Utilizing Beneficial Bacterial Endophytes to Promote Switchgrass Growth in Low-input Agricultural Production Systems

By

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ABSTRACT

The US Department of Energy has focused research efforts on developing switchgrass into a bioenergy feedstock, helping to offset the use of non-renewable fossil fuels and make the US more energy independent. Bacterial endophytes, which reside inside plant tissues, are proven to increase yield and stress resistance in a number of plants. The primary objective of this dissertation was to explore the use of endophytes to improve biomass yields of switchgrass on lands not suitable for food crops and better understand the underlying mechanisms of the plant-endophyte interaction. Integration of this research into K-12 STEM education to increase interest in plant sciences and create the next generation of scientists with the motivation to help solve the challenges facing society in the twenty first century was the objective of the outreach component of this project. Chapter one demonstrates the ability of *Burkholderia phytofirmans* strain PsJN to colonize switchgrass and promote plant growth under *in vitro* (approximately 50% higher), and growth chamber and greenhouse (48.6% higher biomass yields) conditions. The objectives of Chapter two were to determine stand establishment in the field with different nutrient levels. PsJN bacterization positively benefited growth and development of switchgrass seedlings in the field with both low and high nutrient content. Highly significant ($p<0.001$) stimulation of root and shoot growth, lateral root formation and number of tillers was recorded on soil with low fertility. PsJN bacterization also enhanced biomass accumulation during the two seasons of growth on both poor ($p<0.001$) and rich ($p<0.05$) soil, indicating the potential for the use of PsJN in a low-input switchgrass feedstock production system. Chapter three outlines differences in gene expression patterns upon bacterization, between the responsive cv. Alamo, and a non-responsive cv. Cave-in-Rock. Using EST microarrays and quantitative PCR up- and down-regulated genes were identified in both cultivars. One of the key genes identified was a member of the tau class, glutathione S-transferase (GST). GST enzymes are known to be involved in plants responses to stress. Using overexpression and knockout/knockdown techniques we demonstrated that GST is likely involved in the bacterization induced early plant growth promotion in switchgrass. Chapter four describes the potential for the utilization of beneficial

bacterial endophytes capable of fixing atmospheric nitrogen in a free-living state in the development of low-input switchgrass feedstock production systems. *Sphingomonas* sp. strain NSL isolated from switchgrass tissue was able to grow on nitrogen free medium and stimulated growth of switchgrass cv. Alamo under nitrogen deficient conditions. The ability to fix atmospheric nitrogen was also moved to *Burkholderia phytofirmans* strain PsJN via horizontal gene transfer from the legume nodulating *Burkholderia phymatum*. The transformed PsJN was able to fix nitrogen and promote plant growth under nitrogen limited conditions. At every step of the research described in this dissertation efforts were made to include its elements into K-12 education. Chapter five describes four case studies aiming at the enhancement of youth interest in plant sciences in the socieoeconomically depressed areas of Southside Virginia.

DEDICATION

I dedicate this dissertation to my parents

Robert Preston Lowman, Jr. and Peggy West Lowman

And

My wonderful wife Susan, for her strength and support throughout this endeavor

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Potential for the use of endophytes in switchgrass biomass production*

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Introduction

Switchgrass (*Panicum virgatum*), a native warm season perennial grass found throughout the US, characteristically produces high biomass yields annually with low inputs and can grow on marginal land. Since the introduction of the Department of Energy's Bioenergy Feedstock Development Program over 3 decades ago, switchgrass has been the subject of intensive study, yielding a plethora of data regarding plant growth and stress resistance. As a C4 species, switchgrass is efficient at converting the sun's energy into carbohydrate compounds, and combined with being perennial, the plant offers much promise for future biomass production on a large scale, helping to offset the use of fossil fuels. In fact, switchgrass yielded 504% the energy consumed in a large, multi-farm study in the Central Plains (Schmer et al., 2008), and stands can produce for more than a decade. Furthermore, compared with other bioenergy crops, switchgrass cultivation is relatively simple and requires no specialized equipment by the producer. While yields are high, much more could be improved for bioenergy purposes. Beneficial plant-microbe interactions, a field of study generating much interest in the past two decades, offer new solutions to improve biomass yields, stress tolerance, first-year establishment, and sustainability.

Both bacterial and fungal microorganisms form ancient and mutually beneficial symbiosis with plants, and mycorrhizal fungi in particular are associated with the initial colonization of land by plants (Ryan et al., 2008; Wang and Qiu, 2006). A cultivated field of plants represents a complex community of microbes, interacting, competing, and often assisting with plant growth promotion

and stress resistance. Generally, beneficial plant-microbe interactions provide plant growth promotion via production of plant hormones, such as auxin, aiding in stress resistance to abiotic stresses including drought and salinity, production of antimicrobial compounds against plant pathogens, and nutrient acquisition such as atmospheric nitrogen fixation and solubilization of phosphorus in soil (Reviewed in Berg, 2009). These interactions are intricate and multifaceted, often dependent on time of development, genotype, environmental conditions, and native soil communities. Although switchgrass has been intensively studied (Parrish and Fike, 2005), only a few articles have been published focusing on endophytes in switchgrass and their influence on growth promotion (Ghimire et al., 2009; Kim et al., 2012). Together, beneficial microorganisms could have the potential to help in the development of a low input and sustainable switchgrass production system (Nowak et al., 2011) and offer a practical way to improve plant growth and disease resistance.

Nomenclature, diversity, and classification

The term 'endophyte' is derived from the Greek term 'endo' (within) and 'phyte' (plant), and may apply to both fungi and bacteria that reside in plant tissues during all or part of their life cycle and cause no apparent harm (Wilson, 1995). It is estimated that every plant species has at least one associated bacterial endophyte (Strobel et al., 2004) and they belong to diverse classes of bacteria including Alpha, Beta, and Gamma proteobacteria, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* (Rosenblueth and Martinez-Romero, 2006). These bacteria thrive within plants where they successfully colonize roots, translocate to leaves, stems, and even to reproductive organs where they may be vertically transmitted to the next generation, ensuring a stable interaction with its host plant. The number of microorganisms present in natural ecosystems is tremendous; in fact, estimates of the number of bacterial endophytes in the Brazilian Atlantic forest indicate the possibility of 2-13 million species in the above ground plant parts alone (Lambais et al., 2006). Of the bacterial species identified, 97% were previously not described. A single plant species may also have a wide range of different bacterial genera associated. In wheat, culture based studies have shown that 88 bacterial species representing 37 genera inhabit the above ground plant tissue (Legard et al., 1994). Culture based studies likely underestimate the number of microorganisms as molecular studies yield much larger population numbers (Rasche et al., 2006). Both culture based and molecular based analyses indicate that Alpha and Beta proteobacteria are the most numerous colonizers of the phyllosphere (Thompson et al.,

1993). In total, 853 bacterial endophytes were isolated from aboveground parts of four agronomic crops and 27 prairie plants including switchgrass, and *Cellulomonas*, *Clavibacter*, *Curtobacterium*, and *Microbacterium* isolates showed high levels of colonization and had the ability to persist in host plants (Zinniel et al., 2002).

Diazotrophic, or atmospheric nitrogen-fixing bacteria have been isolated from bioenergy crops, including *Miscanthus* spp. and *Pennisetum purpureum*, where *Herbaspirillum frisingense* sp. nov. (Kirchhof et al., 2001), *Azospirillum doebereinerae* (Eckert et al., 2001), and *Herbaspirillum frisingense* (Rothballer et al., 2008) were found. Similarly, different nitrogen-fixing bacteria belonging to genera *Stenotrophomona*, *Pseudomonas* and *Burkholderia* were isolated from sand dune grasses (*Ammophila arenaria* and *Elymus mollis*) in Oregon, which may biologically fix nitrogen and promote the growth of these plants under poor soil conditions (Dalton et al., 2004). Nitrogen-fixing bacteria have also been isolated from different plant species, such as Kallar grass (*Leptochoa fusa*) growing in the highly saline soils in the Punjab of Pakistan (Reinhold-Hurek et al., 1993), lodgepole pine (*Pinus contorta*), western red cedar (*Thuja plicata*) (Bal et al., 2012), and hybrid poplar (*Populous trichocarpa*) (Taghavi et al., 2010). While general surveys of endophytic populations in switchgrass have been undertaken (Zinniel et al., 2002), there are no detailed analysis on native bacterial endophytic interactions in switchgrass.

Fungal endophytic populations may also be substantial, particularly in longer lived plants, as 340 genetically distinct taxa were recovered from two tropical understory plant species (Arnold et al., 2000). Endophytic fungi can also have a significant beneficial impact on switchgrass performance (Kleczewski et al., 2012). While much emphasis has been placed on the study of fungal endophytes from the Clavicipitaceae family (*Neotyphodium*/*Epichloë*) with cool- and warm-season grasses (Rodriguez et al., 2009), 2 recent surveys of switchgrass endophytes have failed to identify members of this family (Ghimire et al., 2011b; Kleczewski et al., 2012), suggesting that the major endophytic fungi inhabiting switchgrass are of the non-clavicipitaceous type, representing primarily *ascomycetous* fungi (Kleczewski et al., 2012). These endophytes may be found colonizing tissues above- and/or below-ground (Rodriguez et al., 2009). Recently, 18 taxonomic orders of fungal endophytes were isolated from switchgrass plants in northern Oklahoma belonging to the genera *Alternaria*, *Codinaeopsis*, *Fusarium*, *Gibberella*, *Hypoerea*

and *Periconia*, and switchgrass shoot tissues showed a significantly higher diversity of fungal endophytic species compared to the root tissues (Ghimire et al., 2011b). Similar fungal endophytic genera,such as *Alternaria*, *Epicoccum*, *Phoma*, *Phaeosphaeria* and *Stagonospora*, were isolated from switchgrass plants growing in a range of habitats across Indiana and Illinois (Kleczewski et al., 2012). Since switchgrass is one of the most promising bioenergy crops, several laboratories in the US have been working on isolation and characterization of bacterial and fungal endophytes from switchgrass. Identifying and harnessing beneficial endophytic microorganisms that have a broad spectrum of plant growth promotion traits and possess various mechanisms for stress tolerance may aid in the development of a low input and sustainable switchgrass feedstock production system, particularly on marginal land.

Colonization of plant tissues and organs by bacteria

The ability of some endophytes to colonize the xylem provides the opportunity for their systemic spread throughout the rest of the plant, via the transpirational stream in the xylem lumen. However, not all endophytes are capable of colonizing the aerial parts of plants. This may reflect the inability of some to adapt and survive the different niches represented by aerial tissues and organs (Compant et al., 2010). In switchgrass, *B. phytofirmans* strain PsJN titers were higher in the root than in the leaves 7 days post-inoculation of the roots. By 14 days post-inoculation, titers were higher in leaves and sheaths than in the roots, indicating translocation to these tissues (Kim et al., 2012). Generally, bacterial endophyte titers in the aerial plant tissues are reported to be lower than in the root (Rosenbleuth and Martínez-Romero, 2006; Compant et al., 2008). In addition, a fair amount of variation can be observed in these tissues. Compant et al. (2008) reported that PsJN could be found in only 10 - 60% of grape inflorescence stalks and grape berries following initial inoculation of roots. These were localized to xylem vessels, and only a single or few cells were observed. These results further indicated the importance of the xylem for systemic spread of endophytes, allowing them to reach as far as the reproductive tissues. However, this spread was very slow, taking 5 weeks to reach inflorescence tissues. The very low titers of PsJN that ended up in these tissues was attributed to competition with other co-localized endophytes, which can inhabit different tissues and organs, reflecting different niches of colonization (Compant et al., 2011). Bacterial colonization, in general, varies from one cultivar to another and depends on many factors. For example, in soybean, plant genotype, tissue age,

season of isolation, and herbicide application, all effected colonization (Kuklinsky-Sobral et al., 2004).

Plant growth promotion

One of the most well-studied bacterial-endophyte associations is atmospheric nitrogen fixation by specific endophytes. This symbiosis is well known in leguminous plants (Stacey et al., 2006) where the soil bacteria Rhizobia infect the roots of the host plants, inducing the formation of nodules where they fix atmospheric nitrogen and provide it to the host plant in exchange for carbon compounds (Lodewyckx et al., 2002). However, mutualistic associations through the fixation of nitrogen can also be observed in non-leguminous plants, such as rice (Mattos et al., 2008), maize (Montañez et al., 2012), sugarcane (Oliveira et al., 2009), wheat (Webster et al., 1997), strawberries (de Melo Pereira et al., 2012), and grasses (Kirchhof et al., 2001; Reinhold-Hurek et al., 1993). Nitrogen-fixing bacteria have been studied extensively in the bioenergy crop sugar cane, and include *Gluconacetobacter* spp., *Azospirillum* spp., *Herbaspirillum* spp. and *Burkholderia* spp. (de Carvalho et al., 2012; James et al., 2001; Montañez et al., 2012; Suman et al., 2005). In fact, cultivation of sugarcane in Brazil, when combined with the use of nitrogen fixing bacteria, uses only a small amount of fertilizer (de Carvalho et al., 2011) without showing nitrogen deficiency symptoms (Rosenblueth and Martinez-Romero, 2006), and there is evidence that a significant amount of nitrogen is obtained from plants associated with bacterial endophytes (de Carvalho et al., 2011). In switchgrass, young seedlings of the cultivar Alamo inoculated with *Burkholderia phytofirmans* strain PsJN, isolated from onion roots by Frommel et al. (1991), showed significant growth promotion with an increase of root and shoot length of 35.6 % and 32.8 %, respectively, as well as an increase of fresh weight of 83.6 % compared with control plants (non-inoculated) after one month under *in vitro* conditions (Kim et al., 2012). The same pattern was observed under growth chamber and greenhouse conditions, where plants inoculated with the *B. phytofirmans* strain PsJN showed persistent growth vigor with significant increases in fresh and dry weights, and an increase in the number of early tillers (Kim et al., 2012). Also, results showed that *B. phytofirmans* strain PsJN has potential in the development of a low input and sustainable switchgrass feedstock production system on marginal lands as higher biomass yields were observed under sub-optimal conditions with PsJN inoculated plants vs control (Kim et al., 2012). However, PsJN growth promotion is genotype specific in switchgrass as the upland cultivar, Cave-in-Rock did not respond to inoculation.

Fungal endophytes are most commonly found living in above-ground plant tissues and occasionally in roots (Saikkonen et al., 1998). Plants infected with fungal endophytes gain growth promotion, stress tolerance, water use efficiency, and protection against vertebrate herbivores and root nematodes (Rodriguez et al., 2008; Rodriguez et al., 2009; Schardl et al., 2004). During the interaction, endophytes obtain shelter, nutrition and dissemination through propagules of the host plants (Schardl et al., 2004). Like bacterial endophytes, fungal endophytes also promote host plant growth, such as increased root growth and longer root hairs (Malinowski et al., 1999), which may contribute to enhanced nutrient uptake. For instance, the root and shoot biomass of poplar, maize, tobacco, bacopa, *Artemisia*, and parsley was doubled compared with their respective controls after four weeks of *Piriformospora indica* inoculation (Varma et al., 1999).

Fungal endophytes of the genus Neotyphodium (an asexual form of Epichloë spp.) have been well studied for their symbiotic associations with different grass species, especially the family Pooideae, which includes many important species of forage and turf grasses (Clay, 1990; Schardl et al., 2004; Sugawara, 2011). Through this symbiosis, grasses have exhibited increased growth, reproduction, tolerance to stress and resistance to herbivores (Faeth et al., 2010; Schardl et al., 2004). For instance, plant growth, biomass yield and tiller number increased when ryegrass (*Lolium perenne*) was inoculated with *N. lolii* (Spiering et al., 2006), and Dahurian wild rye (*Elymus dahuricus*) with *Neotyphodium* spp. (Zhang et al., 2007). Endophyte-infected plants showed a higher survival rate, regrowth rate, and more biomass seed production compared to non-infected plants after a year in the field (Iannone et al., 2012). In switchgrass, NF/GA-993 (a synthetic lowland switchgrass cultivar) inoculated with six strains of *Sebacina vermifera* fungal endophytes showed increased plant growth, root length, and biomass production (Ghimire et al., 2009a). Recently, Sasan et al. (2012) found that the fungal endophyte *Metarhizium robertsii* was able to endophytically colonize the roots of switchgrass and promoted growth and increased the density of root hairs. However, fungal endophytes recently isolated from switchgrass plants had both beneficial and detrimental effects on switchgrass biomass yields in greenhouse conditions. *Phaeosphaeria pontiformis*, *Epicoccum nigrum*, *Alternaria* spp and *Colletotrichum* spp. increased total biomass by 25-33%, *Stagonospora* spp. increased shoot biomass by 22%, and *Colletotrichum* sp. increased root biomass by 45%, but over 60% of isolates tested reduced switchgrass growth (Kleczewski et al., 2012).

Many microorganism related factors affect plant growth promotion because plants exist in a community of bacteria, fungi, algae and/or viruses (Rodriguez et al., 2008) and plants could be associated with more than one microorganism. Inoculation of switchgrass seedlings with multiple types of rhizosphere microflora increased the yield of shoots and roots up to 15-fold and also increased nitrogen uptake 6-fold and phosphorus uptake 37-fold, compared with the control plants infected with rhizosphere bacteria only (Brejda et al., 1998). Environmental factors, such as nutrients and stress, also influence symbiosis between endophytes and host plants. Under high nutrient availability, symbiotic *Neotyphodium occultans* - *Lolium multiflorum* showed higher seed weight than that of non-symbiotic plants (Gundel et al., 2012).

Abiotic stress tolerance

Plant growth is usually limited by abiotic stresses. Abiotic stress includes various environmental stresses, such as drought, temperature, salinity, air pollution, heavy metals, pesticides and soil pH. Symbiotic relationships with endophytes have been shown to increase stress tolerance in host plants (Gibert et al., 2011). Drought is one of the most wide spread and common abiotic stresses and causes economically important losses in agriculture and forestry crops every year. The mutualistic symbiosis between bacterial or fungal endophytes and host plants could enhance host plant drought tolerance. The evergreen tree *Theobroma cacao* infected with the endophytic fungus *Trichoderma hamatum* isolate DIS 219b exhibited delayed drought stress by changes in stomatal conductance, water potential, and net photosynthesis (Bae et al., 2009). In grasses, endophytic associations also increased drought tolerance as some accessions of the perennial ryegrass (*Lolium perenne*) infected by *N. lolli* showed more tillers, greater tiller length and higher biomass than non-infected plants (Kane, 2011). Endophytic inoculation of *Epichloë festucae* in *Fetusca eskia* enhanced seedling survival under drought conditions (Gibert et al., 2011). A perennial bunchgrass, *Achnatherum sibiricum* infected with endophytic fungi showed a higher root/shoot ratio and net photosynthetic rate than non-inoculated plants under drought conditions (Han et al., 2011). The symbiosis between *Agrotis hyemalis* and *Epichloe amarillans*, when placed under drought conditions, produced 40% more inflorescences, earlier flowering and greater seed mass than non-inoculated plants (Davitt et al., 2011). However, when *Panicum rigidulum* plants were subjected to drought conditions, endophyte *Balansia benningsiana* infected plants did not show any advantages over control plants during drought stress but endophyte infection helped rapid leaf regrowth during recovery (Ren and Clay, 2009).

Cultivated soils are becoming more saline due to excessive fertilizer use, the use of wastewater from urban and peri-urban areas and agricultural drainage as well as the desertification processes (Bashan et al., 2010). Plant growth promoting bacteria offer the potential to reduce the impact of this stress. For instance, cucumber plants inoculated with *Paecilomyces formosus* showed increased shoot length compared with that of non-inoculated plants under high salinity conditions (Khan et al., 2012). In studies with *Salicornia brachiata*, the most salt-tolerant plant species among *Salicornia* spp., *Brachybacterium saurashtrense* and *Pseudomonas* sp. bacterial endophytes significantly increased plant growth under salt stress conditions. The bacteria *Pseudomonas putida* and *P. pseudialcaligens* inoculation increased plant growth of chickpeas under saline conditions in pot experiments (Patel et al., 2012).

Phytoremediation is the process in which plants can uptake, accumulate, or metabolize toxic compounds, such as heavy metals and other compounds, from contaminated soil (Kumar et al., 1995). The plant-endophyte association has been used at phytoremediation sites to degrade toxic compounds for practical use (Van Aken et al., 2004). *Brassica juncea* inoculated with a plant growth promoting bacteria strain A3R3 showed increased plant fresh and dry weights when grown in soil at different concentrations of nickel, with the increases of fresh and dry weights by 50 and 45%, respectively at 450 mg Ni/kg soil compared with non-inoculated plants (Ma et al., 2011). Many plant growth-promoting endophytes could alleviate plant stress from contaminants by degrading such contaminants, and in return, could provide the products for plant use (Weyens et al., 2009a,b). For phytoremediation of toxic metals, endophytes may have a metal-resistant or sequestration system and could reduce metal toxicity and influence metal translocation to the aboveground plant parts. Metal-resistant endophytic bacteria have been found in the genera *Pseudomonas*, *Methylobacterium*, *Microbacterium* and *Burkholderia* (Weyens et al., 2009a). In tall fescue (*Lolium arundinaceum*) grown under greenhouse conditions in a solution contaminated with cadmium, endophytic fungus (*Neotyphodium coenophialum*) infection enhanced cadmium accumulation and increased cadmium transport from roots to the shoots (Ren et al., 2011). In two grass species, *Festuca arundinacea* and *Festuca pratensis*, grown under high cadmium conditions, results showed higher biomass production and higher levels of cadmium accumulation in the roots and shoots of endophyte-infected plants versus uninfected plants (Soleimani et al., 2010). Under greenhouse conditions, the seedlings of guinea grass (*Panicum*

maximum) cultivars inoculated with *Pantoea* spp. Jp3-3 exhibited significant alleviation from the negative effect caused by the stress of 300 μM copper (Huo et al., 2012).

Biotic stress tolerance

Biotic refers to living organisms that cause diseases, such as bacterial and fungal pathogens, pests, insects, viruses, and nematodes. Endophytes inhibit plant pathogen growth and prevent or reduce disease development through the production of toxic alkaloids or by occupying the same ecological niche as the pathogen (Clay, 1990). Studies found that three *Bacillus* strains and two *Pseudomonas fluorescens* strains decreased up to 60% of the disease symptoms caused by *Pseudomonas syringae*, a powdery mildew and angular leaf spot, and increased the fresh weight of inoculated melon plants compared with non-inoculated controls (García-Gutiérrez et al., 2012). In tomato plants, bio-control of *Bacillus subtillis* S499 was tested for antagonism against *Fusarium spp*. by treating the seeds with a formulated powder containing different concentrations of viable spores of *B. subtillis* S499, and results showed that all treatments significantly reduced disease severity up to 65-70% compared with control plants (non-inoculated seeds) (Nihorimbere et al., 2010).

Since endophytes have the ability to inhibit or prevent pathogen growth, they have been considered as biological control agents. In the interaction of Italian ryegrass (*Lolium multiflorum Lam*) with the fungal endophyte *Neotyphodium*, the ryegrass exhibited increased resistance to *Trigonotylus caelestialium* (Shiba et al., 2011). Additionally, the bird cherry oat-aphid (*Rhopalosiphum padi*), a notorious pest of forage and cereal grasses, showed a preference to non-infected plants of Alpine timothy (*Pleum alpinum*) over the plants infected with *Neotyphodium spp*. (Clement et al., 2011). Perennial ryegrass (*L. perenne*) plants colonized by *N. lolii* exhibited reduced aphid populations, and in some cases the aphids exhibited reduced adult life-span and fecundity (Meister et al., 2006). Tall fescue plants inoculated with *Neotyphodium coenophialum* decreased the survival rate and feeding of the corn flea beetle, *Chaetocnema pilucaria* (Ball et al., 2011). Similar preferences were observed in *Achnatherum inebrians* (drunken horse grass) where *Neotyphodium gansuense*-infected plants decreased the preference of herbivores such as bird cherry-oat aphid (*Rhopalosiphum padi*), carmine spider mite (*Tetranychus cinnabarius*), grasshopper (*Oedaleus decorus*) and seed-harvesting ant (*Messor aciculatus*) due to high levels of ergine, ergonovine and ergoit alkaloids produced by the fungal endophyte (Zhang et al.,

2011). Recently endophytic bacteria isolated from root tissue of six plants growing in a tidal flat area of Korea showed antagonistic potential toward the pathogenic oomycete fungi *Phytophtore capsici* and *Pythium ultimim*, and some of them were able to degrade biopolymers, such as cellulose and β-1,3-glucan, which are major components of the cell wall of oomycetes (Bibi et al., 2012). In switchgrass production, it was found that large-scale planting of switchgrass could be devastated by *Puccinia emaculata* Schwein, a rust fungus (Zhao B. http://hayandforage.com/biofuels/rust-resistant-switchgrass-research-goal-0323). In the future, it may be possible to identify endophytes which produce antifungal compounds to help offset losses caused by biotic stresses.

Mechanisms of growth promotion

As plants are sessile organisms, the wide diversity of mutually beneficial plant-microbe interactions represents an ancient evolutionary partnership, helping the host plant survive and thrive, even in some of the harshest environments on the planet. Mechanisms of growth promotion by bacterial and fungal endophytes have been investigated in grasses for decades, and various mechanisms play roles in promoting plant growth and development. Bacterial endophytes are capable of producing or regulating plant hormones, helping acquire vital nutrients, and bio-control of pathogens (reviewed in Sturz et al., 2000). Furthermore, a particular bacterial endophyte may utilize one or more mechanisms to promote plant growth and may even utilize different mechanisms at various points during the life cycle of plants. While it is clear that endophytes can benefit the host plant in many ways, establishing clear-cut growth promotion in the field can be difficult due to a number of factors including the diversity of native microorganisms in the soil and soil conditions. A more profound understanding of these mechanisms is allowing scientists to discover new ways to integrate their use into increasing yields of bioenergy crops like switchgrass. Also, by utilizing tools of modern molecular biology and functional genomics to understand the complexity of growth promotion at the genetic level, additional light will be shed on these complex interactions. As more is learned about the biochemistry, molecular biology, and physiology of microbe-plant interactions, it is evident that bacterial and fungal microorganisms will be important components for sustainable bioenergy feedstock production in the future.

Plant growth promotion can generally be achieved directly by interactions between the microorganism and host and/or indirectly through antagonistic activity against plant and environmental pathogens (Berg, 2009). In this section, we will discuss both mechanisms and how different beneficial microbes may work together to benefit the host plant simultaneously (Muller et al., 2009), as well as how microorganisms, especially bacteria, may share mechanisms of actions genetically through horizontal gene transfer.

Phytohormone production and regulation

Plant tissues produce or regulate different hormones to respond to internal and external cues during practically every aspect of plant growth and development. Bacterial endophytes have the ability to produce plant hormones and regulate their balance as well. Auxins, a group of hormones associated with plant growth promotion, influences many plant cellular functions and are important regulators of growth and development. Bacterial endophytes are commonly capable of production of auxin which, at the genetic level, may either be constituently expressed or inducible (Mattos et al., 2008). Auxin producing bacterial endophytes increased the number and length of lateral roots in wheat (Barbieri et al., 1993). Increased root length, root surface area and the number of root tips were observed in hybrid poplar inoculated with auxin producing bacteria, resulting in enhanced uptake of nitrate and phosphorus and boosting biomass by 60% compared with non-inoculated plantlets (Taghavi et al., 2009). *Pseudomona flourescen*s significantly increased the growth of maize plant radicles under laboratory conditions via the production of auxin (Montañez et al., 2012). To date, multiple auxin biosynthesis pathways have been identified in bacteria, and their regulation is influenced by several different genetic and environmental factors (Bertalan et al., 2009). The production of native auxin, indole-3-acetic acid (IAA) by bacteria has been documented in species such as *Rhizobium*, *Pseudomonas*, *Azospirillum*, *Azotobacter* and *Bacillus* families (Hayat et al., 2010).

Cytokinins are a diverse range of compounds that, like other plant hormones, are involved in many activities of plant growth and development. As a group, they have been shown to regulate cell division, seed dormancy and germination, senescence, new bud formation, and leaf expansion. They also play roles in controlling plant organ development, mediating responses to various extrinsic factors and the response to biotic and abiotic stresses (reviewed in Spichal, 2012). Researchers have demonstrated that certain endophytic bacteria are able to produce

cytokinins and promote lateral root growth (Senthilkumar et al., 2009). Zeatin, a native plant growth promotive hormone, belonging to the cytokinin family, has been found in significantly higher levels in the beneficial bacteria *B. subtilis* and *P. putida* (Sgroy et al., 2009).

Gibberellins are native plant growth promotive hormones. Many plant growth promoting endophytes also produce gibberellins to enhance host plant growth (Fernando et al., 2010; Joo et al., 2009). For example, one *Penicillium citrinum* isolate, IR-3-3 from the sand dune flora, produced higher physiologically active gibberellins and stimulated Waito-c rice and *Atriplex gemelinii* seedling growth (Khan et al., 2008). GA3 levels were also high in the plant associated bacteria *Lysinibacillus fusiformis*, *Achromobacter xylosoxidans*, *Brevibacterium halotolerans*, and *Bacillus licheniformis* (Sgroy et al. 2009).

Ethylene, a simple organic molecule (CH_2=CH_2), is commonly thought to be a growth inhibitive hormone. It is typically produced when plants are exposed to environmental stress, repressing plant growth and development until the stress disappears or the levels of ethylene decrease (Gamalero et al., 2012). Ethylene inhibits stem elongation, promotes lateral swelling of stems, and causes stems to lose their sensitivity to gravi-trophic stimulation (reviewed in Glick, 2005). In biomass production as in agriculture generally, it is important to keep ethylene low in order to maximize yields. An enzyme, 1-aminocyclopropane-1-carboxylate (ACC) deaminase produced by bacteria, interferes with the physiological processes of the host plant by decreasing ethylene levels (Hardoim et al., 2008) via metabolizing ACC, a precursor to ethylene. By metabolizing this precursor, ethylene levels are reduced in plants thereby reducing the effects of ethylene. Activity of ACC deaminase is a common feature found in plant-growth promoting bacteria such as *Enterobacter*, *Pseudomonas* and *Burkholderia* (Govindasamy et al., 2008; Sessitsch et al., 2005b; Shah et al., 1998). *Burkholderia phytofirmans* strain PsJN stimulates growth of many plant species, including potato, tomato, grapevine, and switchgrass (Barka et al., 2002; Nowak et al., 1998; Pillay et al., 1997; Kim et al., 2012) and was reported to have a high activity of ACC deaminase (Sessitsch et al., 2005a). Endophytes that produce ACC deaminase have also been shown to increase host plant growth in soils with high salinity (Egamberdieva, 2012; Siddikee et al. 2012) and increase drought tolerance (Arshad et al., 2008; Belimov et al., 2009). *Pseudomonas* strain A3R3 showed higher ACC deaminase activity and increased plant growth in Ni contaminated soil (Ma et al., 2011).

Abscisic acid (ABA) is involved in responses to environmental stresses such as heat, water, and salt, and is also produced by endophytes. Endophytic bacterial strains SF2, SF3, and SF4 isolated from sunflowers (*Helianthus annuus*) had the ability to produce ABA and jasmonic acid (JA), which increased under drought conditions (Forchetti et al., 2007), implying these endophytes enhance stress tolerance of host plants. Two strains of *Azospirillum brasilensis*, successfully used to increase the yield of maize and wheat in field conditions, were both able to produce different plant growth regulators such as IAA, GA3, zeatin and ABA (Perrig et al., 2007), highlighting the ability of endophytes to confer multiple mechanisms of growth promotion.

Atmospheric nitrogen-fixation

Endophytic bacteria that live freely in the internal tissues of plants and cause no apparent harm have a diverse range of growth promotion mechanisms including nitrogen fixation in grasses. Although 78 percent of the earth's atmosphere is nitrogen, nitrogen is often a limiting factor in agriculture since it is not readily available to plants. Bacteria and Archea are the only organisms that can fix atmospheric di-nitrogen, thereby making it available for plant growth. This activity is termed biological nitrogen fixation (BNF) and is catalyzed by the oxygen sensitive nitrogenase enzyme to convert N2 to bio-available NH3. Nitrogenases are complex metalloenzymes with highly conserved structural and mechanistic features (reviewed in Alberty, 1994; Burgess and Lowe, 1996; Rees et al., 2000). The enzyme is oxygen sensitive, which imposes physiological constraints on the organism. Additionally, the enzyme has a relatively slow turnover time (Thornely and Lowe, 1985), which requires the microbe to synthesize large quantities of the protein, up to twenty percent of protein in the cell (reviewed in Dixon and Khan, 2004). Also, the conversion of atmospheric di-nitrogen to a form that can be used by plants requires 16ATP to reduce one molecule of N2, making it one of the most energy demanding reactions identified in bacterial organisms (Thornely and Lowe, 1985). Together, the amount of energy, the low oxygen requirement, and the amount of protein required to create the nitrogenase enzyme, place a large burden on a nitrogen fixing endophyte. As a result, the synthesis of the nitrogenase complex is stringently regulated at the genetic level (Dixon and Khan, 2004). It has been suggested that bacterial endophytes are placed in a more favorable environment compared to rhizospheric bacteria because they are less vulnerable to competition from native soil bacteria and are shielded from various biotic and abiotic stresses (Reinhold-Hurek et al., 1998). Perhaps the most-studied grass inoculated with free living nitrogen-fixing endophytes is sugarcane.

Burkholderia MG43 inoculated sugarcane plantlets produced a 20% increase in yield over un-inoculated control (Govindarajan et al., 2006), and it was demonstrated that 60 to 80% of nitrogen accumulated in sugarcane came from atmospheric nitrogen fixation (Boddey et al., 1995). The authors also noted that farmers in Brazil have observed some varieties of sugarcane grown in fields for decades, even up to a century without showing any decline in soil N reserve or yield, despite the supply deficit of nitrogen (Boddey et al., 1995). Rice has also been studied in the context of its relationship with free-living nitrogen-fixing *Burkholderia* spp. In one field experiment, 31% of plant nitrogen was derived from BNF, and inoculation resulted in as high as a 69% increase in biomass compared to the un-inoculated control (Baldani et al., 2000). Researchers also found *Burkholderia vietnamiensis* inoculated rice seedlings increased yield by 5.6 to 12.16%, and 42% of nitrogen found in the inoculated plants came from atmospheric nitrogen fixation (Govindarajan et al., 2008). In addition to rice, *Burkholderia* were found to be among the most common nitrogen-fixing isolates from maize plants cultivated in Mexico, and many were reported to be new species (Estrada et al., 2002). These findings support the use of free-living nitrogen-fixing endophytes in the effort to reduce the use of synthetic nitrogen fertilizer and offer hope in creating high-yielding, low-input agricultural production systems.

Bio-control of pathogens

Another mechanism of plant growth promotion by endophytes is bio-control of pathogens. Endophytes have evolved a diverse range of bio-control mechanisms including production of antibiotics, both antifungal and antibacterial, siderophore secretion, and enzyme production (reviewed by Compant et al., 2005b). Together, these bio-control properties enable endophytes to outcompete pathogens for their niche and limit damages caused by phytopathogens as well as protect their host plant, resulting in increased survival and growth.

Fungal endophytic colonization confers a positive impact on resistance to pests, mites, and nematodes in grasses (Schardl et al., 2004). Perennial ryegrass (*L. perenne*) plants colonized by *N. lolii* reduced aphid populations, adult life span and fecundity (Meister et al., 2006). *Neotyphodium spp*. form mutualistic associations with several grass genera and produce a range of bio-control agents, some of which have insecticidal properties whereas others are associated with health and welfare issues for grazing animals. Through selection, several novel endophytes that produce predominantly insecticidal bio-control agents have now been successfully

commercialized in many temperate grassland areas in New Zealand, Australia, USA, and South America (Easton, 2007).

One of the most commonly recognized bio-control mechanisms associated with endophytic plant growth promoting bacteria and fungi is the production of antibiotics. Agents produced include but are not limited to pyrrolnitrin, phenazines, herbicolin, and oomycin. Furthermore, many endophytic organisms are able to produce multiple agents, which have bio-cidal properties towards various organisms. Pyrrolnitrin, a secondary metabolite isolated from *B. cepacia*, was shown to have activities against both phytopathogenic fungi and bacteria (El-Banna et al. 1998). The gene cluster regulating the production of pyrrolnitrin is similar to the gene cluster in *Pseudomonas* and was suggested to have been acquired by horizontal gene transfer (de Souza et al., 2003). Other strains of *Burkholderia* were reported to produce a large variety of anti-fungal agents such as occidiofungin and burkholdinesn (Lu et al., 2009; Tawfik et al., 2010). *Burkholderia* MP-1 produces at least four anti-fungal compounds including phenylacetic acid, hydrocinnamic acid, 4-hydroxyphenylacetic acid, and 4-hydroxyphenylacetate methyl ester (Mao et al., 2006). The small size of genes encoding antibacterial agents and the relatively small number of genes in bacteria and fungi may allow genes encoding antibiotic agents to be transformed to various growth promoting endophytes.

Siderophore secretion

Iron, one of the most abundant minerals on the planet, is not readily available to bacteria because its most commonly found form, ferric iron (Fe+3), is only slightly soluble and tightly bound to many particles in the soil. To gather iron needed for growth, bacteria and fungi secrete low molecular weight compounds called siderophores. Bacterial siderophores generally act to inhibit pathogenic fungi as a result of their siderophores having more affinity to iron than fungal siderophores (Ordentlich et al., 1988). Like many mechanisms of action in bacteria and fungi, environmental factors such as pH, nutrient levels including iron may affect synthesis of siderophores. Siderophore secretion has been confirmed in a number of bacterial taxa including *Bacillus*, *Pseudomonas*, *Rhodococcus*, *Serratia*, *Obesumbacterium* and *Lysinibacillus* (Czajkowski et al., 2012) as well as the fungal endophyte *actinomycetes* (Nimnoi et al., 2010). Genes encoding siderophores may be more difficult to introduce to other plant growth promoting

endophytes since studies have shown that they are located in multiple loci (Osullivan et al., 1990) and have complex control mechanisms (Ovadis et al., 2004).

Abiotic stress tolerance mechanisms

Abiotic stresses include various environmental factors such as hot and cold extremes, drought, salinity, metal contamination and synthetic chemicals, among others, and all may decrease performance of bioenergy crops like switchgrass in the field. To help the host plant tolerate abiotic stresses, endophytes have evolved a number of mechanisms that improve plant growth and health. Symbiotic microorganisms help with drought tolerance through the production of peroxidase, ascorbate, and proline (Fan and Liu, 2011; Ruíz-Sánchez et al., 2011). Plant associated microbes may also benefit the host plant by changing stomatal conductance, water potential, and net photosynthesis during drought (Bae et al., 2009).

Endophytes may modify carbohydrate metabolism, photosynthesis, or produce beneficial compounds, to enhance cold tolerance in the host plant. When grapevine plants were exposed for five days to chilling conditions, net photosynthesis was higher compared with the levels of the control plants helping them to withstand long periods of cold exposure (Fernandez et al., 2012a). Recently, it was found that *B. phytofirmans* PsJN modified trehalose metabolism may be a part of mechanism under which *B. phytofirmans* PsJN increased chilling tolerance in grapevine, which was higher in the roots and leaves of bacterized plants, compared to non-bacterized plants (Fernandez et al., 2012b). Beneficial microbes could offer host plant tolerance to high salinity to aid in plant growth. To achieve increased tolerance to high salinity soils, beneficial organisms, both bacterial and fungal, may display a combination of traits such as the production of IAA, phosphate solubilisation, siderophore production, and ACC deaminase activity (Jha et al., 2011). The salt-tolerant *Azospirillum brasilenses* isolate NH produced IAA under salt-stress conditions, and it is believed that the production of this plant growth regulator may contribute to the increase in salt tolerance of inoculated wheat plants (Nabti et al., 2010). Under similar conditions, the endophytic strains, *B. subtilis*, *B. pumilus*, and *P. putida* isolated from the roots of *Prosopis strombulifera* (Argentine screwbean) produced significantly higher IAA (Sgroy et al., 2009).

Genetic modifications and functional genomics

Both bacterial and fungal endophyte-plant interactions involve modifications of plant gene expression and overall plant physiology/biochemistry to beneficially impact growth and stress tolerance. While monitoring specific gene expression during beneficial endophyte-sugarcane interactions, Arencibia et al. (2006) identified 47 differentially expressed sequence tags (EST) using cDNA-AFLP analysis. The transcripts showed significant genetic homologies to major signaling pathways such as the ethylene signaling pathway. For example, PYK10 encodes for a root- and hypocotyl-specific β-glucosidase/myrosinase and is important during the endophyte *P. indica* and Arabidopsis beneficial bio-control against herbivores and pathogens (Sherameti et al., 2008). Functional genomics research will help scientists understand and elucidate mechanisms under which beneficial microorganisms promote host plant growth and enhance stress tolerance. Currently we are carrying out studies of mechanisms of plant growth promotion by bacterial endophytes using the responsive switchgrass cultivar Alamo and non-responsive cultivar Cave-in-Rock to *Burkholderia phytofirmans* strain PsJN (Kim et al., 2012). Comparative global gene expression profiling is being conducted using both cultivars following *B. phytofirmans* strain PsJN inoculation with DOE-funded switchgrass EST microarray chips by Genomics Core Facility in the Noble Foundation. Approximately 35,200 switchgrass ID probes were identified to show significant differences between switchgrass cultivars Alamo and Cave-In-Rock after B. phytofirmans strain PsJN inoculation. Using the rice genome as a model for the analysis of the data along with the MapMan (Usadel et al., 2005) and the PageMap (Usadel et al., 2006) software, we are currently analyzing this large data set. Results showed that in Alamo almost 2000 genes were unique up-regulated at 0.5 day (unpublished data). On the other hand, in Cave-in-Rock, the number of unique up-regulated genes for 0.5 day was only 901. The significant changes are found in transcription factor genes, plant hormone, and cell wall metabolism.

Bacterial and fungal endophytes exhibit a diverse range of growth promoting mechanisms. In many cases, endophytes, primarily bacteria, possess multiple mechanisms of action and differentially express these traits at different stages of plant growth and development. Under stress conditions, endophytes help the host plant survive and flourish, as in the case of ACC deaminase activity and bio-control compound production. Under normal conditions, endophytes help fix atmospheric di-nitrogen and produce plant hormones to help the plant grow to its

maximum potential. Together, under both stress and normal conditions, endophytes ensure its host plant thrives, and its nutrient rich environment is maintained.

