixing bacteria are actively fixing N_2 in plant root systems compared to shoots (Ito et al., 1980).

The data presented indicated a greater potential for N_2-fixation to occur in the energycane root and the switchgrass shoot system. These results suggest the N_2 fixed in the root system of switchgrass can translocate into the shoots fairly quickly or the ^{15}N diffuse into shoot system, which may account for increased in the activity of N_2-fixing bacteria. Moreover, the discrimination of ^{15}N over ^{14}N causes differences in N_2-fixation in root and shoot systems (Shearer et al., 1980).

These data clearly indicate that ^{15}N was incorporated into plants fairly rapidly (24 h) and that fixed ^{15}N translocated from the roots to the shoots of bioenergy grasses However, this short time period was not sufficient to translocate $^{15}N_2$ from root to shoot in all plant systems tested (Morris et al., 1985). Hence, energycane and giant miscanthus were grasses not shown to have greater N_2-fixation and translocation to shoot compared to switchgrass. A nitrogen concentration in shoots was also greater in switchgrass compared to energycane, suggesting the above ground plant may have had greater translocation from roots or switchgrass may have greater abundance of N_2-fixing bacteria compared to energycane.

These results confirm of the existence of significantly greater contribution of plant-associated biological N_2-fixation into energycane, switchgrass and giant miscanthus accordingly; it is essential to implement these grasses for high yield consumption from marginal lands. Moreover, understanding which microorganisms are responsible for the ANF immediately after $^{15}N_2$ injection and their mechanisms are essential to understanding increasing biomass yield of energycane, switchgrass and giant miscanthus.

3.6 Conclusions

A comparative analysis of the efficiency of bioenergy grasses to fix N_2 has not been examined. Here, the ability of giant miscanthus, switchgrass, and energycane to associate with N_2-fixing microorganisms, and benefit from N_2-fixation, was determine using an isotopic $^{15}N_2$ reduction assay in a controlled greenhouse experiment. Atmospheric N_2 from air ($\delta^{15}N=0$) and $^{15}N_2$ can both be fixed by grass-associated diazotrophs and incorporated into plant and soil pools. Therefore, by measuring the $\delta^{15}N$ after $^{15}N_2$ reduction provided a direct measurement of the plant's ability to fix N_2.

Energycane, as expected, showed evidence for the greatest amounts of N derived from the atmosphere, followed closely by switchgrass, and to a lesser extent giant miscanthus. These results indicate that energycane may be capable of associating with the greatest populations of N_2-fixing bacteria in marginal lands. Further research is needed to determine how specific associative N_2-fixing bacteria respond upon enrichment of $^{15}N_2$ and their activity and contribution to bioenergy grasses yield.

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CHAPTER IV

IDENTIFICATION AND CHARACTERIZATION OF PUTATIVE ENDOPHYTIC AND N2 -FIXING BACTERIA ASSOCIATED WITH THREE FEEDSTOCK GRASSES

4.1 Abstract

Certain bioenergy grasses have been found to grow successfully in marginal lands without the need N fertilizer. This suggests that these grasses are associated with diazotrophic bacterial communities. Variations in the biomass yields and rates of N_2-fixation were previously found between the three energy grasses switchgrass, energycane, and giant miscanthus, with energycane yielding the greatest biomass in comparison to switchgrass and giant miscanthus. It is known that variations exist in the composition of the bacterial communities between different grasses, which can influence the potential yield of biomass obtainable. Therefore, the objective of this study was to characterize the diversity in ANF bacterial populations associated with these three perennial bioenergy grasses. Endophytic, as well as rhizosphere N_2-fixing bacteria, were identified in all three grasses through direct sequencing of the *nifH* gene in the communities. The N_2-fixing bacteria *Azospirillum sp.*, *Ramlibacter spp.*, and *Burkholderia spp.* were identified in the roots of energycane, switchgrass, and giant miscanthus, respectively. Additionally, the diversity of the microbial communities associated with these grasses was analyzed by denaturation gradient gel electrophoresis (DGGE) based on the 16S rRNA gene. Specific

bacteria identified in the endophytes of these grasses were *Flavobacterium sp.* and *Pseudomonas sp.* in switchgrass and *Rahnella sp*. in energycane. These results suggest that the diversity of the putative ANF bacteria associated with bioenergy grasses growing in marginal lands may contributes to N_2 -fixation and hence biomass yields. The community associated with energycane may have the greatest influence on N_2-fixation and warrants further investigation.

4.2 Introduction

Soil is a heterogeneous, complex and dynamic environment that consists of diverse microorganisms influenced by above ground vegetation. Soil microorganisms are highly concentrated near the rhizosphere regions of plants, which are nutrient rich regions of soil directly adjacent to the roots (Batten et al., 2006). Microorganisms within this region can infect the plants and exist as endophytes (obligate or facultative), or exist as free-living associative microbes with close relation to plants. This relationship is important as the plants provide carbon for soil microorganisms in the form of residues and root exudates (Butler et al., 2003; Wardle et al., 2004; Williams et al., 2007; Yarwood et al., 2009) and associative N_2 fixers provide N in a usable form to the plants (Boddey et al., 2000).

Much research has been conducted to analyze the relationship of the microbial communities associated with the rhizosphere and endophytes with non-legumes. Diverse ANF in more than six genera and nine species have been identified in sugarcane, rice, kallar grass and maize (Triplett, 1996; Baldani et al., 1997; James, 2000). However, limited studies have been conducted to analyze the microbial populations of bioenergy grasses.

Bioenergy can be used to produce energy in the biofuel industry. Switchgrass, *Miscanthus* and sugarcane are a few of the major bioenergy grasses that produce high biomass yields in marginal lands (Lapola et al., 2009; Jones et al., 2014). Energycane is a hybrid of sugarcane and contains high lignocellulose raw material useful for biofuel production. These grasses have been shown to be capable of growing in marginal lands with little to no N input from fertilizers, indicating that they are associated with N_2-fixing bacteria. Several N_2-fixing bacteria, such as *Paenibacillus polymyxa* (Ker et al., 2014), *Azospirillum sp.* (Eckert et al., 2001), *Herbaspirillum spp.* (Kirchhof et al., 2001), and *Clostridium spp.* (Saito and Minamisawa, 2006), have been identified in miscanthus and were also found to have plant growth promoting characters. This indicates the importance of these bacteria on the growth of the plant.

Although N_2-fixing bacteria have been identified within several grasses, the microbial diversity and potential populations of N_2-fixing bacteria of these bioenergy grasses have not been characterized. Therefore, the objective of this study was to understand the composition and diversity of N_2-fixing bacteria associated as either endophyte or rhizosphere communities of the three energy grasses giant miscanthus, energycane, and switchgrass when grown in without supplement N. An assessment of the bacterial populations within and surrounding the roots of these important grasses could provide information needed to predict the biomass yield of feedstock grasses in marginal lands and could lead to ways to increase these yields through alterations in the microbial community structure.

4.3 Experimental procedure

4.3.1 Sample collection

The three perennial bioenergy grasses energycane (HO 02-147, hybrid of *Saccharum officinarum* and *Saccharum spontaneum*), switchgrass ('Alamo', *Panicum virgatum*), and miscanthus (giant miscanthus, *Miscanthus x giganteus*) were planted in a randomized block design in four replicated plots at the Agronomy Unit 1 of the Leveck Animal Research Center located at Mississippi State University, Mississippi, USA (33° 28' N and 88° 47' W). The annual grass sorghum (M81-E, *Sorghum bicolor*) was analyzed as a negative control from an adjacent plot. During the initial planting year NH_4NO_3 was applied (54 kg he^{-1}) as a soluble fertilizer to ensure rapid plant establishment. Approximately 500 g of soil (6 cores obtained with a Hoeffer soil probe with 2 cm in diameter) was collected from four plots in the third summer season at rhizosphere. Soil was collected in Whirl-Pak bags, sealed, stored on ice for transport to the laboratory, and subsequently stored at -80°C until use. Soil particles loosely attached to the roots were removed. The roots were washed with sterile distilled water five times, immersed in 70% ethanol for 1 min, then finally washed two times with sterile distilled water (Chelius and Triplett, 2001; Ker et al., 2012). The last wash was plated on N free LGI plates to confirm the removal of N_2-fixing bacteria on the surface of roots. Roots were immersed in liquid N_2 and crushed using a mortar and pestle (Briones et al., 2002) prior to culturing and molecular analyses.