Future perspectives

Bioenergy production will become increasingly important in the future to relieve dependence on fossil fuels and lower greenhouse gas emissions because fossil-based energy is limited and its demand is continually increasing due to economic and population growth around the world. Switchgrass is one of the most promising bioenergy crops due to persistent high yields and its ability to grow on marginal land. Development of a low input and sustainable switchgrass feedstock production system is imperative as the use of chemical fertilizers causes deleterious environmental effects, such as water pollution and N_2O release to atmosphere, a potential greenhouse gas. Endophytes have the potential to help address these challenges due to their enhancement of nutrient acquisition, including nitrogen fixation and mobilization of mineral nutrients as well as increased biotic and abiotic stress tolerance, which together will reduce the amount of fertilizer application and/or pesticide and fungicide use. It will also open a door to growing potential bioenergy crops, such as switchgrass on marginal land or achieving the same yield while reducing fertilizer use, resulting in lower cost and contributing to sustainable rural development.

Plants live in complex environmental conditions containing various microorganisms, both beneficial and detrimental. Although endophytes could benefit plant growth, other microorganisms may have negative effects, and different endophytes may not be compatible, therefore the specific functional compatibility of endophytes needs to be further investigated to develop multi-functional bio-inoculants (Podile and Kishore, 2007) in switchgrass production. Additionally, while studies with endophytes as well as other plant growth promoting microorganisms in laboratories have been encouraging, there have also been reports of a general decrease in performance from the laboratory to the field (Gyaneshwar et al., 2002; Riggs et al., 2001). As with any ecosystem, the variables of field conditions and native microbial populations will have to be addressed to maximize the beneficial effects of bacteria and fungi. Therefore, screening endophytes having a broad spectrum of growth promotion that continues throughout the life of the plant will be another topic for endophyte application.

Genotype specific responses of host plants to endophytes are also a large barrier in application. For example, in poplar, different cultivars had different responses to different endophytes (Taghavi et al., 2009). One of the most studied plant growth promoting bacterium, *B. phytofirmans* strain PsJN, has a beneficial effect on many species, such as potato, tomato, and grape. However, PsJN is also genotype specific. In switchgrass, PsJN promoted growth of the lowland cultivar Alamo but not the upland cultivar Cave-in-Rock (Kim et al., 2012). Understanding these differences will also help in developing a more reliable, stable, and broad spectrum of growth promotion in plants.

Complete understanding of the mechanisms of various beneficial symbioses is the foundation for effectively applying these microorganisms in a sustainable switchgrass feedstock production and to achieve their synergistic activities (Podile and Kishore, 2007). As more is learned from functional genomics of endophytic microorganisms in growth promotion, it may be possible to share these important genes between similar microorganisms through horizontal gene transfer via transformation, conjugation, or transduction, all common occurrences in the bacterial world. Researchers first reported *in planta* horizontal gene transfer in the bioenergy crop hybrid poplar when they found *Burkholderia cepacia* VM1468 transferred its toluene degradation gene to other endophytes (Taghavi et al., 2005). This suggests that such transfer may be used to modify and improve the growth-promoting effects of other endophytes via gene sharing. The phenomenon of horizontal gene transfer may also occur in nature between different genera as the gene encoding the anti-fungal agent pyrrolnitrin in *Burkholderia* was likely horizontally transferred from *Pseudomonas* (de Souza et al., 2003).

Compared with plant genetic engineering, it is much easier for microorganisms to be genetically modified. One could easily transform some useful foreign genes into bacteria or fungi. For instance, the *Bacillus thuringiensis cry1Ac7* and *Serratia marcescens chiA* genes were transformed to sugarcane-associated endophytic bacteria, which helped increase the tolerance of sugarcane plant to the sugarcane borer *Eldana saccharina* (Downing et al., 2000). These applications indicate that we may be able to genetically engineer endophytes with useful genes, such as the *Bacillus thuringiensis* toxin gene, to protect host plants from herbivorous insects, herbicide resistance genes to impart host plant resistance to herbicides, and genes related to abiotic stress tolerance to enhance host plant tolerance to abiotic stresses. An efficient endophyte

transformation method by Agrobacterium was developed by Abello et al. (2008), which will help in the transfer and expression of important genes in host plants via endophytes. As functional genomics research is continually advanced, scientists will better understand the mechanisms under which beneficial microorganisms promote host plant growth and enhance stress tolerance to effectively utilize these microbes in bioenergy crop production. For example, endophytes having the ability to fix atmospheric nitrogen could be combined with endophytes having the ability to enhance host plant tolerance to abiotic stresses or inhibit pathogen growth or to improve nutrient uptake or, possibly, all could be combined.

Since 1999, over 15 new patents have been registered for microbial endophytes (Mei and Flinn, 2010). The worldwide market for microbial inoculants is experiencing an annual growth rate of approximately 10% (Berg, 2009). As world population demand for food is continually increasing, bioenergy crops should be grown on poor or marginal lands or contaminated soil, not competing with food crops for fertile lands. The use of endophytes may help bioenergy crops, such as switchgrass, grow on these lands via their normal mechanisms of action or genetic modification by introducing nitrogen fixation genes, heavy metal accumulation genes, or contaminated compound degradation genes.

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Chapter 1

Growth promotion and colonization of switchgrass (*Panicum virgatum*) cv. Alamo by bacterial endophyte *Burkholderia phytofirmans* strain PsJN

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Abstract

Switchgrass is one of the most promising bioenergy crop candidates for the US. It gives relatively high biomass yield and can grow on marginal land. However, the biomass yield varies from year to year and from location to location. It is imperative to develop a low input and sustainable switchgrass feedstock system. One of the most practical and feasible ways to increase biomass yield is to utilize beneficial endophytes. We demonstrate that one of the most studied plant growth promoting bacterial endophytes, *Burkholderia phytofirmans* strain PsJN, is able to colonize and significantly promote the growth of switchgrass cv. Alamo under *in vitro*, growth chamber, and greenhouse conditions. In several *in vitro* experiments, the average fresh weight of PsJN-inoculated plants was approximately 50% higher than non-inoculated plants. When one-month-old seedlings were grown in a growth chamber for 30 days, the PsJN-inoculated Alamo plants had significantly higher shoot and root biomass compared to controls. Biomass yield (dry weight) averaged from five experiments was 54.1% higher in the inoculated treatment compared to non-inoculated control. Similar results were obtained in greenhouse experiments with transplants grown in 4-gallon pots for two months. The inoculated plants exhibited more early tillers and persistent growth vigor with 48.6% higher biomass yields than controls. We also found that PsJN could significantly promote switchgrass cv. Alamo growth under sub-optimal conditions. However, we also found that PsJN-mediated growth promotion in switchgrass is genotype specific.

Keywords: Plant microbe interactions, Growth Promotion, Endophyte, Switchgrass, *Burkholderia phytofirmans* strain PsJN

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Introduction

Increasing concern over foreign energy supplies, global greenhouse gas emissions and the need for rural economic development has driven the interest in sustainable biomass production as a feedstock for bioenergy and bio-products. It has been suggested that by 2025, the world energy demand will likely be increased by more than 50% (Hamelinck, et al., 2005; Erahin et al., 2011). This demand and societal concerns about the environmental impact of burning fossil fuels are key factors stimulating the development of national and regional strategies aimed at the growth of renewable energy supplies, primarily focused on biofuels. To reduce the reliance on fossil fuels, the USA, the world's major energy consumer, released the Energy Independence and Security Act of 2007 that projects an increase in the production of renewable fuels from 9.0 billion gallons in 2008 to 36 billion gallons by 2022 (Sissine, 2007).The recent USDA/DOE National Biofuels Action Plan (http://www1.eere.energy.gov/biomass/pdfs/nbap.pdf) has helped to delineate the priority areas required to accelerate sustainable biofuel industry development. Within this document, Action Area 2 was identified as feedstock production and improvement. Various feedstocks, such as perennial rhizomatous grasses, can provide sources of lignocellulosic biomass, serving as new sources of crop growth and income for regional farmers. One of the most promising feedstocks capable of contributing to the realization of US renewable energy goals is the common perennial grass, switchgrass (*Panicum virgatum* L.) (Sanderson et al., 2006). This native prairie grass, consisting of a diverse germplasm (McLaughlin and Kszos, 2005), can grow on marginal lands under low inputs of water and agrochemicals (Hill et al., 2006), so that its cultivation does not compete with food crops for land. Due to its large root system and fast stand regrowth, switchgrass has other positive environmental effects, including the prevention of surface runoff and soil erosion, carbon sequestration, and the provision of a wildlife habitat (Humphreys, 1999; Sanderson et al., 2006). Switchgrass cultivated lands also had much higher total soil organic carbon deposits than lands cultivated with annual crops, such as corn and wheat (McLaughlin et al., 2002; Liebig et al., 2005).

The economics of biofuel production is highly dependent on feedstock cost and conversion technology (Hamelinck et al., 2005; McLaughlin and Kszos, 2005). The development of improved switchgrass varieties for low-cost production on marginal lands is one prerequisite for the success of the bioenergy program (Sanderson et al., 2006; Dyer et al., 2008). One such approach involves the use of beneficial microorganisms, such as endophytes, which form

intimate associations with plants (Sturz el al., 2000). Endophytes, both fungal and bacterial, have been targeted as mechanisms to enhance plant characteristics for commercial uses (Mei and Flinn, 2010). The colonization of grasses by fungal endophytes for performance enhancement is well documented (Funk et al., 1993), including their use with switchgrass (Ghimire et al., 2009). However, to our knowledge only one study has been reported on growth promotion of a bioenergy feedstock grass (*Miscanthus x giganteous* seedlings) by a bacterial endophyte (*Herbaspirillum frisingense*) (Kahn et al., 2008). One key component of our bioenergy crop research program involves the utilization of beneficial bacterial endophytes that form stable and persistent associations with switchgrass, as the mechanism to improve biomass yield and enhance stress tolerance under low-input production systems (Nowak et al., 2011). Although the molecular mechanisms of beneficial endophyte-host plant interaction are largely unknown, several studies have demonstrated that endophytes can promote plant growth by enhancing the plant's capacity for nutrient acquisition, better water management, and/or resistance to abiotic and biotic stresses via regulation of hormones (Sturz et al., 2000; Berg, 2009; Mei and Flinn 2010; Welbaum et al., 2004; Compant et al., 2005). For instance, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase produced by endophytes lowers the ethylene levels in host plants, reducing their response to abiotic and biotic stress, and by changing root morphology, leading to promotion of plant growth (Berg, 2009; Glick et al., 1998; Glick, 2004). Many known endophytes also promote plant growth by producing gibberellic acid (GA3), indole-3-acetic acid (IAA) (Khan et al., 2008; Mattos et al., 2008), or cytokinins (Arshad and Frankenberger, 1991; Lazarovits and Nowak, 1996).

Burkholderia phytofirmans strain PsJN has been found to be a highly effective plant growth promoting bacterial endophyte, with a broad host range including potatoes, tomatoes, and grape vines (Lazarovits and Nowak, 1996; Conn et al., 1997; Barka et al., 2000; Nowak et al., 2003; Compant et al., 2005; Sessitsch et al., 2005; Wang et al., 2006). In addition, its genome has recently been sequenced (Weilharter et al., 2011), providing the genomic resources needed to develop an understanding of the mechanisms associated with this endophyte's ability to promote plant growth. PsJN produces a high level of ACC deaminase (Sessitsch et al., 2005), enhances host plant cold (Barka et al., 2002) and heat (Bensalim et al., 1998) stress tolerance, improves water management (Frommel et al., 1991) and plant resistance to pathogens (Sharma and Nowak, 1998). In this study, we report growth promotion of switchgrass cv. Alamo by

Burkholderia phytofirmans strain PsJN under *in vitro*, growth chamber, and greenhouse conditions. To our knowledge, this is the first report detailing the switchgrass-PsJN interaction.

Materials and methods

Plant materials

Switchgrass (*Panicum virgatum L.*) cvs. Alamo and Cave-in-Rock seeds were purchased from Warner Brothers Seed Co. (Lawton, OK), and other switchgrass seeds were kindly provided by Dr. Bingyu Zhao (Department of Horticulture - Virginia Tech, Blacksburg, VA). Switchgrass seeds were surface-sterilized by treatment with 70% ethanol for 2 min, rinsed 3X with distilled water, de-husked for 30 min with 60% H2SO4 with stirring, washed 3X with distilled water, sterilized with 0.4 M sodium hypochlorite (50% commercial bleach solution containing 6% sodium hypochlorite) containing 0.1% Triton 100 for 30 min followed by 5X rinse with sterile, deionized, distilled water (ddH2O).

Bacterial endophytes and culture conditions

Burkholderia phytofirmans strain PsJN (Sessitsch et al., 2005) and its PsJN-GFP derivative (Compant et al., 2005) were obtained from Dr. Angela Sessitsch (Austrian Institute of Technology, Seibersdorf, Austria). The cultures were streaked on King's B (KB) solid medium as described in (Pillay and Nowak, 1997). Inoculum was produced by transferring one loop of PsJN from 2-day-old cultures to 5 ml KB broth in a 15-ml culture tube, followed by incubation at 28˚C on a shaker (150 rpm) overnight. Five ml of the overnight PsJN culture was added to 45 ml KB broth in a 250-ml Erlenmeyer flask and grown to 0.7 OD_{600}. Bacterial cells were then collected by centrifugation at 3,500 rpm for 7 minutes at 4 ˚C, and re-suspended in PBS buffer (10 mM NaH2PO4 containing 0.8% NaC1, pH 6.5) after which the OD_{600} was adjusted with PBS buffer to 0.5, unless described otherwise.

Seedling inoculation with PsJN and plant growth responses

Surface-sterilized seeds were germinated in petri-dishes for 7 days at 25 ˚C, under white fluorescent light (67 µmol m-2s-1), 16 h photoperiod, on a switchgrass growth medium consisting of MS salts and vitamins (Murashige and Skoog, 1962), 30 g/l maltose and 3 g/l phytogel, pH 5.8. The root tips of the young seedlings were cut prior to PsJN inoculation to facilitate bacterial penetration (Pillay and Nowak, 1997). For the direct seed inoculation surface-

sterilized seeds were placed on wet filter paper for 3-5 days in an incubator at 25 ˚C with 16 h photoperiod (white fluorescent bulbs at 67 µmol m-2s-1) followed by soaking in PsJN suspension (0.5 of OD_{600}) (approx. 10^8 cfu)for 1 min. Control seedlings/seeds were treated with PBS buffer alone. The treated seedling/seeds were blot-dried with sterile paper towel, placed on switchgrass growth medium in GA7 Magenta vessels (Sigma-Aldrich) containing 50 ml of media and 5 seedlings or germinating seeds per vessel, and grown for one month in the incubator as above. Root and shoot length, and seedling fresh weight were then determined, and the plants transferred to a soil mix composed of 2/3 Miracle-Gro® Potting Mix (Scotts Miracle-Gro Company, Marysville, Ohio) and 1/3 Arabidopsis growing media (Lehle Seeds, Round Rock, Texas). Plants were grown in 72-cavity trays in a growth chamber at a 28/22°C day/night temperature, 16 h photoperiod (white fluorescent bulbs at 88 µmol m-2s-1) for 30 days, or at 4-gallon pots in the greenhouse.

PsJN colonization

The plants inoculated with PsJN-GFP were examined under a fluorescent stereomicroscope (Model SZX-ILLD2-100; Olympus, Tokyo, Japan) equipped with a GFP filter (BP460-490, Olympus, Tokyo, Japan) and the Zeiss 510 laser scanning confocal microscope (LSCM) (Carl Zeiss, Inc., Thornwood, NY) to observe colonization.

For bioassays, the control and PsJN-GFP inoculated plants were surface-sterilized with 0.032 M sodium hypochlorite for 1 min, then washed 4X with sterile distilled water. Fifty µl of the final wash was plated on solid KB medium to confirm effectiveness of surface sterilization. Root, leaf and sheath parts were then separated, each weighed, and ground with mortar and pestle in 1 ml sterile distilled water. The homogenates were then centrifuged at 2000 rpm for 3 min, and the supernatant serially diluted with distilled water. Fifty µl samples of the serially diluted solutions were spread on solid KB medium. The plates were incubated for 3 days at 28˚C in the dark and the number of GFP-positive colonies determined using fluorescence stereomicroscopy as described above.

Results

PsJN endophytic association with switchgrass Alamo

The endophytic colonization of switchgrass by *Burkholderia phytofirmans* strain PsJN-GFP was visualized using confocal microscopy (**Figure 1.1**). Under the appropriate illumination, the PsJN-GFP could be clearly observed inside the roots of PsJN-inoculated plants 3 days after inoculation, while no fluorescence was observed in roots of buffer-inoculated control plants. The titer of PsJN-GFP in inoculated plants was also determined using tissue homogenates from various tissues (root, leaf and sheath) at different times (**Table 1.1**). The endophyte initially infected and colonized plant roots, and by 7 days post-inoculation, PsJN titers were still highest in the root. However, the titers increased significantly in other tissues by day 14, indicating translocation to leaves and sheaths.

Effects of PsJN on Alamo growth *in vitro*

Young switchgrass seedlings were prepared and inoculated as described in the Materials and Methods, and the non-inoculated and inoculated plants were analyzed after growth *in vitro* for one month. The result showed that PsJN significantly and repeatedly promoted Alamo root and shoot growth, with a 35.6% increase in shoot length, a 32.8% increase in root length, and an 83.6% increase in fresh weight, compared to the non-inoculated plants (**Figure 1.2**). After several replications, the average fresh weight of the PsJN-inoculated plants was always approximately 50% higher than non-inoculated plants.

Effects of PsJN on Alamo growth in a growth chamber environment

As described above, PsJN significantly enhanced switchgrass cv. Alamo growth *in vitro*. We next assessed the impact of PsJN on growth under soil conditions. One-month-old *in vitro* grown Alamo (control and PsJN-inoculated seedlings) were transferred to a flat with 72 cavities filled with soil and grown in a growth chamber under 28/22°C day/night temperatures with 16 h light period for one month. The PsJN-inoculated plants showed significant growth increases compared to control plants in shoot length and fresh/dry weights (**Figure 1.3**). The growth chamber experiments were repeated 5 times, and the average data from 5 experiments showed significant growth promotion by PsJN, with a 46.3%, and a 54.1% increase in fresh weight and dry weight, respectively. The total dry weight increase (54.1%) by PsJN was more than the total fresh weight increase (46.3%), indicating that the PsJN-inoculated plants accumulated more biomass.

Effects of PsJN on Alamo growth in the greenhouse

Non-inoculated and PsJN-inoculated plants were also grown under greenhouse conditions to determine growth enhancement persistence. The plants inoculated with PsJN and grown *in vitro* for 25 days were transferred to 4-gallon pots with 5 plants in each pot and grown in the greenhouse. The plants inoculated with PsJN exhibited sustained growth vigor, as they were significantly taller, and more tillers developed early compared with the non-inoculated control plants (**Figure 1.4**). Following one month of growth in the greenhouse, the PsJN-inoculated plants had 76.2% more tillers than the control plants. The plants were harvested following growth for two months, and the biomass yield determined (**Figure 1.5**). The PsJN-inoculated plants were repeatedly significantly higher in biomass yield, with a 36.8% increase in fresh weight and a 57.1% increase in dry weight.

Effects of PsJN on Alamo growth in sub-optimal conditions

In order to develop a low input and sustainable switchgrass feedstock production system utilizing the beneficial bacterial endophyte, we tested growth performance of PsJN-inoculated plant with unfertilized field soil, in a glasshouse under ambient conditions during the Fall, when the temperature was not optimal for switchgrass growth. The results showed that PsJN-inoculated plants produced twice the total biomass of controls (**Figure 1.6**).

Direct inoculation of switchgrass seeds with PsJN

In order to explore a practical way to inoculate switchgrass with the bacterial endophyte, we sterilized switchgrass seeds as described in Materials and Methods, placed the sterilized seeds on wet filter paper for 3-5 days in an incubator at 25˚C, and then inoculated the germinating seeds with different concentrations of endophyte inoculum to determine the optimal inoculation concentration (OD600 at 0.1-0.5). The plants inoculated with PsJN at OD600 of 0.1, 0.25, and 0.5 exhibited 28.7%, 55.0% and 80.1% increases in dry weight, respectively, compared to non-inoculated plants after grown *in vitro* for 25 days and in growth chamber for another month. A PsJN concentration of 0.5 was the most effective at promoting biomass increase (**Figure 1.7**), and no biomass difference was observed between the 0.1 treatment and control.

Endophyte infection and colonization of seeds are dependent on endophyte concentration and the status of seed imbibitions. So, in order to facilitate infection and colonization by the bacterial endophyte, the sterilized seeds were imbibed in water for 1, 2, 3, or 4 days, and then inoculated

with PsJN at an OD600 of 0.5 or 1.0, since an OD600 of 0.5 was the most effective as described above. The PsJN-inoculated seeds were placed in an incubator at 25˚C with a 16 h light period for 25 days, and transferred to soil and grown in a growth chamber for 30 days. The results indicated that plants from the seeds imbibed for 2 days and then inoculated with an OD600 of 0.5 had the highest dry weight, with a 55% increase compared to un-inoculated control plants (**Figure 1.7**).

Genotypic responses to PsJN

As described above, PsJN was able to stimulate growth in switchgrass cv. Alamo. To assess the influence of plant genotype on this response, seven other switchgrass cultivars were tested for their growth responses to PsJN. As shown in **Table 1.2**, growth promotion by PsJN was genotype-dependent. The switchgrass cvs. Forestburg, Nebraska, and Blackwell were all responsive to PsJN, with measured significant growth increases of 60.1%, 26.8%, and 23.0%, respectively, while the cvs. Cave-in-Rock, Sunburst, Shelton, and Shawnee did not exhibit growth promotion in response to PsJN under similar conditions. Preliminary result from Cave-in-Rock bioassay indicated that PsJN titers were not sustained after inoculation and were much lower in the non-responsive plants following inoculation.

Discussion

In the present study, we demonstrated the ability of *B. phytofirmans* PsJN to colonize and promote growth in switchgrass cv. Alamo. Three days following PsJN inoculation, we could clearly visualize bacterial cell colonization inside the roots under confocal microscopy. The bacterial population inside the roots was initially much higher than that of the leaves and sheaths, and the bacterial endophyte was subsequently transmitted vertically to the upper leaves through the leaf sheath. These results were similar to that reported for grapevine (Compant et al., 2005) and potato (Reiter et al., 2002), where PsJN was transported through the interior vasculature system, from root xylem vessels to the upper parts of the plants. This is a critical first step in the endophytic bacteria-plant interaction (Whipps, 2001). We observed significant growth promotion of cv. Alamo by PsJN, under both *in vitro* and soil conditions. Our study showed total fresh weight and total dry weight of the inoculated plants was increased by 45% and 55% respectively compared with the non-inoculated control plants when the inoculated seedlings were grown *in*

vitro and then transferred to soil and grown in growth chamber for one month. Similar results have been obtained in 4-gallon pots under our greenhouse conditions. Other studies have reported levels of growth promotion by PsJN, with grapevine showing a 6-fold increase in total biomass (Barka et al, 2006), and potato showing an approximate 2-fold increase in root and haulm biomass (Frommel et al., 1991) over controls. The mechanism of plant growth by *B. phytofirmans* PsJN has been reported (Compant et al., 2005) and attributed to the ability of PsJN to produce high levels of ACC deaminase activity, which degrades ACC to ammonia and α-ketobutyrate (Long et al., 2008), which is a common characteristic of plant growth promoting bacteria. ACC is the precursor to ethylene, a plant stress hormone; hence, the reduced ethylene level in PsJN-colonized plants will promote plant growth. According to the report by Penrose and Glick (2003), ACC activity over 20 nmol α-ketobutyrate/h/mg is sufficient to promote host plant growth, and PsJN has been shown to contain 308 nmol α-ketobutyrate/h/mg of ACC deaminase activity (Sessitsch et al., 2005). Although several studies have reported the interaction between this endophyte and host plants for growth promotion, most studies have reported *in vitro* data (Compant et al., 2005; Lazarovits and Nowak, 1996; Conn et al., 1997; Frommel et al., 1991). Our results with unfertilized field soil, in a glasshouse under ambient conditions during the Fall, when the temperature was not optimal for switchgrass growth (**Figure 1.6**) implied the potential benefit of switchgrass inoculated with PsJN for growth on marginal lands and sub-optimal growth conditions.

While our initial studies were carried out with cv. Alamo, we tested the utility of PsJN as a growth-promoting endophyte with other switchgrass cultivars. Our results indicated that specific genotype effects existed, with some genotypes being highly responsive to the growth promotive effects of PsJN, and others not. Similar genotype effects have been reported by others. It was reported that the potato response to PsJN involves some form of genetic control, as some potato cultivars display the beneficial response to the endophyte, while others do not (Bensalim et al, 1998; Nowak et al., 1998; Nowak et al., 2007). The typical *in vitro* phenotype for a strongly responsive cultivar was characterized by a massive, well-branched root system and after the first 3-4 weeks in culture, the plantlet was developmentally more advanced than the non-bacterized controls. Stems were sturdier, with more lignin deposits around the vascular system, more root hairs and more and larger leaf trichomes (Nowak et al., 1998). We also noticed PsJN-inoculated switchgrass plants were developmentally advanced (unpublished data). Such enhancements were

not apparent for the poorly-responsive cultivars. We also observed some of these phenotypic differences between PsJN-responsive and non-responsive switchgrass cultivars. Additional work illustrating the genetic control of the beneficial response to the endophyte used monoploid lines derived from anther culture of an adapted diploid *Solanum phureja* clone, BARD 1-3 (Nowak et al., 2007). The diploid anther donor, BARD 1-3, exhibited a bacterization response comparable to Red Pontiac, while monoploid lines exhibited a response to PsJN ranging from favorable to unfavorable to neutral. The assumption here was that the response range of the monoploid population was due to the segregation of alleles for genes involved in regulating the positive or negative interaction with PsJN.

The potato/PsJN studies have been the most characterized, and carried out with material clonally propagated via nodal sections, in which a single inoculation is sufficient to initiate colonization through the xylem tissue, eventually spreading to the upper leaves (Frommel et al., 1991). Bacterial levels must reach a threshold population within the plant before they are effective (Pillay and Nowak, 1997) with a direct relationship between plantlet growth enhancement and PsJN colonization of both interior and exterior surfaces (Nowak et al., 2007). The PsJN colonization profiles for a responsive and poorly responsive cultivar over 8 tissue culture generations revealed bacterial loads one order of magnitude greater for shoot/root surface and interior colonization in the responsive compared to the poorly responsive cultivar. Furthermore, the responsive cultivar exhibited increased colonization over successive generations, while the poorly responsive cultivar exhibited declining bacterial populations over successive generations. We are currently assessing the level of colonization in switchgrass cultivars responsive and non-responsive to PsJN to determine the degree of similarity between switchgrass and potato responses to the endophyte. At present, the mechanisms governing *B. phytofirmans* PsJN genotype-specificity in growth promotion of switchgrass (and other plants) are unknown, although we are currently using large scale gene expression analyses to determine the differences in the switchgrass molecular responses between differently-responding cultivars. In summary, the results reported here illustrate the ability of *B. phytofirmans* PsJN to infect and colonize responsive switchgrass (*Panicum virgatum*) cultivars like Alamo, and to promote plant growth. This study lays the foundation to develop a low input and sustainable switchgrass feedstock production system on marginal lands using this, and other, beneficial bacterial endophytes. Results obtained with switchgrass cv. Alamo growth promotion by *B. phytofirmans* PsJN under

different conditions, particularly in sub-optimal conditions, indicate that we could apply the beneficial bacterial endophyte in switchgrass practical management to help switchgrass establishment in the first year and in developing a low input and sustainable switchgrass feedstock production system. In the future, the mechanisms of growth promotion need to be elucidated with molecular biology and function genomics. Our results show *B. phytofirmans* strain PsJN significantly promotes switchgrass cv. Alamo growth under different conditions, especially in early growth stages yielding more early tillers, which may benefit switchgrass establishment in the first year. Also, PsJN could significantly stimulate switchgrass cv. Alamo growth in sub-optimal conditions, indicating the use of the beneficial bacterial endophyte to boost switchgrass growth on marginal lands and to develop a low input and sustainable switchgrass feedstock production system.

Acknowledgements

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Figures and Tables

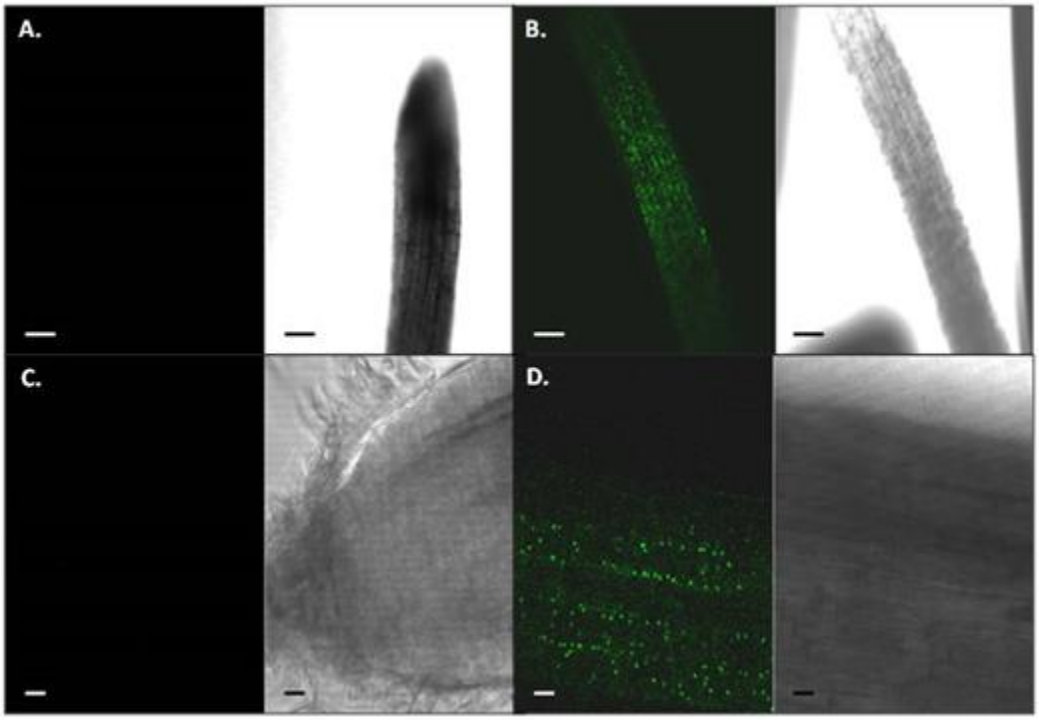

Figure 1.1 Confocal microscope images of roots. Images captured 3 days following switchgrass cv. Alamo inoculation with PsJN-GFP, showing bacterial colonization inside the roots. (A): Control and (B): PsJN- inoculated plants. (C): Control and (D): PsJN-inoculated plants. The bars represent 100 µm (A and B) and 20 µm (C and D).

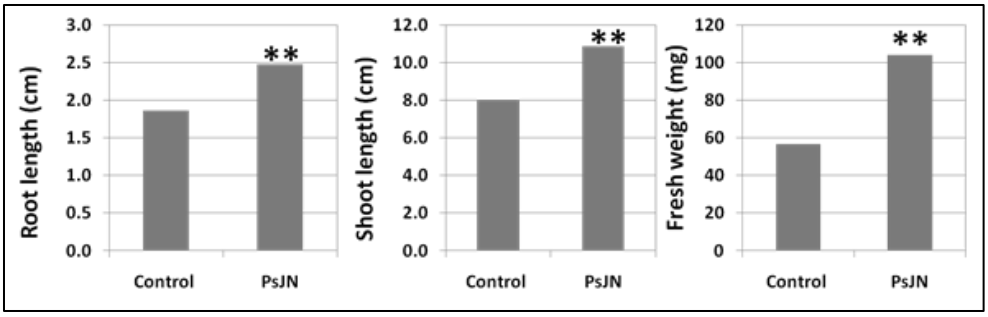

Figure 1.2 Effects of PsJN inoculation on switchgrass cv. Alamo growth *in vitro*. Data were obtained after plants were grown in incubator for 36 days. Sample number was 25, and ** means significant difference at 0.01 level between PsJN and control using student T-test.

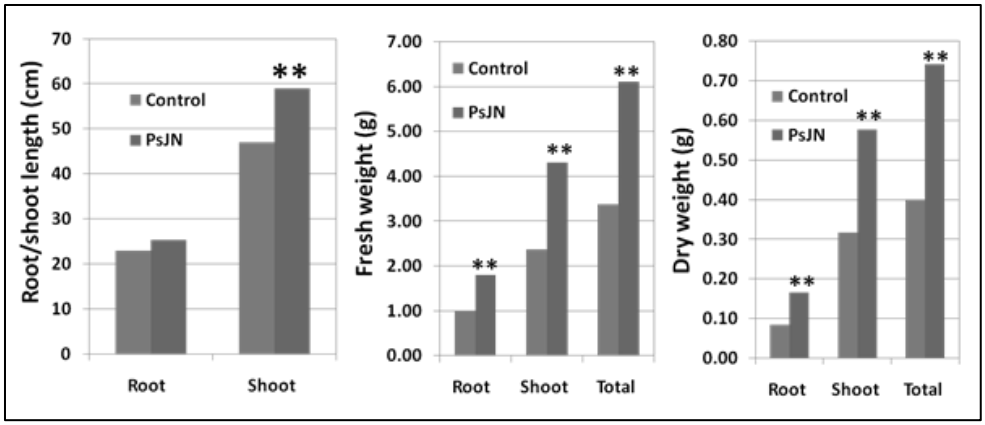

Figure 1.3 Effects of PsJN inoculation on switchgrass cv. Alamo in growth chamber. The seedlings were inoculated with PsJN and grown *in vitro* for one month, then transferred to soil and grown in growth chamber for another month. Dry weight was determined after samples were dried in oven at 65□C for one day. Sample number was 36, and ** means significant difference at 0.01 level between PsJN and control using student T-test.

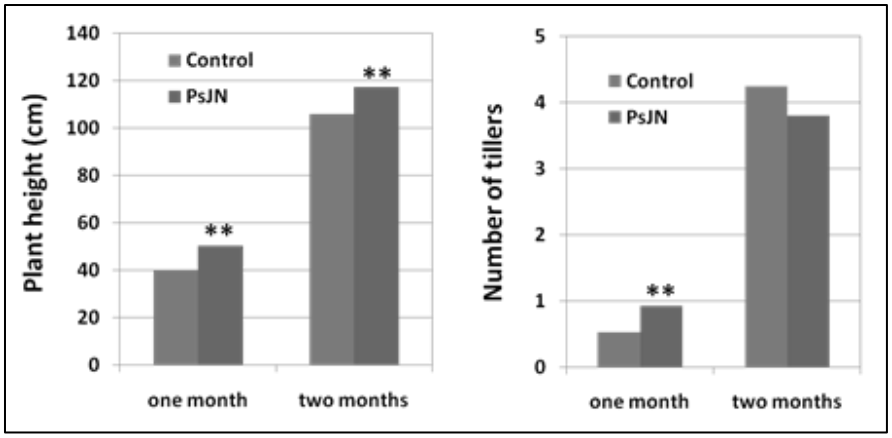

Figure 1.4 Plant growth and tiller development. Measurements were recorded after control and PsJN inoculated plants were transferred to 4-gallon pots and grown in greenhouse.

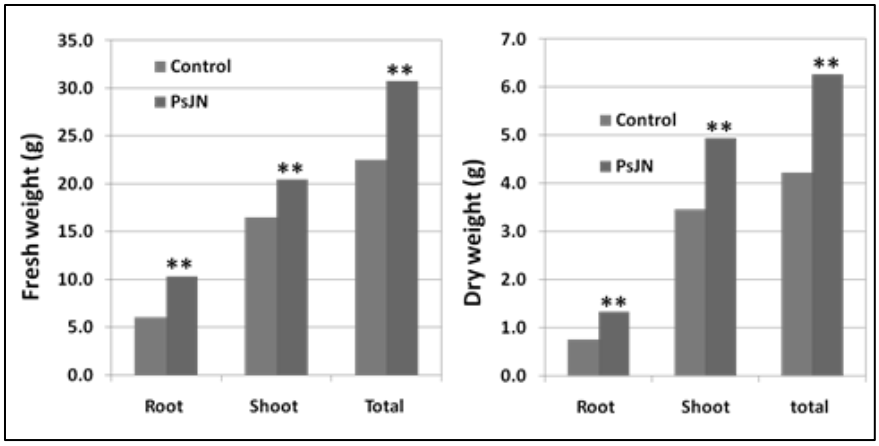

Figure 1.5 Persistence of growth promotion increase of Alamo in greenhouse after PsJN bacterization. The seedlings were inoculated with PsJN and grown *in vitro* for one month, then transferred to 4-gallon pot with 5 plants/pot and grown in greenhouse for two months. Dry weight was determined after samples were dried in oven at 65°C for one day. Sample number was 25, and ** means significant difference at 0.01 level between PsJN and control using student T-test.

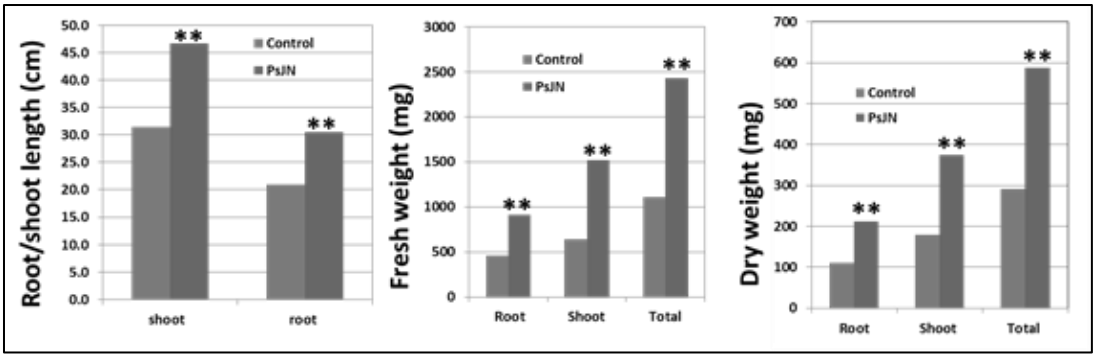

Figure 1.6 Growth promotion of Alamo by PsJN inoculation in sub-optimal conditions. The seedlings were inoculated with PsJN and grown *in vitro* for one month, then transferred to 4-gallon pot with 5 plants/pot with unfertilized field soil and grown in glasshouse in ambient environment for 2.5 months in the late Fall of 2010. Dry weight was determined after samples were dried in oven at 65°C for one day. Sample number was 25, and ** means significant difference at 0.01 level between PsJN and control using student T-test.

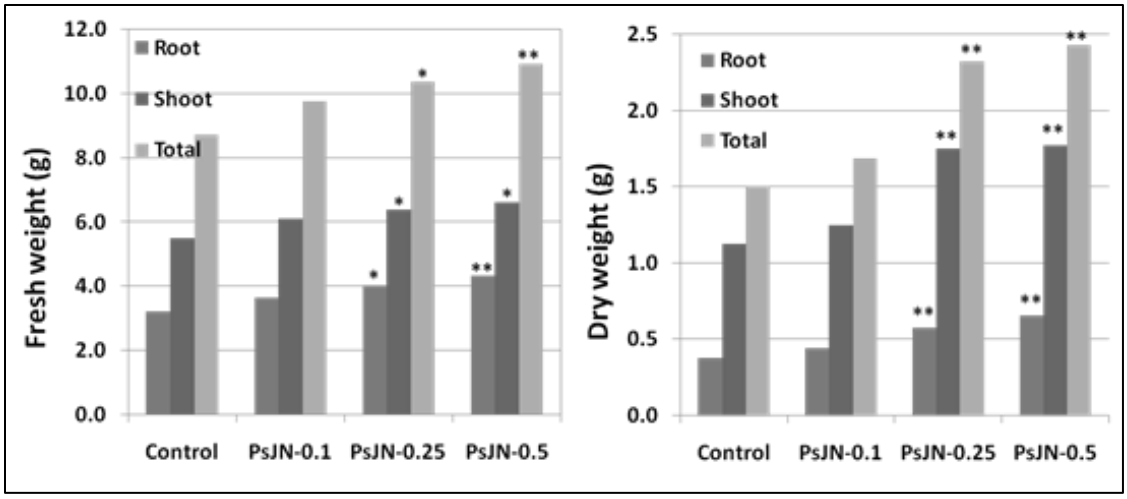

Figure 1.7 Effects of PsJN on growth of swtchgrass cv. Alamo after direct seed inoculation. The surface-sterilized seeds were infected with different concentrations of PsJN and grown *in vitro* for 17 days, then transferred to 72-cavity trays and grown in a growth chamber for 50 days. * and ** mean significant difference at 0.05 and 0.01 levels respectively between PsJN and control using student T-test.

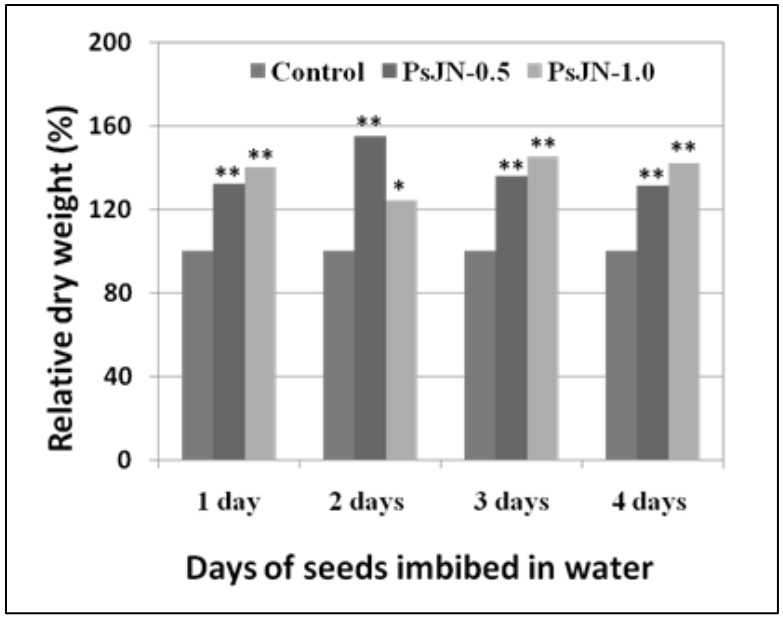

Figure 1.8 Effects of PsJN on switchgrass growth after inoculation of seeds imbibed in water. The seeds were infected with different concentrations of PsJN and grown *in vitro* for 25 days, then transferred to 72-cavity trays and grown in a growth chamber for 37 days. Sample number was 72 for each treatment. * and ** mean significant difference at 0.05 and 0.01 levels respectively between PsJN and control using student T-test.