4.3.2 Detection of *nifH* and 16S sequencing from root and soil samples

The DNA was isolated from 0.5 g of sterilized crushed roots and 1.0 g of rhizosphere soil of switchgrass, giant miscanthus, energycane and sorghum using ZR soil

microbe DNA isolation kit (Zymo Research). Gene amplification was performed using PCR in 1500 bp region of 16S rRNA gene using 27F (5'-CATCTCAGTGCAACTAAA-3') and 1492R (5'-CAGGAAACAGCTATGAC-3') primers and 600-750 bp fragment (cluster I) in functional N_2-fixing gene *nifH* (*nifH* forward 5- GTTTT ACGGC AAGGG CGGTA TCGGCA -3 and *nifH* reverse 5- TCCTC CAGCT CTCCA TGGTG ATCG -3) (Kumari and Kumar, 2009), which is used to identify aerobic and anaerobic proteobacteria and N2 -fixing bacteria (Ueda et al., 1995; Bergmann et al., 2009; Gaby and Buckley, 2014). A standard PCR protocol was used to amplify the 16S rRNA gene: initial denaturation of 5 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1.5 min at 54°C, 1 min at 72°C, and a final extension of 5 min at 72°C. Polymerase chain reaction conditions for the amplification of the *nifH* gene was as follows: initial denaturation for 5 min at 94°C, followed by 30 cycles of 1 min at 94°C, 45 sec at 55°C, 1 min at 72°C, and a final extension of 5 min at 72°C. The amplified products were visualized in a 0.8% agarose gel. Products from PCR were cloned into Topo TA (pCR ® 2.1 TOPO® Invitrogen™). Inserts from correct clones were sequenced using the M13 reverse and forward primers by standard Sanger sequencing at Arizona State University. The sequence chromatograms were trimmed and edited using Codon Code Aligner. Sequences were aligned using ClustalW for chimera check by Mallard and Pintail programs. The chimera free sequences were then analyzed using NCBI BlastN. Total of 24 samples (13 rhizosphere soils and 11 roots samples) were analyzed in quadruplicate for each bioenergy grass.

4.3.3 Cultivation of Nitrogen-Fixing Bacteria

Nitrogen free LGI plates were used to enrich for N_2 fixers (Cavalcante and Dobereiner, 1988). The composition of the medium was as follows: 5 g/L sucrose, 0.2 g/L K_2HPO_4, 0.6 g/L KH_2PO_4, 0.2 g/L $MgSO_4.7H_2O$, 0.02 g/L $CaCl_2.2H_2O$, 0.002 g/L $Na_2MoO_4.2H_2O$, 5% bromothymol blue in 0.2 N KOH, FeEDTA 1.64% (w/v) solution 4 ml, 15 g/L agar, 0.03 g/L yeast extract, with the pH adjusted to 6-6.2 with H_2SO_4 (Cavalcante and Dobereiner, 1988). Soil and root samples (0.5 g) were diluted in PBS and plated onto LGI agar. Plates were incubated aerobically at 29°C for 5 d prior to enumeration. Three replicates were performed for each sample.

4.3.4 DGGE analysis

The DNA isolated from soil and root samples were analyzed by differential gradient gel electrophoresis (DGGE) (Muyzer et al., 1993). Briefly, the bacterial specific PCR primers to a conserved region flanking the variable V3 region of 16S rRNA genes were used in a 50 µL total reaction volume. Primers (50 pmol of each; primer 2: 5'-ATTACCGCGGCTGCTGG-3′; primer 3: with a 40-bp GC clamp 5’-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCA CGGGGGGCCTACGGGAGGCAGCAG-3’), were mixed with Jump Start RedTaq Ready Mix (Sigma Chemical Company, St. Louis, MO) according to manufacturer’s instructions, with 100 ng (50 ng of DNA pooled from 2 samples each) of template DNA (Sheffield et al., 1989; Muyzer et al., 1993). Amplification was performed on a PTC-200 Peltier Thermal Cycler (MJ Research Inc., Waltham, MA) with denaturation at 94°C for 2 min, followed by 17 cycles of denaturation at 94°C for 1 min; annealing at 67°C for 45 s (decreasing by 0.5°C per cycle to minimize spurious by-products); (Don et al., 1991;

Wawer et al., 1997), and extension at 72°C for 2 min. The second stage consisted of 12 cycles of denaturation at 94°C for 1 min; annealing at 58°C for 45 s; then a final extension at 72.0°C for 7 min. Polyacrylamide gels (8% v/v; acrylamide-bisacrylamide ratio of 37.5:1) were cast with a 35 to 60% urea deionized formamide gradient. Amplified samples were mixed with an equal volume of 2X loading buffer (0.05% (wt/vol) bromophenol blue, 0.05% (wt/vol) xylene cyanol, and 70% (vol/vol) glycerol. Gels were loaded in a DCode Universal Mutation Detection System (Bio-Rad Laboratories, Richmond, CA) for electrophoresis in 1X Tris-Acetate buffer at 59°C for 17 h at 60 V. Gels were stained with SYBR Green 1 (1:10,000 dilution) for 40 min. Amplified fragment pattern relatedness of samples was determined with molecular analysis fingerprinting software (Bio-Rad Laboratories, v. 1.610) based on the Dice similarity coefficient and the unweighted pair group method using arithmetic averages (UPGMA) for clustering and dendrogram construction. Two individual samples were analyzed in duplicate by DGGE.

4.3.5 Scanning Electron Microscopic (SEM) analysis

Longitudinal slices of roots from energycane, giant miscanthus, switchgrass, and sorghum were fixed in 3% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 3 h at room temperature and post-fixed in 1% (v/v) osmium tetraoxide in the same buffer. Specimens were washed three times in sterile distilled water and treated with aqueous solution of uranyl acetate 2% (w/v) for 40 min. After fixation, samples were dehydrated through a graded ethanol series (30% - 100%) followed by acetone (100%), critical point dried, mounted on aluminum stubs, coated with gold, and examined with a ZEISS EVO-

50 (Carl Zeiss Co., Germany) scanning electron microscope. Three to four surfaces were analyzed from two samples of each bioenergy grass and control plants.

4.4 Results

4.4.1 *nifH* gene identification

The *nifH* gene is the most conserved gene in the N_2-fixing pathway (Rösch et al., 2002). Therefore, DNA isolated from the roots and soil from each bioenergy grass was analyzed for the presence of this gene (Fig. 4.1). The positive control *Gluconacetobacter diazotrophicus* was used to identify the size of *nifH* gene (Eskin et al., 2014). Sorghum was used as the negative control.