Table 1.1 PsJN colony-forming units (CFU) in root, leaf, and sheath tissues.

Days after PsJN-GFP inoculation	Plant Tissues	Average CFU/g fresh weight
3	All (Roots, leaves, and sheath)	$4.2X10^5$
7	Roots	$7.6X10^5$
	Leaves	$2.6X10^3$
14	Roots	$3X10^4$
	Sheaths	$1.3X10^5$
	Leaves	$1.2X10^5$

Table 1.2 Effects of *B. phytofirmans* PsJN on plant growth in different switchgrass cultivars.

Cultivars	Treatment	No. plants	Root length (cm)	Shoot length (cm)	Total fresh weight (mg)	PsJN/ control (%)[a]
Shawnee	Control	24	2.3	8.4	50.1	
	PsJN	24	2.1	7.8	58.8	117.4
	p-value[b]		0.2037	0.2049	0.0717	
Nebraska	Control	24	1.5	10.3	40.9	
	PsJN	24	1.5	11.6	51.9	126.8
	p-value		0.4160	0.0656	0.0055	
Forestburg	Control	34	1.6	8.6	33.7	
	PsJN	30	1.8	12.7	54.0	160.1
	p-value		0.1986	0.0000002	0.0000001	
Shelton	Control	28	3.3	14.1	117.7	
	PsJN	28	3.0	12.0	135.3	115.0
	p-value		0.1579	0.0067	0.0907	
Blackwell	Control	28	1.4	9.9	52.6	
	PsJN	28	1.5	11.3	64.7	123.0
	p-value		0.2970	0.0998	0.0543	
Sunburst	Control	30	0.8	8.5	33.0	
	PsJN	28	0.9	10.4	26.4	80.0
	p-value		0.2985	0.0137	0.0731	
Cave-in-Rock	Control	33	2.8	14.7	107.8	
	PsJN	34	3.1	13.8	113.4	105.2
	p-value		0.2317	0.4052	0.5613	

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Chapter 2

The effect of *Burkholderia phytofirmans* strain PsJN bacterization of switchgrass seedlings cv. Alamo on plant performance in the field on fertile and poor soils

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Abstract

Switchgrass (*Panicum virgatum* L.), a native perennial warm season grass, has been identified as a promising bioenergy feedstock, capable of growth on marginal lands unsuitable for food crops without high inputs of fertilizer and irrigation. Improving stand establishment, stress resistance, and biomass yield are the main areas of effort in the development of low input switchgrass production systems. Our program focuses on the utilization of beneficial bacterial endophytes, which reside within plant tissues, to enhance its performance on poor soils. In earlier studies, we demonstrated that inoculation of switchgrass cv. Alamo with a growth promoting endophyte, *Burkholderia phytofirmans* strain PsJN (PsJN), can significantly enhance its growth under both *in vitro* and greenhouse conditions. In this study, we tested the effect of PsJN bacterization of switchgrass seedlings on cv. Alamo stand establishment, growth, and biomass yield in 3 field experiments. The experiments were conducted for two years on highly fertile prime field soil and on soil of a former tobacco farm with low fertility. PsJN bacterization affected growth and development of switchgrass seedlings, including significant stimulation ($p<0.001$) of root and shoot growth on soil with low fertility, lateral root formation, and a number of tillers. It also enhanced biomass accumulation during the two seasons of growth on both poor ($p<0.001$) and rich ($p<0.05$) soil, indicating the potential for the use of PsJN in a low-input switchgrass feedstock production system.

Keywords: Growth Promotion, Endophytes, *Burkholderia phytofirmans* strain PsJN, Field Studies, Bioenergy, Marginal Lands, Switchgrass

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Introduction

Fossil fuels have driven world economics since the beginning of the industrial revolution. However, their supply is limited, and peak petroleum production is estimated to have passed (Murray and King, 2012). Moving into the future, access to fossil fuels will become increasingly difficult to maintain as world energy demand is estimated to increase by more than 50% in the next two decades (reviewed in Hamelinck et al., 2005). Moreover the growing use of fossil fuels will further affect climate change through greenhouse gas emissions. The development and use of renewable forms of energy including solar, wind, and bioenergy, became one of the major drivers of innovation in our generation. In the United States, switchgrass (*Panicum virgatum* L.) has been identified as a model renewable bioenergy crop (Sanderson et al., 2006; Wright, 1994) due to its high water use efficiency, good carbon sequestration capacity, and ability to grow on marginal lands under low input of agrochemicals (McLaughlin and Kszos, 2005). On-farm evaluation of switchgrass production in the Mid-West highlighted production potential on marginal lands of 10 separate farms and demonstrated that switchgrass produced 504% more energy than consumed. Authors estimated that further increases were likely achievable with the expansion of breeding programs and improved management (Schmer et al., 2008).

Because of economics and transportation logistics, biofuel industries will likely be regional, and the primary feedstock grown will be suitable to a particular locale (Bouton, 2004). Therefore, the development of switchgrass production on marginal land without competition for fertile soils used for food crops can also potentially to benefit agricultural producers. Southside and Central Virginia have a rich farming tradition, primarily built upon the production of tobacco. Global demand for tobacco has fallen dramatically in the last few decades, leaving many fields empty and often depleted of nutrients. The emerging field of bioenergy feedstock production may utilize these fields, with little investment in new machinery as conventional farm forage equipment can be used (McLaugnlin and Kszos, 2005).

Beneficial bacterial endophytes (endophytes) have been utilized to increase production of other graminaecious bioenergy crops, such as corn and surgarcane (Boddey, 1995; Riggs et al., 2001). Endophytes are naturally occurring soil microorganisms that can penetrate plant roots and translocate to the above ground organs and, upon colonization, affect plant growth, health, and productivity (Reviewed in Sturz et al., 2000; Welbaum et al., 2004; Mei and Flinn, 2010).

Multiple mechanisms of plant growth promotion by beneficial bacterial endophytes have been reported over the past 30 years including, generally, production of plant hormones to directly stimulate growth, synthesis of antimicrobial compounds to increase resistance to plant pathogens, and helping the host plant acquire nutrients through mechanisms such as atmospheric nitrogen fixation and secretion of siderophores (reviewed in Compant et al., 2008). A particular endophyte may also convey multiple mechanisms of growth enhancement. For example, *Burkholderia phytofirmans* strain PsJN (PsJN) has been shown to secrete siderophores for iron acquisition, induce plant host's stress resistance via production of trehalose and ACC deaminase, and stimulate plant growth by enhanced production of phytohormones (Lazarovits and Nowak, 1997; Barka et al., 2002; Sessitsch et al., 2005; Weilharter et al., 2011). PsJN also effectively colonizes tissues of a broad range of plants including tomato (Nowak et al., 2004; Pillay and Nowak, 1997; Sharma and Nowak, 1998), potato (Frommel et al., 1991), sweet pepper (Nowak et al., 2004), and grapevine (Compant et al., 2005, 2008). Under drought conditions, PsJN inoculation increases photosynthesis, chlorophyll content, and efficiency of photosystem II compared to the control treatment (Naveed et al., 2013).

In switchgrass cv. Alamo, PsJN was shown to increase fresh weight by 57, 46 and 37% under *in vitro*, growth chamber, and greenhouse conditions, respectively (Kim et al., 2012). In the field, however, the large abundance and diversity of native soil bacteria may out-compete introduced microorganisms and diminish the gains often seen in the lab (Sturz et al., 2000). Over time, larger populations of endophytes were found in older stands of switchgrass compared to younger stands, indicating they may change over time (Gagne-Bourgue et al., 2012). The primary objective of this study was to determine if growth promotion shown in the lab by *Burkholderia phytofirmans* strain PsJN persists in the field during the important seedling establishment year (Parish and Fike, 2005) and subsequent years with different fertilities of soil and different planting times.

Materials and methods

Plant material and bacterization

Switchgrass (*Panicum virgatum* L.) seeds of cv. Alamo were purchased from Warner Brothers Seed Co. (Lawton, OK). Seeds were surface sterilized as described previously (Kim et al., 2012) and germinated for 5-7 days on sterile filter paper in petri-dishes at 25°C. *B. phytofirmans* strain

PsJN was obtained from Dr. Angela Sessitsch (Austrian Institute of Technology, Seibersdorf, Austria). PsJN cultures were streaked on King's B (KB) solid medium as described (Pillay and Nowak, 1997). Inoculum was produced by transferring one loop of bacteria from 2-day-old cultures to 5 ml KB broth in a 15-ml culture tube, followed by incubation at 28˚C on a shaker (220 rpm) overnight. Five ml of the overnight culture was added to 45 ml KB broth in a 250-ml Erlenmeyer flask and grown to 0.7 OD_{600}. Bacterial cells were then collected by centrifugation at 3,500 rpm for 7 min at 4˚C, and re-suspended in PBS buffer (10 mM NaH_2PO_4 containing 0.8% NaC1, pH 6.5) after which the OD_{600} was adjusted with PBS buffer to 0.5 (approx. 10^8 cfu). Surface-sterilized seeds were germinated in petri-dishes for 7 days at 25 ˚C, under white fluorescent light (67 µmol m-2s-1), 16 h photoperiod, on sterile filter paper followed by soaking the emerging plantlets in PsJN suspension for 1 minute. Control seedlings/seeds were treated with PBS buffer alone. The treated seedling/seeds were blot-dried with sterile paper towel and transferred to GA-7 Magenta containers with Murashige and Skoog basal salts plus vitimans (MS + V) (M519, Phytotech Labs, Shawnee Mission, KS) containing 3% maltose (RPI Inc.) and 0.3% phytagel (Phytotech labs) and pH 5.8. The plantlets were grown in GA-7 Magenta containers at 25°C (16hr photoperiod, fluorescent light (67 µM $m^{-2}s^{-1}$)). After three weeks growth *in vitro*, PsJN inoculated and non-inoculated seedlings were then transferred to a 72-cavity flat filled with Miracle-Gro® Potting Mix (Scotts Miracle-Gro® Company, Marysville, Ohio) and grown in a growth chamber under 28/22°C day/night temperatures with 16h light photo period for two weeks before being transferred to the field or to 4 gallon pots with field soil.

Field trials

Table 2.1 describes two field experiment sites and their soil characteristics. Experiment 1 was conducted in Lynchburg, Virginia, at Lynchburg Grows Urban Farm and Environmental Education Center (37°23'26"N, 79°9'57"W) on Cecil-appling association soil: deep, well drained, with 2-15% slopes, and firm clay subsoil. Experiment 2 (plots 1 and 2) were conducted in Danville, Virginia, at Walden Farm (36°36'42"N, 79°19'32"W) on Cecil-sandy loam soil: deep, well drained, with a 2 to 7% slope (NRCS, http://websoilsurvey.nrcs.usda.gov). Both soils are classified as Prime Farmland. The field in Lynchburg was historically managed grassland, and no crops were planted five years before the study began. The field in Danville was historically planted with tobacco, and no crops were planted five years before the study began. A

broad spectrum herbicide (Roundup®, Scotts Miracle-Gro® Company, Marysville, Ohio) was applied before the growing season according to manufacturers recomendation. Both were previously managed by yearly mowing. Sites were cultivated mechanically and hand weeded, no herbicide was applied after planting. To test soil fertility, five soil samples, approximately 15cm deep, were taken each plot and combined to form a composite sample for analyses. Nitrogen and Carbon analysis was done by Environmental and Agricultural Testing Service (EATS; http://www.soil.ncsu.edu/services/asl/) at the Soil Science Department at NC State University in Raleigh, NC. Additional soil analyses were done at the Department of Crop and Soil Environmental Sciences - Virginia Tech Soil Testing Lab (http://www.soiltest.vt.edu/).

The trial site in Lynchburg (**Figure 2.1a**) was 25' x 50', divided into 10 rows spaced 2.5' apart, and 20 trransplants were planted in each row with a 2.5' spacing. In Danville, plot 1 was 22.5' x 60', divided into 9 rows spaced 2.5' with 16 transplants spaced 2.5' apart. Plot 2 was 20' x 20', divided into 8 rows, 8 transplants per row spaced as above. The Lynchburg field experiment was planted in early spring (5/17/2012) and two subsequent harvests of above ground biomass were performed by cutting plants at a 5 cm stubble height in pairs; the first was during vegetative growth at the beginning of the summer (7/6/2012, n=25), and the final harvest was completed at the end of the growing season at full dormancy (1/10/2013, n=50). Fresh weight and number of tillers were recorded after the first harvest. The plant material was dried for 2 weeks at 70°F before dry weights were taken. Second year harvests were done on June 5th (n=23) and November 20th 11/20/2013 (n=75). Dry weights were determined as above.

The Danville experiment plots 1 and 2 were planted on 8/20/2012. **Figure 2.1b** illustrates the layouts and designs of the plots. Plants in plot 1 were bacterized on 7/3/2012 and plants in plot 2 were bacterized on 6/21/2012. Height of each tiller and tiller number were measured at the end of the growing season (11/26/2012). During the second year, root and shoot growth was determined during vegetative growth by digging the entire plant up (6/17/2013, n=10) and washing the plant roots with tap water. Fresh weights were determined, and the plants were allowed to dry in a humidity controlled room for 3 weeks. Dry root and shoot weights were then recorded, and statistical analysis was performed using the student's *t*-test. The final second year harvest was performed on 12/04/2013 after the plants were dormant. Fourteen pairs of plants were harvested from plot 1 randomly and 12 pairs of plants were harvested from plot 2

randomly. The dormant plants were dug in an 18-inch diameter around the roots and 10-inches deep. The roots were washed with tap water as described above and the entire plants were allowed to dry for two weeks. The roots were cut from the shoots and each was labeled and weighed.

Values were recorded, and statistical analysis was performed as described above. Values were assigned to each group and reported at 95%, 99%, or 99.9% confidence levels. Biologically interesting numerical differences are also presented and labeled with the p-value. Experimental design was paired, and plants were compared side-by-side to determine the effects of bacterization. Sites were selected to conduct switchgrass performance on high nutrient content soil versus low nutrient soil to test the hypothesis that PsJN inoculation would promote growth in the field under diverse soil conditions.

Field soil pot experiments

To test the effect of PsJN bacterization on growth and development of switchgrass cv. Alamo in a greenhouse in 4 gallon pots filled with field soil of medium nutrition level (**Table 2.1**) Plantlets were bacterized as above and transplanted five plants per pot, total of 11 pots per treatment, on 9/17/2011 and grown in Lynchburg Grows greenhouse at ambient temperature. The pots were watered equally with an above ground spray system every three days delivering 50 ml of tap water per pot. Growth stages were determined using the method of Sanderson (1992).

Root morphology experiment

To determine the effect of PsJN bacterization on root growth and morphology, bacterized transplants and and non-bacterized controls were planted in 4 gallon pots containing Miracle Gro® soil mix on 3/28/2013 in a temperature controlled greenhouse and harvested on 5/14/2013. The entire plants were harvested and roots were washed. Fresh and dry weights of roots and shoots were determined as described above. The number of seminal roots was determined and lateral roots counted on each seminal root down to 3 cm from the top.

Photosynthesis

Four measurements of photosynthesis were performed on the second fully formed leaf from the top (Exp. A;n=10, Exp. B;n=10, Exp. C;n=10, Exp. D;n=20) using Li-COR photosynthesis system (Li-COR® Lincoln, Nebraska) at light of 1500 µMol/m^2.second, 380 ppm CO_2 25°C.

Results

Lynchburg field trial in high nutrient soil

Table 2.1 indicates the relative nitrogen, carbon, and nutrient profile as well as soil type of this field trial at Lynchburg Grows Urban Farm and Environmental Education Center where the field has been managed grassland for more than 50 years. To investigate growth promotion at different stages of switchgrass growth during the establishment year, two harvests were performed. During vegetative stem development at 52 days (**Figure 2.2**) significant ($p<0.05$) increases in both above ground fresh and dry weight were recorded from 25 pairs of plants harvested randomly. The final harvest of the remaining 100 plants was after seed shattering. **Figure 2.3** represents the above-ground dry weights of the two harvests, with the first and last harvests achieving 0.001% and 0.05% significance levels, respectively. A second year subsampling was utilized to determine if growth promotion was maintained during vegetative growth in the second year. **Figure 2.4** illustrates results of plants at vegetative stem development in the second season, with significant differences ($p<0.05$, $n=25$) of both fresh and dry weights recorded. **Figure 2.5** represents the dry weights recorded at the end of the second season after dormancy. A statistical difference of $p=0.019$ ($n=75$) of weights were recorded.

Walden Farm field trials in low nutrient soil

To test the effects of PsJN bacterization on switchgrass cv. Alamo in the field with low nutrient soil (**Table 2.1)**, two plots were established on a former tobacco farm in Southern Virginia. Plots were planted in August of 2012, and tiller and height measurements were taken after two months growth. Tiller numbers (**Figure 2.6**) were significantly greater in plot 1 ($p<0.01$, $n=73$) and plot 2 ($p<0.001$, $n=32$) for PsJN bacterized plants. The height of each tiller was also recorded and added together to get an estimate of biomass, which is referred to as sum of tillers or total height (**Figure 2.7**). Total heights of PsJN bacterized plants were significantly greater in plot 1 ($p<0.001$, $n=73$) and plot 2 ($p<0.001$, $n=32$). During the second year of growth, entire plants were harvested in mid-season during vegetative growth (roots and shoots, $n=10$) from both plots to determine the effects of PsJN bacterization on both root and shoot growth in low nutrient soils (**Figures 2.8 and 2.9)**. Significant differences were recorded for plot 1 plants in fresh root weights ($p<0.05$, $n=10$), dry shoot weight ($p<0.05$, $n=10$), and dry root weight ($p<0.01$, $n=10$). PsJN bacterized plants in Plot 2 exhibited significantly more fresh shoot and root weight and dry

shoot and root weight with $p<0.05$,$p<0.05$, $p<0.01$, $p<0.05$, respectively). A larger number of plants were harvested at the end of the second season, and significant differences were obtained in both dry shoot and root weight in both plots 1 and 2 (**Figures 2.10 and 2.11**). Together, these results indicated that PsJN inoculation increased yields of switchgrass cv. Alamo in the low fertility field during the establishment and second year of growth.

Tiller production under different conditions

To explore the effects of PsJN bacterization on switchgrass establishment, tiller production was recorded after 3 months of growth. **Figure 2.12** illustrates that bacterization significantly increases tiller number during early vegetative growth stage in different soil nutrient levels (See **Table 2.1**). The field trial in Lynchburg clearly produced more tillers compared to pots with field soil, and the field trial at Walden farm, with almost 5 times the number of tillers produced in PsJN bacterized plants in high nutrient soil in Lynchburg. All PsJN bacterized plants produced significantly more tillers compared to control plants, and results are similar to those recorded growth chamber and greenhouse (Kim et al., 2012).

Growth stage

Accelerated growth stages of above ground switchgrass were recorded during two experiments to determine if accelerated growth occurred in PsJN bacterized plants at 2.5 months growth (**Figure 2.13**). During the test of growth promotion in pots with field soil, growth stage was measured by the number of leaves formed and the maturity of the new leaf according to the method of Sanderson (1992). During the test, significant ($p<0.001$) advances in growth stages was recorded in PsJN bacterized plants with almost an entire new leaf forming in the PsJN treatment. The study was repeated to confirm the results.

Root growth and morphology

Figure 2.14 represents the results of a 2.5 month pot study in a temperature controlled greenhouse to determine the effects of PsJN inoculation on root growth. Plants were grown in Miracle-Gro® potting mix. Significant increases ($p<0.001$) were observed in both fresh and dry root weight as well as root length. These results confirmed earlier studies which also demonstrated significant increases in root weight and length. To further study root morphology, the number of seminal roots was counted on PsJN and control plants as well as the number of lateral roots per cm on seminal roots were estimated by counting lateral roots in a 3 cm portion

of a randomly selected seminal root and dividing by 3. **Figure 2.15** illustrates these findings along with photos for reference. PsJN bacterized plants produced significantly more seminal roots ($p<0.001$) and the seminal roots produced significantly more lateral roots per cm compared to control plants ($p<0.001$). Together, these results indicate that PsJN bacterized switchgrass may have a greater capability to gather moisture and nutrients.

Photosynthesis rates

Photosynthesis rates were measured on both PsJN bacterized and control plants in pots with field soil and in the field (**Figure 2.16**). While rates were not significantly different, photosynthesis rates were higher in PsJN in all four experiments compared to controls.

Discussion

Changing global patterns of temperature and water supply combined with decreasing supplies of non-renewable fossil fuels have prompted interest in switchgrass, a native warm season perennial capable of growth with little inputs, as a potential bioenergy crop to offset the use of fossil fuels in a sustainable manner. Studies have shown that bacteria which reside in the internal tissues of plants without causing apparent harm, known as endophytes, have the ability to promote plant growth of a number of graminaceous energy crops (Boddey, 1995; Riggs et al., 2001; Mei and Flinn, 2010) including switchgrass (Kim et al., 2012; Gagne-Bourgue et al., 2012; Xia et al., 2013). Switchgrass is taxonomically divided into two ecotypes: cold tolerant upland which are short stature and yield lower biomass, and lowland cultivars, found in milder wet areas and are higher biomass producers (Vogel, 2004). Lowland cv. Alamo is a prime candidate for bioenergy production in the US southeast because of its high biomass production (Bouton, 2002). However, switchgrass establishment, like that for most warm season perennials, is a primary challenge during the first two years because of seed dormancy and weed competition (Moser and Vogel, 1995). It is critical, therefore, to improve switchgrass establishment during the first two years for overall healthy stand development and economics (Parish and Fikes, 2005).

Naturally occurring bacterial endophytes, isolated from switchgrass growing in the field, have been shown to promote switchgrass growth (Gagne-Bourgue et al., 2012; Xia et al., 2013). The well-studied beneficial bacterial endophyte, *Burkholderia phytofirmans* strain PsJN, isolated from onion roots (Frommel et al., 1991) was shown to increase the growth and tiller

development of switchgrass cv. Alamo in the growth chamber and greenhouse (Kim et al., 2012), but was not investigated in the field. The objective of this study was to explore the effects of *Burkholderia phytofirmans* strain PsJN bacterization on switchgrass cv. Alamo seedlings during the first two years of growth in the field, including the impact on tiller number and above and below ground biomass in two soil conditions; one was in a field with prime soil and another was in a former tobacco field common in Southern Virginia. Across the two sites, overall above ground biomass was significantly greater at mid-season, during vegetative growth and before seedhead excertion and in the second year of growth ($p<0.05$).

At the high nutrient soil site in Lynchburg, two harvests were performed during different stages of plant development to determine if growth promotion was persistent in the field. Other experiments have shown that the longer switchgrass is grown in the field, the more endophytic bacteria become associated with it (Gagne-Bourgue et al., 2012). Based on these observations, growth promotion seen with inoculations of a single endophytic bacterium may be diminished when the plant was grown in the field for a longer time. However, in soil with high nutrients, fresh weight was significantly higher through out the first season, at each of the two harvests. The highest effects of PsJN bacterization for both fresh and dry weight were recorded at the first sub-sampling during vegetative growth (52 days of growth, $p=0.002$). It must be noted that switchgrass cv. Alamo has high genetic variances for yield (Burton, 2002), making statistical significance harder to achieve. At the final harvest, which was undertaken after the plant had completed senescence and primarily dry tissue remained, revealed a small but significant ($p<0.05$, $n=50$) increase in above ground biomass. During the second year, a harvest was performed during vegetative growth at mid-season, and the results showed a significant ($p<0.05$) increase in both fresh and dry weights. The final harvest at the end of the second season also demonstrated a significant difference in weights in PsJN bacterized plants vs. controls. Together, this data indicates that in fertile field soil, switchgrass cv. Alamo growth is increased throughout seedling establishment in the first year and persists through the second year.

In both prime soil and soil from a former tobacco farm, tiller numbers were increased significantly even when plants were transplanted at different times and in different locations. Increased tiller numbers are important during establishment as an early indicator of future biomass production potential in perennial grass systems (Boe and Beck, 2008). For example,

increased tiller numbers have been observed in winter wheat inoculated with *A. brasilense* (Dobbelaere et al., 2001). In the tobacco field with marginal soil, when tiller height was measured for each tiller and added together to get a total height measurement for the plant, PsJN bacterized plants clearly outperformed ($p<0.001$) control plants, indicating help in establishment during the first year of growth. During the second year of growth, in order to determine both above and below ground biomass, entire plants were dug, washed and weighed. In previous experiments, root size and weight were increased significantly in pots with field soil (Kim et al., 2012). In both plots, PsJN bacterized plants produced significantly higher dry and fresh root biomass, indicating that bacterization increases root size, allowing the plant to have access to more nutrients and water, important to establishment. Dry shoot weights were also significantly increased at the second year subsampling, indicating an increase in biomass. The only comparison that did not achieve a p value of <0.05 was fresh shoot weight in plot 1which, during this vegetative growth period, achieved a p value of 0.07, a biologically important number, likely due to the small sample size of the harvest. However, plot 2 shoot weight did achieve a statistically significant value of $p = 0.02$.

PsJN bacterization was shown to accelerate development in Arabidopsis (Poupin et al., 2013). Data from the pot experiment with field soil support this hypothesis as growth stage was advanced in PsJN bacterized plants (**Figure 2.13**). To confirm findings in the field regarding root growth and to further explore the effects of PsJN bacterization on root development, a 2.5 month study was initiated in pots with Miracle-Gro® potting mix in the greenhouse (**Figure 2.15)**. This study indicated that PsJN bacterization significantly increased root weight ($p<0.001$) as was found in the field trial. Root morphology was also changed as PsJN bacterized plants had more seminal roots and more lateral root branches per cm compared to controls, supporting earlier findings of increased lateral branching of roots in Arabidopsis (Poupin et al., 2013). This data indicates that PsJN not only increases root size, but also changes morphology to allow the plant to penetrate the soil more completely to gain better access to nutrients and water through increased lateral roots and increased number of seminal roots. These morphology changes could help the plant tolerate drought compared to controls, an effect shown to occur in PsJN inoculated maize (Naveed et al., 2013). Root growth promotion has been reported in other plants with use of bacteria that produce IAA-like substances (Glick, 1995). Similarly, increased root dry weight was demonstrated in greenhouse studies with maize by bacteria producing IAA-like compounds

(Mehnaz and Lazarovits, 2006). Recently, it was reported that increased root development contributes to greater shoot numbers in the field in switchgrass inoculated with a mixture of endophytes (Ker et al., 2012).

Overall, *Burkholderia phytofirmans* strain PsJN bacterization of switchgrass cv. Alamo results in growth promotion in the field, in different soil types, during the first establishment year. The growth promotion effects are sustained through the second year in soils with both high and low nutrient levels. Potentially as a result of increased root growth, more tillers were produced, and growth stage was advanced. Other studies have demonstrated that a mixture of plant growth promoting bacteria, originally isolated from switchgrass, increased production by 40% compared with the bacteria alone (Ker et al., 2012). The mixture of bacteria the authors used had a range of growth promoting abilities including the ability to solubilize P, produce IAA-like substances, and potentially fix atmospheric nitrogen. In this study, we characterized root morphology in addition to biomass, tiller production, and growth stage acceleration by bacterization with a single bacterium capable of multiple mechanisms of action (Barka et al., 2002; Lazarovits and Nowak, 1997; Sessitsch et al., 2005; Weilharter et al., 2011). In the future, continued monitoring of production will be performed to confirm sustained production. Interactions between an endophyte and its host plant are complex, and multidimensional and further field research is needed to understand how use of these beneficial microorganisms is to benefit sustainable agriculture.

Acknowledgements

We would like to thank Dr. John Seiler and Bingxue Wang for the photosynthesis measurements.

Figures and Tables

Figure 2.1a Field trial design for the 2012 experiment at Lynchburg Grows Urban Farm. Twenty seedlings were planted in 10 rows and the plot was established on 05/17/12.

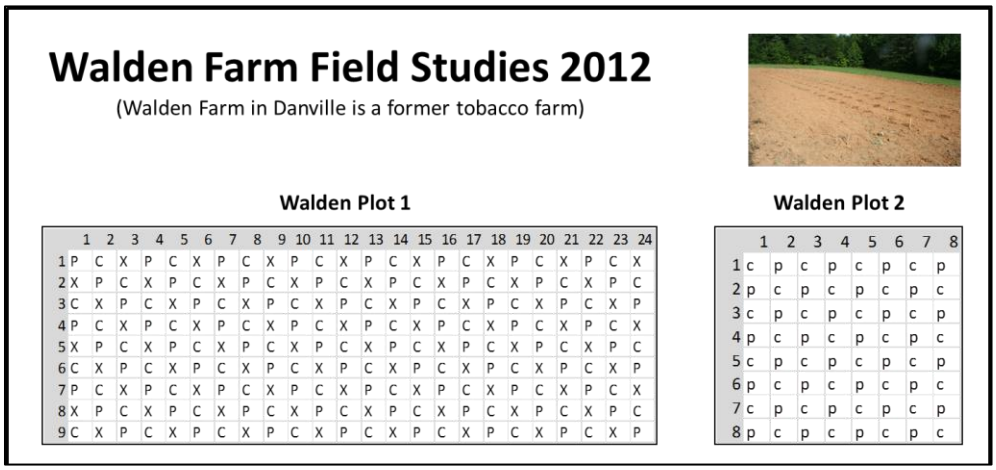

Figure 2.1b Descriptions of the 2 research plots at Walden Farm. p=*Burkholderia phytofirmans* strain PsJN bacterized switchgrass cv. Alamo, c= control or buffer inoculated switchgrass cv. Alamo and x= switchgrass cv. Alamo bacterized with a different bacteria. Plots 1 and 2 were planted on 08/27/2012.

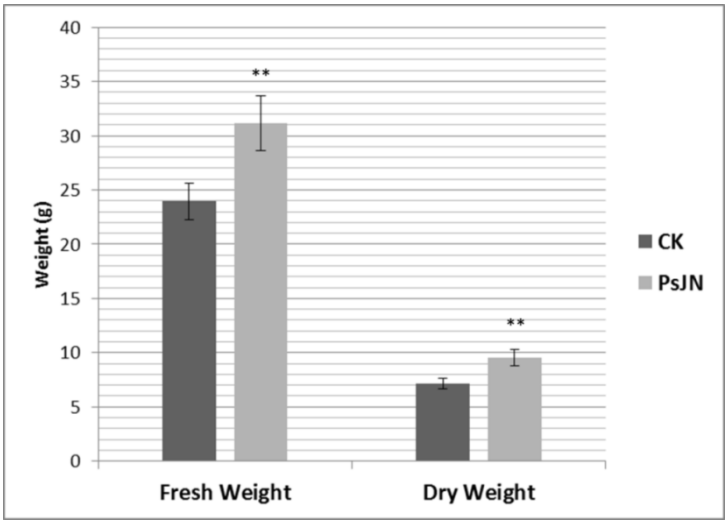

Figure 2.2 Results of the first switchgrass harvest in Lynchburg . Above ground tissues were gathered in Lynchburg on 07/06/2012, at 52 days of growth (n=25, USDA prime farmland, **p<0.01).

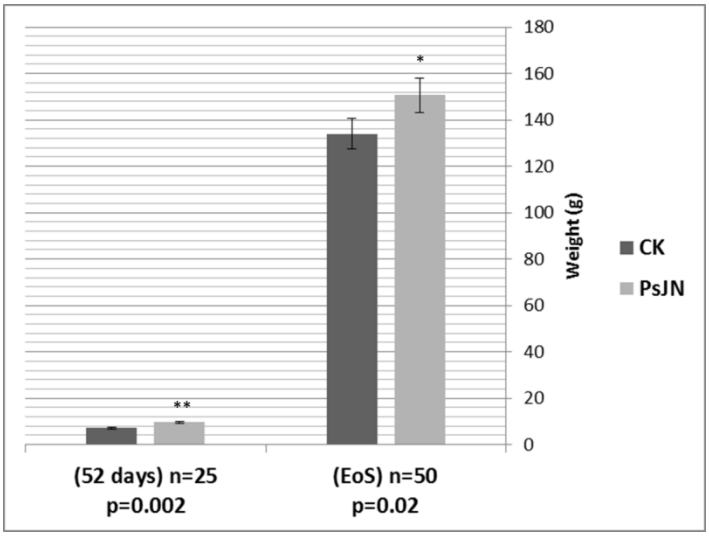

Figure 2.3 Summary of dry weights of the two switchgrass harvests in Lynchburg. Plants were allowed to dry for 2 weeks at 70°F before weight was measured (**p<0.01, *p<0.05).

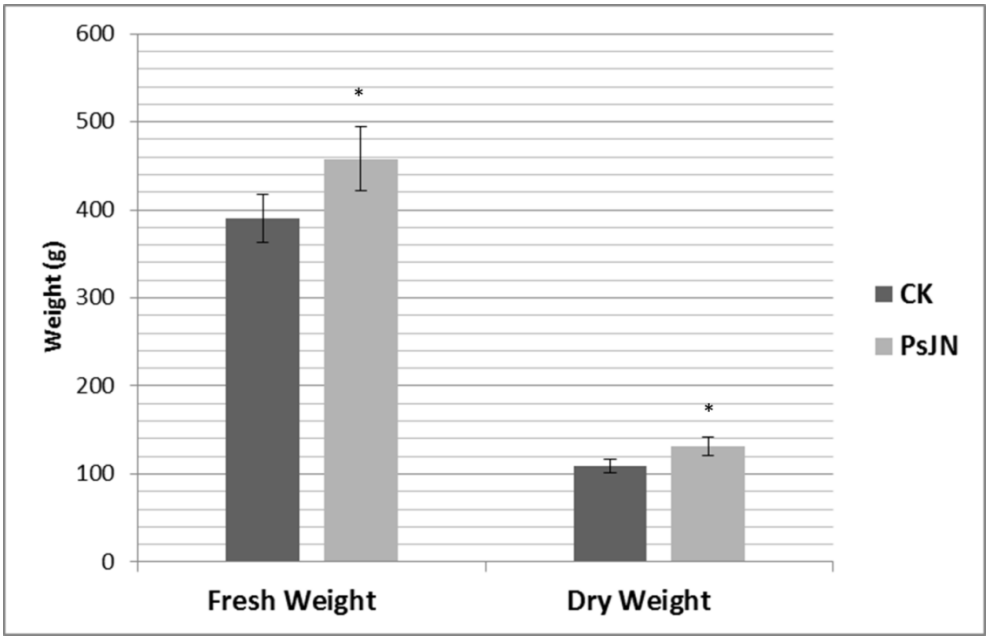

Figure 2.4 First harvest in the second season (06/05/2013) in Lynchburg. Twenty three pairs of plants were randomly selected, harvested, and weighed for fresh weight. Plants were allowed to dry in a humidity controlled room for 2 weeks and dry weight was recorded (*$p<0.05$). Bars represent standard error.

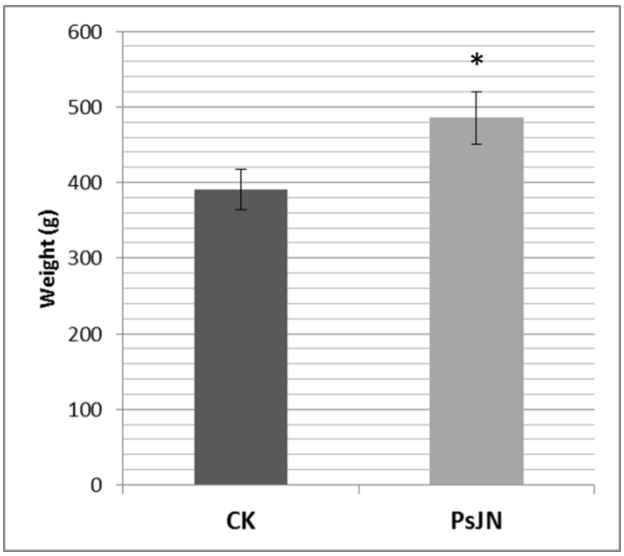

Figure 2.5 Final harvest in second season in Lynchburg (11/20/2013). The remaining plants (75 pairs) were harvested, allowed to dry in a humidity controlled room for 2 weeks, and dry weight was recorded (*$p<0.05$). Bars represent standard error.

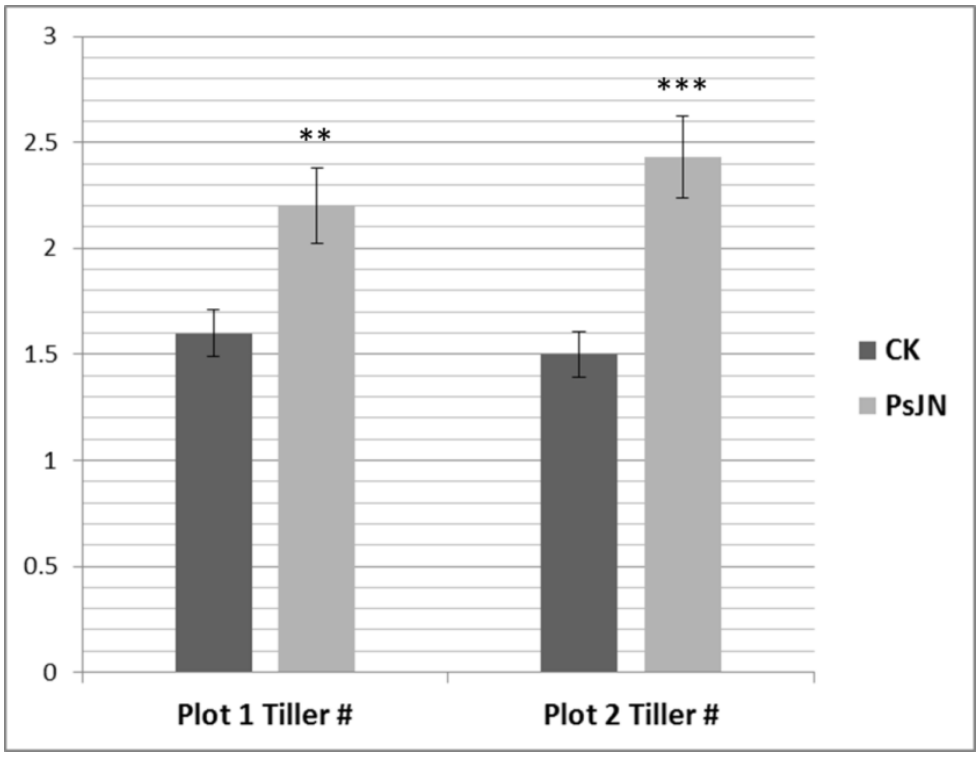

Figure 2.6 Switchgrass tillering after two months at Walden Farm. The plots were planted on 09/27/2012 and the data was taken on 11/27/2012, at the end of the first season. Plot 1 (n=73, tobacco farm soil, **p<0.01) Plot 2 (n=32, tobacco farm soil, ***p<0.001).

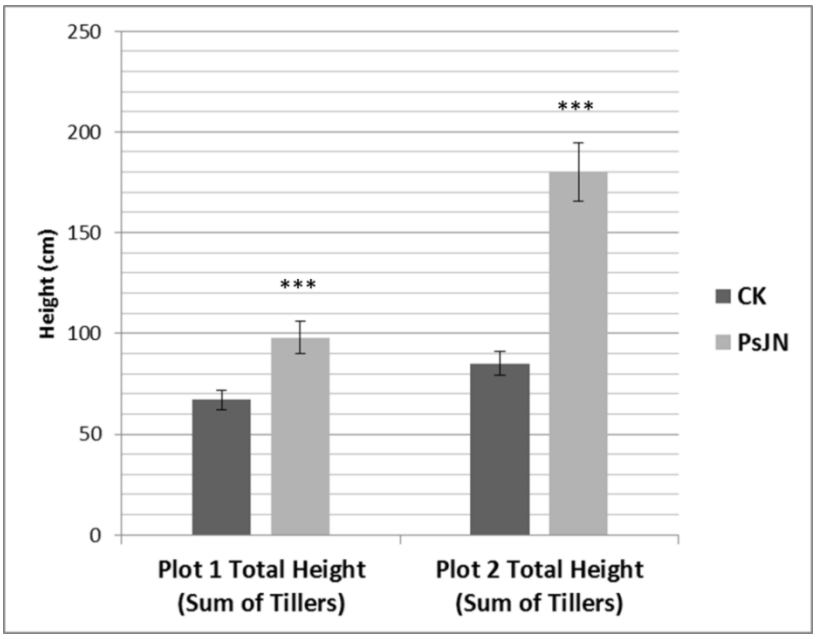

Figure 2.7 Total heights of switchgrass (sum of the height of each tiller) at Walden Farm. Measurements were recorded 2 months after planting. The plots were planted on 09/27/2012 and the data was taken on 11/27/2012, at the end of the first season. Plot 1 (n=73, tobacco farm soil, ***$p<0.01$) Plot 2 (n=32, tobacco farm soil, ***$p<0.001$).

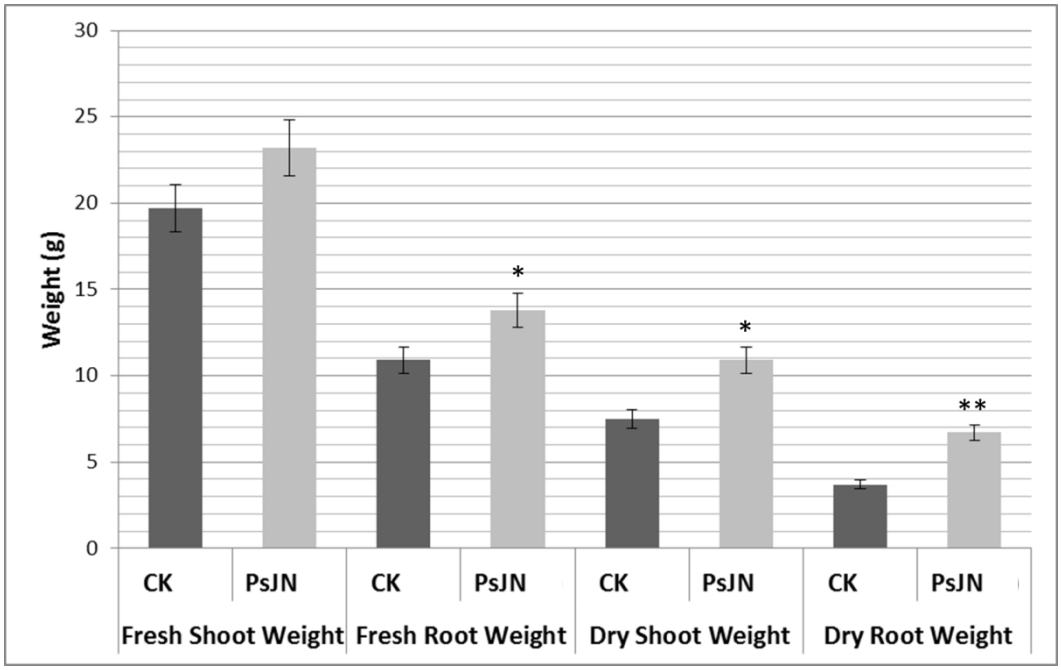

Figure 2.8 Plot 1 second year mid-season harvest of cv. Alamo from Walden Farm. Measurements were recorded on 06/17/2013. Ten pairs of plants were dug out completely; the roots were washed and allowed to dry, and then the above ground portion was separated and labeled. Fresh weights were taken within 8 hours and the plants were dried at 28°C for two weeks. Statistical analysis was performed using a paired students T test (**$p<0.01$, *$p<0.05$).