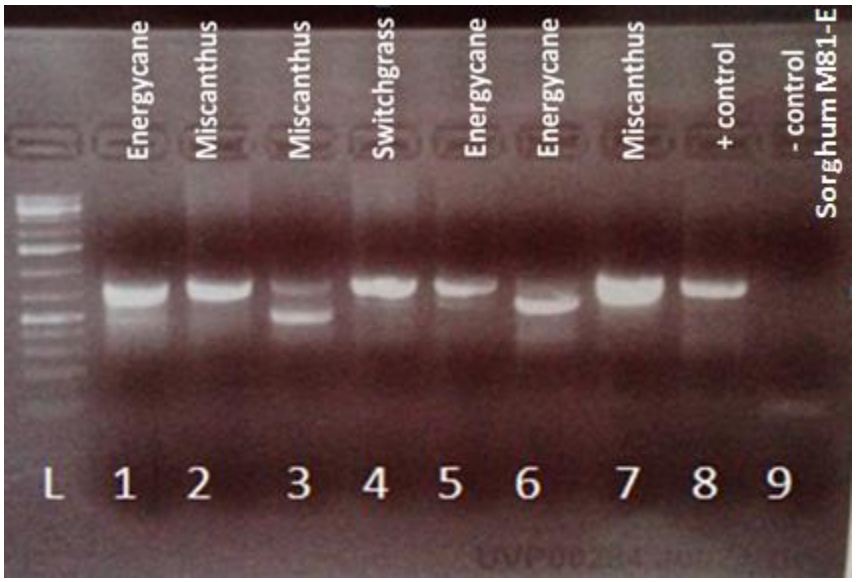

Figure 4.1 Electrophoretic analysis of PCR products (amplified with *nifH* primers) using DNA from soils and roots of energycane, giant miscanthus, switchgrass and sorghum

Lane-L, 100 bp ladder (company); lanes 1 and 2 rhizosphere; lanes 3, 4, 5, 6, and 7 roots; and lane 8 positive control *Gluconoceobacter diazotrophicus* (ATCC 49037); lane 9 root of sorghum (negative control). Bands were present in 600-750 bp regions

The DNA sequences were obtained from roots (11 clones) and rhizosphere soils (13 clones). Nine clones from energycane (5 from rhizosphere soil and 4 from roots), 8 from giant miscanthus (4 from rhizosphere and 4 from roots) and 7 from switchgrass (4 from rhizosphere soil and 3 from roots) were used to identify bacteria associated with these bioenergy grasses. Diverse groups of N_2-fixing bacteria were observed in both roots and rhizosphere regions of three different bioenergy grasses among *nifH* sequencing analysis. These culture independent methods revealed the presence of obligate endophytes and rhizosphere bacteria, as well as facultative N_2-fixing bacteria associated with these grasses (Table 4.1). Energycane is a hybrid of sugarcane; however, we could not identify some of the bacteria associated with sugarcane in this hybrid plant. *Azospirillum spp.* was identified as an obligate endophyte (22.2%), *Pseudomonas was* identified as an obligate associative fixer (22.2%) and *Bacillus spp.* was identified as facultative N_2 fixer in energycane. *Beijerinckia indica* and *Sinorhizobium melilot* were also identified associated with energycane (Table 4.2). *Ramlibacter sp.* was identified as endophyte in switchgrass roots and *Bradyrhizobium japonicum* were found in the rhizosphere region. Niroge-fixing and plant growth promoting bacteria *Burkholderia spp*. were identified in roots of giant miscanthus.

Table 4.1 Associative N_2-fixing bacteria identified in roots of energycane, switchgrass and giant miscanthus by *nifH* gene analysis

	Switchgrass	Giant miscanthus	Energycane
***Bacillus sp.* IHB B 2269**	+	+	+
Ramlibacter sp.	+		
Burkholderia sp.		+	
Azospirillum lipoferum			+
Azospirillum sp. B510			+
Beijerinckia indica			+

(+) Indicates the presence of bacteria associated with energy grass

Table 4.2 Associative N_2-fixing bacteria identified in rhizosphere region of energycane, switchgrass and giant miscanthus by *nifH* gene analysis

	Switchgrass	Giant miscanthus	Energycane
Bradyrhizobium japonicum	+	+	
Bacillus spp.	+	+	+
Pseudomonas sp.			+
Sinorhizobium melilot			+

(+) Indicates the presence of bacteria associated with energy grass

4.4.2 Analysis of bacterial communities using 16S rRNA gene analysis

Universal primers (27F and 1498R) were used to identify bacteria associated with rhizosphere and as endophytes within the three bioenergy grasses. A total of 24 clones in rhizosphere soil and roots were generated and subsequently analyzed. Several clones were clustered with sequences from uncultured bacterial clones (16/24) in 16S rRNA genes. *Rahnella aquatilis* (Berge et al., 1991) has been previously identified as a putative endophytic N2 fixer in maize and wheat and was identified through sequencing to be associated with energycane (Table 4.3). The putative N_2 fixers *Flavobacterium spp.,*

Pseudomonas spp. and some *Bacillus spp.* were also identified in switchgrass and giant miscanthus.

Table 4.3 The most closely related described bacteria to the sequences retrieved in 16S rRNA analysis, analyzed by NCBI BlastN

Source	Putative bacteria Identified	Accession number	Sequence % Identity
Energycane - Endophyte	Uncultured gamma proteobacterium clone ATB-LH-5964 16S ribosomal RNA gene	FJ535196.1	91
	Rahnella sp. 'CDC 2987-79' 16S ribosomal RNA gene	U88436.1	91
	Rahnella aquatilis strain TB-143 16S ribosomal RNA gene	KF817754.1	77
Switchgrass - endophyte	*Flavobacterium sp*. HLT2-26 16S ribosomal RNA gene	JX949340.1	83
	Flavobacterium sp. T93L.09.P.BKT.AS.H.Gill.N 16S ribosomal RNA gene	JX287835.1	90
	Pseudomonas sp. PP007 16S ribosomal RNA gene	KF153212.1	82
Giant miscanthus - endophytes	*Bacillus flexus* strain Sur5 16S ribosomal RNA gene	KC462189.1	74
	Bacillus aryabhattai strain IARI-IHD-34 16S ribosomal RNA gene	KF054900.1	96
	Bacillus aryabhattai strain IARI-IHD-34 16S ribosomal RNA gene	KF054900.1	91
Energycane – Rhizosphere soil	Uncultured *Acidobacterium sp*. clone A2 16S ribosomal RNA gene	EF125937.1	82
	Uncultured *Rahnella sp*. clone spike_2.17 16S ribosomal RNA gene	HQ111164.1	77
	Uncultured planctomycete clone EB1047 16S ribosomal RNA gene	AY395366.1	80

Table 4.3 (Continued)

Switchgrass-Rhizophere soil	Uncultured planctomycete clone EB1038 16S ribosomal RNA gene	AY395357.1	95
	Uncultured Myxococcales bacterium clone Plot4-F03 16S ribosomal RNA gene	EU449598.1	80
	Uncultured organism clone ELU00568-T370-S-NIPCRAMgANa_000392 small subunit ribosomal RNA gene	HQ764930.1	85
Giant miscanthus – rhizosphere soil	Uncultured bacterium clone kab243 16S ribosomal RNA gene	FJ936960.1	77
	Uncultured bacterium clone p11f23ok 16S ribosomal RNA gene	FJ478874.1	91
	Uncultured bacterium clone FFCH15672 16S ribosomal RNA gene	EU134489.1	82
Sorghum - Endophyte	Uncultured bacterium clone p9i17ok 16S ribosomal RNA gene	FJ478844.1	94
	Uncultured bacterium clone p9i17ok 16S ribosomal RNA gene	FJ478844.1	75
	Uncultured bacterium clone PA137 16S ribosomal RNA gene	JX013304.1	88
Sorghum – rhizosphere soil	Uncultured bacterium clone FCPS498 16S ribosomal RNA gene	EF515992.1	85
	Uncultured bacterium clone KGB200711-189 16S ribosomal RNA gene	EU881273.1	78
	Uncultured bacterium partial 16S rRNA gene	FN860865.1	95

4.4.3 Community analysis

Different banding patterns of DGGE analysis showed the diverse community associated with three bioenergy grasses and negative control in rhizosphere soil and plant root material. Instead of the universal primers, primers for the V3 conserved region of 16S rRNA were used to identify bacteria present (Table 4.4). The soil of three bioenergy grasses (energycane, giant miscanthus, and switchgrass) as well as negative control (sorghum) appeared to have a more diverse community compared to their root communities. However, the roots of the sorghum contained a more diverse community than the roots of the bioenergy grasses. This banding pattern was highly reproducible, but the intensity was varied between the two replicated samples. DGGE profile was slightly different within same plants in different plots (Fig. 4.2.).