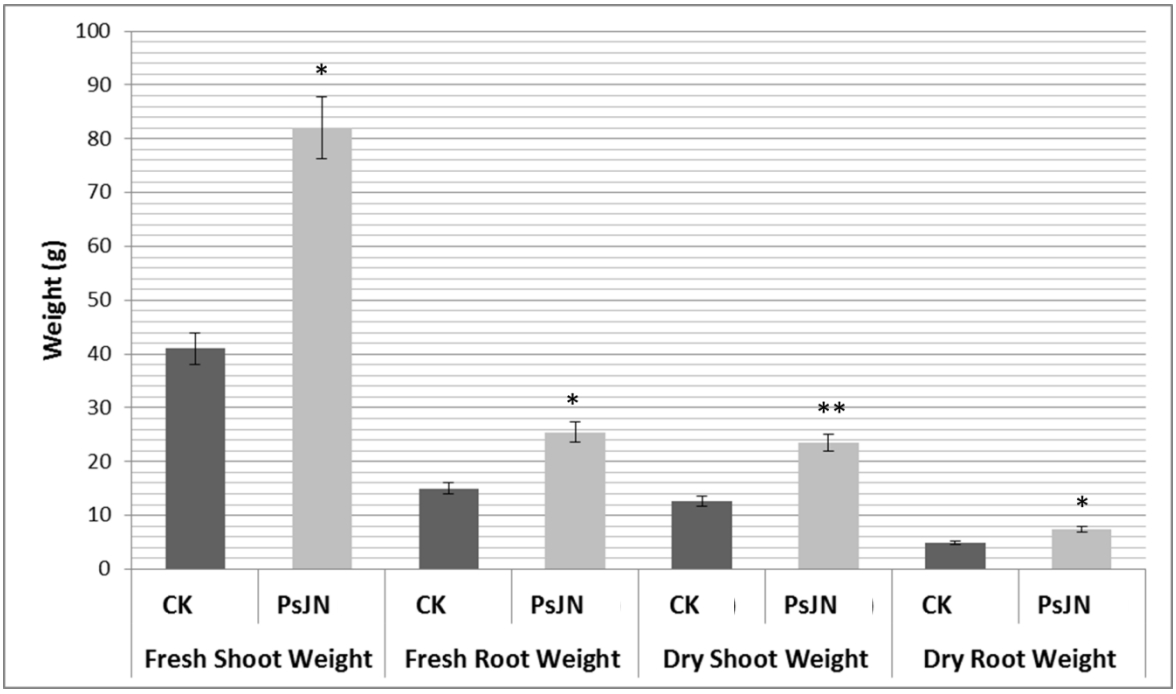

Figure 2.9 Walden Farm plot 2 second year mid-season harvest. Ten pairs of switchgrass plants were harvested on 06/28/2013. Ten pairs of plants were dug out completely; the roots were washed and allowed to dry, and then the above ground portion was separated and labeled. Fresh weights were taken within 8 hours and the plants were dried at 28°C for two weeks. Statistical analysis was performed using student's t test (**$p<0.01$, *$p<0.05$).

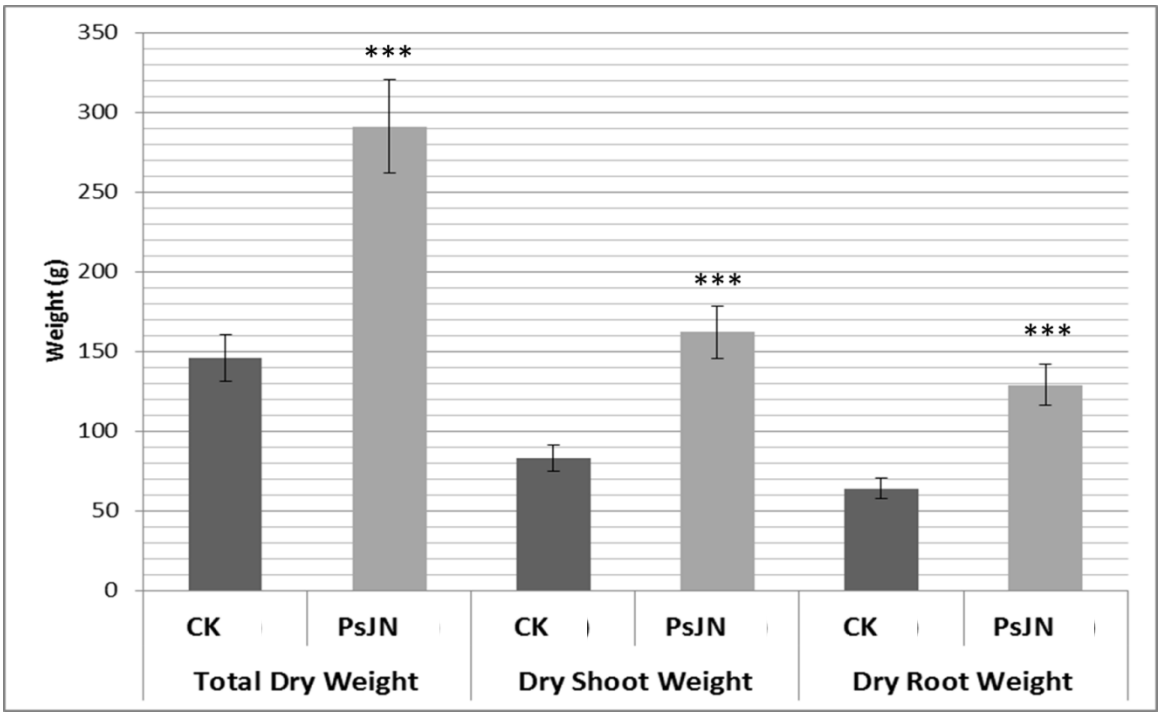

Figure 2.10 Walden Farm plot 1 second year end of season harvest. 14 pairs of switchgrass plants were harvested on 12/04/2013. Plants were dug out completely; the roots were washed and allowed to dry, and then the above ground portion was separated and labeled. Dry weights were recorded after plants were dried for two weeks. Statistical analysis was performed using student's t test (***$p<0.001$).

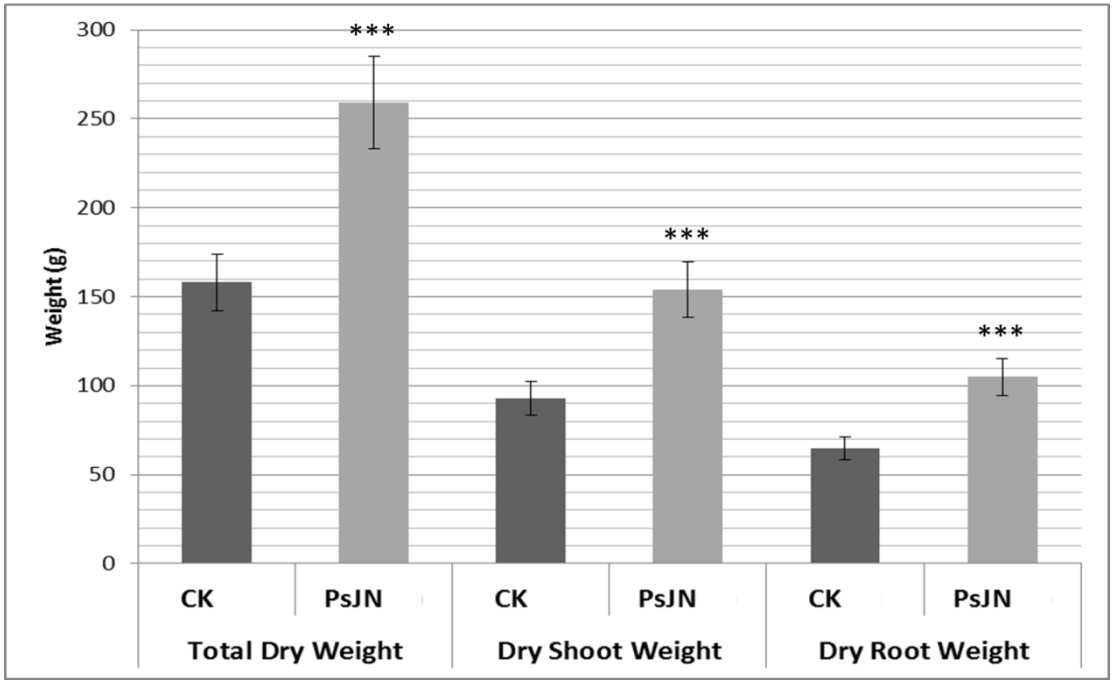

Figure 2.11 Walden Farm plot 2 second year end of season harvest. 12 pairs of switchgrass plants were harvested on 12/04/2013. Plants were dug out completely; the roots were washed and allowed to dry, and then the above ground portion was separated and labeled. Dry weights were recorded after plants were dried for two weeks. Statistical analysis was performed using student's t test (***$p<0.001$).

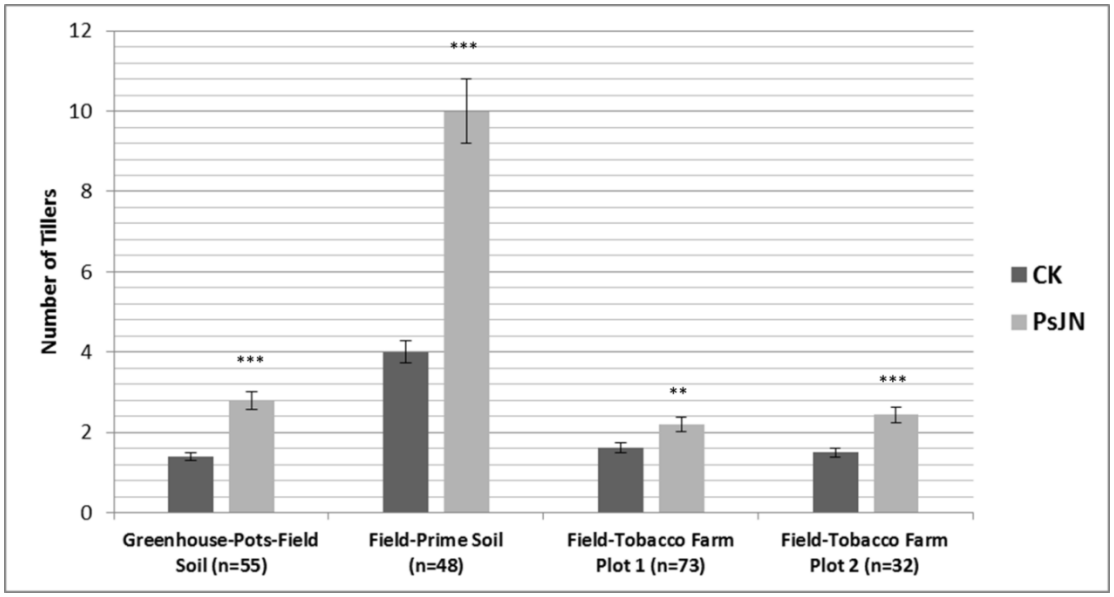

Figure 2.12 Switchgrass tiller number during first year establishment**.** Measurements of tiller number of control (CK) and *Burkholderia phytofirmans* strain PsJN bacterized switchgrass were recorded during first year establishment in different types of field soil (*** $p<0.001$, ** $p<0.01$).

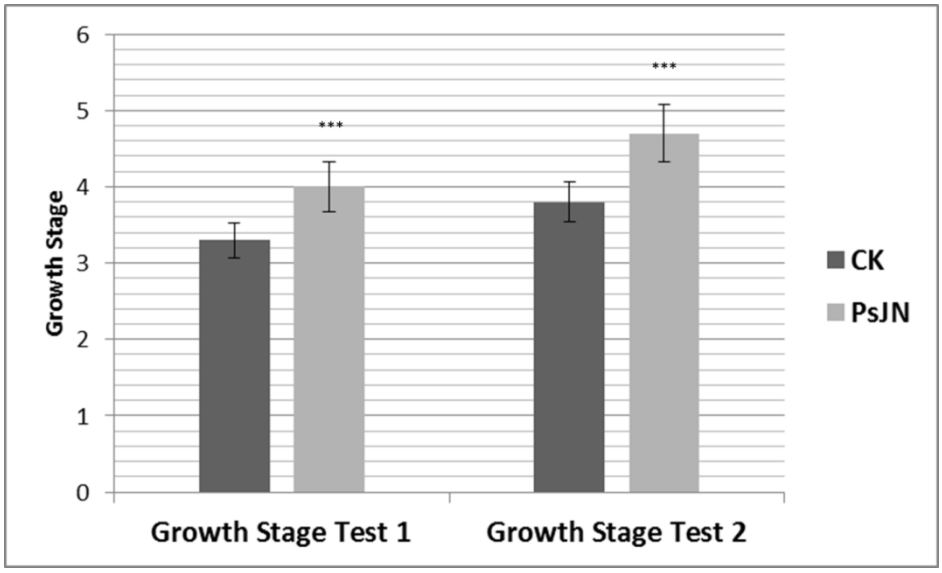

Figure 2.13 PsJN bacterized plants exhibit advanced growth stage. Measurements were recorded at 2.5 months growth, ***$p<0.001$, $n=50$. Test 1 was performed in 2010 and Test 2 was performed in 2011.

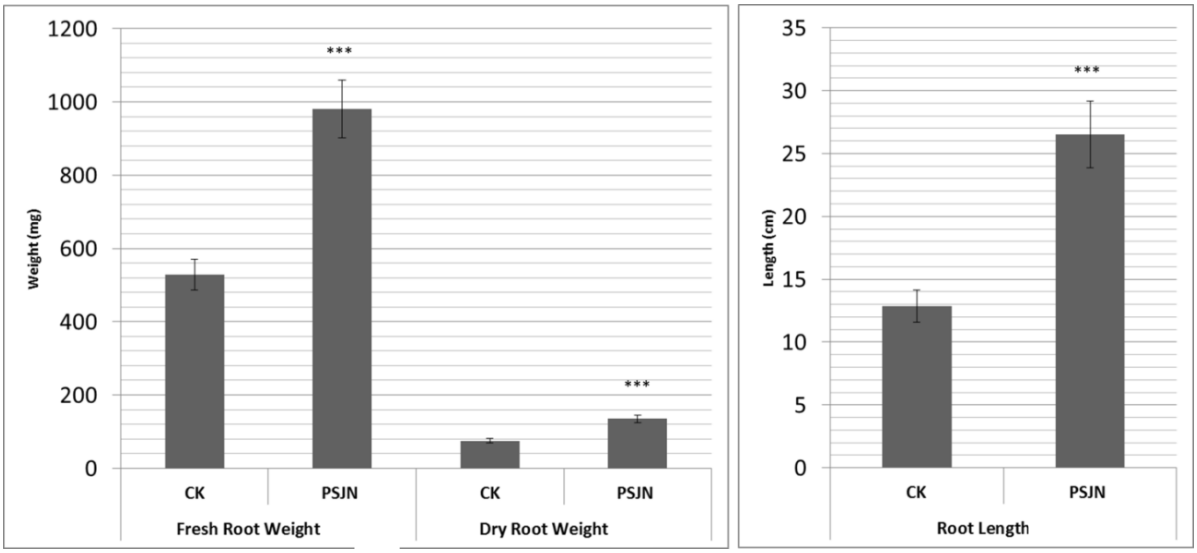

Figure 2.14 Root biomass and length at 2.5 months growth in pots. *Burkholderia phytofirmans* strain PsJN bacterized switchgrass increased root biomass and length (***$p<0.001$) at 2.5 months growth in pots with Miracle Gro® soil mix in a temperature controlled greenhouse.

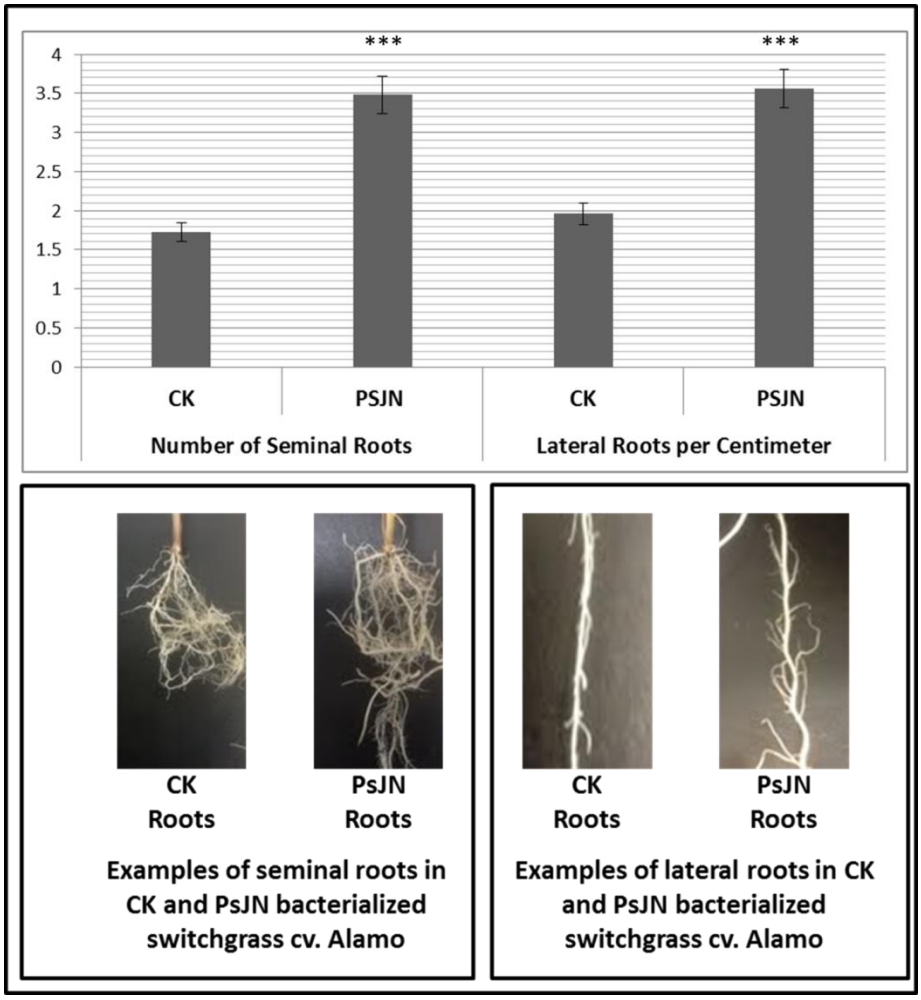

Figure 2.15 Root morphology comparison of control (CK) and PsJN bacterized switchgrass. Measurements were recorded at 2.5 months (n=25, ***p<0.001).

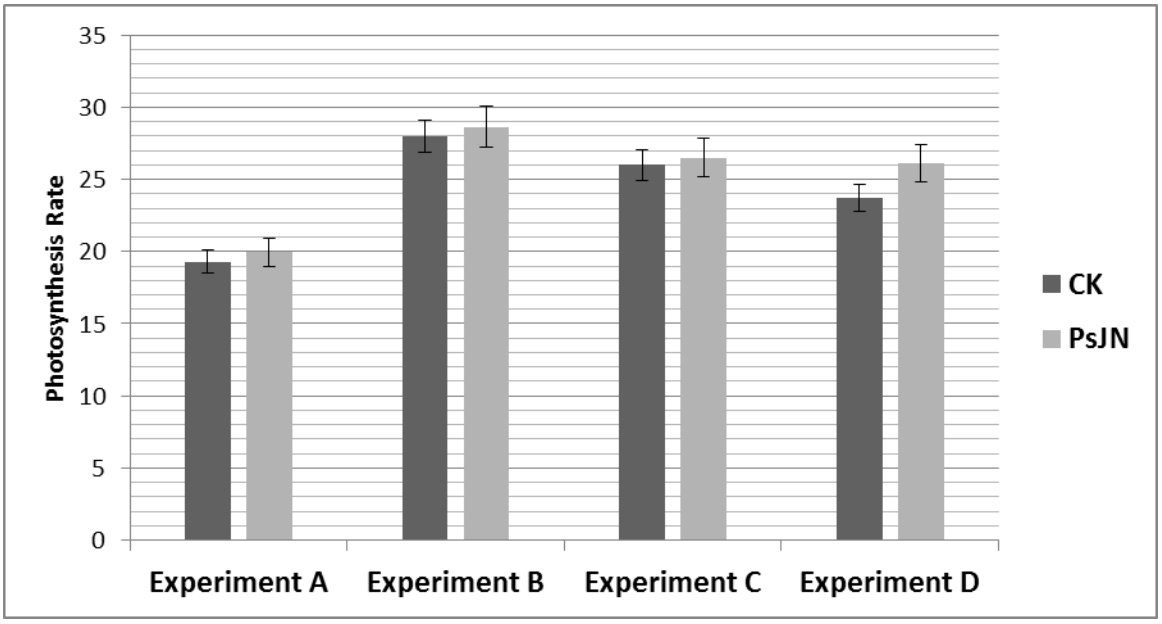

Figure 2.16 Measurements of photosynthesis rates. Experiment A, n=10, 3 month old plants, planted in pots with field soil in a greenhouse: Experiment B, n=10, 3 month old plants, planted in USDA prime field soil: Experiment C, n=10, 5 month old plants, planted in USDA prime field soil: Experiment D, n=20, 3 month old plants, planted in USDA prime field soil. Experiments were conducted with graduate student Bingxue Wang.

Table 2.1 Trial descriptions and soil characteristics. Ratings are in parenthesis (VH=Very High, H=High, M=Medium, L=Low) All trace minerals were rated as sufficient.

Field Soil Parameters	Field Trial Site 1 (Lynchburg field trial)	Field Trial Site 2 (Walden Farm field trial)	Pot Trial (Pots with field soil)
Location	Lynchburg, VA (37°23'26"N, 79°9'57"W)	Danville, VA (36°36'42"N, 79°19'32"W)	Lynchburg, VA (37°23'26"N, 79°9'57"W)
Description	Cecil-appling association	Cecil-sandy loam soil	NA
Classification	Prime farmland	Prime farmland	NA
Crop History	Managed grassland	Historically tobacco	Rose nursery production
Last Planted	Fallow for more than 20 years	Fallow for more than 5 years	Fallow for more than 5 years
Previous Crops	Managed Grassland	Tobacco	Cut Roses
Slope	2-15%	2-7%	NA
pH	6.0	5.7	6.6
% Nitrogen	0.54	0.07	0.20
% Carbon	7.30	0.85	2.84
P (lb/A)	2044(VH)	4 (L)	1063 (VH)
K (lb/A)	393 (VH)	76 (M-)	924 (VH)
Ca (lb/A)	9979 (VH)	510 (L+)	5880 (VH)
Mg (lb/A)	995 (VH)	175 (H)	1043 (VH)
Zn (ppm)	54.3	0.5	49.3
Mn (ppm)	39.8	2.4	107.9
Cu (ppm)	0.7	0.3	2.7
Fe (ppm)	19	9.9	23.6
B (ppm)	1.4	0.1	1.2
Buffer Index	6.17	6.20	6.45
Acidity (%)	4.9	36.2	3.3

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Chapter 3

Burkholderia phytofirmans strain PsJN bacterization effect on differential expression of a tau class glutathione-S-transferase (GST) in switchgrass cultivars Alamo and Cave-in-Rock seedlings

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Abstract

Molecular mechanisms underlying interactions between growth promoting bacterial endophytes and their host plants during colonization and early growth are not well understood. To identify molecular determinants of these interactions, responsive cv. Alamo and non-responsive cv. Cave-in-Rock were inoculated with *Burkholderia phytofirmans* strain PsJN and genotype specific responses determined. Comparative global gene expression profiling was conducted by using EST microarrays in collaboration with the Genomics Core Facility at the Noble Foundation. Approximately 50 genes were selected based on the apparent differences in the expression levels between these two genotypes. One of the key genes, a member of the tau class of glutathione S-transferase (GST), an enzyme known to be involved in stress responses in plants, was selected for functional studies using overexpression and RNAi knockout/knockdown techniques. GST overexpression line 26 had significantly stimulated growth at one month after PsJN bacterization ($p<0.01$) compared to either line 26 controls or p1300S controls which were also bacterized. All GST RNAi lines exhibited reduced growth promotion compared to controls. Together these results indicate that the GST enzyme is likely involved in early colonization and growth promotion in cv. Alamo.

Key Words: Glutathione-S-transferase, Plant Microbe Interactions, *Burkholderia phytofirmans* strain PsJN, Switchgrass, Overexpression and RNAi Transgenic Plants, Enzyme Activity

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Introduction

Bacterial endophytes reside in plant tissues during all or part of their life cycle and cause no apparent harm (Wilson, 1995). These tiny organisms are widespread as it is estimated that every plant species has at least one associated endophyte (Strobel et al., 2004), and they are diverse, belonging to multiple classes of bacteria (Rosenblueth and Martinez-Romero, 2006). Furthermore, a single plant species can harbor a range of endophytes. For example, above ground plant tissues from wheat were shown, through culture based studies, to be occupied by 88 bacterial species representing 37 genera (Legard et al., 1994). Research has revealed that the interactions between the host plant and its endophytes are diverse and multifaceted, often dependent on developmental stages, environmental conditions, and genotypes (reviewed in Mei and Flinn, 2010). Multiple mechanisms of plant growth promotion by endophytes have been reported over the past 30 years, including production of plant hormones, synthesis of antimicrobial compounds to increase resistance to plant pathogens, and helping the host plant acquire nutrients through mechanisms such as atmospheric nitrogen fixation and secretion of siderophores (reviewed in Compant et al., 2008; Mei and Flinn, 2010). A particular bacterium may also convey multiple mechanisms of growth enhancement, demonstrated by research into one of the most studied endophytes, *Burkholderia phytofirmans* strain PsJN, which can secrete siderophores for nutrient acquisition, aid in plant resistance through the induced systemic response (ISR), produce trehalose to increase tolerance to abiotic stress, and through production of ACC deaminase, metabolize a precursor of ethylene, a plant growth inhibiting hormone, resulting in plant growth promotion (Lazarovits and Nowak, 1997; Barka et al., 2002; Sessitsch et al., 2005; Weilharter et al., 2011).

Functionally, GSTs are an ancient class of catalytic and binding proteins, often associated with stress tolerance and induced by a wide variety of biotic and abiotic stresses. GSTs also have the ability to detoxify herbicides, pollutants, and toxins, often in concert with the plant stress reaction (Reviewed by Frova, 2003) by catalyzing the nucleophilic addition of glutathione (GSH) to a variety of molecules, thereby targeting them to vacuoles for destruction (Armstrong, 1997). Plant glutathione-S-transferases (GSTs) are also involved in stress-induced signaling, and their gene expression may be stress responsive (Loyall et al., 2000). In plants, there are six distinct recognized classes (phi, tau, theta, zeta, lambda and dehydroascorbate reductase), many similar to those found in other organisms, and two, phi and tau, are found in plants only (Dixon

et al., 2002). Genes of different isoforms of the tau GST family may also be expressed constituently or induced only in response to stress (Lo Piero et al., 2009). While the majority of plant GSTs belong to the tau and phi classes, sequence identity can be less than 50% within a class and, surprisingly, less than 25% between classes, despite the recognition that protein structure is conserved (Dixon et al., 2002). The most common GSTs in plants are cytosolic and may account for up to 2% of soluble proteins (Scalla and Rulet, 2002). Genetically, plant GSTs have been shown to be up-regulated in response to nodulation factors secreted by *Sinorhizobium meliloti* (formerly *Rhizobium meliloti*) during colonization of the legume *Medicago truncatula* (Ramu et al., 2002).

The complex interactions between an endophyte and its host can be very specific, even at the host genotype level. Genotype specificity has been documented in interactions between *Burkholderia phytofirmans* strain PsJN and different genotypes of potato (Conn et al., 1997; Frommel et al., 1993), tomato (Pillay and Nowak, 1997), and switchgrass (Kim et al., 2012). In switchgrass, a promising bioenergy crop identified by the US Department of Energy, bacterization of the cultivar (cv.) Alamo resulted in growth promotion while cv. Cave-in-Rock bacterization had no increase in growth compared to controls (Kim et al., 2012). Seedlings of bacterized cv. Alamo exhibited an increase of root and shoot length of 35.6 % and 32.8 %, respectively, as well as an increase of fresh weight of 83.6 % compared with control plants (non-bacterized) after one month under *in vitro* conditions. This genotype specificity provides the opportunity to compare the molecular mechanisms through molecular techniques such as real time PCR and EST microarray chips, between responsive and non-responsive genotypes during colonization and growth promotion, possibly contributing new information on molecular plant-microbe interactions. This chapter focuses on a functional study of a plant glutathione S-transferase (GST) belonging to the tau class, shown through microarray analysis to be up-regulated in the responsive switchgrass cv. Alamo while the non-responsive cv. Cave-in-Rock remained unchanged. In *Citrus sinensis*, tau class GST gene expression was demonstrated to be cultivar related specificity (Lo Piero et al., 2009). The goal of this chapter was to characterize the role of GST during PsJN colonization and early growth promotion in switchgrass through generation of both GST overexpression and RNAi transgenic plants and analysis of gene expression levels, growth promotion responses, and GST enzyme activities.

Materials and methods

Plant material preparation and PsJN bacterization

Switchgrass (*Panicum virgatum* L.) seeds of cvs. Alamo and Cave-in-Rock were purchased from Warner Brothers Seed Co. (Lawton, OK). Seeds were surface sterilized as described previously (Kim et al., 2012) and germinated for 5-7 days on sterile filter paper in petri-dishes at 25°C. *B. phytofirmans* strain PsJN and PsJN-GFP were obtained from Dr. Angela Sessitsch (Austrian Institute of Technology, Seibersdorf, Austria). PsJN cultures were streaked on King's B (KB) solid medium as described (Pillay and Nowak, 1997). Inoculum was produced by transferring one loop of bacteria from 2-day-old cultures to 5 ml KB broth in a 15-ml culture tube, and followed by incubation at 28˚C on a shaker (220 rpm) overnight. Five ml of the overnight culture was added to 45 ml KB broth in a 250-ml Erlenmeyer flask and grown to OD_{600} around 0.7. Bacterial cells were then collected by centrifugation at 3,500 rpm for 7 minutes at 4 ˚C, and re-suspended in PBS buffer (10 mM NaH_2PO_4 containing 0.8% NaC1, pH 6.5) and adjusted OD_{600} to 0.5 (approx. 10^8 cfu). Germinated seeds were bacterized as described previously (Kim et al., 2012), and transferred to GA-7 Magenta containers with Murashige and Skoog basal salts plus vitimans (MS + V) (M519, Phytotech Labs, Shawnee Mission, KS) with 3% maltose (RPI Inc.) and 0.3% phytagel (Phytotech labs) pH 5.8. The plantlets were grown in GA-7 Magenta containers at 25˚C (16 hr photoperiod, fluorescent light at 67µmol $m^{-2}s^{-1}$).

RNA isolation

Both Alamo and Cave-in-Rock seedlings were inoculated and grown as described above, and tissues were collected at 0.5, 2, 4, and 8 days with controls inoculated with PBS buffer alone and collected at 0 day. Three biological replicates were used for each time point. Tissues were frozen in liquid nitrogen immediately and stored at -80ºC until use. Total RNA was extracted using Qiagen RNeasy Plant Mini Kit following manufacturer's instruction (Chatsworth, CA).

Microarray analysis

Purified RNA (500 ng) was amplified and labeled using IVT Express Kit (Affymetrix, Santa Clara, CA). Microarray chips were hybridized, washed, and stained following the manufacturer's recommendations. Data normalization was conducted by using the robust multi-array average (RMA)(Irizarry et al., 2003). The Affymetrix platform switchgrass EST microarray chip was developed by BESC (the DOE BioEnergy Science Center, Zhang et al., 2013). This chip contains

more than 100,000 probe set for putative genes. Differentially expressed genes among the sample groups were selected using Associative Analysis as described by Dozmorov and Centola (2003) using an expression value cutoff of 2 and Bonferroni corrected p value of 4.0659E-07, which is derived from 0.05/N where N is number of probe set on the chip which for switchgrass chip is 122,972. For each sample group, the treated samples were compared against the untreated control samples to select significant genes. Ratio generated by comparing the PsJN inoculated treatment with control was subjected to the Log2 of the ratio and then plotted into MapMan software (Usadel et al., 2005) for visualization of metabolic pathways. Additionally, PageMan (Usadel et al., 2006) was used to show significant up- and down-regulated genes. Number of total genes, or unique genes which were either up- or down-regulated at the specific pathways was generated by the Venn diagram tool at ±1.0 threshold of the MapMan software. Selected switchgrass probes were annotated using the "*Oryza sativa*, OSA_AFFY_150909" map identifiers downloaded from the MapMan website (mapman.gabipd.org).

qPCR verification

RNA was extracted as described above after PsJN bacterization at 0, 0.5, 2, 4, and 8 days and stored at -80°C until use. GST specific gene primers were designed using the sequence provided by the Noble Foundation (CCGN10868.b1 CCGN *Panicum virgatum* etiolated seedlings) with the Primer 3 software (Untergrasser et al., 2012) (Table 3.1). DNase treatment was performed with a DNA-free kit (Ambion; Foster City, CA). cDNA synthesis was performed using SuperScript III (Invitrogen; Carlsbad, CA, USA) from 1µg total RNA following manufacturer's protocol. Final volume was adjusted 1:10 (cDNA:H_2O) and stored at -20° C until use. Real time polymerase chain reaction (qPCR) was performed with gene specific primers (Table 3.1) with equal amounts of cDNA using the $2^{-\Delta\Delta C}{}_{T}$ method (Livak and Schmittgen, 2001). Ubiquitin gene expression was used as a normalizer. qPCR was performed with a Bio-Rad iQ™5 Multicolor Real-Time PCR Detection System.

Generation of GST transgenic plants

GST gene synthesis

The GST gene described above was synthesized by GENEWIZ, Inc. (Plainfield, NJ, USA) with Kpn I and Sal I enzyme sites designed in the N- and C- terminals, respectively for cloning.

Overexpression and RNAi construct creation

For GST overexpression, the 732 bp synthesized gene was inserted into the pCAMBIA1300S vector (kindly provided by Dr. Yinong Yang in Penn State University) with Kpn I and Sal I enzymes. Both the pCAMBIA1300S vector and the GST gene fragment in pUC57 were digested with Kpn I and Sal I , gel extracted using a QIAquick Gel Extraction Kit (Qiagen, Chatsworth, CA) and ligated with T4 DNA ligase (New England Biolabs, Ipswich, MA, USA) according to manufacturer's protocols. Ligated constructs were transformed into *Escherichia coli* strain DH5α competent cells by heat shock (42°C, 90 sec.), plated on Luria broth (LB) plus Kanamycin (50 mg/L) plus 1.5% agar for growth overnight at 37°C. Colonies were picked and grown overnight in LB plus Kanamycin (50 mg/L) overnight at 37°C. With the Quicklyse Miniprep kit (Qiagen, Chatsworth, CA), plasmid DNA was isolated and digested with Kpn I and Sal I to confirm gene presence.

For RNAi construct deveopment, RNAi primers were designed using the same software as described above with the longer fragment having BamH I and Kpn I enzyme sites and the shorter fragment contained BamH I and Sal I enzyme sites (Table 3.1). After which, the longer and shorter fragments were PCR-amplified with above primers, respectively. Then the shorter fragment was cloned into the p1300S plasmid with BamH I and Sal I enzymes, and followed by cloning the longer fragment into p1300S plus the shorter fragment with BamH I and Kpn I enzymes.

Both the overexpression construct and the RNAi constructs were then transformed into *Agrobacterium tumefaciens* strain EHA 105 using the freeze-thaw method. Briefly, 1 µg of above each plasmid DNA was added to thawed competent EHA105 cells, the mixture was then placed on ice for 5 min, followed by liquid nitrogen for 5 min, and 37°C for 5 min, and then 700 ul LB was added, and the mixture was incubated at 28°C for 2-4 hours for recovery. The solution was then spun down at 7000 rpm for 3 min to pellet, re-suspend, and plated on LB plus kanamycin (50 mg/L) and grown at 28°C for 2-3days. Colonies were then chosen, placed in 5 ml KB plus 50 mg/L kanamycin and grown overnight at 28°C while shaken at 220 rpm. Plasmids were isolated as described above and digested with appropriate enzymes to confirm inserts.

Sequencing to confirm presence of GST genes

Genes were sequenced to confirm that the correct gene was present using GenomeLab™ Dye Terminator Cycle Sequencing with Quick Start Kit following manufacturers' protocol on a Beckman Coulter CEQ™ 8800 Genetic Analysis System (Brea, CA). Approximately 30 ng of DNA template was used for the sequencing reaction.

Callus induction and maintenance

Switchgrass seeds were surface-sterilized by treatment with 70% ethanol for 2 min, rinsed 3X with distilled water, de-husked for 30 min with 60% H2SO4 with stirring, washed 3X with distilled water, sterilized with 0.4 M sodium hypochlorite (50% commercial bleach solution containing 6% sodium hypochlorite) containing 0.1% Triton 100 for 30 min, followed by 5X rinse with sterile, distilled water (ddH2O). Surface-sterilized seeds were placed on callus induction medium (4.43 g/L MS +V (Phytotech Labs# M519, Shawnee Mission, KS), 45 µ*M* 2,4-D, 5 µ*M* BA, 30 g/L maltose, 3 g/L Phytagel®) for one month. The calli with embryogenic tissue formation were then chosen and placed on callus maintenance media (same as induction medium except for 22.5 µ*M* 2,4-D) and sub-cultured monthly under dark conditions.

Switchgrass transformation using the agrobacterium-mediated method

Agrobacterium tumefaciens strain EHA 105 cells containing either the overexpression or the RNAi construct were plated as described above and incubated at 28°C for 48 hours. Fresh colonies were then picked and inoculated in 5 ml LB medium plus 50 mg/L kanamycin and shaken at 220 rpm overnight. Next day, 5 ml of the overnight culture was added to 45 ml LB medium in a sterile flask, incubated at 28°C, and shaken at 220 rpm, until an OD 600 achieved 0.5 to 0.7. Cells were then pelleted by centrifugation at 4°C, 3000 rpm, for 15 min. Supernatant was discarded, and the pellet was re-suspended in approximately 20 ml suspension medium (4.43 g/L MS+V, 30 g/L maltose, pH 5.5 containing 100 µ*M* acetosyringone) in sterile 50 ml tubes. Freshly growing embryogenic switchgrass cv. Alamo calli were added and gently shaken for 30 min at room temperature. The calli were then blot dried on sterile paper towels to remove excess bacteria and placed on co-cultivation medium (4.43g MS+V, 30 g maltose, 22.5 µ*M* 2,4D, 5 µ*M* BA, 100 µ*M* acetosyringone, 3 g/L Phytagel®, pH 5.5) for 5-7 days. The co-cultivated calli were then washed in sterile ddH_2O and transferred to selection medium (same as induction medium with the addition of 300 mg/L cefotaxime and 50 mg/L hygromycin) for one month. The

hygromycin-resistant calli were then transferred to regeneration medium. Plantlets were transferred to liquid shoot multiplication medium for propagation. Empty pCambia1300S vector was also transformed into cv. Alamo, and used as a control in RNAi and overexpression experiments. Individual transformation events were labeled and later used to determine relative expression.

GST gene expression analysis of transgenic plants with qPCR

RNA was extracted as described above and stored at -80°C until use and qPCR was performed as described above

Response of transgenic lines to PsJN bacterization

Growth promotion

After confirmation of relative expression levels, transgenic lines were chosen for PsJN growth promotion experiments. PsJN bacterization was performed as described above, and thirty to sixty uniform plantlets were chosen from both RNAi and overexpression transgenic event lines. Plantlets were trimmed to approximately 2.5 cm in length and dipped in either the PsJN or the PBS buffer alone for one min, blot-dried with sterile paper towel, and placed on switchgrass growth medium in GA7 Magenta vessels (Sigma-Aldrich) containing 50 ml of media (4.43 g/L MS +V, 30 g/L maltose, and 3 g/L phytogel, pH 5.8), with 5 plantlets per vessel, and grown for one month in the incubator as above. Root and shoot lengths and plantlet fresh weights were then determined.

GST assay

Both bacterized (PsJN) and buffer treated (CK) transgenic RNAi and overexpression lines were assayed for GST activity at day 0, 2, and 7 days post treatment using the GST Colorimetric Activity Assay kit (KT-204, Kamiya Biomedical Company, Seattle, WA, USA). The tissues were ground with motor and pestle with 2 ml GST buffer per gram of tissues. The samples were then centrifuged at 10,000g, 4°C for 15 min, and the supernatant was removed and placed in a new tube. The assay was performed in 96 well plates duplicately, according to the manufacturer's protocol. Absorbance was measured at 340 nm at 0, 5, 10, 15, and 20 min after initiation of reaction. Absorbance values were plotted, and the slope (rate) was determined for the linear portion of the curve (ΔA340). GST activity was calculated using the GS-DNB

extinction coefficient at 340 nm 0.0096 μM^{-1} cm^{-1}. This value has been adjusted for the path length of the solution of the well (0.262 cm) with a volume of 0.1 mL). Protein concentration was determined using Bio-Rad Protein Assay using BSA as a standard.