It is possible that the PCR for the bioenergy grasses were inhibited, as only a few faint bands were visible on the DGGE gels in replicate samples of bioenergy grasses from replicate plots. However, endophytic communities of energycane and rhizosphere soils from the three bioenergy grasses had dominant banding patterns (Fig. 4.2a and 4.2c).

According to the dendrogram (Fig. 4.3), bacterial diversity was divided into six distinct groups. Root samples of giant miscanthus and sorghum formed a cluster with 38% correlation, whereas soil of energycane and giant miscanthus formed a cluster with a 60% correlation. All the others were segregated from each other. Roots of switchgrass have separate from all the others, energycane roots had 40% similarity with soils of four samples, soils of switchgrass had 50% similarity with other soils, and sorghum had 51% similarity with other soils. The highest amount of similarity was observed between soil of energycane and giant miscanthus. Though the rhizosphere soil bacteria associated with

energycane and giant miscanthus were related, the rhizosphere soil community in switchgrass and sorghum were different than energycane and giant miscanthus. Moreover, endophytic communities in sorghum and giant miscanthus were 38% similar, while the switchgrass and energycane were different. Bacterial communities in switchgrass isolated from soil and inside the roots were clustered far apart.

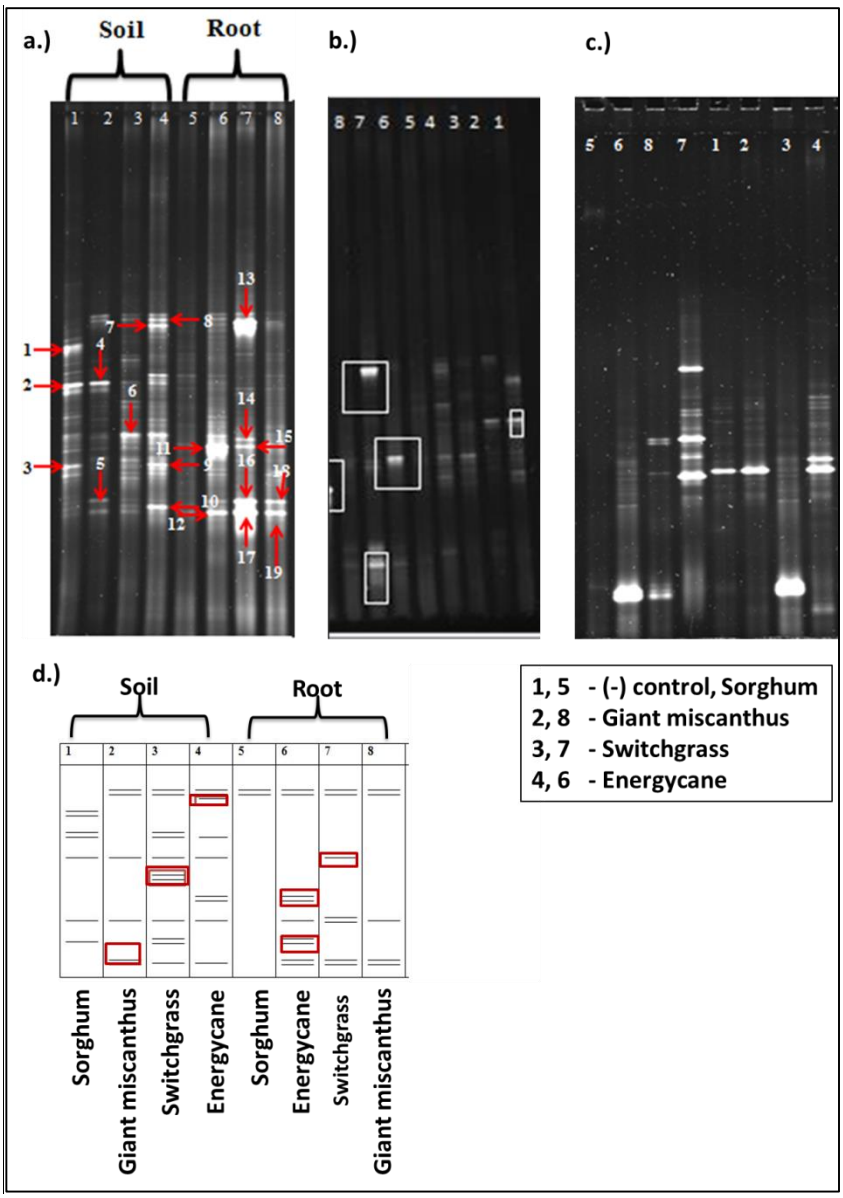

Figure 4.2 Denaturation Gradient Gel Electrophoresis (DGGE) profiles of polymerase chain reaction (PCR) amplified V3 region of 16S rRNA gene in bacterial DNA from three bioenergy grasses; 1-4 rhizosphere soil and 5-8 roots

Each bioenergy grass has unique banding patterns, although many energy grasses have common bands. a.) DGGE gel image of four plant type in soil and root b.) Replicate of gel a. c.) DGGE gel image of four plant type in soil and root in different plot at same area d.) Line image of DGGE gel

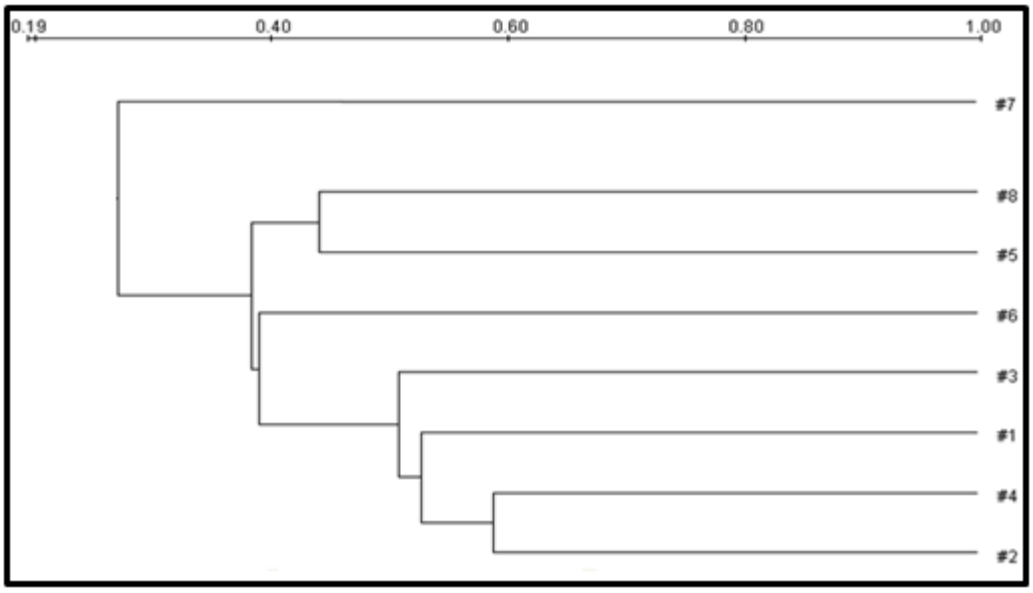

Figure 4.3 Phylogenetic analysis switchgrass, giant miscanthus and energycane along with negative control sorghum, rhizosphere soil and root of bacterial 16S rRNA gene

Numbers indicate as follows: 1-4 were rhizosphere soil and 5-8 were roots of three bioenergy grasses. 1 and 5 were sorghum, 2 and 8 were giant miscanthus, 3 and 7 were switchgrass, 4 and 6 were energycane. The amount of similarity was reflected by the relatively closeness or grouping and was indicated by the percentage similarity coefficient bar located above each dendrogram

Table 4.4 The most closely related described bacteria to the sequences retrieved in DGGE , analyzed by NCBI BlastN

Well no/Gel band no:		Accession number	% identity	Source
1-	1	JX105427.1	88%	Uncultured bacterium isolate DGGE gel band 9-15 16S ribosomal RNA gene
2		KC570918.1	99%	Uncultured bacterium isolate DGGE gel band A2 16S ribosomal RNA gene
3		FJ406571.1	84%	Uncultured bacterium isolate DGGE gel band EA-28 16S ribosomal RNA gene
2-	4	KC570918.1	96%	Uncultured bacterium isolate DGGE gel band A2 16S ribosomal RNA gene, Actinomycetales spp.
3-	6	GU365996.1	99%	Uncultured soil bacterium clone CRS5552T-1 16S ribosomal RNA gene
4-	9	JF361271.1	91%	Uncultured soil bacterium clone GO0VNXF07ILE5M 16S ribosomal RNA gene, Pseudomonas putida, Actinomycetales bacterium
5-	0			None
6-	12	KF981558.1	94%	*Luteibacter sp*. 11638940 16S ribosomal RNA gene
7-	13	KJ395366.1	97%	*Acinetobacter calcoaceticus* strain Fe10 16S ribosomal RNA gene
15		JX936827.1	100%	Uncultured bacterium clone GXTJ5A301ADNA8 16S ribosomal RNA gene
		FN178349.1	93%	Uncultured *Bifidobacterium sp.*
		FJ406568.1	91%	Uncultured bacterium isolate DGGE gel band EA-29 16S ribosomal RNA gene
8-	1	JQ282813.1	99%	Micrococcus luteus strain RKHC-71A 16S ribosomal RNA gene
9-	2	KC477604.1	96%	Uncultured Streptomyces sp. clone G-26-6-3 16S ribosomal RNA gene
10-	2	EF554974.1	98%	Uncultured *Clavibacter sp*. isolate DGGE gel band AtR16 16S ribosomal RNA gene
11-	2	JX317731.1	94%	*Bacillus sp*. RKBH-B55 16S ribosomal RNA gene
	4	EU919224.1	99%	Uncultured *Trichococcus sp.* clone QRSYY9 16S ribosomal RNA gene
	5	KF220427.1	99%	*Bacillus sp.* A1-4 16S ribosomal RNA gene

4.4.4 Isolation of N_2-fixing bacteria through enrichment culturing techniques and SEM imaging

To identify the culturable N_2-fixing bacteria, soil and sterilized root samples were plated onto LGI media. This media allows for the cultivation of N_2-fixing bacteria. In general, greater populations were observed in soil samples in comparison to roots.

However, the populations isolated from energycane (soil 126 x10^5 CFU/ml, root 115 x10^5 CFU/ml) were greater in comparison to switchgrass (soil 85 x10^5 CFU/ml, root 74 x10^5 CFU/ml) and giant miscanthus (soil 51 x10^5 CFU/ml, root 32 x10^5 CFU/ml). No colonies were observed with soil and roots of sorghum in N free LGI medium (Table 4.5).

Table 4.5 Number of colonies in LGI plate in 10^{-5} dilution factor

Plant type	Soil (means x10^5 CFU/g)	Root (means x10^5 CFU/g)
Energycane	126	115
Switchgrass	85	74
Giant miscanthus	51	32

(values represent for means of three replicates)

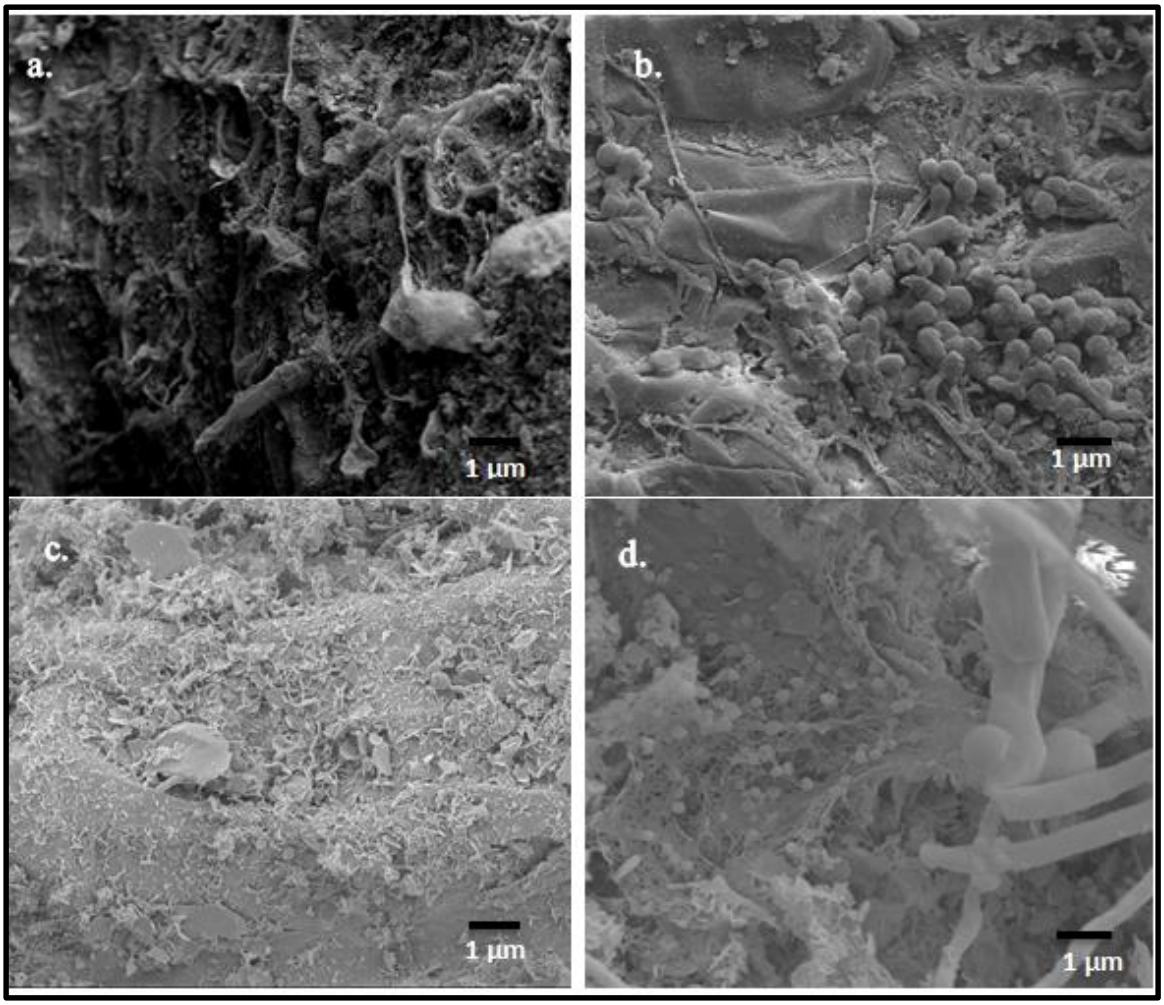

Figure 4.4 Scanning electron micrograph of perennial bioenergy grasses roots colonized by bacteria

Root surface of (a.) annual grass sorghum (b.) switchgrass; (c.) giant miscanthus; and (d.) energycane. No potential microbes were identified in annual grass sorghum. Scale bars represent 1 µm. A minimum of three fields were examined on the SEM.

Interactions between energy grass root surface and bacteria were observed by scanning electron microscopy (SEM). Rod-shaped bacteria were detected, mostly as microcolonies covering the root surface of switchgrass, energycane, and giant miscanthus (Fig.4.4b, c, d). Bacteria appeared adhered to the root epidermal cells, forming microaggregates throughout the surface of energycane and switchgrass. There were

visually fewer bacterial aggregates in giant miscanthus compared to energycane and switchgrass. However, no such clumps of microorganisms were observed in root surfaces of sorghum (Fig. 4.4a). These results are in agreement with the populations identified by the enrichment culture.