Results

GST expression - microarray data

To identify important changes in gene expression during colonization and early growth promotion, two switchgrass cultivars, responsive Alamo and non-responsive Cave-in-Rock, were chosen for global gene expression profiling comparisons based on previous results with *Burkholderia phytofirmans* strain PsJN (Kim et al., 2012). Samples were taken at 0 (control), 0.5, 2, 4 and 8 days after PsJN inoculation, and global gene expression analysis was performed at the Genomics Core Facility at the Noble Foundation using EST microarrays. From the microarray data, approximately 50 genes were initially chosen showing apparent differences in the expression levels between PsJN-inoculated Alamo and Cave-in-Rock. Among these genes, glutathione S-transferase (GST) was chosen for further functional studies using overexpression and RNAi knockout/knockdown techniques **(Figure 3.1)**. This data indicates that GST expression is up-regulated in cv. Alamo at 0.5 days and continued to increase in expression levels at 2 and 4 days, and remained high by 8 days after bacterization. In contrast, GST expression in switchgrass cv. Cave-in-Rock was not up-regulated after PsJN bacterization. The GST gene was next explored using Blast alignment (http://www.ncbi.nlm.nih.gov/) revealing closest match to GST protein in corn (*Zea mays*) of the tau subfamily, specific to plants **(Figure 3.2a and b)**, and primers were designed for further study **(Table 3.1)**.

Real time PCR confirmation of GST gene expression

Since the microarray data indicated cv. Alamo GST expression increases in response to bacterization, this gene expression pattern was verified through quantitative PCR (qPCR) **(Figure 3.3)**. qPCR relative transcript patterns were similar to the reported microarray data. The largest differences noted between Cave-in-Rock and Alamo was immediately post bacterization and then, to a lesser level, at day 8.

GST transgenic plants

Overexpression and RNAi transgenic generation

To study GST role during growth promotion by PsJN, GST gene overexpression and RNAi constructs were created for switchgrass transformation (**Figure 3.4)**. Both the constructs **(Figure 3.5 and 3.6)** were used to transform switchgrass cv. Alamo embryogenic calli, and transgenic plants were generated.

qPCR analysis of transgenic lines

A total of 3 RNAi transgenic lines and 8 overexpression lines were generated, and qPCR was performed to determine gene expression levels relative to control plants, which were transformed with the p1300S vector alone. GST RNAi transgenic plants **(Figure 3.7)** all had lower expression levels compared to control plants at approximately 20% of the control plants levels. GST overexpression lines exhibited more variation **(Figure 3.8)** with lines #33 and #26 showing the highest expression levels (9.1 and 8.7-fold respectively); lines #7, 17 and 29, intermediate expression levels , ranging from 5 to 2.8-fold expression levels; and lines #9, 6, and 36 exhibiting only 1.3 to 2 relative GST expression levels, compared to control plants..

GST enzyme activity of transgenic plants after challenged with PsJN

Figure 3.9 represents the summary of GST enzyme activities of control (buffer inoculated), GST RNAi transgenic plant lines, GST overexpression plant lines, and plant lines transformed with an empty p1300S vector. Overall, the trends are similar to the gene expression data found in both the microarray and qPCR analysis. The first time point at day two was lower overall than the next time point at day 7 post bacterization. Finally, the last time point measured at day 14 was similar to day 7 indicating the activity had leveled off. Interestingly, GST enzyme activity in RNAi lines #2 and 3 were higher than p1300S control transgenic plants and overexpression plants, which were similar to control.

Growth experiments of transgenic plants after PsJN challenge

Initial experiments were conducted with a group of plants from the overexpression lines combined. Biomass data **(Figure 3.10)** indicated the group exhibited no difference in growth trends compared to either buffer inoculated plants or p1300S control transgenic plants. However, when shoot and root lengths were measured, shoot lengths of GST overexpression plants were significantly longer compared to controls **(Figure 3.11)**. To explore these differences further, PsJN bacterization studies were undertaken with both RNAi and overexpression transgenic plant line clones. **Figure 3.12** are the results of the RNAi transgenic lines with bacterization and

demonstrated lost ability in growth response of RNAi lines to PsJN bacterization compared to controls of the same transgenic lines as well as in general compared to p1300S control plants. Results of GST overexpression lines with bacterization were somewhat different **(Figure 3.13)**, with all but one line tested showing increased growth compared to control in the same transgenic lines. Line 26 plants grew significantly larger than p1300S after PsJN inoculation.

Discussion

Tau class GSTs, one of the most prominent plant specific GSTs, play an important role in stress responses to a variety of both endogenous and exogenous challenges, including pathogen attack, wounding, and oxidative and temperature stresses (reviewed in Frova, 2003). The enzyme class overall responds to diverse biotic and abiotic challenges, and an important commonality exists, the generation of active oxygen species (Reviewed in Marrs, 1996). This is supported by the findings that plant GSTs also have GSH-dependent peroxidase activity, are widely distributed among plants, and are found in every tissue examined and at every stage of plant development (Cummings et al., 1999). During endophytic colonization of the host plant, some of these same pathways have been shown to be involved with successful colonization and/or pathogen recognition (Ramu et al., 2002). However, the molecular mechanisms regulating GST expression in plants are still largely unknown (reviewed in Frova, 2003). Because of these factors, the *GST* gene which was shown to be up-regulated in switchgrass cv. Alamo during colonization and during early stages of growth promotion was chosen for further characterization. Based on alignments **(Figure 3.2b)**, the GST tau enzyme appears to be a dimeric enzyme where the N-terminal domain of one subunit is adjacent to the N-terminal domain of its partner, and tau class dimers are hydrophilic (Armstrong, 1997).

Our results of microarray analysis indicated that, in switchgrass cv. Alamo, expression of the *GST* gene post PsJN bacterization increases at day 0.5, 2, and 4 and then declines slightly at day 8 compared to cv. Cave-in-Rock, which showed no response in gene expression **(Figure 3.1)**. Real-time PCR data was similar to the microarray findings **(Figure 3.3)**. Together, this report indicates that GST may be involved in successful colonization and early growth promotion in a genotype specific manner in switchgrass cv. Alamo compared to cv. Cave-in-Rock. When transgenic plants were created which both overexpressed (**Figure 3.8)** and decreased expression **(Figure 3.7)** and subsequently bacterized with PsJN, a range of GST activity was observed in

both overexpression and RNAi lines, indicating the plants may be compensating for either gene expression changes as artificial changes in gene expression may spur pronounced secondary effects in the genome (Teng et al., 2013). To test if overexpression or RNAi transgenic lines exhibited changes in growth promotion by PsJN bacterization, the three RNAi transgenic lines and four overexpression lines were bacterized, grown for one month, and the fresh weight was determined **(Figure 3.12 and 3.13, respectively)**. Plants transformed with an empty vector were also tested with PsJN and demonstrated the normal growth promotion responses typically seen with PsJN bacterization (Kim et al., 2012). RNAi lines generally demonstrated ability lost in growth promotion by PsJN compared to control p1300S transgenic plants. The two overexpression lines with the highest levels of expression (#33 and #26) performed similar to or outperformed controls, respectively. Line 26 was significantly larger after PsJN bacterization ($p<0.01$) compared to either line 26 controls or p1300S controls which were also bacterized. Much of the increases driving these differences were from root growth. However, overexpression lines with moderate levels of overexpression (#7 and #29) showed a decrease in response compared to control p1300S transgenic plants. Together, this report demonstrates that glutathione-S-transferase from the tau class may have a genotypic effect on early colonization and growth promotion observed between Alamo and Cave-in-Rock switchgrass cultivars. More work is needed to clarify these observations.

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Figures and Tables

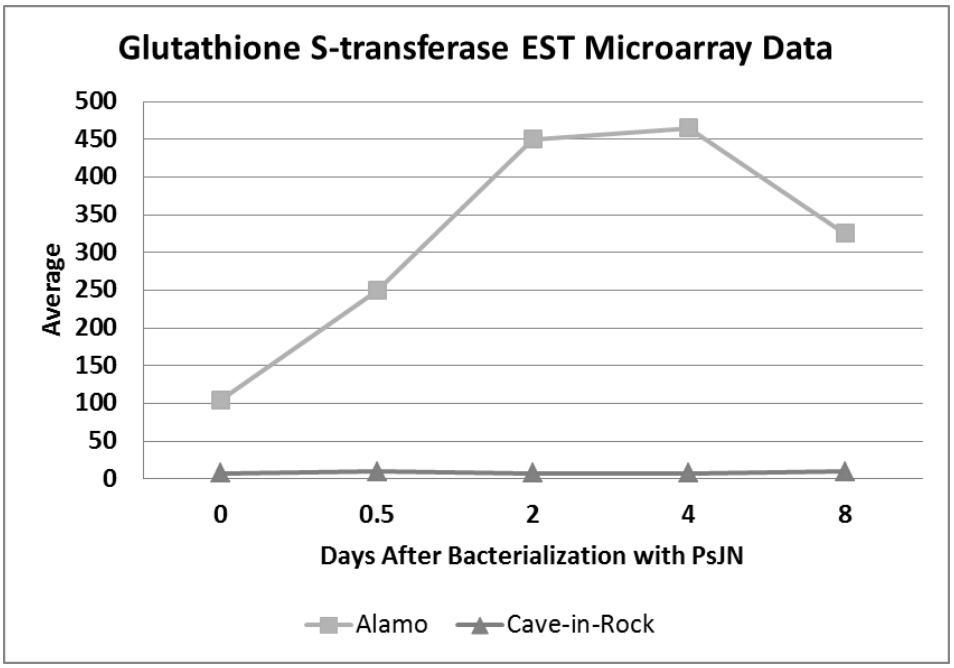

Figure 3.1 EST microarray data from switchgrass cvs. Alamo and Cave-in-Rock. Points represent the average expression levels of the glutathione S-transferase gene 0, 0.5, 2, 4, and 8 days after bacterization with *Burkholderia phytofirmans* strain PsJN. Values equal the average of three biological replicates.

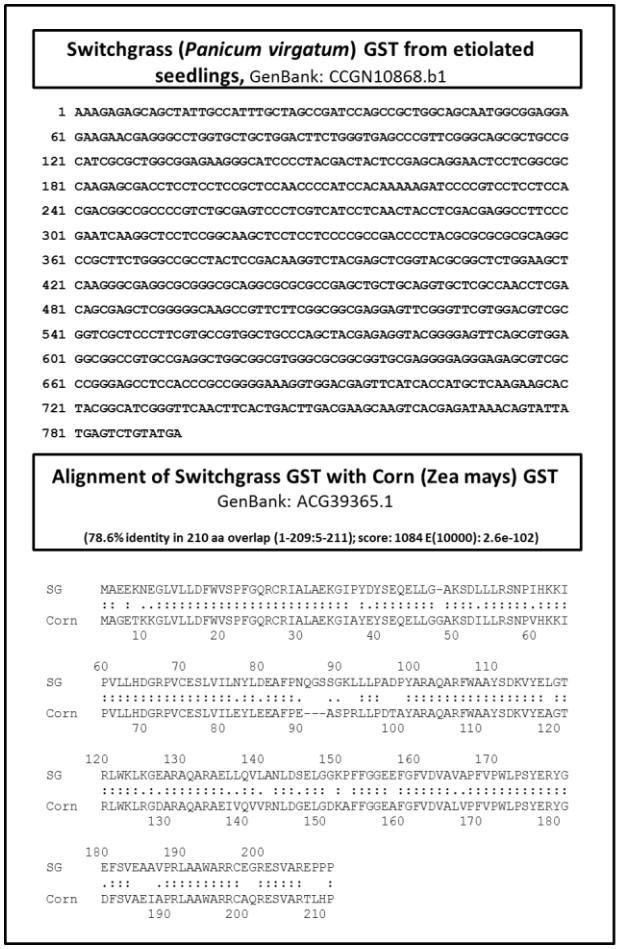

Figure 3.2a Switchgrass GST gene and alignment with corn GST.

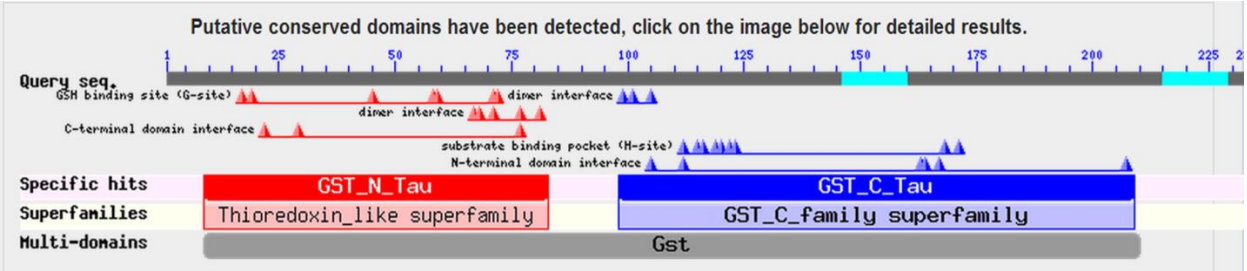

Figure 3.2b Protein blast using translated switchgrass GST gene. The protein most similarly matches the tau GST family and contains the GSH binding site.

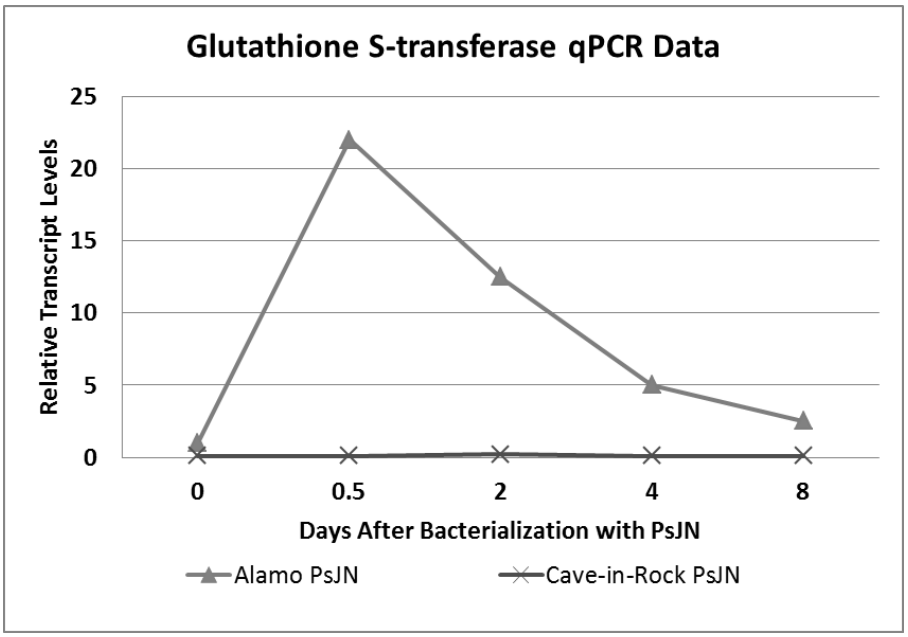

Figure 3.3 qPCR to determine relative transcript levels. Values are at 0, 0.5, 2, 4, and 8 days after bacterization with *Burkholderia phytofirmans* strain PsJN or buffer alone (CK). Relative transcript levels were normalized using UBQ-10 as the endogenous control, and the value of 0 days was set at 1. Each value represents a mean of two technical replicates and three biological replicates.

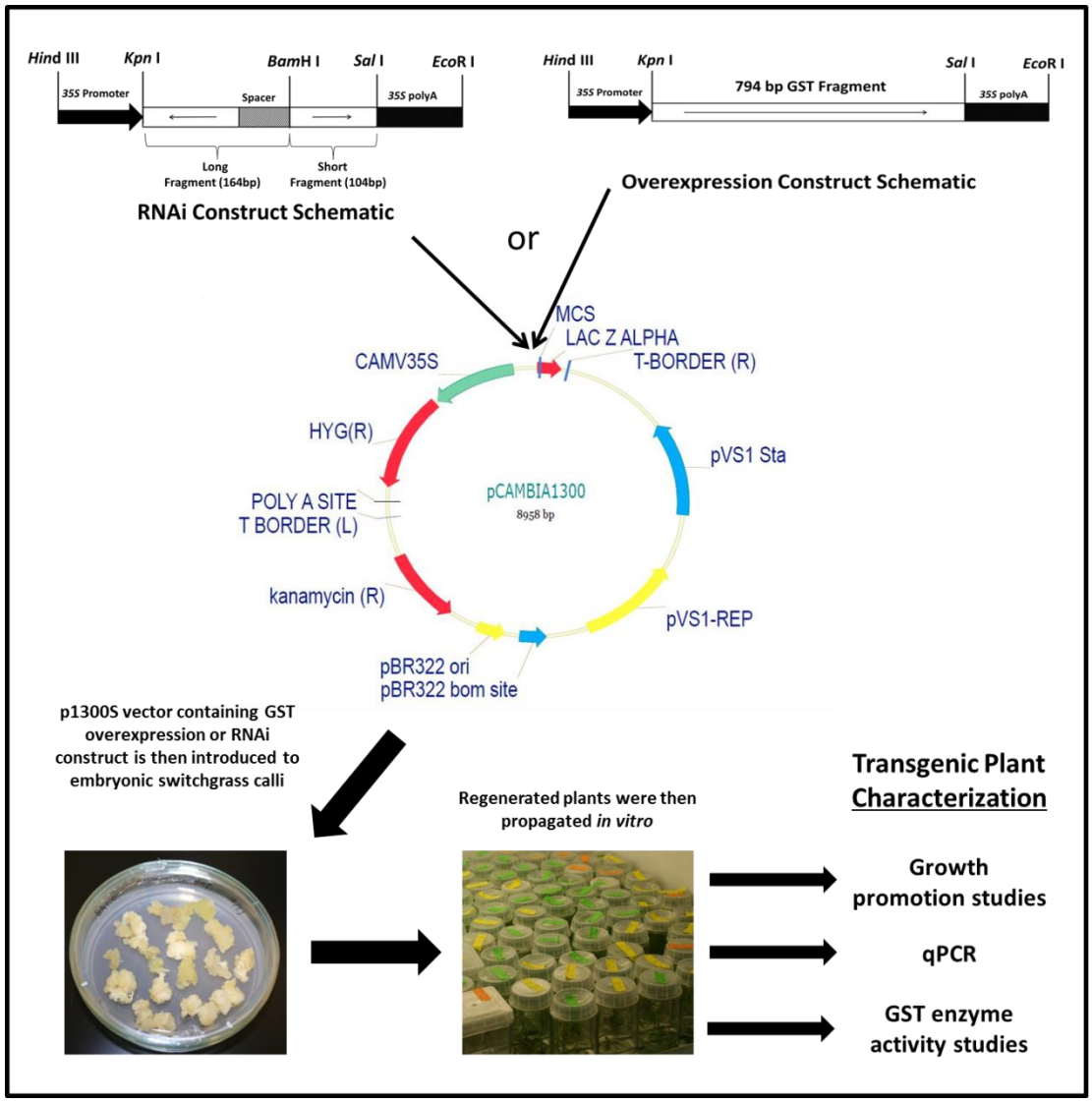

Figure 3.4 Schematic of RNAi and overexpression constructs. Highlighted are places of insertion in the pCambia1300S multiple cloning site, and subsequent transformation and transgenic plant characterization

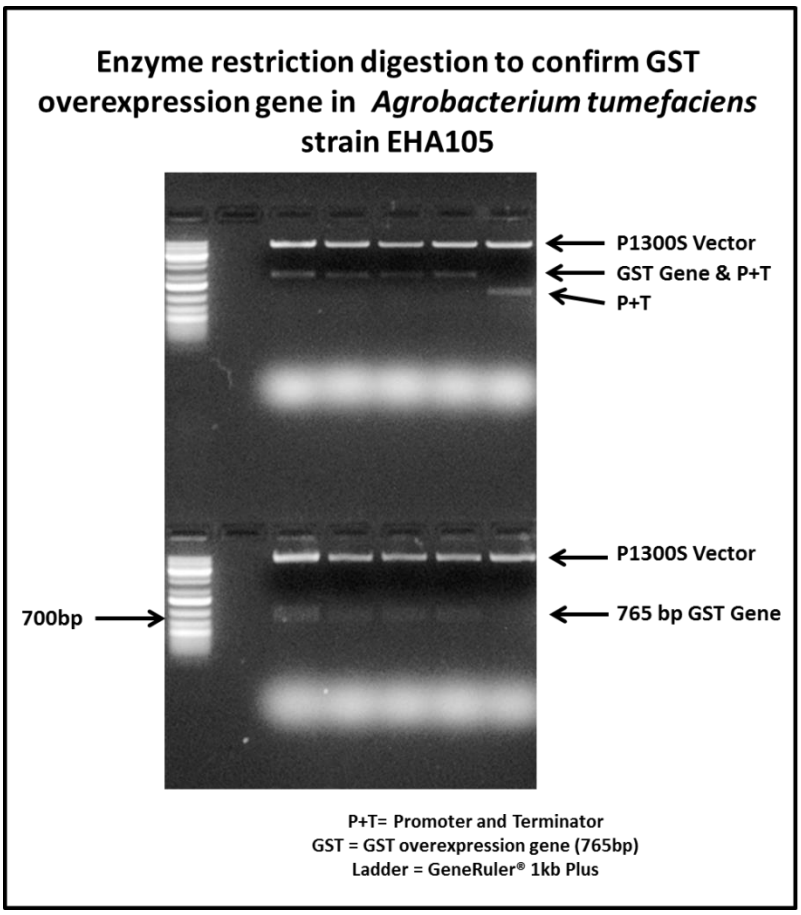

Figure 3.5 Gel confirmation of overexpression GST gene transformation. *Hin*d III and *Eco*R I double restriction digestion was used to release the promoter and terminator (P+T) and GST gene and *Kpn* I and *Sal* I double restriction digestion was used to release GST gene alone.

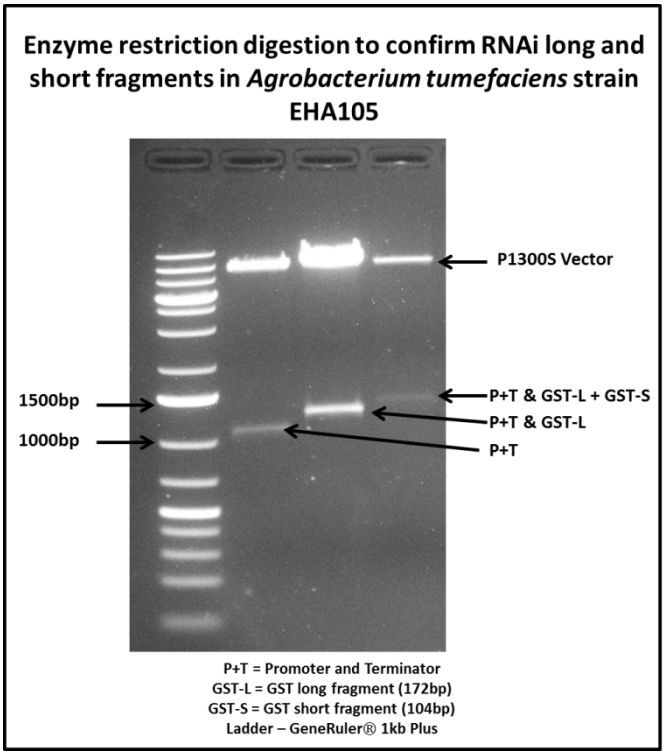

Figure 3.6 Gel confirmation of RNAi GST gene transformation. *Hin*d III and *Eco*R I double restriction digest was used to release the promoter and terminator (P+T) + GST-L and (P+T) + GST-L + GST-S genes.

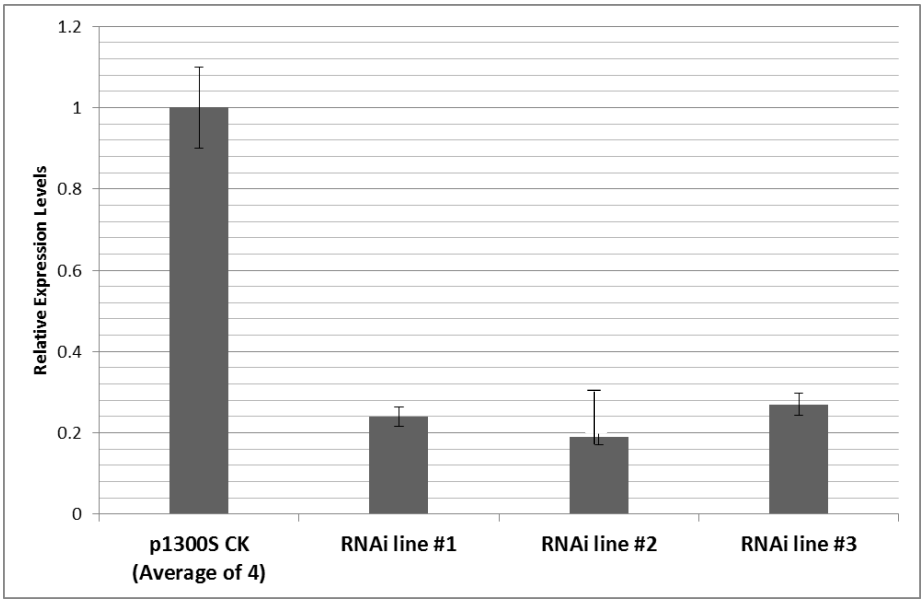

Figure 3.7 Gene expression was measured using qPCR in RNAi transgenic plant lines. Relative transcript levels were normalized using UBQ-10 as the endogenous control, and the value of control was set at 1. Each value represents a mean of three biological replicates. Values were determined using the method of Livak and Schmittgen (2001).

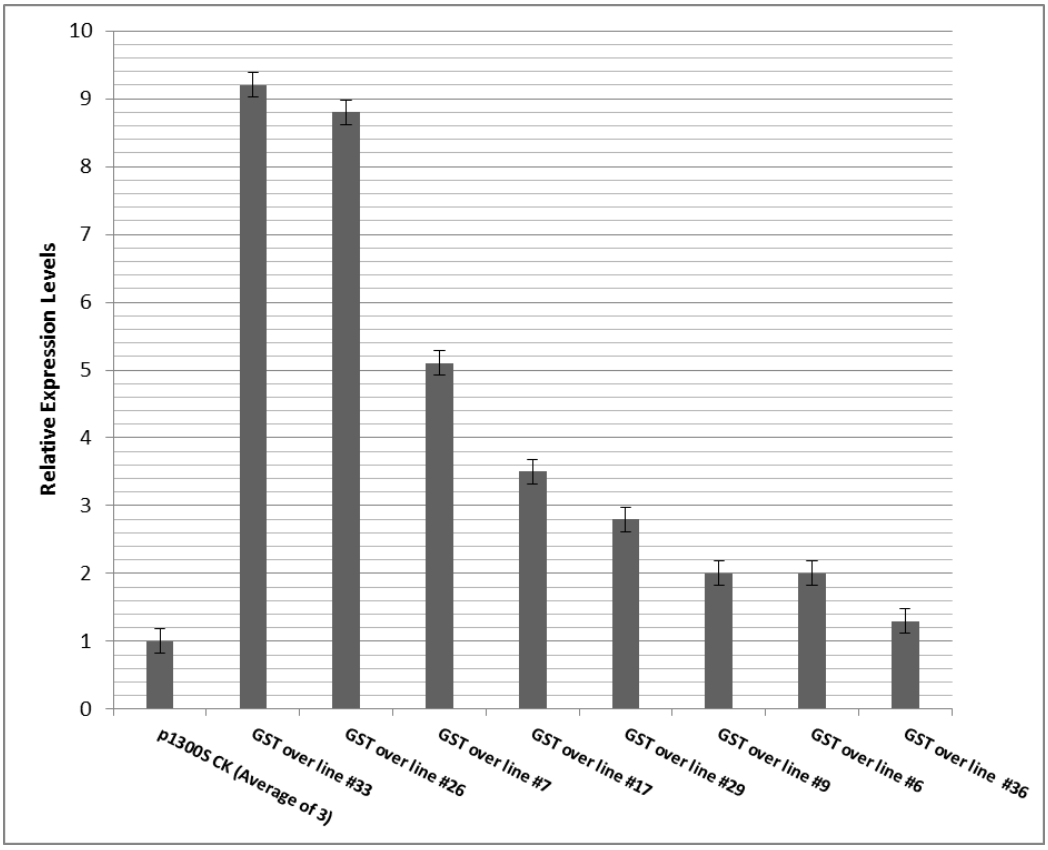

Figure 3.8 Expression levels of switchgrass GST overexpression plants. Relative transcript levels were normalized using UBQ-10 as the endogenous control, and the value of control was set at 1. Each value represents a mean of three biological replicates. Values were determined using the method of Livak and Schmittgen (2001).

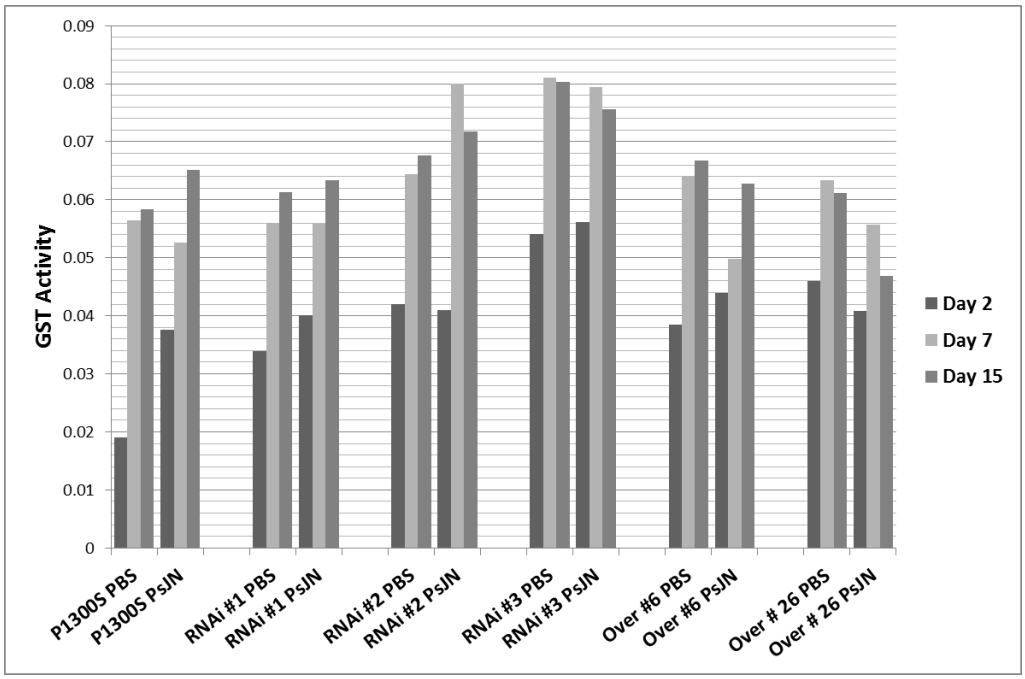

Figure 3.9 GST activity in transgenic lines after PsJN bacterization. GST activity was measured at days 2, 7, and 15 in transgenic lines after PsJN bacterization. Each point represents the average of two biological replicates.

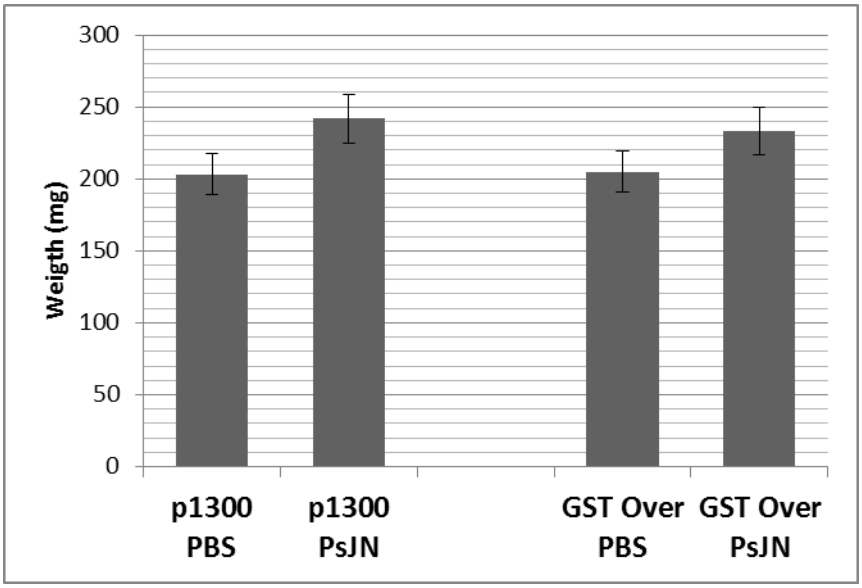

Figure 3.10 Initial GST transgenic growth data. Plants were inoculated as described, and growth data was recorded at 2.5 months.

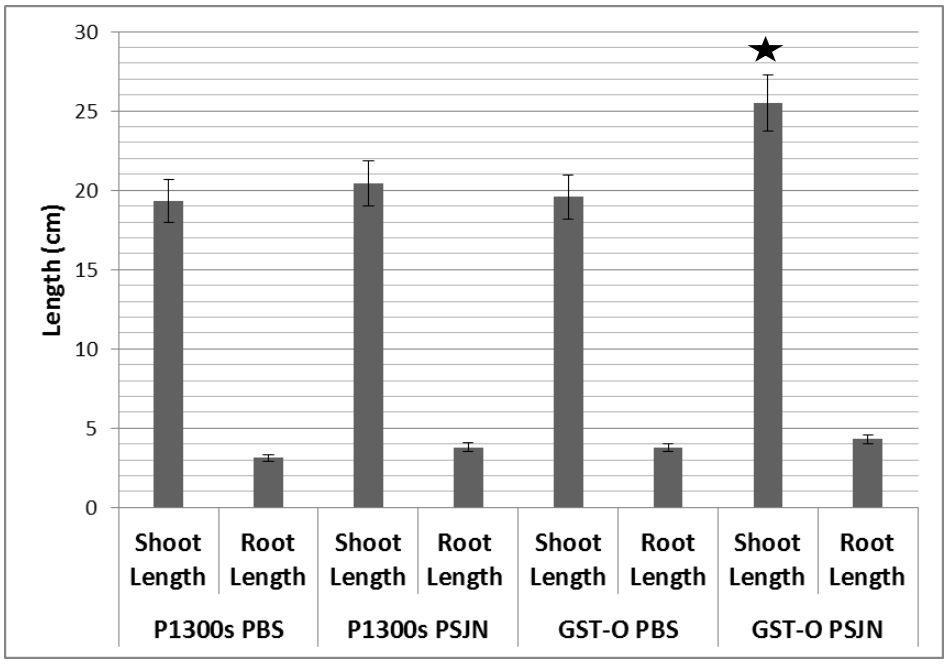

Figure 3.11 Initial growth data from transgenic GST overexpression plants. Inoculations were performend with either CK (buffer) or PsJN. ★ Statistically significant ($p<0.05$)

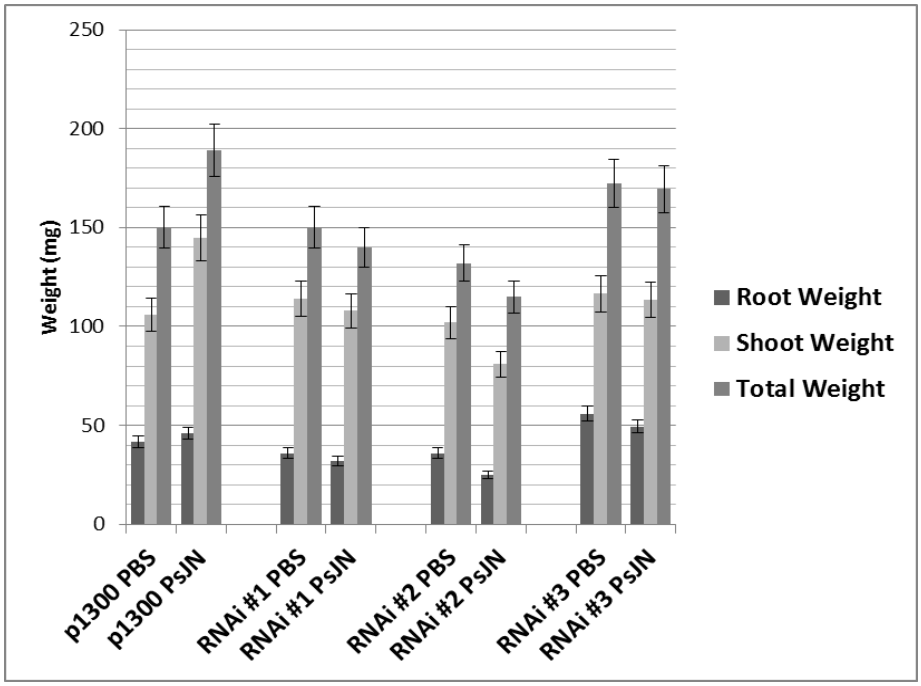

Figure 3.12 Growth at 30 days in GST RNAi transgenic plants after bacterization. Measurements were taken after control (p1300S) and RNAi transgenic plants were challenged with either buffer alone (PBS) or PsJN and plants were grown for 1 month.

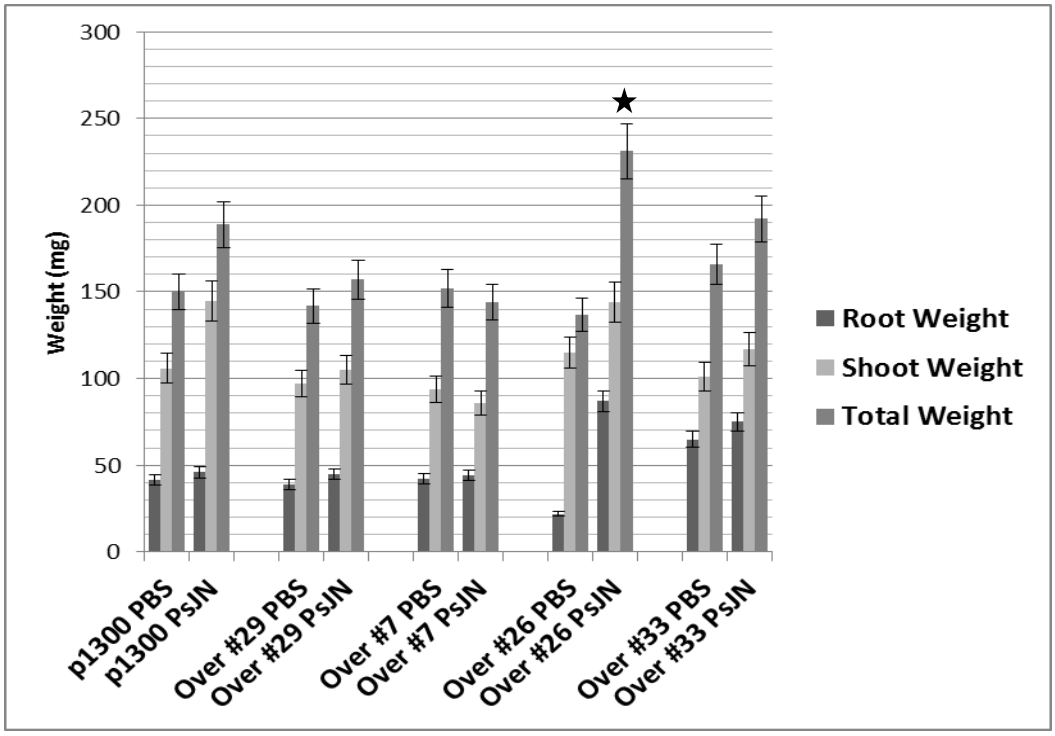

Figure 3.13 Growth at 30 days of the GST overexpression transgenic plants. Measurements were taken one monthafter plants were bacterized with PsJN. ★ Statistically significant ($p<0.05$)

Table 3.1 Primers for GST cloning and qPCR.

Description	Switchgrass ID probe	Rice Locus	Annotation	Primers	Product Size (bp)
qPCR primers	AP13ITG69022	LOC_Os07g28480	Glutathione S-transferase, putative, expressed (GST)	TCCATTCACGGAATCACTCA GAAGCACTACGGCATCGAGT	118
Overexpression primers	AP13ITG69022	LOC_Os07g28480	Glutathione S-transferase, putative, expressed (GST)	Gene was synthesized	794
RNAi primers	AP13ITG69022	LOC_Os07g28480	Glutathione S-transferase, putative, expressed (GST)	GST-BamHI-F1: GGATCCACGAGAGGTACGGGGAGTTC GST-KpnI-R1: GGTACCAGTGAAGTTGAACCCGATGC	164
				GST-BamHI-F2: GGATCCGAGGGGAGGGAGAGCGTCG GST-SalI-R2: GTCGACAGTGAAGTTGAACCCGATGC	104

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Chapter 4

Strategies for enhancement of switchgrass (*Panicum virgatum* L.) performance under limited nitrogen supply based on N-fixation by bacterial endophytes

J. Scott Lowman[1,2] Chuansheng Mei[1,2*], and Jerzy Nowak[2*]

Abstract

Sustainable agricultural production in the 21st century requires new approaches to reduce the use of synthetic nitrogen fertilizers to prevent pollution from runoff and nitrification. Free living atmospheric nitrogen fixing bacteria which are present throughout the environment and have the ability to tap into the vast environmental reserve of di-nitrogen in air have been overlooked in the development of sustainable cropping systems. A newly recognized option for supplying plants with fixed nitrogen is through the use of N-fixing beneficial bacterial endophytes. The objective of the presented study were to explore strategies for nitrogen supply to plants via 1) identification, isolation, and utilization of naturally occurring N-fixing endophytes, and 2) harnessing the ability of horizontal gene transfer between bacterial endophytes to fix atmospheric nitrogen and promote plat growth. We have identified and isolated a strain of *Sphingomonas* sp. from surface sterilized switchgrass cv. Alamo grown in nitrogen-free hydroponic medium. The bacterium significantly ($P<0.01$) promoted plant growth in nitrogen deficient conditions. The capacity to promote switchgrass growth under nitrogen deficient conditions was also shared via transformation of the ability to fix nitrogen from *Burkholderia phymatum* to the plant growth promoting *Burkholderia phytofirmans* strain PsJN. The transformed PsJN was able to promote growth under nitrogen deficient conditions compared to the wild type and non-bacterized control.

Keywords: Atmospheric Nitrogen Fixation, Endophytes, Vertical Transmission, Horizontal Gene transfer, *Burkholderia phytofirmans* strain PsJN, Growth Promotion, Bioenergy, Marginal Lands, Switchgrass

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*Both contributed to project development and supervision.