4.5 Discussion

In the present study, both molecular and cultivation approaches were used to identify ANF bacteria in the rhizosphere and inside the roots of three energy grasses. This study demonstrated that diverse N_2-fixing bacteria colonize the rhizosphere and the roots of energycane, switchgrass and giant miscanthus.

There are several *nif* genes, such as *nifH*, *nifD*, *nifK,* that are associated with N_2-fixing microorganisms. The *nifH* is the most widely conserved gene among N_2-fixing bacteria and has been used in previous studies to classify the species associated (Mao et al., 2011; Mao et al., 2013). Therefore, the *nifH* functional gene was used to identify N_2-fixing bacteria associated with three bioenergy grasses. All three energy grasses analyzed had the presence of the *nifH* gene. The *nif*H gene was not identified in the bacterial DNA extracted from the interior of sorghum plant roots, thus providing support that it serves as a valid proxy for soil and not atmospheric sources of N.

Several studies have shown that *Azospirillum*, which has been previously identified in sugarcane (Okon and Itzigsohn, 1995), can be used as an inoculum in wheat, maize, and corn to increase the crop yield (Kapulnik et al., 1982; Hungria et al., 2010) by increasing rates of N_2-fixation (Kennedy et al., 1997). In this study, *Azospirillum* was observed in energycane, which is a hybrid of sugarcane. The most common endophytic N_2-fixing bacteria reported in sugarcane are *Gluconacetobacter diazotrophicus*, and

Herbaspirillum spp. (James, 2000; Baldani et al., 2002; Boddey et al., 2003). However, in the current study, these bacteria were not identified as endophytes in energycane. Endophytic bacterial diversity is dependent upon the host, which most likely accounts for this discrepancy. We have identified putative N_2 fixers in switchgrass (*Ramlibacter sp.*) and in giant miscanthus (*Burkholderia)*. *Bukholderia* species are known to have growth promoting abilities in addition to N_2-fixation mechanisms (Vessey, 2003). Therefore, the increased yield in giant miscanthus could be due to both growth promoting bacteria and N_2-fixation bacteria associated with the plant. Moreover, *Bacillus sp.* were identified in all three energy grasses and can be responsible for N_2-fixation or plant growth promoting activity (de los Milagros Orberá Ratón et al., 2012). Few putative N_2 fixers, such as *Rahnella sp.,* were identified associated with energycane that have also been found in wheat and maize plant systems as ANF bacteria (Berge et al., 1991). Moreover, *Pseudomonas sp.* were identified from 16S rRNA sequencing from switchgrass samples and these bacteria have ability to fix N_2 in rice rhizosphere system (Habibi et al., 2014).

In addition to functional gene analysis, DGGE is frequently used to characterize soil and plant root microbial communities. In this study, the highly variable V3 region of 16S rRNA gene was used to characterize the soil microbial community (Vasileiadis et al., 2012). Even though we could not identify associative N_2 fixers, different banding patterns were indicated the different bacterial communities associated with three different grasses, while the individual discrete band refers to an unique sequences type of each grass (Muyzer et al., 1995). Several faint bands were detected by DGGE and it is possible that these could represent, at least in part, N_2-fixing bacteria. Nevertheless, diverse banding patterns were observed in three different bioenergy grasses in soil and roots.

SEM images of washed roots were obtained to identify tightly attached microorganisms with roots. Bacteria were not found attached to the root surface of the negative control sweet sorghum variety M81-E, whereas all three bioenergy grasses appeared to have tightly attached bacteria. Therefore, SEM imaging suggested the presence of bacteria associated with rhizosphere zone, which could potentially penetrate into roots or be present in rhizosphere region to contribute to plant growth through N_2-fixation.

Energycane was previously found to have significantly greater biomass yield (23.1 Mg h^{-1}) in marginal land after three years of plant establishment without addition of N fertilizer in comparison to switchgrass and miscanthus. Switchgrass had a biomass yield of 15.1 Mg h^{-1} and giant miscanthus had a biomass yield of 13.5 Mg h^{-1}, which is also significantly greater than the negative control sorghum (8.5 Mg h^{-1}). As diversity was observed in the N_2-fixing bacteria associated with these three grasses, it is possible that this difference contributes to the difference in growth in low N environments. Variations exist in the populations of N_2-fixation bacteria associated with different grasses and at different environmental conditions (Boddey and Dobereiner, 1995; Oliveira et al., 2004). Therefore the *Azospirillum sp.* isolated from energycane has greater potential to adapt to marginal lands to potentially contribute to a greater biomass yield. Further research is needed to identify the mechanisms and the rates of N_2-fixation associated with *Azospirillum* in order to increase biomass yield for bioenergy grasses.

4.6 Conclusions

In this study the bacterial communities were characterized for three different bioenergy grasses grown on marginal lands. Previously, energycane was found to have

the highest rate of N_2-fixation and produce the highest yield of biomass. This information, when coupled with the microbial community analysis provided, suggest that the endophytic relationship with *Azospirillum lipoferum* could be contributes to the potential increase in biomass observed with energycane. Additional growth promoting and N_2 fixing bacteria, such as *Burkholderia,* may also contribute to plant growth in low N environments. Further research is needed to determine the bacterial community required to promote the growth of bioenergy grasses to obtain increased biomass yield.

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CHAPTER V

EVALUATION OF THE GROWTH OF THE THREE FEEDSTOCK GRASSES INOCULATED WITH *Azospirillum lipoferum* ISOLATED FROM ENERGYCANE

5.1 Abstract

Energycane, switchgrass and giant miscanthus are widely used as feed stocks in the biofuel industry. To increase the cost effectiveness of their use in the biofuel industry, much research has been done to increase the biomass yield of bioenergy crops without the need for expensive N fertilizers. One method is to introduce N_2-fixing bacteria in order to reduce the need for N fertilizer to optimize production. Energycane produces greater biomass yields in marginal lands in comparison to switchgrass and giant miscanthus. Previous research from our laboratory identified that the N_2-fixing bacterium *Azospirillum lipoferum* is found associated with energycane roots, but not other bioenergy grasses, suggesting that this microbe influences the high biomass yield observed. The aim of this project was to determine whether inoculating this N_2-fixing bacterium isolated from energycane could improve the growth response of other bioenergy grasses. *Azospirillum lipoferum* was inoculated into switchgrass, giant miscanthus and energycane plants grown under greenhouse conditions to measure growth responses such as root and shoot length and total biomass yield. Control plants were grown without the addition of *A. lipoferum* and triplicate trials were conducted in each

condition for each plant type. No significant differences were observed upon inoculation in biomass or shoot lengths. However, root lengths were significantly increased in giant miscanthus provided *A. lipoferum* in comparison to those not inoculated. Nutrient deficiencies in marginal soil may cause lack of adaptation of inoculated bacteria. Moreover, endophytic bacteria isolated from energycane may not successfully survive in soils. A further modification of this method is needed to successfully improve the growth of other grasses through synthetic inoculation with N_2-fixing bacteria.

5.2 Introduction

Nitrogen is an essential and key element needed by plants to improve crop productivity (Nasholm et al., 1998). Plants cannot utilize molecular N (Gyurján et al., 1995; Döbereiner, 1997). However, N_2-fixing bacteria can convert molecular N_2 into NH_3, which is then used to supply N to the plant system. These N_2 fixing bacteria are known as diazotrophs and belong to the genera *Azotobacter, Azospirillum, Burkholderia, Beijerinckia, Herbespirillum* and *Pseudomonas* (Nasholm et al., 1998).

Energycane, switchgrass and giant miscanthus are well known second generation biofuel feedstock grasses that produce substantial biomass yields (Arundale et al., 2014; Iqbal and Lewandowski, 2014). However, in order to improve cost efficiency, biomass yields need to be improved with little to no input of N from fertilizers (Arundale et al., 2014).

Azospirillum is a gram negative rod shaped facultative endophytic bacterium that colonizes the interior and surface of certain grasses' roots (James, 2000). These bacteria are N_2-fixing bacteria that also promote plant growth through increasing the efficiency of water and nutrient absorption (Boddey et al., 1986; Bashan and Holguin, 1997; Reis et

al., 2000). Not only the N economy, *Azospirillum spp.* can contribute to promotion of plant growth by producing growth hormones (Okon and Labandera-Gonzalez, 1994). *Azospirillum sp.* isolated from sugarcane was inoculated into seed of rice, wheat and maize to evaluate growth responses (Fallik et al., 1988; Dalla Santa et al., 2004; Pedraza et al., 2009). Root surfaces of maize increased significantly upon inoculation of *Azospirillum* and significant increases occurred in grain yield in wheat and rice. However, some studies have found that, there were no significant changes in biomass yields due to low survival of inoculated bacteria, physical and chemical characteristics of the soil, physiological states of bacteria, plant genotypes and the presence of high number of native microorganisms (Bashan and Levanony, 1990).

Hence previous studies have suggested that the inoculation of N_2-fixing bacteria may help to improve the growth of other plants. Moreover, we have observed greater biomass yield in energycane and also identified that this grass is associated with *Azospirillum lipoferum*. Therefore, the present study focusses on inoculation studies in pot cultures with *Azospirillum,* directly isolated from energycane to determine the effects on growth of switchgrass and giant miscanthus.

5.3 Experimental procedure

5.3.1 Bacterial isolation and identification

Roots and rhizomes were collected from surface soil (0-10 cm) of matured Energycane grown plots (4 plots, roots contained in 6-10 core samples) at the Agronomy Unit 1 of the Leveck Animal Research Center located at Mississippi State University, Mississippi, USA (33° 28’ N and 88° 47’ W) then sterilized (refer to chapter III) and crushed with liquid N_2. Crushed roots (0.5 g) were mixed in 10 ml of sterile distilled

water, shaken vigorously to dislodge bacteria and plated (1 ml) on LGI plates (Cavalcante and Dobereiner, 1988). Plates were incubated at 29°C for 5 days and consecutively streaked (5 times) to isolate single colonies. After 3 to 4 weeks, isolates from the LGI plates were streaked on Luria Bertani (LB) plates. The DNA was extracted from isolated colonies from energycane samples using a modified yeast genomic DNA preparation protocol (Looke et al., 2011). N2 -fixing bacteria were identified through using PCR specific for the *nifH* gene as described in Chapter III. Products after PCR were cloned into TOPA TA (pCR® 2.1 TOPO® Invitrogen™) and sequenced using the M13 F and R primers using Sanger sequencing methods at Arizona State University. The colonies identified from energycane were 89% similar to *Azospirillum lipoferum*.

5.3.2 Inoculation

The experiments with *Miscanthus x giganteus* (giant miscanthus), *Panicum virgatum* (switchgrass, 'Almo') and *Saccharum sp.* (energycane, HO 02-147) for biofuel production were implemented in a greenhouse at the Department of Plant and Soil Sciences at Mississippi State University. Soils collected from the field (Agronomy Unit 1 of the Leveck Animal Research Center located at Mississippi State University, Mississippi, USA, 33° 28' N and 88° 47' W) were sieved and mixed with sand (50:50) to fill the pots (~2500 g).

Isolated *Azospirillum* from energycane was grown in LGI broth for 2-3 days in a rotary shaker, 2000 x g at 29°C. After 2-3 days of incubation, cultures were centrifuged (12 000 g), cell pellets were washed two times with 0.85% (5 g of NaCl in 1 L sterile distilled water) saline solution and re-suspend in saline at a concentration of 10^6 colony forming units/ 10 mL (CFU) (Bashan, 1986). Treatment pots were irrigated with 10 mL

of the bacterial suspension for one week and control plots were provided an equal volume of saline water without bacterial inoculation.

5.3.3 Growth parameters

Plants were harvested after 75 days and growth parameters such as plant height (from base to tip of the plant) and root length were measured. Plant materials were dried at 80°C for 72 h in an oven to obtain the total dry matter yield.

5.3.4 Experimental design and statistical analysis

Pots were arranged in a completely randomized design in a greenhouse; three replicates were performed for each control and treatment. The statistical analyses were performed using Statistical Analysis System (SAS 9.2, Carey, NC) The analysis of variance was performed with the application of the F test with p=0.05.

5.4 Results and discussion

In this work we assessed the *Azospirillum* inoculation effect on plant growth of three bioenergy grasses. We selected the roots of energycane to isolate *Azospirillum* and inoculated into soils of three grasses grown in greenhouse. Inoculation with *Azospirillum* did not provide a significant increase in root length (Fig. 5.1), shoot length (Fig. 5.2), or biomass yield (Fig. 5.3). However root lengths of giant miscanthus were significantly increased in inoculated plants compared with uninoculated plants (Fig. 5.1). In other experiments, different cultivars as well as among the different cultures, specificity in the association of the plant and bacteria was observed (Millet et al., 1984). The intensive uses or lack of the inoculants with ANF bacteria were lead to give insignificant amount results in growth measurements (Bashan, 1986). However, we could not obtain a significant

increase in biomass, which may be due to a low rate of inoculation, low survival of inoculated bacteria, physical chemical characters of soil plant genotype, or comptition withnative microorganisms.

Agronomic use of *Azospirillum* is being extensively tested and results are inconsistent. More consistent results are necessary for the commercial development of inoculants with *Azospirillum* to obtain greater biomass yield from bioenergy grasses. We have observed *Azospirillum* only inside the roots of energycane. However in this experiment we have inoculated bacteria into soil. Therefore it is possible that the insignificant results are due to the low survival of inoculated bacteria. Reports have shown that the constant exposure to *Azospirillum* increases the yield above 20%, which is considered commercially viable for the current agriculture (Bashan and Levanony, 1990). Though the giant miscanthus increased root lengths 28%, switchgrass and energycane were not showed increase in root lengths upon inoculation. Therefore, further experiments should be administered to improve the inoculation procedure.

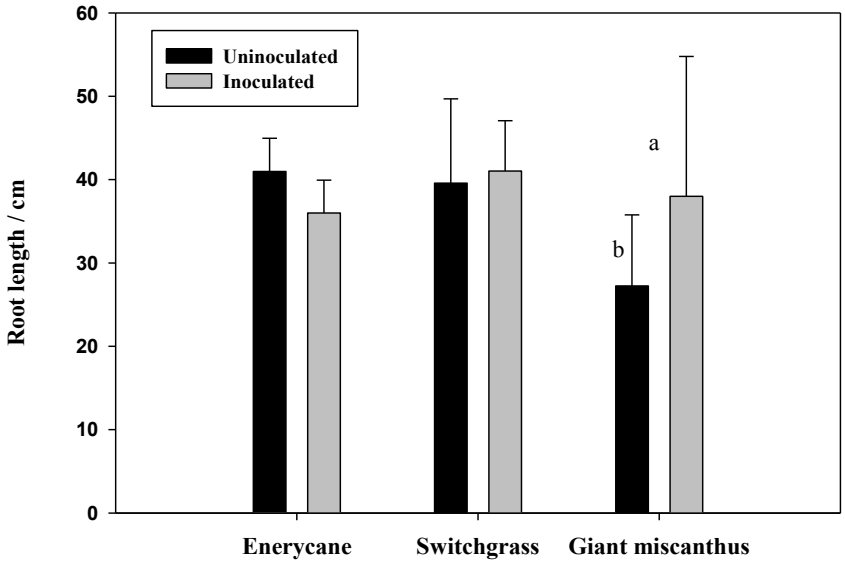

Figure 5.1 Effect of inoculation of *Azospirillum* on soil for roots lengths of energycane, switchgrass and giant miscanthus

Letters (a,b) denote significant difference between inoculated and uninoculated pots of same species and error bars represent standard error (n=3; $p<0.05$)