Introduction

While the Green Revolution yielded significant increases in plant production over the past fifty years, based on advances in crop breeding for high input production systems, little concern was given to the impact of excessive use of fertilizer and irrigation on the environment (Rejesus and Hornbaker, 1999). It is estimated that fifty to sixty percent of soil surface application of synthetic nitrogen fertilizer is not utilized by target plants, thereby contaminating the surrounding ecosystem through surface runoff and erosion into streams, which cause eutrophic dead zones (Tonitto et al., 2006; Rejesus and Hornbaker, 1999) and through denitrification, which contributes to greenhouse effects (Tilman et al., 2002). Plants utilize a number of different forms of nitrogen, including nitrate (NO_3), ammonium (NH_4), and organic nitrogen ($C\text{-}NH_2$), but are unable to break the strong triple bond in atmospheric N_2 (Alberty, 1994). Of the approximately 170 Tg (teragrams) of total farmland nitrogen utilized each year, only about 40 Tg comes from biological nitrogen fixation (BNF); the remaining comes synthetically produced by the Haber-Bosch process (reviewed in Fields, 2004), mining of minerals (Erickson, 1983) and mining of guano (Clark and Foster, 2009). Furthermore, prices of nitrogen fertilizers are linked to commodity prices which likely fueled a steep increase in prices in 2007 (FAO, 2009) and guano, which is highly prized in the organic fertilizer market, has seen prices double recently and supplies are likely to be exhausted in the next two decades (Clark and Foster, 2009). It is becoming clear that future sustainable agricultural practices need to reduce the use of synthetic nitrogen fertilizers (Donner and Kucharik, 2008; Weekley et al, 2012) and develop cropping systems that better integrate biological nitrogen fixation (BNF), the predominant supplier of nitrogen in the worlds' natural ecosystem, should be a primary goal (Roesch et al., 2008).

Prokaryotes are the only organisms that can fix atmospheric di-nitrogen (Markmann, 2009) and make it available to plants and they can be symbiotic, free-living, or associative, forming casual associations with plants (reviewed in Welbaum et al., 2004). Associative endophytic nitrogen fixing bacteria, which reside in the internal tissues of plants, can enhance plant growth by supplying small, but adequate amounts of fixed nitrogen directly to the plant (reviewed in Welbaum et al., 2004). While bacteria that form nodules in a symbiotic relationship with plants are the most widely studied nitrogen fixing associations, the study of associative nitrogen fixing endophytes continues to gain momentum (Riggs et al., 2002). Nitrogen fixing bacteria also live

in association with plants in the rhizosphere, where they trade fixed nitrogen for carbon compounds in root exudates (reviewed in Cocking, 2003).

Nitrogenases, the enzymes utilized by prokaryotes to convert atmospheric N_2 to NH_3, which can be utilized by plants, are complex metalloenzymes with highly conserved structural and mechanistic features (reviewed in Alberty, 1994; Burgess and Lowe, 1996; Rees and Howard, 2000). The enzyme contains two components; the smaller component, encoded by the *nifH* gene, is known as the Fe protein, is a dimer and an ATP dependent electron donor; and the larger component, encoded by *nifDK*, known as the MoFe protein, is a heterotetramer that contains the enzyme catalytic site (reviewed by Dixon and Kahn, 2004). The coding sequences for the nitrogenase enzyme operon are located in a conserved *nif* cluster, and include over 50 genes involved in its expression, biosynthesis, maturation, and assembly (Menard et al., 2007). Nitrogenase is limited by several important physiological constraints. First, the Fe protein, one of the two subunits which make up the nitrogenase enzyme, is denatured by oxygen (Thornely, 1985), however, endophytes have evolved compounds such as triterpenes, involved in the protection of nitrogenase from oxidation in a free-living state (Santhi et al., 2012). Second, the nitrogenase enzyme has a relatively slow turnover rate (Thornely, 1985), requiring the bacteria to synthesize large quantities, comprising of up to twenty percent of the total protein in the cell (reviewed in Dixon and Kahn, 2004). Finally, reduction of atmospheric di-nitrogen requires 16ATP, making it one of the most energy demanding reactions identified in bacterial organisms (Thornely, 1985). All combined, the amount of protein required to form an active enzyme complex, its protection from oxygen, and the high energy requirement for driving nitrogen fixation place a large burden on nitrogen fixing bacteria. Thus, the synthesis and subsequent activation of the nitrogenase enzyme is stringently regulated at the genetic level (reviewed in Raymond et al., 2004). While nitrogenase is sensitive to oxygen, oxygen is required for ATP production, therefore O2 intercellular concentration is regulated by mitochondrial respiration and conformational protection of the nitrogenase enzyme because of the location of nitrogenase inside the cell membrane (Gausch et al., 2001).

To benefit from endophytes capable of biological nitrogen fixation (BNF), several strategies may be employed to ensure the presence of BNF *in planta*, and when combined with breeding programs which emphasize BNF, together may increase the sustainability of agriculture by

reducing reliance on synthetic N fertilizer. Two strategies in particular are explored in this study: 1) vertical transmission of free living BNF endophytes via the seeds, and 2) sharing of the genes involved in BNF with other bacterial endophytes through horizontal gene transfer.

Strategy One: Identification of vertically transmitted nitrogen fixing endophytes capable of improving switchgrass growth *in vitro*

Introduction to vertical transmission

Research has shown inconsistent plant performance by bacterial inoculants for a number of reasons, including failure to establish proper population sizes, to colonize appropriate plant parts, or to express proper traits at the appropriate time (Compant et al., 2005). There is a need to understand and develop new mechanisms to ensure the stability of promising beneficial endophytes *in planta* generation after generation, and vertical transference from parent to offspring is considered here as one possible solution. Vertical transmission of microorganisms could potentially occur through the external seed coat, vegetative tissue as in the case of tubers, fruit, and those that have been identified to occupy the internal tissue of seed. Although many different endophytic bacteria have demonstrated beneficial interactions with plants (Compant et al., 2005), studies of naturally-occurring beneficial endophytes that are transmitted vertically to seeds are limited. For example, plant growth promoting bacteria have been isolated from surface sterilized seeds of Norway spruce (Cankar et al., 2005), eucalyptus (Ferreira et al., 2008), tobacco (Mastretta et al., 2009) and rice (Mukhopadhyay et al., 1996). Bacterial taxa such as *Bacillus, Erwinia, Flavobacterium, Pseudomonas, Cytophaga*, *Leuconostoc*, *Micrococcus*, and *Xanthomonas* were isolated from surface-sterilized seeds of a variety of plants in earlier studies (Mundt and Hinkle, 1976; Bacon and Hinton, 1996). In Norway spruce seeds, *Pseudomonas* and *Rahnella* were isolated (Cankar et al., 2005). Bacterial genera isolated from the seeds of Norway spruce and eucalyptus differed indicating diverse bacterial populations may inhabit seeds from different species (Cankar et al., 2005). These interactions are likely complex and varied, and the role of endophytes in seeds may be controversial (Hallmann et al., 1997).

The first step in understanding vertical transference, particularly with endophytes that are able to occupy the internal seed embryonic tissue, is to study the mechanisms of seed population establishment. Much of the work regarding this question has focused on plant and human pathogenic microorganisms that are vertically transferred to the host plant offspring.

Additionally, several factors complicate this understanding, including the low percentage of culturable bacteria in general, the finding that some vertically transmitted pathogens may remain asymptomatic throughout the hosts' life cycle (Darrasse et al., 2007), the low number of microorganisms often found in seed (Lemaire et al., 2011), and inconsistent or imperfect efficiency of vertical transmission (Mundt and Hinkle, 1976). If, however, research reveals the essential mechanisms of vertical transference, either with pathogenic or beneficial microorganisms, it may be possible to select for organisms that provide consistent advantages, including direct growth promotion, stress resistance, and bio-control.

While little work to date has focused on vertical transmission, endophytic bacteria isolated from seeds of Norway Spruce collected from different locations have been shown to occupy the same genera, indicating established, vertically transmitted endophytes for this species *in vivo* (Cankar et al., 2003). These observations are consistent with findings of endophyte-host interactions in general, because due to specific secondary metabolism and morphology, bacteria show a certain degree of specificity for each plant species, even different genotypes within the same species (Kim et al., 2012; Berg and Smalla, 2009). In a broader survey, 15% of surface sterilized seeds of herbaceous plants including okra, pawpaw, radish, squash, maize, barley, rye and wheat and 16% of seeds of woody plants yielded culturable bacteria (Mundt and Hinkle, 1976). Bacteria were also identified in 30 – 40% of maize and pea seeds (Samish et al., 1963) and coffee seeds (Vega et al., 2005).

Importantly, research has demonstrated that vertically transmitted endophytes may remain in supply generation after generation (Bacon et al., 2001). *Pantoea agglomerans*, an endophytic bacterium associated with providing benefits to plants, isolated from surface sterilized seeds of Eucalyptus, was gfp tagged, inoculated into seeds, and re-isolated from seedlings (Ferreira et al., 2008). When endophytes are faithfully transmitted from parent to progeny, they may be generally under selection pressure to maintain and even enhance their contributions to plant growth promotion and bio-control because they are also dependent on the performance of their host plant (Darsonval et al., 2008). By extension, properties including bio-control, associated with faithfully transmitted endophytic populations may improve over time when these populations flourish (Struz and Matheson, 1996). As a result, vertically transmitted endophytes may exhibit multiple mechanisms of action. When endophytes were isolated from seeds of 12

different soybean cultivars, 18% of them were able to inhibit growth of pathogenic fungi, 39% were able to help acquire phosphates via solubilization, and 100% were able to produce IAA, a plant growth promotive hormone (Assumpcao et al., 2009). *Rahnella aquatilis* was isolated from seeds of *Brassica napus* and demonstrated the ability to fix atmospheric nitrogen (Grane'r et al., 2003). *Rahnella* and *Pseudomonas* were isolated from surface sterilized Norway Spruce seeds, and each has been associated with both plant growth promoting activities and bio-control (Katarina et al., 2005). Tubers, another potential vessel for vertical transmission, also harbor beneficial bacteria as 11 different endophytes were isolated from store brought sweet potatoes, and growth promotive mechanisms identified included BNF and IAA production (Kahn and Doty, 2009). In tomato, approximately 20% of seedlings derived from the seeds of a nitrogen-fixing endophyte containing mother plant were able to fix atmospheric nitrogen 2 months after germination (Varga et al., 1994). Some cases of dependence of the endophyte-host interactions are extreme, as in the interactions between *Psychotria leptophylla* and its leaf nodule endosymbiont, where vertical transference is critical for proper plant growth, with endophyte free seedlings resulting in a dwarf phenotype (Miller, 1990). These endophyte free seedlings developed normally until the fourth pair of leaves emerged, and at that stage, differentiation ceased and the shoot tips degenerated into callus. The plants remained at this stage for several years until the plants perish (Miller, 1990).

Seeds harboring multiple endophytic bacteria have also been identified. *Bacillus, Enterococcus*, *Paenibacillus* and *Methylobacterium* were isolated from seeds and seedlings of 10 Eucalyptus species and two hybrids (Ferreira et al., 2008). Norway spruce seeds contained *Pseudomonas* and *Rhanella* sp. identified by 16s rDNA (Cankar et al., 2005). In earlier studies, five percent of potato seeds contained multiple culturable bacteria (Hollis, 1951). Vertically transmitted endophytes may eventually be able to deliver desirable genes to other host plant endophytes or vice versa, via lateral genomics, a common occurrence in microbial populations and a prospect that has been identified under experimental conditions (Taghavi et al., 2005).

Materials and methods – vertical transmission

Plant material

Switchgrass (*Panicum virgatum* L.) cultivar Alamo seeds were purchased from Warner Brothers Seed Co. (Lawton, OK), and other switchgrass cultivars and accessions were obtained from Dr.

Bingyu Zhao's (Department of Horticulture - Virginia Tech, Blacksburg, VA) field trial at Kentland Farm. Seeds were collected from a population cross of accessions 073, 037, 066, and 070 collected from Kentland Farm (P1).

Bacterial endophyte and culture conditions

Two types of strains were used for growth promotion and horizontal gene transfer experiments. The first consisted of 2 type and reference strains belonging to *Burkholderia* species; *Burkholderia phytofirmans* strain PsJN, an endophytic bacterium isolated from onion roots by Jerzy Nowak (Frommel et al., 1991). *B. phytofirmans* strain PsJN and its PsJN-GFP derivative was obtained from Dr. Angela Sessitsch (Austrian Institute of Technology, Seibersdorf, Austria). The second was isolated from surface sterilized switchgrass shoots, grown from surface sterilized F1 seeds that were produced from a cross of plants grown from seeds obtained from Dr. Bingyu Zhao's field trial at Virginia Tech's Kentland Farm and grown in nitrogen free media on a 3'x6'ebb-flow hydroponic table containing 100 liters of nitrogen-free Hoagland's solution (bioWORLD.com # 30630037-2 (759991)).

All cultures were streaked on King's B (KB) solid medium as described (Pillay and Nowak, 1997). Inoculum was produced by transferring one loop of bacterium from 2-day-old cultures to 5 ml KB broth in a 15-ml culture tube, followed by incubation at 28˚C on a shaker (220 rpm) overnight. Five ml of the overnight culture was added to 45 ml KB broth in a 250-ml Erlenmeyer flask and grown to 0.7 OD_{600}. Bacterial cells were then collected by centrifugation at 3,500 rpm for 7 min. at 4˚C, and re-suspended in PBS buffer (10 mM NaH_2PO_4 containing 0.8% NaC1, pH 6.5) after which the OD_{600} was adjusted with PBS buffer to 0.5 (approx. 10^8 cfu), unless described otherwise.

Nitrogen-free hydroponic table experiments

Seeds collected from Kentland Farm were wet-chill cold stratified to break dormancy by placing in 15 ml Falcon tubes overnight at 5°C to chill, then each tube was filled with sterile H_2O and placed again at 5°C for 24 hours, the water was then removed with a pipette and the 15ml tube was placed at 5°C for 2 weeks. The caps of the 15 ml tubes were then removed and allowed to dry for 5 days. The seeds were surface sterilized by treatment with 70% ethanol for 2 min, rinsed 3X with distilled water, de-husked for 30 min with 60% H2SO4 with stirring, washed 3X with distilled water, sterilized with 0.4 M sodium hypochlorite (50% commercial bleach solution

containing 6% sodium hypochlorite) containing 0.1% Triton 100 for 30 min followed by 5X rinse with sterile, deionized, distilled water (ddH2O). Twelve seeds of each accession were planted ¼ inch deep in vermiculite (THERMOROCK # 489702) on a 3'x6' ebb-flow hydroponic table containing 100 liters of nitrogen-free Hoagland's solution. The table was flooded 5 min., three times daily. Population crosses between cultivars, grown in N-free media for three months and transferred to 4 gallon pots containing Miracle Gro®, were performed by grouping flowering inflorescence with a brown paper bag for a period of two weeks, lightly shaken 3 times per week. The population cross was successful and seeds were collected for further analysis. One hundred F1 seeds were wet/chill cold stratified as described earlier, and a germination test for seed viability was performed by placing 25 seeds in each petri-dish containing two filter papers with 5 ml of sterilized H_20 for 1 week.

Analysis of tissue and seed endophyte populations

Approximately two grams of tissues collected from F1 plants grown from surface sterilized seeds in N-free media for 2 months and plants from the second hydroponic table experiment were surface sterilized as previously described (Kim et al., 2012). Leaf and root samples were collected, weighed, labeled, and placed in sterile 1.5ml Eppendorf tubes. The samples were washed with 1 ml 1% sodium hypochlorite plus .05% Triton X-100 for 2 min. The tissue samples were washed twice with 1 ml sterile water and then submerged for 1 min in 15% H_2O_2. The H_2O_2 was decanted, and the samples were again washed twice with sterile distilled H2O. To determine if contamination was present, the 0.05 ml of the final wash was plated on LB media. The samples were then homogenized in 1.5 ml tubes with small pestles in 1ml sterile H2O. The homogenized samples were then spun at 200 rpm for 1 min. One hundred µl of the supernatant was removed and serially diluted to get a final concentration of 1:1000 with H_2O. One hundred µl of diluted solutions was plated on Norris Glucose Nitrogen Free Medium M712 (Ranganayaki and Mohan, 1981) (HiMedia Laboratories) used for the cultivation of chemoheterotrophic bacteria capable of fixing atmospheric nitrogen; the ingredients include glucose (10 g/L), dipotasium phosphate (1 g/L), magnesium sulphate (0.2 g/L), calcium carbonate (1 g/L), sodium chloride (0.2 g/L), sodium molybate (0.005 g/L), ferrous sulphate (0.1 g/L), with a final pH of 7.0 at 25º C.

Switchgrass seeds were surface-sterilized as described above. Surface sterilized seeds from P1 and F1 were also homogenized in sterile 1.5 ml tubes with 1 ml of sterilized H_2O and plated in serial dilutions along with the final wash as described above on Norris Glucose Nitrogen Free Medium M712. Growth on the plate was re-plated twice to confirm. Finally, the best growing colony was placed in glycerol stock for future use and also sent off for identification to MIDI LABS (Newark, DE).

Seedling inoculation and plant growth response

Surface-sterilized seeds were germinated in petri-dishes for 7 days at 25˚C, under white fluorescent light (67 µmol m-2s-1) with a 16 h photoperiod followed by soaking in PsJN suspension for 1 min. Control seedlings/seeds were treated with PBS buffer alone. The treated seedling/seeds were blot-dried with sterile paper towel, placed on media consisting of pre-mixed Hoagland Nitrogen Free Media (bio-World.com, Dublin Ohio) 1.9 g/L, phytogel 3 g/L, and ammonium sulfate at 10 – 375 mg/L, pH 5.8 in GA7 Magenta vessels (Sigma-Aldrich) containing 50 ml of media and 5 seedlings, and grown for one month in the incubator as above.

Establishment of a nitrogen growth curve of switchgrass cv. Alamo

Initial experiments demonstrated that switchgrass cv. Alamo growth in N-free Hoagland's media with no addition of nitrogen was very limited, regardless of bacterial inoculation. However, when 200 mg/L ammonium sulfate was added, switchgrass growth for one month was equal to that produced with a media containing adequate nitrogen. In order to determine when switchgrass growth is limited, and how the stress of low nitrogen affects plant growth, an experiment was developed to test growth in a range of levels of nitrogen. The test included measuring both root and shoot biomass at 1.5 months in nitrogen levels (mg/L) at 0, 10, 25, 50, 75, 100, 125, 250, 375, and 500 added to pre-mixed Hoagland's Nitrogen Free Media as described above.

Results – Vertical transference

Hydroponic table experiments and identification of bacteria

To determine if accessions of switchgrass seeds harbor vertically transmitted nitrogen fixing endophytes, seeds from 168 accessions of both upland and lowland switchgrass accessions were collected in the fall of 2009 from Kentland farm at Virginia Tech and screened for growth in N-

free media. During collection, the plants from each accession were rated for height on a scale of one to ten, and accounted for the number of tillers as an estimate of potential biomass production (**Figure 4.1**) To screen various accessions' seeds for potential nitrogen fixing endophytes, seeds from the 48 highest rated accessions were wet-chill cold stratified to break dormancy, surface sterilized, and planted in vermiculite. A hydroponic table was built to assess growth of a large number of switchgrass plantlets in N-free media (**Figure 4.2**) at the Center for Sustainable and Renewable Resources in Danville, Virginia and Central Virginia's Governors' School for Science and Technology in Lynchburg, Virginia. The first test was performed in Danville, VA for three months as described above. During this test, accessions were rated by height (cm) weekly during a three-month test. The experiment was repeated at Central Virginia Governor's School for Science and Technology as an outreach opportunity to confirm results. At the end of the experiment, plant height was measured on 12/07/2010 (**Figure 4.3**). The best performing accessions (073, 037, 066, and 070) were selected, and tissue was collected for analysis. Four plants of each accession were transplanted in 3 gallon pots for a population cross. Leaf and root tissues from each accession were washed and surface sterilized as described (Kim et al., 2012), and the extract was plated on Norris Glucose N-free media, with no colonies resulting.

A second hydroponic table experiment was undertaken during the population cross described above to determine variation of growth within accessions. This was done to determine if vertically transmitted endophytes may be present only in a small number of plants from the same accession. Sixty plants each from accessions 006, 009, 014, 013, 016, and 005 were grown from surface sterilized seeds for 3 months and rated by height (**Figure 4.4**) and survival rate (**Figure 4.5)**. The three best growing plants were selected from each accession and replanted together in one gallon pots with five plants each pot, in vermiculite and allowed to continue growing for plant tissue screens for N-fixing endophytes. After 6 months of growth in vermiculite and only watered with half-strength Hoagland's N-free media, tissue was collected, surface sterilized as described (Pillay and Nowak, 1997), and plated on Norris Glucose N-Free media. No colonies resulted after three repeats. The plants were continued to be watered, and were still growing after 1.5 years in vermiculite with no addition of nitrogen indicating the plants may be attaining nitrogen from the atmosphere via BNF, although the nitrogen fixing bacteria could not be cultured (**Figure 4.6**).

An experiment was next designed to determine if F1 seeds or switchgrass plants grown from F1 seeds resulting from a cross of the best performing accessions in the first N-free hydroponic table experiment harbor atmospheric nitrogen fixing endophytes. F1 seeds were surface sterilized, germinated in a sterile environment as described and planted in one gallon pots with vermiculite, watered with half-strength Hoagland's N-free media for analysis for the presence of N-fixing endophytes. After 6 months of growth, plant tissue was collected, and surface sterilized as described (Pillay and Nowak, 1997). Simultaneously, F1 seeds were surface sterilized and ground with small pestle in 1.5 ml tubes. Extract from both were plated on Norris Glucose N-free media. After 5 days of incubation, 4 colonies of two morphologies resulted from the F1 plant tissue. These colonies were re-plated on separate N-free plates, with only one colony morphology surviving two additional cultures. The colony morphology, after repeat plating on N-free media, was grown overnight in KB media, placed in glycerol stock, and sent off for identification (Attach Identification). FAME Matches identified the bacterium as *Sphingomonas herbicidovorans*, a species with the common ability to fix atmospheric nitrogen. The bacterium was then tested for plant growth promotion in N-free media.

Switchgrass nitrogen level growth promotion experiments

Morphology and root and shoot biomass of the plants changed in the ranges of nitrogen levels (**Figure 4.7**) with roots making the obvious transition from red to green between 100 mg/L and 125 mg/L, matching the change in biomass noted earlier. Shoot weight increased throughout the curve, with the exception between 125 mg/L and 250 mg/L. Root weight increased from 0 mg/L to 100 mg/L and then decreased from 100 mg/L to 125 mg/L and remained almost the same from 125 mg/L to 500 mg/L (**Figure 4.8**). Additionally, although total weight of plants grown in 100 mg/L was not significantly different compared to 500 mg/L, the difference in the morphology of the plant was clear as the shoots were much taller in the latter.

Growth promotion under a range of nitrogen levels

In order to determine the effects of the *S. herbicidovorans* isolate (NSL) bacterization on switchgrass growth (**Figure 4.9**), plantlets were tested in three levels of nitrogen; 10 mg/L, 75 mg/L, and 375 mg/L. Results from the 10 mg/L test demonstrated no significant differences between treatments, likely because of either the length of the test or because nitrogen deficiency negates the effects normally seen by these bacteria. NSL outperformed control (CK) in both

nitrogen deficient (75mg/L) and adequate nitrogen levels (375mg/L) indicating that this bacterium may have multiple mechanisms of action, a characteristic noted in diazotrophs isolated from grasses by other authors (Riggs et al., 2002). A second test was undertaken, similar to the first experiment, where root, shoot, and total biomass were measured at one month with similar results (**Figure 4.10**). A third test was performed with total weights recorded after one month with similar results (**Figure 4.11**).

Discussion - Vertical transference

Endophytic microbes can inhabit various parts of the plant, such as the root, stem and leaves, and can also be found in flowers, fruits and seeds (Zakria et al., 2008; Rodriguez et al., 2009; Compant et al., 2011; Kim et al., 2012). Microorganisms, including viruses, fungi, and bacteria, may be transmitted to seed internally by the host xylem and subsequently through the hilum (Agarwal et al., 1987). *Burkholderia phytofirmins* strain PSJN, a well characterized beneficial bacterial endophyte, was tagged with green fluorescent protein and microscopically visualized spreading through grapevine stalks, pedicels, and to immature berries through xylem vessels (Compant et al., 2007). Complicating the matter of determining mechanisms of vertical transmission is the fact that most bacteria are not culturable in general, and in the case of foliar bacterial nodules, the bacterial partner could not be cultivated at all, suggesting that endosymbionts may be dependent on their host for growth (Lebard and Belin-Depoux, 2003).

This research highlights potential methods of isolating and utilizing vertically transferred microorganisms as an alternative external application of beneficial microorganisms since externally applied inoculum may change the makeup of the soil (Conn and Franco, 2004), or is unable to effectively colonize and promote the growth of target plants (Sturz et al., 2000). Conversely, identifying microorganisms that are faithfully vertically transmitted, generation after generation provides the opportunity to introduce stable and predictable effects in the field, regardless of native soil population. As knowledge of vertical transference of bacteria is limited, an approach of isolating a model organism, known to occupy and consistently be transmitted to the next generation should be developed. Other conditions such as soil composition, temperature, plant genotype, and stress need to be taken into account to ensure consistency of transmission, even under adverse conditions. Once established, beneficial traits could be naturally transferred through lateral genomics to the model organism and followed throughout its life cycle. Plant

breeding and genomics may also be employed to identify hosts with genotypic compatibility to the endophyte. Together, such a system may yield valuable data to contribute to the understanding of this little studied phenomenon. Furthermore, targeting particular bacteria that form spores, are capable of energy storage, or are commonly vertically transmitted to progeny, may allow delivery of certain traits such as atmospheric nitrogen fixation on a consistent basis. These endophytic organisms may then share these traits *in planta* with other endophytic microorganisms, especially under challenging conditions. Vertical transmission is also an option to maintain stable natural populations.

Strategy Two: Utilizing horizontal gene transfer in *Burkholderia* sp. to improve switchgrass growth under nitrogen limited conditions

Introduction

Understanding the nitrogenase enzyme on both the molecular and genetic levels requires consideration of the complex mechanisms of lateral genomics (Raymond et al., 2004) which include the phenomena of genes selectively lost, horizontally transferred, duplicated, and merging with other important biochemical pathways (Xiong et al., 2000). Genetic transfer and rearrangement in bacteria often involve plasmids, some of which contain genes specifically responsible for gene sharing. For example, in alpha-proteobacteria, conserved segments in *Rhizobium* and *Agrobacterium* contain the *vir*, *tra*, and *sym* regions required for conjugative T-DNA gene transfer and symbiosis (Gonzalez, 2006). As the analysis of complete bacterial genomes progresses, the nitrogenase (*nif*) operon and the associated suite of proteins involved in regulation and activation of the nitrogenase enzyme in diverse bacterial genera are coming to light (Reviewed in Bently and Parkhill, 2004).

To explore horizontal gene transfer between endophytes, the well documented *Burkholderia* taxa was chosen because of its proven beneficial endophytic interactions with a variety of plants and it is commonly capable of BNF. In general, *Burkholderia* are gram-negative beta-proteobacteria comprised by over 50 species commonly found in the environment and can colonize soil, water, plants, and animals (reviewed in Compant et al., 2008). In addition to bacteria capable of BNF, *Burkholderia* sp. is also known to enhance plant stress tolerance, disease resistance, aid nutrient uptake, and enhance its hosts' capacity for metabolic adaptation to diverse environments (reviewed in Compant et al., 2008). Some of these responses are linked to the bacterial

production and secretion of siderophores, rock-phosphate solubilization, ACC deaminase activity, quinolinate phosphoribosyl transferase activity, and production of phytohormones (Sturz et al., 2000; Sturz and Nowak, 2000; Bhattacharjee et al., 2008). Free-living endophytic *Burkholderia* can inhabit practically every plant part (De Costa and Erabadupitiya, 2005; Kim et al., 2012). For example, *B. tropica* was found in the stems and roots of pineapples (Cruz et al., 2001), *B. gladioli* in the roots, stems, seeds, and berries of coffee (Vega et al., 2005), and *B. phytofirmans* strain PsJN, isolated from onions, was able to colonize several unrelated crop species (reviewed in Nowak and Shulaev, 2003).

Interactions between endophytic *Burkholderia* sp. and its plant host can be obligate, where the plant suffers greatly in its absence. For example, plant tissue cultures of *Psychotria* that do not contain *Burkholderia* endophyte had distorted leaves, stunted growth, and could not survive for a long term (Van Oevelen, 2003). *Burkholderia* has also been found to form extended niches in fungi. For example, the arbuscular mycorrizal fungus *Gigaspora margarita* has a resident population of as many as 250,000 *Burkholderia* spp. in the cytoplasm of a single fungal cell (Ruiz-Lozano and Bonfante, 2000). A nitrogen-fixing bacterium closely related to *Burkholderia* (Bianciotto et al., 1996) has been discovered within the spores of *Gigaspora margarita* (Jargeat et al., 2004), where more than 20,000 individuals were estimated to inhabit every spore (Bianciotto et al., 2004), indicating the important evolutionary ability to be transferred vertically to the next generation and ensuring a stable interaction.

Ecologically, the versatility of *Burkholderia* is likely due to its large genome which ranges in size from 4.7 to 9Mb (Bellenger et al., 2011), or twice the size of *Escherichia coli* (Parke and Gurian-Sherman, 2001). Over 20 percent of its DNA sequences may have been acquired by horizontal gene transfer (Chain et al., 2006), an adaptation mechanism driven by bacteriophage and transposon elements (Konstantinidis and Tiedje, 2005). To determine the adaptability of an organism with such a large genome, researchers experimentally adapted 12 populations of *B. cenocepacia* to an onion medium for 1000 generations (Ellis and Cooper, 2010). They found that 78 percent of all populations increased in fitness compared to their ancestors, and significant variations among lines were observed. Populations also varied in several phenotypes related to the association with the host, including quorum-sensing function, motility, and bio-film formation (Ellis and Cooper, 2010).

Complete genome sequencing of other bacterial endophytes along with the accompanying genetic analysis has led to differing conclusions about the stability and genetic differences of these diverse bacteria. For example, the genome sequence of *Azoarcus* BH72 revealed a relatively stable genome, attributed to the bacteria adapting to the stable internal environment of plants (Krause et al., 2008). Conversely, the sequence and comparative analysis of *Gluconacetobacter diazotrophicus* Pal5, a nitrogen-fixing bacterium associated with sugarcane, reveals a high number of transposable elements, an indication of a recent evolutionary bottleneck possibly due to a recent change in niche (Bertalan et al., 2009). The latter bacteria also had multiple plant growth promoting properties compared to its soil borne counterpart, indicating that the transposable elements could have been acquired from other bacteria inhabiting the same environment through lateral genomics, again indicating the common occurrence of horizontal gene transfer (Bertalan et al., 2009).

Free-living endophytic *Burkholderia* capable of BNF can colonize plant organs and internal tissues of a broad range of non-legume plant species (Elliott et al., 2007; Bontemps et al., 2010; Martinez-Aguilar, 2008). It has been suggested that free-living endophytes capable of BNF are placed in a more favorable environment compared to free-living rhizospheric bacteria because they are less vulnerable to competition with native soil bacteria and are shielded from various biotic and abiotic stresses (Reinhold-Hurek and Hurek, 1998). The low oxygen environment created in plant tissue also optimizes nitrogenase activity, the enzyme responsible for BNF (Dixon and Kahn, 2004). Free-living BNF *Burkholderia* spp. have been identified in field grown tomato (*B. tropica* and *B. xenovorans*) and sorghum (Wong-Villarreal 2010) where *B. tropica* was found almost exclusively.

The genome structure and nitrogen fixing efficiency of 7 free-living rhizoshperic and endophytic *Burkholderia* (*B. unamae, B. tropica, B. silvatlantica, B. xenovorans, B. vietnamiensis, B. kururiensis,* and *B. sacchari*) was analyzed (Martines-Aguilar, 2008). The authors noted that the *nifH* gene was located on different chromosomes, ranging from 2 to 5, and the gene sequences showed tight clusters and were clearly different from other nitrogen fixing bacteria. To confirm atmospheric nitrogen fixation, the authors utilized the $^{15}N_2$ isotopic dilution assay and found amounts of N fixed ranged from less than 100 ng to more than 3,000 ng in *B. vietnamiensis*. Even strains within species such as *B. unamae* exhibited a 20-fold difference in the amount of N_2

fixed. In *B. vietnamiensis* G4, NtrB is part of two-component system, likely involved in the regulatory response to both nitrogen and oxygen status (Mendard et al., 2007).

Together, the large and dynamic genome, its ability to occupy many niches, the evidence of common occurrences of horizontal gene transfer, its multiple proven growth promotion mechanisms including free-living BNF, multiple genomes sequenced, and availability of strains, all indicated *Burkholderia* would be a good taxa to study potential horizontal gene transfer of BNF. The artificial freeze-thaw method utilizing gDNA from a bacterium capable of BNF and natural co-cultivation with a bacterium capable of BNF are explored below to introduce BNF to *B. phytofirmans* PsJN, a strain with its genome sequenced which lacks the ability to fix atmospheric nitrogen.

Materials and methods - Horizontal gene transfer

Plant material

Switchgrass (*Panicum virgatum* L.) cultivar Alamo seeds were purchased from Warner Brothers Seed Co. (Lawton, OK).

Bacterial endophyte and culture conditions

Two types of strains were used for growth promotion and horizontal gene transfer experiments. The first consisted of reference strains belonging to *Burkholderia* species; *Burkholderia phytofirmans* strain PsJN, an endophytic bacteria isolated from onion roots by Jerzy Nowak (Frommel et al., 1991) and the second, *Burkholderia phymatum* STM 815, a nodule forming bacterium isolated from Mimosa (Elliott et al., 2007). *B. phytofirmans* strain PsJN and its PsJN-GFP derivative was obtained from Dr. Angela Sessitsch (Austrian Institute of Technology, Seibersdorf, Austria). *Burkholderia phymatum* STM 815 was purchased from DSMZ, Braunschweig, Germany. Genomic DNA from *B. phymatum* STM815 was kindy provided by Dr. Lionel Moulin, Laboratoire des Symbioses Tropicales et Mediterraneennes, Montellier, France. All cultures were streaked on King's B (KB) solid medium as described (Pillay and Nowak, 1997). Inoculum was produced by transferring one loop of bacteria from 2-day-old cultures to 5 ml KB broth in a 15-ml culture tube, followed by incubation at 28˚C on a shaker (220 rpm) overnight. Five ml of the overnight culture was added to 45 ml KB broth in a 250-ml Erlenmeyer flask and grown to 0.7 OD_{600}. Bacterial cells were then collected by centrifugation at

3,500 rpm for 7 min at 4 ˚C, and re-suspended in PBS buffer (10 mM NaH_2PO4 containing 0.8% NaC1, pH 6.5) with OD_{600} at 0.5 (approx.. 10^8 cfu) , unless described otherwise.

***Burkholderia* genome analysis**

Search of published *Burkholderia* genomes was performed using BLAST alignment tool using *Burkholderia vietnamenisis* as the reference strain due to its documented abilities to both fix atmospheric di-nitrogen and contribute fixed nitrogen to plant growth in a free living state.

Transformation of gDNA from *B. phymatum* STM 815 to *B. phytofirmans* strain PsJN

Three µl of *B. phymatum* STM 815 gDNA was added to 100 µl of competent *B. phytofirmans* strain PsJN cells and placed on ice for 5 min. The mixture was then placed in liquid nitrogen for 5 min followed by incubation at 37ºC for 5 min. Next, 700 µl of LB was added for recovery for 4 hours at 28ºC on a shaker at 220 rpm. The suspension was then spun at 7000rpm for 3 min to pellet. All but 100µl was decanted and the pellet was re-suspended on and plated on solid LB for 2-3 days.

Bacterial genomic DNA isolation

Bacterial genomic DNA was isolated (Lemanceau et al., 1995) by growing for 21 hr at 28ºC on a shaker at 220 rpm in Luria-Bertani (LB) medium (Miller, 1972). The cells were pelleted by centrifugation at 4000 rpm for 10 min, washed by re-suspending in sterile H2O and repeating centrifugation at 4000 rmp for 10 min. The final pellet was then suspended to an optical density of 2 at 600 nm by dilution with sterile distilled H2O. Then, in a 1.5 ml tube, 100 ul of 10 mM Tris-HCL (pH 8.3) and 13 ul of proteinase K (1 mg/ml in H2O)(Sigma) were added to 100 ul of bacterial cell suspension and incubated at 55ºC overnight. Proteinase K was inactivated by incubation of the cell suspension for 10 min at 100ºC. gDNA was stored at -20ºC until use. Primers were designed for *B. Phymatum* STM 815 NifH: Forward primer: 5'– GGGTGTGATCCAAAGGCTGA-3', Reverse primer: 5'– ATTCGTCAGGCGGTCAGTTC-3'. Oligonucleotides were synthesized by IDT. PCR was carried out with 5 ul aliquots of cell suspension under PCR conditions; 25 ul: 50 pmol each primer, 1.25 mM DNTPs and 2U *Taq* DNA polymerase. Cycles as follows: 1 cycle at 95ºC for 7 min, 30 cycles at 94ºC for 1 min, at 52ºC for 1 min, and at 65ºC for 8 min; 1 cycle at 65ºC for 16 min and a final step at 4ºC. 10ul of PCR products were run on a 1% agarose gel, stained with cyber green, and photographed.

Seedling inoculation and plant growth response

Surface-sterilized seeds were germinated in petri-dishes for 7 days at 25˚C, under white fluorescent light (67 µmol m-2s-1) with a 16 h photoperiod. Surface-sterilized seeds were placed on wet filter paper for 3-5 days in an incubator at 25 ˚C with 16 h photoperiod (white fluorescent bulbs at 67 µmol m-2s-1) followed by soaking in PsJN solution (0.5 of OD600) for 1 min. Control seedlings/seeds were treated with PBS buffer alone. The treated seedling/seeds were blot-dried with sterile paper towel, placed on media consisting of pre-mixed Hoagland Nitrogen Free Media (bioWorld # 30630038-2) 1.9 g/L, phytogel 3 g/L, and ammonium sulfate at 10 – 375 mg/L, pH 5.8 in GA7 Magenta vessels (Sigma-Aldrich) containing 50 ml of media and 5 seedlings, and grown for one month in the incubator as above.

Establishment of a nitrogen growth curve

Initial experiments demonstrated that switchgrass cv. Alamo growth in N-free Hoagland's media with no addition of nitrogen was very limited, regardless of bacterial inoculation. However, when 200 mg/L ammonium sulfate was added, switchgrass growth for one month was equal to that produced with a media containing adequate nitrogen. In order to determine when switchgrass growth is limited, and how the stress of low nitrogen affects plant growth, an experiment was developed to test growth in a range of levels of nitrogen. The test included measuring both root and shoot biomass at 1.5 months in nitrogen levels (mg/L) at 0, 10, 25, 50, 75, 100, 125, 250, 375, and 500 in pre-mixed Hoagland's Nitrogen Free Media as described above.

Results - Horizontal gene transfer

***Burkholderia* genome survey for potential symbiotic plasmids**

With the recent surge in bacterial genome sequencing, much more information is available regarding both the range of total size of genomes as well as the relative number of chromosomes and plasmids present in a species such as *Burkholderia* (**Table 4.1**). In an effort to identify other Beta-proteobacteria that are able to fix atmospheric nitrogen, and if these bacteria contain a SYM plasmid potentially capable of sharing this property, a survey was undertaken of the *nifH* gene in published *Burkholderia* genomes using BLAST. Results indicated that several *Burkholderia* species possessed the *nifH* gene with high identity to that of *B. vietnamiensis*, a known free-living nitrogen fixing bacterium, and thereby potentially have the ability to fix atmospheric

nitrogen (**Table 4.2**). Most importantly, these surveys indicated that, of the three *Burkholderia* with sequenced genomes identified to possess the *NifH* gene, *B. phymatum* STM 815 had the gene located on a plasmid. This plasmid was then explored further using the annotated genome feature in the NCBI database.

Plasmid pBPHY02 symbiotic gene analysis

B. phymatum, a highly effective bacterium at nodulating Mimosa, has also been the first reported Beta-proteobacteria capable of fixing atmospheric di-nitrogen *ex planta*, in a free-living state (Elliott et al., 2007). Unlike many nodulating bacteria, including other Burkholderia, *B. phymatum* STM815 was found to nodulate a wide range of important legumes (reviewed by Gyaneshwar et al., 2011). In growth promotion experiments, *B. phymatum* STM 815 increased the dry weight of inoculated *Mimosa* plants 5 to 6 times over controls (Elliott et al., 2007).

The complete genome sequence of *B. phymatum* STM815 was released in 2008 (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). It is comprised of one large 3.5Mb chromosome, two smaller (2.7Mb and 1.9Mb) chromosomes, and a 595Kb plasmid. Analysis of the annotated *B. phymatum* 595Kb plasmid pBPH02 revealed that many symbiotic genes, including genes responsible for nodulation, nitrogen fixation, and conjugation were present (**Figure 4.12**) (**Tables 4.3 and 4.4**).

Nodulation genes on the *B. phymatum* pBPHY02 range from 480Kb to 489Kb, and are located upstream from the nitrogen fixing operon. The six genes in this region are involved in nodulation (including *nodT*), protein export (*nodJI*), and in secretion of signal molecules by nodulating bacteria (*nodSAH*) in response to host plant signals (Debelle et al., 2001). The membrane transport complex is encoded by *nodI* and *nodJ* (Fernandez-Lopez, 1996). Together, the genes are required for symbiotic responses for successful infection. The signaling molecules confer differing levels of specificity between the nodulating bacteria and the host plant (Sprent et al., 2009). In an analysis of *nodC* sequences of 143 Burkholderia strains isolated from 47 Mimosa host plants in Brazil, researchers reported a monophyletic origin suggesting a single acquisition of these conserved genes (Bontemps et al., 2010).

The genes responsible for nitrogen fixation are located on the 492Kb to 588Kb fragment of the *B. phymatum* pBPHY02 plasmid and include the complete set of *nifHDKEN* genes encoding for

the nitrogenase heterotetramer (reviewed by Dixon and Kahn, 2002). At 96Kb in length, this operon is twice as long as others reported for *Burkholderia* (Menard et al., 2008). The *nifA* gene, involved in transcriptional regulation of nitrogenase production, is located upstream of the remaining *nif* genes and downstream of the *nod* genes involved with nodulation. The *nifB* gene is separated from *nifA* by at least 6 genes, a configuration similar to the arrangement in *B. tuberum* but differs from that of other *Burkholderia sp*. where the genes are located next to one another (reviewed in Gyaneshwar et al., 2011). The *nifEN* genes are also separated by a number of genes from *nifHDK* which differs from the arrangement in other nitrogen fixing bacteria. A downstream duplication of the *nifHDK* region includes *nifH* as well as *nifT* and *nifZ* genes, involved in the biosynthesis, and maturation of the FeMo protein also exists. The region also contains the PAS signaling domain involved in sensing cell nitrogen status (Menard et al., 2008) and genes encoding the two component LuxR protein sensory transduction system (Birck et al., 2002) that has also been implicated to be involved in quorum sensing (Pappas et al., 2004).

Like the tumor inducing plasmid in *A. tumefaciens,* commonly used as a model plasmid in molecular biology research, and plasmid p42a of the nodulating Alpha-proteobacteria *Rhizobia etli*, the *B. Phymatum* plasmid contains genes encoding the VirB protein involved in the type IV 'adapted conjugation' system for T-DNA transfer to the plant host cell, and the *Tra* and *Trb* genes essential for horizontal DNA transfer in bacterial conjugation (Chen et al., 2002; Gonzalez et al., 2006). In gram-negative bacteria, conjugation systems consist of two surface molecules, the mating channel for DNA and protein translocation, and the conjugal pilus to contact recipient bacteria (reviewed by Christie et al., 2000). The VirB proteins, involved in horizontal transfer in Alpha-proteobacteria, include three functional groups; extracellular proteins which form the pilus and adhesive structures, the mating channel, and ATPases localized in the cytoplasmic membrane (reviewed by Christie et al., 2000). The *B. phymatum* plasmid pBPHY02 contains four *virB* homologs; 6, 8, 9, and 11. Genes present related to phage activity also indicate the potential for horizontal gene transfer (Gonzalez, 2006).

Introduction of *B. phymatum* plasmid to *B. phytofirmans* PSJN

To test the potential of horizontal gene transfer of the *B. phymatum* STM 815 pBPHY02, containing the *Nif* operon, genomic DNA (gDNA) from this bacterium was introduced to *Burkholderia phytofirmans* strain PsJN, one of the most studied beneficial bacterial endophytes,

shown to promote switchgrass growth under normal nitrogen levels. The transformation of *B. phymatum* gDNA was performed as described and the resulting bacterium was plated on Norris Nitrogen free media. Successful colonies were placed in glycerol stock and referred to as PsJN + (plus the ability to fix nitrogen). In a separate experiment, *B. phymatum* STM815 was co-cultured with *B. phytofirmans* strain PsJN with a green fluorescent protein (GFP) tag (PsJN-GFP) in 5ml liquid KB medium overnight, the bacterial cell suspensions were centrifuged and washed twice with H2O, serially diluted from 1:10 to 1:10,000 with sterile water and then plated on Norris N-free media. After 4 days of growth, two colony types were noted; one which possessed GFP and the other did not (**Figure 4.13**). The counted ratio of GFP colonies vs. no GFP was approximately 1 to 100. GFP containing colonies were re-plated and isolated for later analysis.

To determine if the *nifH* gene, a gene required for nitrogen fixation (Dixon and Khan, 2002), was present in *B. phytofirmans* strain PsJN through either co-cultivation or transformation, PCR primers were designed for *nifH* in *B. phymatum* STM 815 (GenBank #CP001043.1). Genomic DNA was isolated from overnight cultures of *B. phytofirmans* strain PsJN, *B. phymatum* STM 815, PsJN + (produced from transformation), and *B. phytofirmans* strain PsJN-GFP with the ability to grow on n-free media. PCR was performed as described above and resulted in band patterns of expected size, indicating the presence of the *nifH* gene in both PsJN + and PsJN-GFP capable of growth on N-free media, as well as *B. phymatum* STM 815 (**Figure 4.14**).

Nitrogen concentrations effect on growth

Initial experiments demonstrated that switchgrass cv. Alamo growth in N-free Hoagland's media with no addition of nitrogen was very limited, regardless of bacterial inoculation. However, when 200 mg/L ammonium sulfate was added, switchgrass growth at one month was near that produced with a media containing full nitrogen (500 mg/L). In order to determine when switchgrass growth is limited, and how the stress of low nitrogen affects plant growth, an experiment was designed to test growth in a range of levels of nitrogen. The test included measuring both root and shoot biomass at 1.5 months in nitrogen levels (mg/L) 0, 10, 25, 50, 75, 100, 125, 250, 375, and 500. Morphology of the plants also changed throughout the curve (**Figure 4.7**) with roots making the obvious transition from red to green between 100 mg/L and 125 mg/L, matching the change in biomass noted earlier. Shoot weight increased throughout the

curve, with the exception between 125 mg/L and 250 mg/L. Root weight increased from 0 mg/L to 100 mg/L and then decreased from 100 mg/L to 125 mg/L and remained almost the same from 125 mg/L to 500 mg/L. (**Figure 4.8**). Additionally, although total weight between 100 mg/L and 500 mg/L was not significantly different, the difference in the morphology of the plant was clear as the shoots were much taller in latter.

Growth promotion experiments

In order to determine the effects of endophytes with or without *Nif* genes on growth, *B. phytofirmans* strain PsJN, PsJN +, *B. phymatum* STM 815 were tested in three levels of nitrogen: 10 mg/L, 75 mg/L, and 375 mg/L. (**Figure 4.15**). Results from the 10 mg/L test demonstrated no significant differences among treatments, likely either because of the length of the test or because nitrogen deficiency negates the effects normally seen by these bacteria. For instance, *B. phytofirmans* strain PsJN was shown to promote switchgrass growth at sufficient nitrogen levels by as much as 50%. At 75 mg/L, PsJN transformed with the *B. phymatum* STM 815 gDNA, clearly outperformed other inoculations. PsJN appeared to have no effect versus control in 75 mg/L ammonium sulfate media. At 375 mg/L, little difference was recorded between PsJN and PsJN+, and both were significantly higher compared to control. To confirm these effects in larger numbers, the tests were repeated in 75 mg/L and 375 mg/L with PsJN, PsJN+, and control (**Figure 4.16**) with similar results. A final test was undertaken, similar to the second experiment, where root and shoot biomass were measured at one month with similar results (**Figure 4.17**). Average percent change vs. control was determined and illustrated in **Figure 4.18**. This graphic clearly shows the effect of inoculation with PsJN + compared to PsJN without the ability to fix nitrogen in 75 mg/L or a nitrogen deficient circumstances. All three tests demonstrate this ability. However, average percent changes with these two were tested in 375 mg/L, or adequate nitrogen levels, were very similar (**Figure 4.19**). These results were demonstrated in all three experiments performed. Together, these tests indicate that PsJN+ out performs PsJN alone when grown in nitrogen deficient media. This benefit is lost when adequate nitrogen is supplied.

Discussion - Horizontal gene transfer

As more is learned about the genetics of *Burkholderia* it may be possible to share these important genes between similar organisms through horizontal gene transfer via transformation, conjugation, or transduction, all common occurrences in the bacterial world. Researchers first

reported *in planta* horizontal gene transfer in hybrid poplar among plant associated bacteria when they found *Burkholderia cepacia* VM1468 transferred its toluene degradation gene to other endogenous endophytes (Taghavi et al., 2005). This suggests that such transfer may be used to modify and improve the growth-promoting effects of other endophytes via gene transfer. The phenomenon of horizontal gene transfer may also occur between *Burkholderia* and different genera. It was suggested by researchers that the gene encoding the anti-fungal agent pyrrolnitrin in *Burkholderia* was horizontally transferred from *Pseudomonas* (de Souza and Raaijmakers, 2003).

Burkholderia represents a remarkably diverse group of plant associated bacteria with unusually complex and plastic genomes (Parke and Gurian-Sherman, 2001; Ellis and Cooper, 2010). This characteristic increases their ability to adapt to diverse environments and helps to explain why they have been found occupying a range of niches, including soil, water, plant, animal, and rhizosphere (Coenye and Vandamme, 2003). As more free-living nitrogen-fixing *Burkholderia* are discovered, and considered as the apparent evolution of the species from a common diazotrophic ancestor (Bontemps et al. 2010), research may lead to the species helping to decreasing synthetic nitrogen fertilizer use, which in turn will help maintain the productivity of farmland. This review supports exploring the use of free-living nitrogen fixing endophytes as an option in the effort to reduce the use of synthetic nitrogen fertilization and offer hope in creating high-yielding, low-input agricultural production systems that do not damage the ecosystem.

Future prospects

In an effort to improve sustainable agriculture in the future, beneficial microorganisms may help to increase yield while decreasing the use of chemical fertilizers and pesticides, each known to have detrimental effects on the environment. While growth promotion utilizing these organisms has been well documented in the lab, field experiments often demonstrate diminished results or no growth promotion at all. Soil applied inoculum has also failed to consistently provide growth promotion to the extent needed. This article highlights the potential of utilizing vertically transferred microorganisms as an alternative to the above mentioned approaches as externally applied inoculum may change the makeup of the soil (Conn and Franco, 2004), or is unable to effectively colonize and promote the growth of target plants (Sturz et al., 2000). Conversely, identifying microorganisms that are faithfully vertically transmitted, generation after generation

provides the opportunity to introduce stable and predictable effects in the field, regardless of native soil population. As knowledge of vertical transference of bacteria is limited, an approach of identifying a model organism, known to occupy and consistently be transmitted to the next generation should be identified. Other conditions such as soil composition, temperature, plant genotype, and stress need to be taken into account to ensure consistency of transmission, even under adverse conditions. Once established, beneficial traits could be naturally transferred through lateral genomics to the model organism and followed throughout its life cycle. Plant breeding and genomics may also be employed to identify hosts with genotypic compatibility to the endophyte. Together, such a system may yield valuable data to contribute to the understanding of this little studied phenomenon. Furthermore, targeting particular bacteria that are commonly vertically transmitted to progeny may allow delivery of certain traits such as atmospheric nitrogen fixation on a consistent basis. These endophytic organisms may then share these traits *in planta* with other endophytic microorganisms, especially under challenging conditions. Vertical transmission with other endophytes is also an option to maintain stable natural populations. The arbuscular mycorrizal (AM) fungus *Gigaspora margarita*, has a resident population of as many as 250,000 Burkholderia spp. in the cytoplasm of a single fungal cell (Ruiz-Lozano and Bonfante, 2000). The gene involved in this interaction is *vacB* and may be used to identify other bacteria that could be introduced to AM. Nitrogen-fixing bacteria closely related to Burkholderia (Bianciotto et al., 1996) have been discovered within the spores of *Gigaspora margarita* (Jargeat et al., 2004), where more than 20,000 individuals were estimated to inhabit every vegetative spore (Bianciotto et al., 2004) indicating the important evolutionary ability to be vertically transferred to the next generation may occur between AM, an obligate endosymbiont itself, and its obligate intercellular bacteria. These interactions all provide potential to better understand and improve the stability of endosymbiotic relationships between endophytes and their hosts.

Since 1999, over 15 new patents have been registered for microbial inoculants (Mei and Flinn, 2010), and the worldwide market is experiencing an annual growth rate of approximately 10% (Berg, 2009). The use of microbial inoculants are an important part of the effort to decrease the use of chemical based fertilizers and to meet the global demand of agricultural crops for food, feed and fuel which are increasing at a rapid pace (Edgerton, 2009). It is clear these tiny organisms contribute, and can be utilized to help maintain or increase productivity of farmland.

Identification of free living, nitrogen-fixing, bacterial endophytes that are transferred vertically from plant to seed could increase plant growth on marginal land in a reliable and predictable fashion while reducing the need for synthetic fertilizers. Breeding in the context of BNF and horizontal transfer of genes encoding beneficial properties, including atmospheric nitrogen fixation, may also be utilized to introduce the property to an endophyte that is vertically transferred via seeds. From nodule forming associations with the common legume Mimosa to endophytic bacteria living freely in the tissues of plants (Elliott et al., 2007; Bontemps et al., 2010), atmospheric nitrogen-fixing bacteria offer hope in the effort to improve agricultural practices in the 21st century by reducing the use of synthetic nitrogen fertilizers.

Acknowledgements

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Figures and Tables

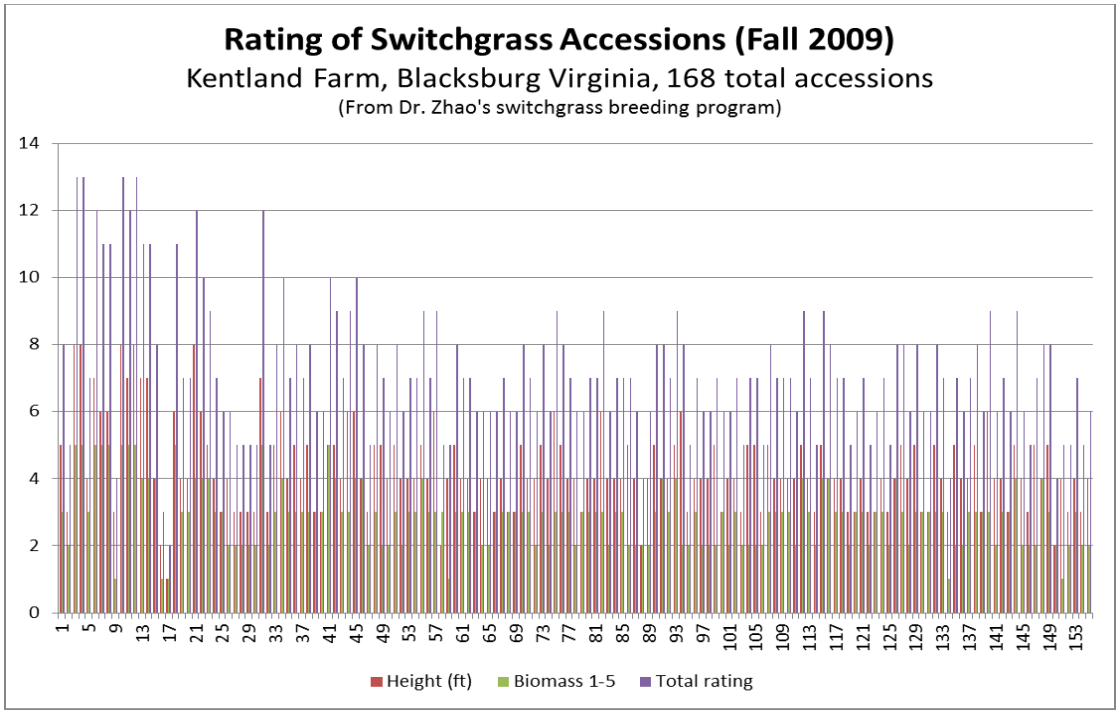

Figure 4.1 Rating of Kentland Farm switchgrass accessions. Values were based on height (ft) and biomass on a scale of one to 5, with 5 producing the most tillers.

3'x6' Ebb-Flow Hydroponic Tables

Highlights:

- Ability to screen 9 trays with 648 plants
- Automated watering
- 18 sq feet of growing area
- Multiple uses for different experiments

Materials	Cost
(4) 2x4x10'	$13.00
(2) 2x8x10'	$14.00
(2) 2x6x10'	$12.00
(2) 4'x8' ¾ inch plywood	$32.00
Timer	$17.00
130 GPM pump	$23.86
Liner	$15.00
Misc.	$15.00
Total cost:	**$141.00**

Growth Solution:

- Type: Nitrogen-free Hoaglands
- Volume @ 2inches = 88 liters
- 100 liters =$ 77.00

Growth Medium:

- Sand or Vermiculite in 1.5" x 1.5" cells

Central Virginia Governor's School for Science and Technology – Lynchburg, VA

The Institute for Sustainable and Renewable Resources – Danville, VA

Figure 4.2 Design and material list for hydroponic table experiments

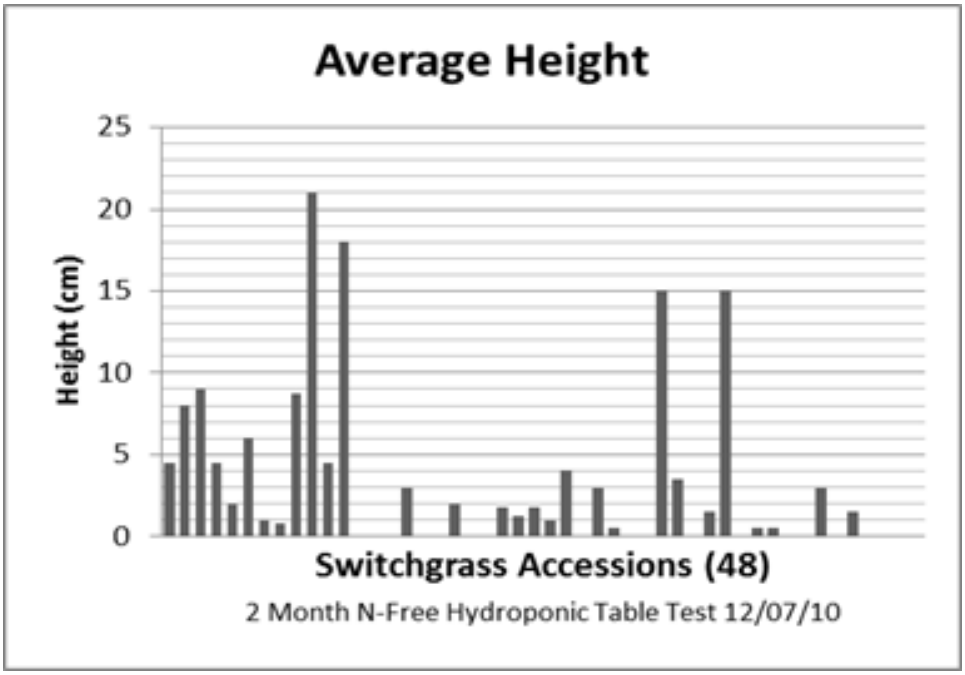

Figure 4.3 Switchgrass height at the end of three months growth in N-free media. Surface sterilized seeds were planted in vermiculite and grown on an ebb-flow hydroponic table containing Hoagland's Nitrogen-free media for 3 months.

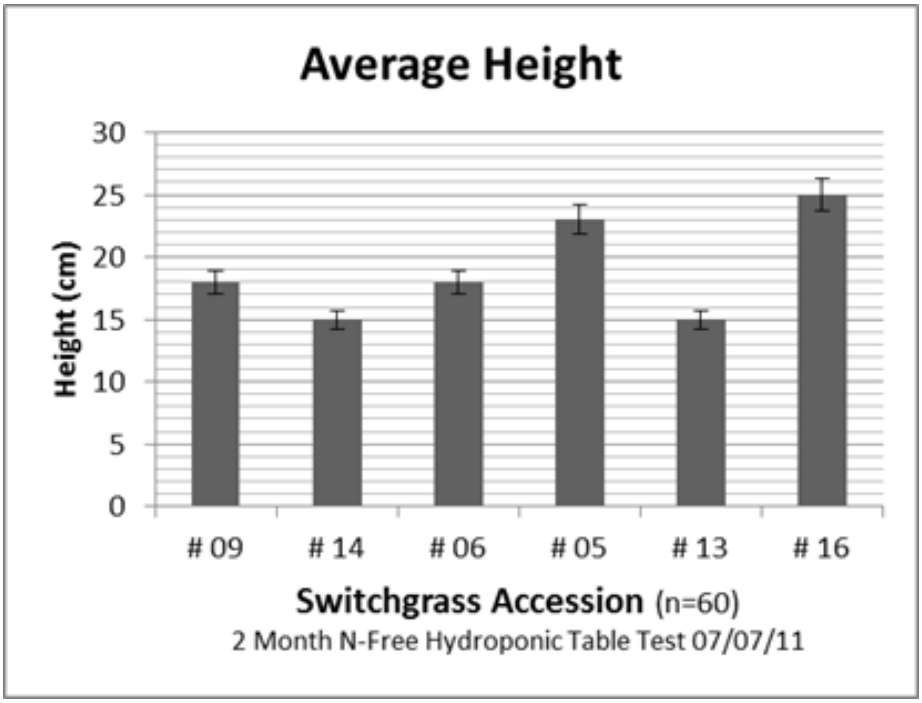

Figure 4.4 Average heights of switchgrass accessions in the 7/7/2011 experiment. Sixty plantlets from surface sterilized seeds of the best performing accessions were planted and grown in vermiculite with N-free liquid media as described in an ebb-flow hydroponic table.

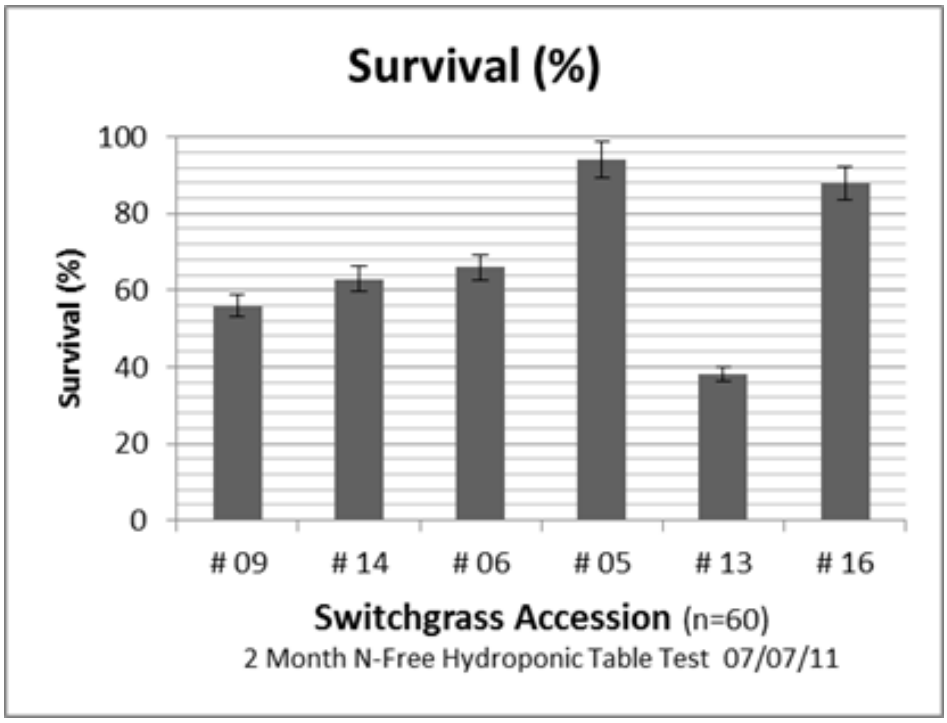

Figure 4.5 Average survival rates of switchgrass accessions in 7/7/2011 experiment. Sixty plantlets from surface sterilized seeds of the best performing accessions were planted and grown in vermiculite with N-free liquid media as described in an ebb-flow hydroponic table.

Figure 4.6 Best growing accessions grown on N-free media after 1.5 years.

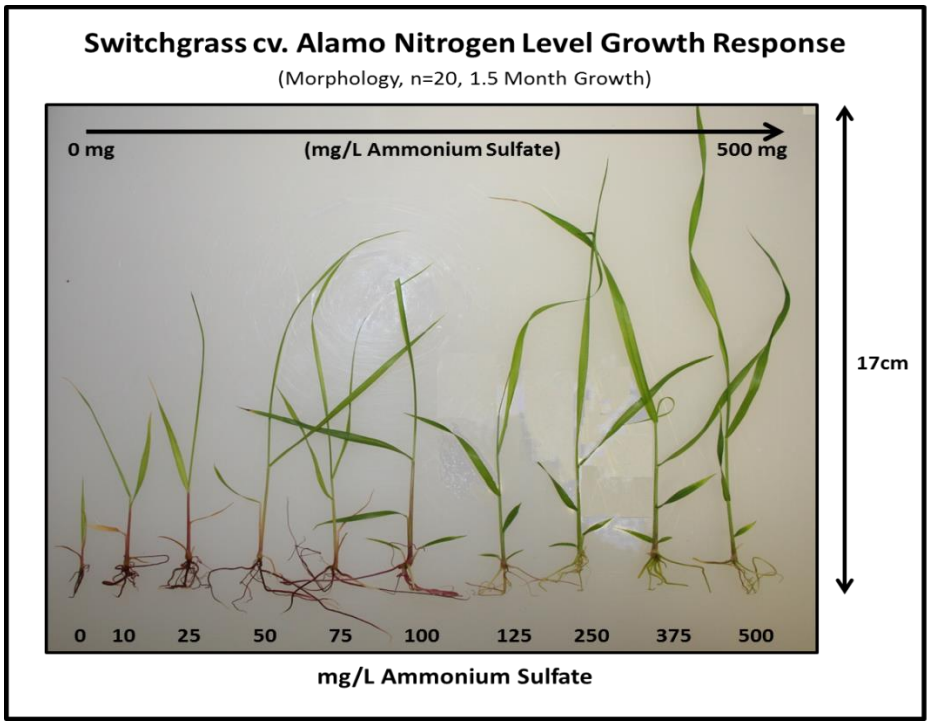

Figure 4.7 Switchgrass morphology in a range of levels of nitrogen. The test included measuring both root and shoot biomass at 1.5 months in nitrogen levels (mg/L) at 0, 10, 25, 50, 75, 100, 125, 250, 375, and 500 added to pre-mixed Hoagland's Nitrogen Free Media

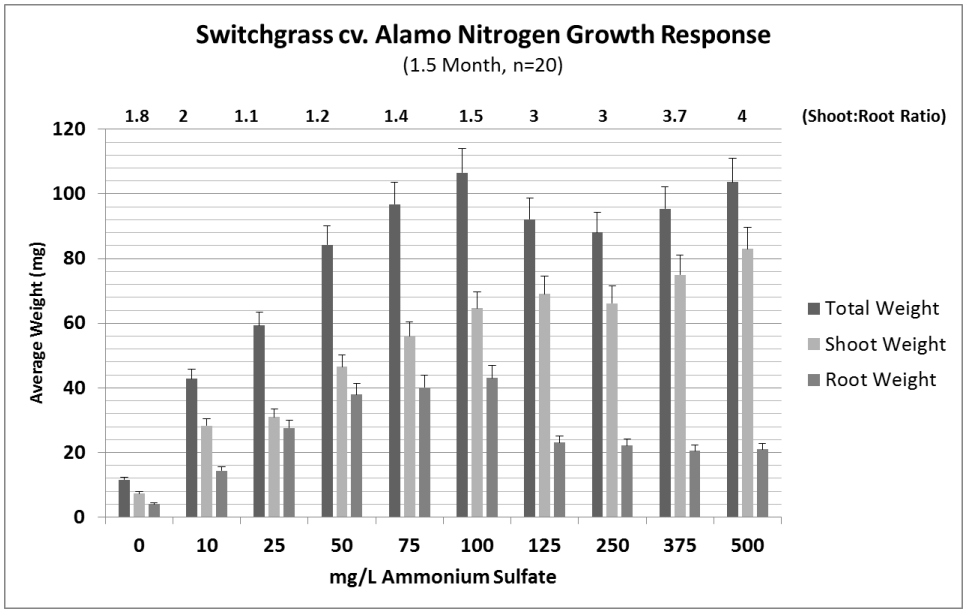

Figure 4.8 Switchgrass growth as affected by nitrogen concentration. The test included measuring both root and shoot biomass of 20 plants per treatment at 1.5 months in nitrogen levels (mg/L) at 0, 10, 25, 50, 75, 100, 125, 250, 375, and 500 added to pre-mixed Hoagland's Nitrogen Free Media. Root to shoot ratio is listed above as an indicator of nitrogen challenge

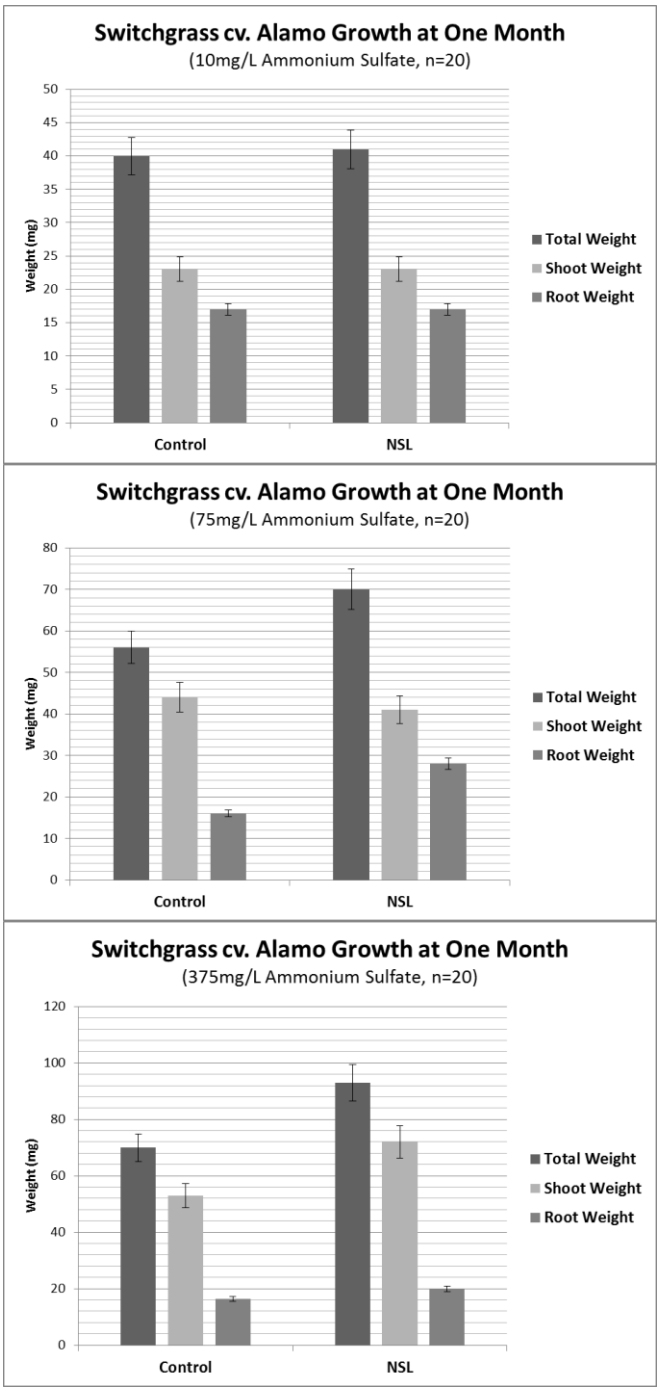

Figure 4.9 Test 1of growth promotion by NSL isolate. Growth was in 10 mg/L (very low nitrogen levels), 75 mg/L (nitrogen deficient), and 375 mg/L (adequate nitrogen). 20 plants were planted per treatment in differing levels of nitrogen in Hoagland's Nitrogen free medium. Plants were grown for one month and the entire plant was harvested. The isolate promoted growth under both nitrogen deficient and adequate nitrogen levels.

Figure 4.10 Test 2 of growth promotion by NSL isolate. Growth was in75 mg/L (nitrogen deficient) and 375 mg/L (adequate nitrogen). Twenty eight plants were planted per treatment in differing levels of nitrogen in Hoagland's Nitrogen free medium. Plants were grown for one month and the entire plant was harvested and root, shoot, and total weights were determined

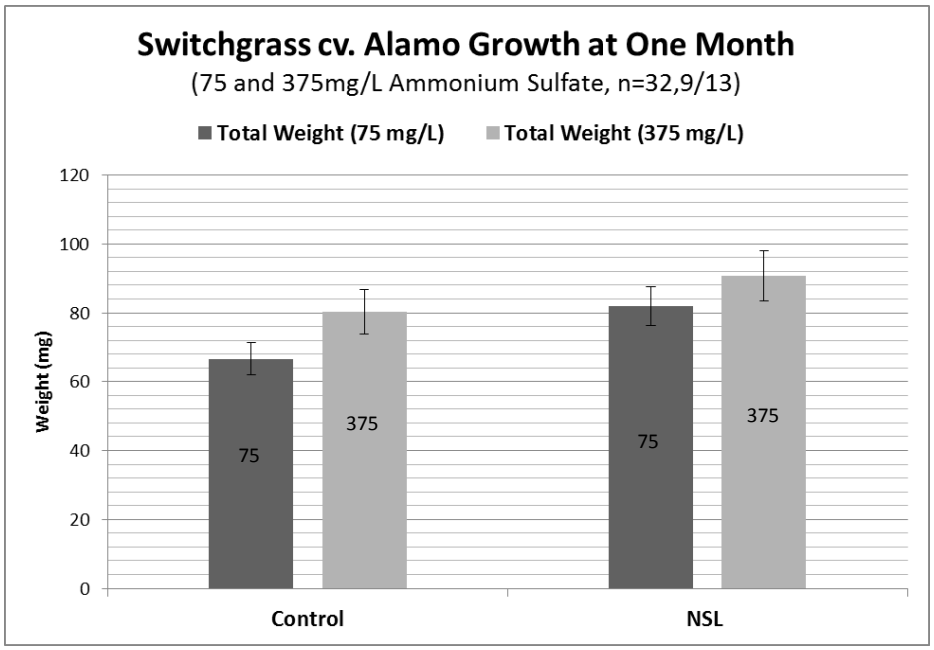

Figure 4.11 Test 3 total weights at one month. Growth was in 75 mg/L (nitrogen deficient) and 375 mg/L (adequate nitrogen). 32 plants were planted per treatment in differing levels of nitrogen in Hoagland's Nitrogen Free Media. Plants were grown for one month and the entire plant was harvested.

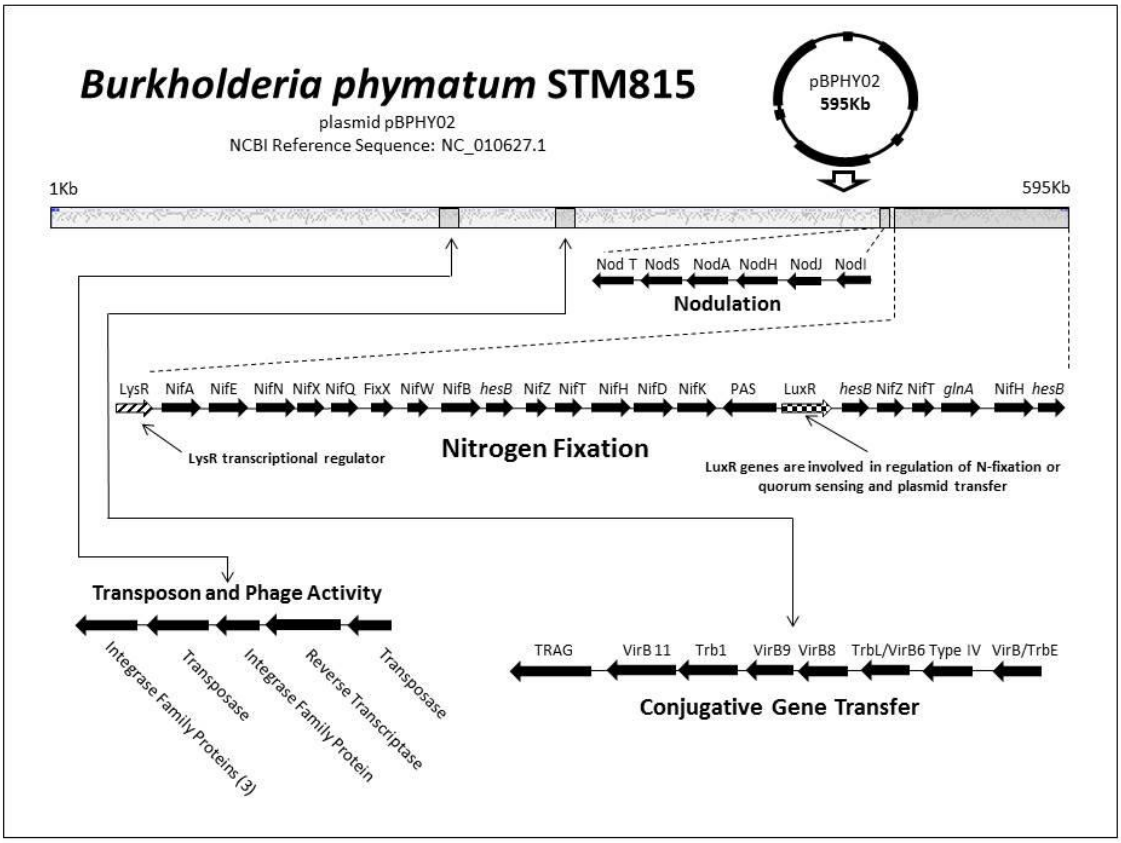

Figure 4.12 A diagram mapping important symbiotic, nitrogen fixing, and nodulating genes on the *B. phymatum* STM 815 pBPHY02 plasmid.

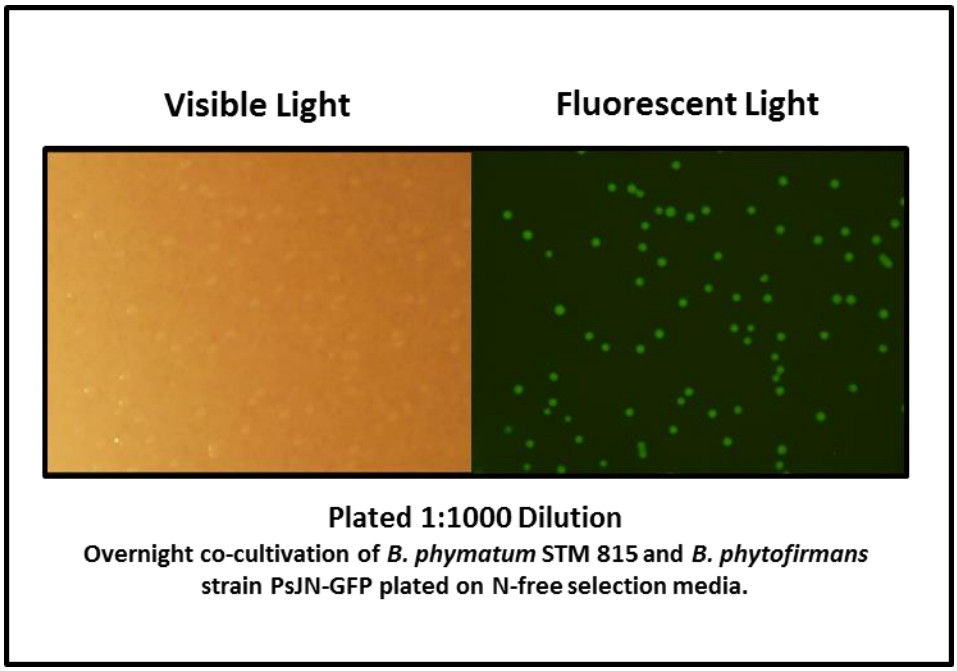

Figure 4.13 Photos of colonies of PsJN-GFP capable of growth on N-free media. Selected GFP containing colonies were streaked and replated three times on N-free selection media to ensure a pure culture.