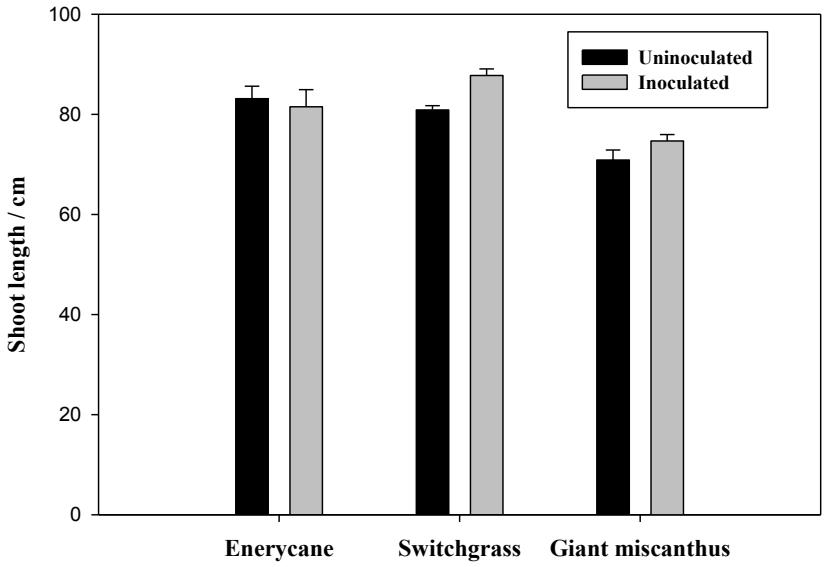

Figure 5.2 Effect of inoculation of *Azospirillum* on soil for shoot lengths of energycane, switchgrass and giant miscanthus

Error bars represent standard error (n=3; $p<0.05$)

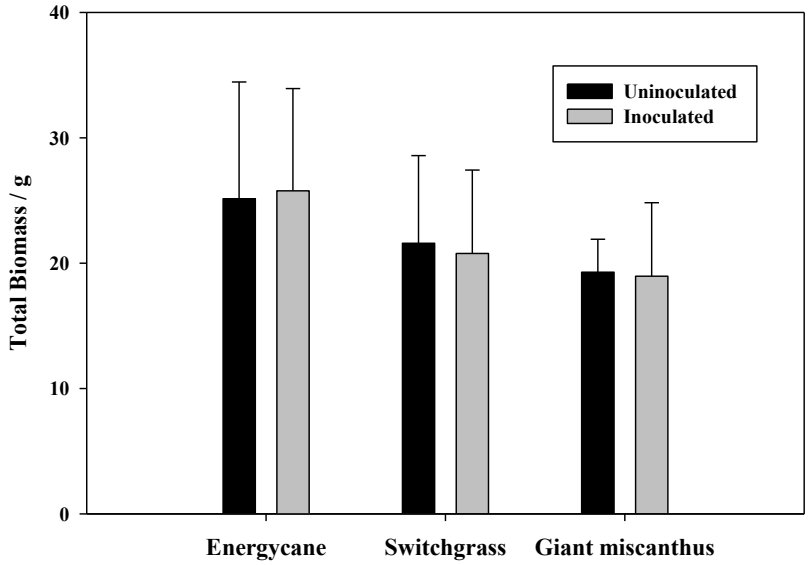

Figure 5.3 Effect of inoculation of *Azospirillum* on soil for biomass yield for energycane, switchgrass and giant miscanthus

Error bars represent standard error (n=3; $p<0.05$)

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CHAPTER VI

CONCLUSIONS

Overall, we can conclude that bioenergy grasses such as energycane, giant miscanthus and switchgrass are associated with nitrogen fixing bacteria and can grow in marginal lands with low or no input of nitrogen. Long time field study with natural abundance technique has showed that the energycane has more potential to support by the ANF bacteria by presenting the lower $\delta^{15}N$ over a three year period time in surface soil, roots and shoots. Moreover, biomass yield and the %Ndfa were greater in energycane compared to switchgrass and giant miscanthus. However, giant miscanthus and switchgrass also showed greater biomass yield compared to annual non fixing sorghum grass. Nitrogen reduction technique was conducted in a greenhouse experiment to determine the nitrogen derived from air for short period of time in bioenergy grasses and found that the greenhouse results was agreement with field results. Further, it was found that enrichment of $^{15}N_2$ actively incorporated to plant root and shoot systems by comparing with the controls. It was supported the idea of bioenergy grasses associated with N_2 fixing bacteria by characterizing bacterial communities and functional *nifH* analysis in field soil rhizosphere and root samples. Endophytic relationship with *Azospirillum lipoferum* could be contributes to the potential increase in biomass observed with energycane. Additional growth promoting and N_2 fixing bacteria, such as *Burkholderia,* may also contribute to plant growth in low N environments. Nitrogen fixing bacteria 85% identical to *Azospirillum*

lipoferum isolated form energycane was inoculated into switchgrass and giant miscanthus. However, no significant results were observed in biomass yield, shoot and root lengths of three bioenergy grasses except the increment in root length of giant miscanthus. Further research is needed to quantify and determine the N_2-fixing bacterial community required to promote the growth of bioenergy grasses to obtain increased biomass of three bioenergy grasses.

APPENDIX A

FIELD SOIL CHARACTERISTICS

AAA712989 **Soil Test Results**

Mississippi State University Extension Service
MIssissippi State University and U.S. Dept. of Agriculture Cooperating

Plant and Soil Sciences-----Soil Testing Lab
Box 9610
Mississippi State, MS 39762

September 28, 2012

Brian S Baldwin
Box 9555
MSU, MS 39762

Oktibbeha County **9353**

Lab#: 1055 -- 1058

Soil Lab No : 9001058

Field ID	Extractable Nutrient Levels lbs/acre										Recommended Lime	Needed Plant Nutrients Pounds Per Acre				Example of Fertilizer Mixture to Supply Recommended Plant Nutrients	
	pH	P	K	CA	MG	S	ZN	NA	CEC	%OM	Tons/Acre	YR	N	P205	K20	Example 1	Example 2
CUT	6.9	179	167	4946	211		2.1	43	15.2		0.0	1	060		30	60# actual N	
		H+	M		H		H									50# 0-0-60	
												2	060		30	60# actual N	
Crop : 40 Small grain for grazing																50# 0-0-60	
												3	060		30	60# actual N	
At Bottom of Report, See Comment Numbers : 400																50# 0-0-60	

Keys :
* pH = Soil Acidity
* P = Phosphorus
* Ca = Calcium
* Mg = Magnesium
* S = Sulfur
* Zn = Zinc
* Na = Sodium
* K = Potassium
* N = Nitrogen
* OM = Organic Matter
* P205 = Phosphate
* K20 = Potash
* CEC = Cation Exchange Capacity
* VL = Very Low
* L = Low
* M = Medium
* H+ = Very High
*EX = Excessive
*TX = Toxic

These are only samples for each mixture; other fertilizer mixtures can be used. Call your county agent if you need assistance determining the amount of other mixtures to be applied.

400 For small grain winter pasture, apply the recommended P and K fertilizer(s) at seeding with 60-80 lbs per acre of N applied 2 to 3 weeks after seed emergence. Apply an additional 60-80 lbs per acre of N between mid-January and mid-February. In south Miss. increased forage yield and more uniform distribution may be realized by 3 applications of N: 60-80 lbs per acre at planting; 60 lbs per acre Dec 1; and 60 lbs per acre Feb 15. These crops should not be grazed until they are about 8 to 12 inches high. The plants should not be grazed closer than 2-3 inches. If soil tests indicate L or M levels for magnesium, use 10-20 lbs per acre of a magnesium source.

Dr. Karl Crouse, Soil Testing Specialist

If you have any questions regarding this report, please call your local county extension office at 662-323-5916.

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Figure A.1 Soil characteristics at the Agronomy Unit 1 of the Leveck Animal Research Center located at Mississippi State University, Mississippi, USA (33° 28' N and 88° 47' W)