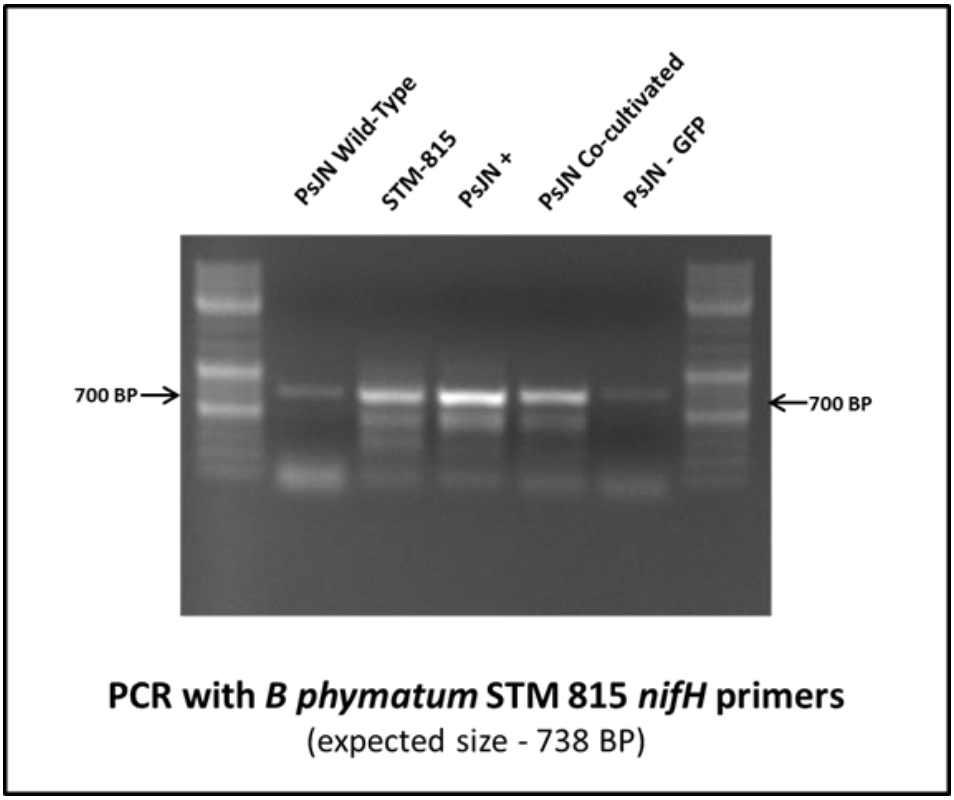

Figure 4.14 PCR results using *B. phymatum* STM 815 *nifH* primers

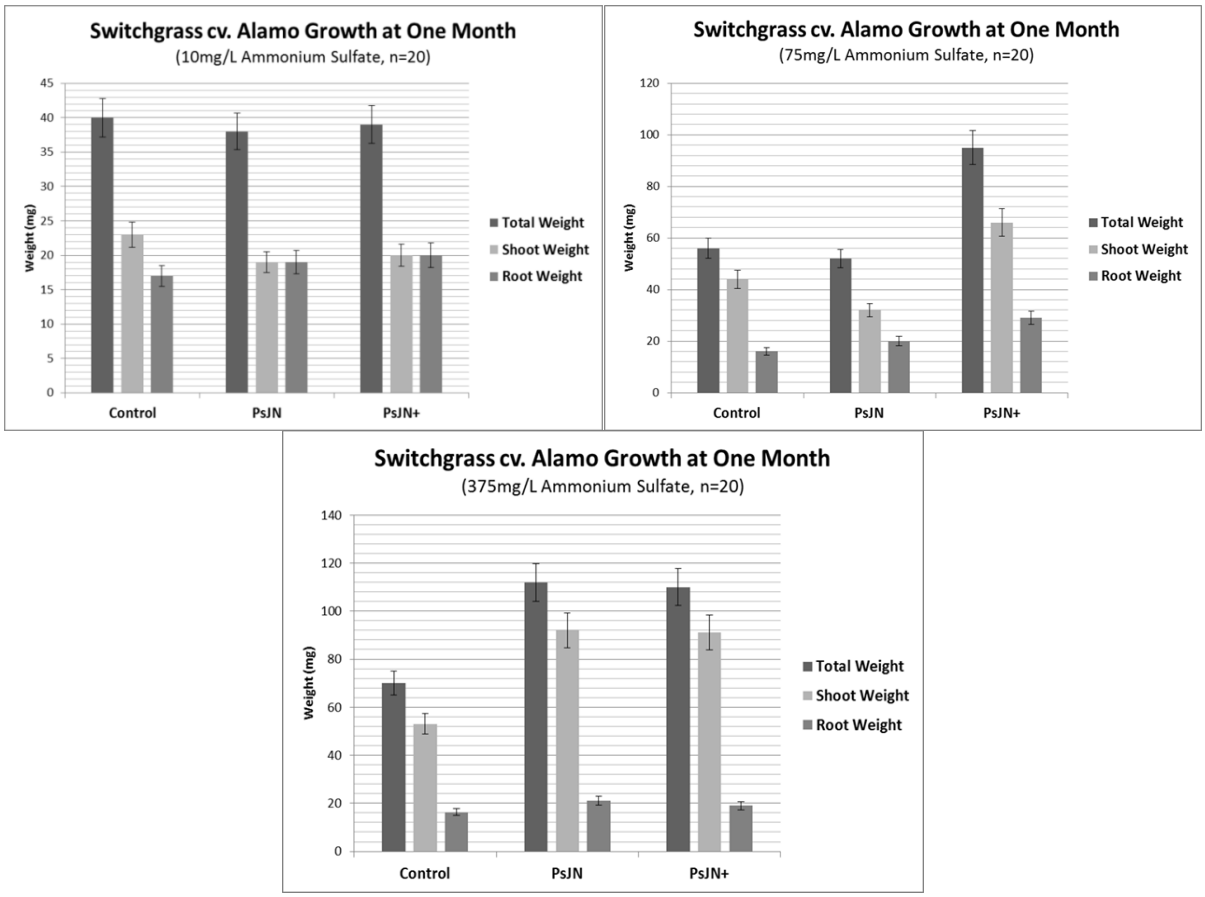

Figure 4.15 Growth promotion by NSL at 10 mg/ml (very low nitrogen levels), 75 mg/L (nitrogen deficient), and 375 mg/L (adequate nitrogen) ammonium sulfate. 20 plants were planted per treatment in differing levels of nitrogen in Hoagland's Nitrogen Free Media. Plants were grown for one month and the entire plant was harvested. Root and shoot weights were then determined

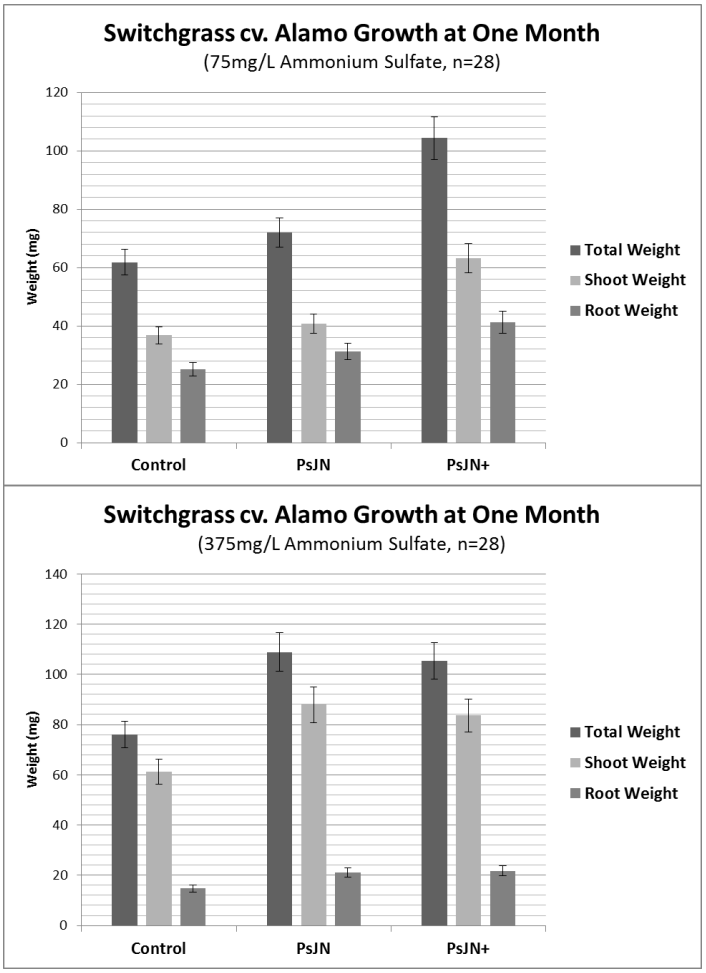

Figure 4.16 Growth promotion by NSL at 75 mg/L (nitrogen deficient) and 375 mg/L (adequate nitrogen) ammonium sulfate. 28 plants were planted per treatment in differing levels of nitrogen in Hoagland's Nitrogen Free Media. Plants were grown for one month and the entire plant was harvested. Root, shoot, and total weights were then determined

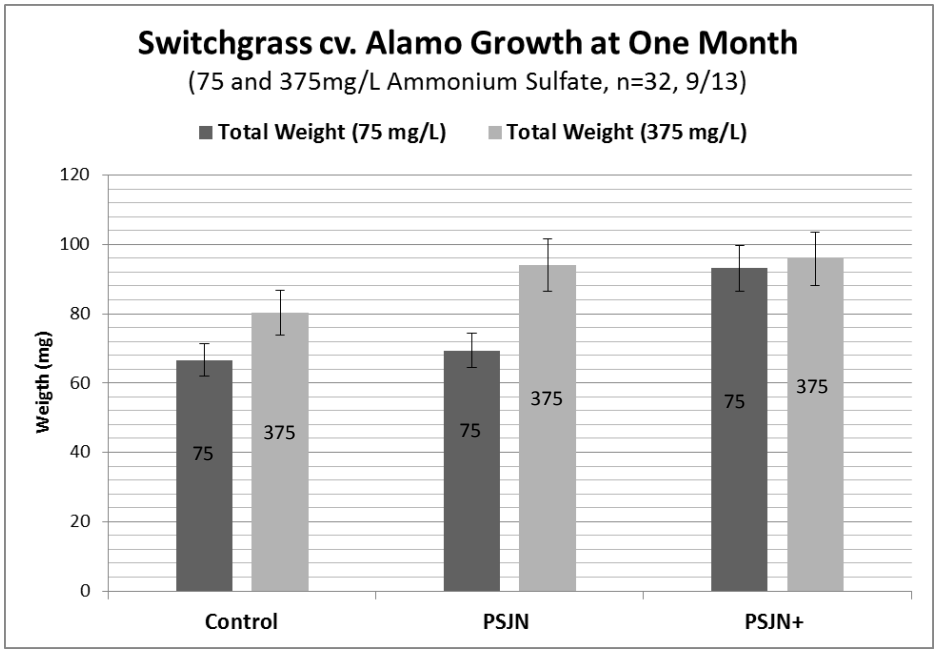

Figure 4.17 Total weight comparisons between plants grown in either 75mg/L or 375mg/L ammonium sulfate. 32 plants were planted per treatment in differing levels of nitrogen in Hoagland's Nitrogen Free Media. Plants were grown for one month and the entire plant was harvested. Root and shoot weight was then recorded.

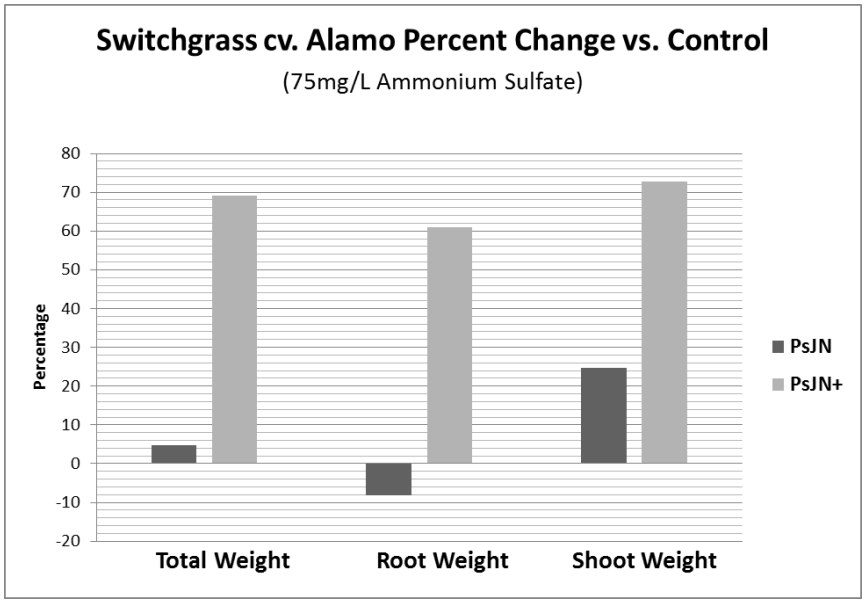

Figure 4.18 Average percent change of PsJN+, PsJN, and control bacterized plants when grown in 75mg/L ammonium sulfate.

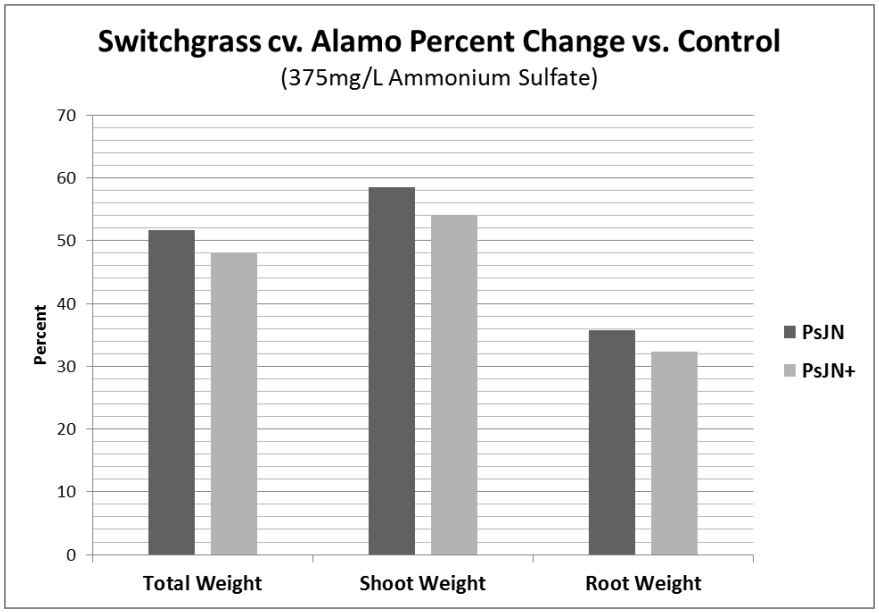

Figure 4.19 Average percent change of PsJN+, PsJN, and control bacterized plants when grown in 375 mg/L ammonium sulfate.

Table 4.1 Sequenced genomes of *Burkholderia* spp.

Organism	Size (Mbp)	GC	#chr	#plsm	GenBank	RefSeq
Burkholderia xenovorans LB400	9.8	62.6	3		Chain et al., 2006	NC_007952.1
Burkholderia phymatum STM815	8.7	62.3	2	2	Elliott et al., 2007	NC_010622.1
Burkholderia sp. 383	8.69	66.3	3		Copeland et al., 2008	NC_007509.1
Burkholderia vietnamiensis G4	8.4	65.7	3	5	Copeland et al., 2008	NC_009254.1
Burkholderia sp. CCGE1002	8.38	63.3			Lucas et al., 2010	NC_014119.1
Burkholderia phytofirmans PsJN	8.22	62.3	2	1	Weilharter et al., 2011	NC_010681.1
Burkholderia cenocepacia J2315	8.07	66.9	3	1	Holden et al., 2009	NC_011000.1
Burkholderia cenocepacia MC0-3	7.9	66.6	3		Copeland, et al., 2008	NC_010508.1
Burkholderia cenocepacia HI2424	7.76	66.8	3	1	Copeland, et al., 2008	NC_008542.1
Burkholderia ambifaria MC40-6	7.6	66.4	3	1	Copeland et al., 2008	NC_010551.1
Burkholderia ambifaria AMMD	7.57	66.8	3	1	Weilharter et al., 2011	NC_008390.1
Burkholderia pseudomallei 1710b	7.31	68.0	2		Holden et al., 2009	NC_007434.1
Burkholderia pseudomallei K96243	7.3	68.1	2		Copeland, et al., 2008	NC_006350.1
Burkholderia cenocepacia AU 1054	7.28	66.9	3		Copeland, et al., 2008	NC_008060.1
Burkholderia glumae BGR1	7.24	67.9	2	4	Copeland et al., 2008	NC_012724.1
Burkholderia pseudomallei 1106a	7.1	68.3	2		Weilharter et al., 2011	NC_009076.1
Burkholderia multivorans ATCC 17616	7.04	66.7	3	1	Holden et al., 2009	NC_010086.1
Burkholderia pseudomallei 668	7	68.3	2		Copeland, et al., 2008	NC_009074.1
Burkholderia multivorans ATCC 17616	6.99	66.7	3	1	Copeland, et al., 2008	NC_010801.1
Burkholderia thailandensis E264	6.71	67.6	2		Copeland et al., 2008	NC_007650.1
Burkholderia mallei NCTC 10247	5.9	68.5	2		Lucas et al., 2010	NC_009079.1
Burkholderia mallei ATCC 23344	5.83	68.5	2		Lucas et al., 2010	NC_006349.2
Burkholderia mallei NCTC 10229	5.8	68.5	2		Holden et al., 2009	NC_008835.1
Burkholderia mallei SAVP1	5.23	68.4	2		Lucas et al., 2010	NC_008784.1

Table 4.2 Presence and location of the *nifH* gene in the genomes of *Burkholderia*

Description	Location of *nifH* gene	Max score	Total score	Query coverage	Max identity	Reference
***B. vietnamiensis* G4**	**chromosome 3**	**1502**	**1502**	**98%**	**98%**	**Copeland et al., 2008**
***B. xenovorans* LB400**	**chromosome 2**	**1009**	**1009**	**100%**	**88%**	**Chain et al., 2006**
***B. phymatum* STM815**	**plasmid pBPHY02**	**942**	**1869**	**100%**	**86%**	**Copeland et al., 2008**

Table 4.3. Overview of nodulation and nitrogen fixation genes in *B. phymatum*.

	Gene or Protein Product	Span (bp)	Product Size (bp)	Description
Nodulation (480Kb-489Kb)	NodT	1599	532	Allows export of a variety of substrates
	NodS	630	209	(Nod) factors are signaling molecules secreted by root-nodulating rhizobia in response to molecules excreted by the host plant. They induce various symbiotic responses on the roots of the host plant at low concentrations, and are required for successful infection(Debellé F et al., 2001)
	Nod/A	591	196	
	NodH	756	251	
	NodJ	792	263	Nod J together with NodI (IPR005978), forms a membrane transport complex involved in the nodulation process (Fernández-López M et al., 1996)
	NodI	915	304	
Nitrogen Fixation (492Kb-588Kb)	LysR	1812	602	Transcriptional regulator (Tyrrell et al., 1997)
	NifA	1644	547	Transcriptional regulator/RNA polymerase sigma factor 54 interaction domain (Morrett and Segovia, 1993)
	NifE	1494	497	Nitrogenase MoFe cofactor biosynthesis proteins (Aguilar et al., 1990)
	NifN	1350	449	
	NifX	417	138	Pathway for the synthesis of the Fe-Mo cofactor (Rangararj et al., 1998)
	NifQ	579	192	
	FixX	300	99	Putative ferredoxin like protein
	NifW	339	112	Essential for the maturation and assembly of nitrogenase (Lee et al., 1998)
	NifB	1608	535	Pathway for the synthesis of the Fe-Mo cofactor (Rangararj et al., 2001)
	hesB	399	132	Involved in Fe-S cluster biogenesis; expressed only under nitrogen fixation (Huang, 1999)
	NifZ	423	140	Required for the maturation of the nitrogenase MoFe protein (Cotton et al., 2009)
	NifT	219	72	Involved in biosynthesis of the FeMo cofactor (Stricker et al., 1997)
	NifH	882	293	Homo-dimer dinitrogenase reductase, or Fe protein (Fani et al., 2000)
	NifD	1464	487	Nitrogenase molybdenum-iron protein alpha chain (Zher et al., 2003)
	NifK	1560	519	Nitrogenase molybdenum-iron protein beta chain (Zher et al., 2003)
	PAS	5430	1809	Signal sensor domain for cell nitrogen status (Menard et al., 2004)
	LuxR	1275	423	1) Regulators which belong to a two-component sensory transduction system (Birck, 2002) 2) Regulators activated by quorum sensing molecules (Pappas et al., 2004)
	glnA	1359	452	Catalyzes the condensation of glutamate and ammonia to form glutamine (Eisenberg et a., 1987)

Table 4.4. Genes involved in conjugative gene transfer, transposon, and phage activity

	Gene or Protein Product	Span (bp)	Product Size (bp)	Description
Conjugative Gene Transfer (295Kb-309Kb)	TraG	1806	601	TraG is essential for DNA transfer in bacterial conjugation (Tomb et al., 1997)
	VirB 11	1038	345	(P-type DNA transfer ATPase VirB11) - a protein that is found in the vir locus of Agrobacterium Ti plasmids where it is involved in the type IV conjugation system for DNA transfer (Li et al., 1999)
	Trb1 VirB9 VirB8 TrbL/VirB6 Type IV VirB/TrbE	1362 918 852 915 678 2574	453 305 283 304 225 857	In the tumor-inducing (Ti) plasmid of *Agrobacterium tumefaciens,* the virB operon is required for the transfer DNA to the plant host and the tra/trb systems are required for the conjugal transfer of the Ti plasmid between cells of Agrobacterium (Wood et al., 2001)
Transposon and Phage Related (228Kb-239Kb)	Integrase family proteins (4)	1008, 957, 1257, 879	355, 318, 418, 292	Cleave DNA by a series of staggered cuts and covalently links to the DNA through a catalytic tyrosine residue at the end of the alignment (Kwon et al., 1997)
	Transposase	1209	402	Needed for efficient transposition of the insertion sequence or transposon DNA (Richter et al., 1998)
	Reverse Transcriptase	1512	503	Uses an RNA template to produce DNA for integration into the host genome and exploitation of a host cell. Occurs in a variety of mobile elements, including retrotransposons, group II introns, and bacterial msDNAs. (Green et al., 1986)
	Transposase IS31/IS911	465	154	Consists of various insertion elements and other bacterial transposases and has been shown to mediate oligomerisation of the transposase components in IS911 (Haren et al., 1999)

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Chapter 5

Connecting research scientists with K-12 education through outreach: concepts, programs, and curricula grounded in design-based STEM education

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Abstract

Modern education reform documents such as the *Framework for K-12 Science Education* released in 2011 by the National Academy of Science support the connection between the practice of science and understanding science. These are departures from past curricula, where science was taught as a collection of facts and the scientific method was presented in a linear and rigid fashion. Now, there is recognition that throughout K-12 education, students should be immersed in scientific experiences to better help them understand the wonders of science and how science can help society address some of the challenges facing civilization in the next century, including predicted shortages of food, feed, and fuel. This chapter seeks to lay a foundation, firmly based in K-12 education literature and reform documents, to encourage more collaborations and partnerships between practicing scientists and the K-12 education community. Reform documents and designed-based STEM curricula are discussed and the potential value of these partnerships is reviewed in the context of successful outreach initiatives. Emphasis is placed on utilization of plants for outreach and the importance of such programs for schools in economically depressed localities. Four case studies are explored including The yearly VT/IALR Plant Molecular Biology Summer Camp, a partnership research experience with 11th grade high school students, the Young Champions partnership for at-risk youth, and finally, a series of short duration outreach activities are highlighted which emphasize renewable energy. Each were developed and delivered by the author during his graduate career.

Key Words: STEM education, Design-Based, At-risk youth, Outreach

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Introduction

Science, engineering, and technology hold the keys to addressing the grand challenges facing our nation in the 21st century, including projected shortages of water, food, and energy. Yet fundamental knowledge and appreciation of the wonder and beauty of these fields is lacking by most US citizens. The connection between each has long been established, with the recognition that all have contributed to advances in one-another and all are tied together almost seamlessly in everyday life (Wells, 2010; NAS, 2011). Education reform is driving the teaching and learning of these subjects in an integrated fashion with a focus on the processes and practices. The National Science Board (2007) clearly states, in its memorandum from the chairman, that the nation is failing to meet the STEM education needs of U.S. students and addressing it is "absolutely essential for the continued economic success of the Nation and its national security". Universities, government granting agencies, museums, professional societies, and corporations are emphasizing that scientists aid and participate in K-12 education (Andrews, et al., 2004) and universities are changing their reward systems for faculty to encourage engagement of the public in their research (Lally et al., 2007). With the release of the new *Framework for K-12 STEM Education* by the National Academy of Science in 2001 which highlight the importance of understanding of the practice of science, now is the time for increased participation from university scientists and engineers as well as those from the private sector to help convey what they do every day with the goal of improving K-12 STEM education. A central theme in increasing these partnerships is to include the involvement of professional scientists in an effort to promote a more informed citizenry and a globally competitive workforce (National Academy of Sciences, 2005).

To convey the importance of a practicing professional's participation, this chapter first seeks to highlight the current focuses of STEM curriculum development in K-12 education, how they are cross connected, and the importance of the integrative and designed based approach. Second, the importance of participation by scientists and engineers, both in and outside the traditional classroom, is highlighted in the context of recent pedagogical recommendations relating to understanding how scientists practice science. Finally, examples of such programs, driven by a partnership between the often separate fields of K-12 education and university research professionals, are presented to emphasize the potential mutual benefits to both. The intent is to

pull together various aspects of both which are mutually beneficial to encourage more participation between each, with the overall goal of improving K-12 STEM education and enriching researchers' broader impacts and professional development. This is a win-win situation using common connections to create a deeper functional understanding of the practices of science and engineering, one of the three core areas presented by the National Academies of Sciences *A Framework for K-12 Science Education* (NAS, 2011).

K-12 STEM education background

Of the STEM subjects, only science and mathematics are established core content areas, while technology and engineering are considered electives and there is a heavy bias towards teaching the former two and between the perceived value of each (Wells, 2010). Additionally, the amount of science and mathematics teaching and learning research, the number of researchers, the number of journals, the number of teachers teaching each, and again, the perceived value of these core areas demonstrate past focuses in K-12 education. However, despite this heavy focus on the "S" in STEM, in 2001 the US Department of Education issued the National Assessment of Educational Progress (NAEP) in science which revealed that average science scores for high school seniors were continuing to decline (US Department of Education, 2001). In the document *A Framework for K-12 Science*, created by the National Academy of Sciences, recognition that in the science content documents created in the mid 1990's, there was much room for improvement. They also recognized that the percentage of students who are motivated by their in school experiences to pursue careers in science and engineering is too low to meet the nation's needs. Perhaps contributing to these observations, traditional science teaching has been discipline specific at the high school level, as in biology or physics or chemistry, focused on facts instead of depth of understanding, lacking in engagement and how science is done (Abell and Lederman, 2007). In fact, in 1996, the National Research Center (NRC) condemned the traditional emphasis on memorization and recitation of facts and instead called on K-12 education to focus on a deeper conceptual understanding with more engagement in authentic scientific practices (NRC, 1996).

At its root, the recent focus on STEM education began with the Excellence Movement in 1983 and highlighted a "Back to the Basics" approach, partially spurred by the politically based document *A Nation At Risk: The Imperative for Educational Reform* by the National

Commission on Excellence in Education (NCEE). Ten years later, the important documents *Science for all Americans (SfAA)* and *Benchmarks for Science Literacy* (BfSL) (AAAS, 1993) were released and highlighted the integrative approach in science, mathematics, and technology (SMT). Integrative STEM education is defined as "the application of technological/engineering design based pedagogical approaches to intentionally teach content and practices of science and mathematics education concurrently with content and processes of technology/engineering education. Integrative STEM Education is equally applicable at the natural intersections of learning within the continuum of content areas, educational environments, and academic levels" (http://www.soe.vt.edu/istemed/). As opposed to the teaching and learning of fact and isolated content - the approach to science and mathematics education in the past - integrative and experiential learning is supported in the literature as matching the way the brain naturally organizes information (Bruning et al., 2004; Shoemaker, 1991). Furthermore, Satchwell and Leopp (2002) found that integrative STEM students were more motivated to learn when content was based in real-life scenarios. Learning this way provides a better understanding of concepts, not just memorized facts, and underscores the importance of an integrative approach to teaching and learning. Student understanding of relationships is also important in problem solving (Benjamin, 1989) and is a core principle in recent STEM curriculum development (NAS, 2011; Wells, 2010) and will surely help in preparing tomorrow's workforce. Standardized tests and other measures show that students in integrative classrooms outperform students in traditional classrooms (Hartzler, 2000; Drake, 2003; Fruger, 2002). Furthermore, applying these concepts from kindergarten to 12th grade, where students are actively engaged in the scientific process and practices, crosscutting across STEM disciplines, will add depth to their understanding of each field's core principles (NAS, 2011).

While the new framework for K-12 science education from the National Academy of Sciences place an emphasis on the practices of science, it must be recognized that, historically speaking, technology education has its roots in hands-on learning and technological design is a well-established component of its pedagogy as Wells (2010, p 202) states "Design-based learning is a pedagogical approach that presents core concepts in a way that concretely demonstrates to students the relevance and utility of content knowledge through an authentic context of need and application". To illustrate this and its grade level specificity in technology education, The Standards of Technological Literacy (2000) produced five categories in the section of

Technology Content Standards that include The Nature of Technology, Technology and Society, Design, Abilities for a Technological World, and The Designed World. The Designed World specifically includes standards 14 – 20 and the subject of biotechnology is specifically included in standard 15: "Students will develop an understanding of and be able to select and use agricultural and related biotechnologies". Content is further divided into sections based on grade levels. Under STL standard 15, the K-2 level includes content based on year-around food availability and conservation of water and parts of ecosystems. Third through fifth graders then address artificial ecosystems, such as aquaculture, where fish and plants are grown together and products of each support the growth of the other. These students also cover the use of agricultural waste and biofuels and that many processes in agriculture require different procedures, products, or systems. In grades 6-8, students begin to learn about and understand how technology has helped reduce labor man-hours and decrease the amount of land needed to grow crops. At this age, students should also begin to understand humans can manipulate living organisms to benefit ourselves. Cause and action is also emphasized at this level and may include farm runoff and pollution of ecosystems. Finally, grades 9-12 begin to use their knowledge of the underlying principles of technology to understand design and systems. Students may consider downstream effects of pollution in aquatic ecosystems, bioremediation, and instruments used to test different parameters. Students also begin to explore business principles. While students participate in designed based exercises, many additional skills are developed, outside the STEM arena, including group approaches to problem solving, an important skill for success in the future. They also develop presentation and organization skills while they share their results. At the same time, they develop writing and the deeper analytical skills, often harder to quantify. Together, integrative and designed based STEM curricula develop the whole student, rather than just a keeper of facts. With this approach, teachers attempting to teach design did not have a grasp of the interconnection between science and technology and they did not have an understanding of the design process, and as a result, they tried to teach it as a linear and context free curriculum, without regard to context. As a result, students were not able to transfer their learning of science (Sidawi, 2009). Compounding the problem of acceptance of inquiry is the underlying belief of many science educators that the balance of teaching inquiry and content is an unachievable goal (Edelson, 2001). However, the poor performance of US students in the past

and new standards which emphasize the practice of science, pressure is now on K-12 education to move away from the teaching of facts and memorization (Sidawi, 2009)

K-12 partnerships with researchers to enhance STEM education

Recommendations from *A Framework for K-12 Science Education* (NAS, 2011 p.17) include three important dimensions: 1) Scientific and engineering practices; 2) Crosscutting concepts that unify the study of science and engineering through their common application across fields, and; 3) Core ideas in four disciplinary areas: physical sciences; life sciences; earth and space sciences; and engineering, technology, and applications of science. Furthermore, the document states that "to support students' meaningful learning in science and engineering, all three dimensions need to be integrated into standards, curriculum, instruction, and assessment." Featuring STEM subjects together, in an integrative fashion reflect the importance of understanding the man-made world and to recognize the value of teaching these subjects, which permeate and influence every aspect of our lives, together (NAS, 2011 p.17). The document consistently recognizes the importance of active engagement of students with crosscutting concepts from early grade levels to achieve a depth of knowledge by the time they graduate high school. Finally, the document states that the "learning experiences provided for students should engage them with the fundamental questions about the world and with how scientists have investigated and found answers to those questions. Throughout grades K-12, students should have the opportunity to carry out scientific investigations and engineering design projects related to the disciplinary core ideas." (NAS, 2011 p.9). It is in this context that professional scientist and engineers, both in industry and academic, should be called upon to make a concerted effort to make a presence in K-12 education, to help train teachers, to engage and explain to students that current scientific understandings of the world are the results of hundreds of years of applying the designed based philosophy.

Currently, higher education institutions are escalating efforts to work with K-12 schools to improve, expand, and supplement educational efforts (Druckman, Peterson, & Thrasher, 2002). Nowhere is the need more evident than in the sciences (National Academy of Sciences, 2006). Furthermore, funding agencies such as the National Science Foundation (NSF) and the National Institutes of Health (NIH) will not consider grant proposals without a clear broader impacts component. A former program manager from the NSF said that while most of their applicant's

scientific merit of their proposal are outstanding, only a few address the broader impact component with seriousness, they are often addressed in a cookie cutter fashion (conference poster and oral presentations, publications, etc.) and this component accounts for 50% of scoring (personal communication). Even the Department of Energy, which traditionally has not addressed broader impacts, is moving towards a model that requires this component (personal communication) and has established an outreach program at its Oakridge Lab in Tennessee. In short, the public is recognizing more and more that these funding agencies should devote increased tax payers' dollars to helping improve K-12 education (which additionally benefits the participating scientists).

Scientist participation in teacher development

Research in the natural sciences typically starts with a hypothesis and a study design is created to control as many variables as possible the hypothesis is then tested and either proven or shown to be false (Gay et al., 2009). While this approach is common in the way a scientist or an engineer addresses a problem every day, delivery of Design Based Learning (DBL) in the K-12 classroom is challenging for a number of reasons including; 1) lack of models, 2) inadequate preparation with content knowledge in the STEM areas and, 3) lack of time for proper preparation and collaborations (Wells, 2010). Programs need to address "methods" of delivery as much as content knowledge as the latter does not necessarily improve teaching abilities (Fennema and Franke, 1992). Roth et al., (1998) suggest that most teachers are not prepared to teach scientific principles advocated by curriculum reform documents citing examples where pre-service teachers perform no better than 8^{th} graders in explaining how science is approached.

To improve knowledge and experiences in science and engineering practices, one emerging viewpoint is also that teachers should have a research experience to foster scientific behavior and thinking (Melear, et al. 2000). In this case, the role of the scientist or engineer in K-12 education may involve teacher development in their facility or lab as teacher developement in hands-on teaching methods that target these higher order thinking skills have been shown to increase student achievement in math and science (Wenglinsky 2002, 2000). The approach to scientific investigation, inquiry, and reason is ingrained in a professional scientist as a result of many years of training. To teach these principles in K-12 requires development of a mindset and often creates tension between teaching of content verses practices (NAS, 2011 p.41) but the past focus

on content lead students to view science as a collection of facts, with a poor understanding of the process of inquiry (Schwab, 1962). There is an importance of understanding how scientists and engineers practice inquiry (NAS, 2011 p.41). For teacher development, one can look to technology educations approach - "Design-based learning combines both practical and theoretical knowledge in a blend of technological design and science inquiry. As a result, students are challenged to employ both vertical and horizontal thinking to synthesize information within learning environments that most closely resemble the context of ill-structured design based problems. In this way designed-based learning creates the need for acquiring integrative understandings in a manner reflective of knowledge requirements in actual practice" (Wells, 2010). The impacts of these approaches have been positively correlated with increased achievement, interest, motivation, attitudes, and problem solving abilities (Reviewed in Wells, 2010). A scientist involved in teacher training can help to communicate the practice of science that both technology education and recent science education curricula emphasized by the Academies of Science (2011).

Scientists as role models to share the wonder of science in the classroom

The "scientist in the classroom" model is also an approach which seeks to bring to schools the content expertise and enthusiasm of professional scientists to stimulate student learning, interest in science, and consideration of science careers is another chance to increase engagement and share the wonder of science (Laursen et al., 2007). Such programs may offer short duration visits to the classroom where the interacts with the students an d can describe what they do in the lab, discuss their careers, or lead a hands on lab activity. Even in this short visit context, the doing of a scientific activity, such as isolating DNA from a strawberry, may increase a students' curiosity or interest and encourage their further study. While both students and the scientists are often enthusiastic about participation (Koehler et al., 1999) few reports are available which provide concrete data on outcomes because the goals are usually broad and long term in scope, as in increasing the number of students who choose science as a career. Designing such a study, controlling for all variables with multiple control groups, enrolling large numbers of participants, and tracking participants though high school and college and into their careers would take many years, and would be excessively expensive with a low chance of assigning influence to any certain outcome (Laursen et al., 2007). While few studies, if any, meet the requirements above due to the vast number variables that are found throughout K-12 education, a review of a long

term program delivered by the University of Colorado, and funded by The Howard Hughes Medical Institute, demonstrated that a scientist in the classroom outreach program has benefits for K-12 students and teachers and "Teachers benefit by learning new content and new ways to teach it, and they feel supported by the presence of interested individuals from the university. We conclude that, when well run and carefully structured, scientist in the classroom programs can have a positive impact on students' interest in science and thus their eagerness to learn it." (Laursen et al., 2007, p.62).

To make programs easier to deliver, with a broader audience, use of modern technology may be employed where a scientist, instead of visiting the classroom physically, may instead videoconference. The Virtual Scientist Program delivered by the Vanderbilt University Center Outreach (CSO) connects university scientists to the K-12 community to enhance and improve science education (McCombs et al., 2006) and delivers 40-50 sessions per year to a national audience. After scientists present a grade level appropriate lesson, teachers, students, and experts complete an anonymous on-line survey that addresses technical and content issues. Students and teachers considered that the program was effective (76% and 89%, respectively) and 97% of students and teachers and 100% of scientists said they would participate in the video conferences again. This program "creates a formal bridge between the science expert and teacher/student audience while promoting an informal interaction open to discussion and exchange. Teachers can select topics suitable to their content needs, with a reasonable time commitment required of the scientist" (McCombs et al., 2006 p66).

Project BioEYES outreach program is also a successful, long term program that features a week-long, grade appropriate, hands-on and inquiry based zebra-fish curriculum that allows students to become the lead scientist while teachers co-teach with university level scientists graduate students nation-wide and internationally (Shuda and Keams-Sixsmith, 2009). Researchers found that students, across all grade levels, showed marked improvements in perception of science, scientific research, and science careers. The authors pointed out that the 5-day program "offers connections between that which was known and that which is being discovered". As the program includes a strong component of teacher training, the authors point out that "Empowering teachers to learn and conduct science, independently, allows for many more students to experience Project BioEYES" (Shuda and Keams-Sixsmith, 2009). This "student co-investigator" model is

also attractive to scientists because data is generated that may be useful and students may more likely think outside the box compared to a scientist who has been focused on the same problem for years with little outside input or ideas injected into their research. Through a hands-on co-investigator approach, students should learn more about other approaches to solving problems, such as modeling, characterizing, discovery based, and reflection – including critique and evaluation - all of which were not emphasized in the past (Schwarz et al., 2009; Abd-El-Khalick et al., 2004). These approaches illustrate the importance of knowing why the wrong answer is wrong can be more of a learning experience than knowing why the right answer is right. The scientist may also explain that practicing science is much more than the typically promoted scientific method, often presented in checklist form where verification of stages is emphasized rather than the creative process practicing science really is (Taylor, 1962). Indeed, after interviewing 52 research scientists, Harwood et al. (2002) quickly found that scientists practiced science in ways very different from what was taught in textbooks and several scientists even provided strong criticism of the traditional scientific method. Practicing scientists and engineers in the classroom can help K-12 students to realize that their work is creative in nature and has the power to change the world (Petroski, 1996). It must again be cautioned that the positive benefits of a "scientist in the classroom" are largely speculative as most of the published literature consists of short outreach program descriptions and advice from experienced program directors (Dolan et al., 2004). These descriptions are valuable in helping to improve existing programs but, again, they are not usually supported by evidence gathered using sound methodology and evaluations (Laursen et al., 2007).

The importance of hands-on experiments in K-12 STEM education in economically depressed areas

While the need to improve and include hands-on and designed based integrative STEM is highlighted throughout recent calls to enhance science education, the *K-12 Farmework for Science Education* states that "concerns about equity should be at the forefront of any effort to improve the goals, structures, and practices that support learning and educational attainment for all students" (NAS, 2011 p.277). This need for educational reform is exacerbated in rural and economically depressed communities. Significant achievement gaps in science do exist in both national and state assessments for low-income and minority students, but these should not be considered an inability of the students to learn complicated topics (NAS, 2011 p.280). "Being

born into a racial majority group with high levels of economic and social resources—or into a group that has historically been marginalized with low levels of economic and social resources—results in very different lived experiences that include unequal learning opportunities, challenges, and potential risks for learning and development" (Banks et al., 2007). Students in such localities are less likely to be exposed to innovative hands-on science learning programs (Lareau, 2003), less social capital mobilized (Lee, 2009), and as a result, often enter formal schooling with less academic vocabularies (Hart and Risley, 1995) compared to more well-funded school systems in proximity to more wealthy metropolitan areas. Providing all students with the foundations of scientific practice will allow them to explore issues related to their communities and personal lives, conduct investigations, and communicate their findings to others (McDermont and Weber, 1998). However, schools in these communities often have low learning expectations and assume little interest in subjects like science and engineering, restricting their educational experiences (Malcom, 1994; Steele, 1997). Studies have shown that children who are identified as "at-risk" because of low family income levels often respond to hands-on educational opportunities verses traditional book and lecture methods (Mccan & Austin, 1988; Cardon, 2000) and engage in more self-directed and creative play outside of the classroom (Lareau, 2003).

A Framework for K-12 Science education points out on p.280 that "While science or engineering institutions can help nearby schools provide high-quality learning experiences for their students (e.g., with experts from industry who visit the classroom, student trips to science centers and aquariums, teacher participation in university programs), access to these assets cannot overcome the effects of inequitable in-school resources across the breadth of schools, and indeed they can reinforce those effects". Importantly, children entering kindergarten from all backgrounds and socioeconomic levels are natural investigators, watching objects fall or plants grow, trying to understand how the world works in sophisticated ways, greater than once recognized (NRC, 2007). Unfortunately, there is almost a total absence of science education in elementary schools with students who are most academically at-risk, students who at this age are often deeply attracted to curriculum related to the natural and designed worlds which provide an important foundation for learning science (NRC, 2009).

Outcomes of programs that are administered by a scientist to disadvantaged school systems are not prominent in the literature. One such study was reported in 2007 and involved graduate students in the sciences who conducted short term outreach in science to K-12 students in schools with greater than 50% minority population in Colorado (Laursen,et al., 2007). Researchers used qualitative data in the form of interviews and chose to solicit observations from both teachers and scientists regarding children's responses to the outreach. Enhanced interest and engagement was reported by 14 of 16 teachers studied. As evidence for these benefits, teachers reported student behaviors such as concentrating on the activities, asking questions, and stating their interest. No teachers reported lack of engagement or interest. Furthermore, the teachers' prior knowledge of their students enabled them to notice responses such as enthusiasm from a student not usually interested in science. The scientists reported on the disparity of educational resources and opportunities for students in the most disadvantaged schools as well as the lack of preparation for college in these same schools. Furthermore, both teachers and scientists reported that the student outcomes may be most important in these high-need schools (Larsen et al., 2007).

Other benefits of a scientist in the classroom may also be realized in these communities of students. They can help at-risk students connect science to local cultural practices, to circumstances in their own lives, and of personal interest (Leuhmann, 2009; Tzou and Bell, 2010). Unfortunately, in schools with little resources, the understanding of basic science skills such as microscopy may be a limitation compared schools with more resources that may already know how to use the instruments (Shuda and Keams-Sixsmith, 2009). Distance between a school in an economically depressed area and a university may also be an obstacle to overcome. Because of this situation, technology, such as videoconferencing, can bridge distances between schools with high proportions of at-risk students not located near universities or teaching institutions, it may provide an opportunity to connect these two entities (Greenburg, 2005). Regardless of how they connect, it is evident that this population of students may not only benefit from a scientist in the classroom, but they may just benefit the most from these types of partnerships.

Programs in plant science

The logistics of studying plants with hands-on activities is difficult due to the timing of the school year. The typical year starts in September and by the time students and teachers are able to initiate a plant biology project, it is often October which is a very difficult time to start almost any type of plant research because of shortening day length and cooling temperatures. Over the long winter break it is often difficult to keep plant research progressing as a modern irrigation system and lights are necessary to continue experiments into the spring semester. However, plant science study in the K-12 classroom has advantages over microbes, because of contamination and scale issues, and animals because of ethical issues, among others. Model plants, such as Arabidopsis, are easy to cultivate, have a fast life cycle, and are easy to care for. The PREP program (Lally et al., 2007) is a large and successful program that allows high school students to design and conduct experiments on mutant lines of Arabidopsis and analyze their phenotypes after they are grown in a number of stress conditions. In partnership with different scientists, this provides a genuine research experience for high school students and teachers while allowing the scientific community to screen and discover functions of a large number of poorly characterized plant genes that may not be otherwise discovered in plants grown under ideal conditions. Through the interactive research experience, students and teachers are mentored by scientists in a 6 to 8week experience developed with the realities of teaching in mind (Lally et al., 2007). Plant scientists are, therefore, in an excellent position to connect with the K-12 education community to improve knowledge, increase interest in science, and help students and teachers understand the practice of science, a focus and direction highlighted in *A Framework for K-12 Science Education* (NAS, 2011). Plant scientists who engage and provide knowledge and positive experiences can help make research visible, accessible, and significant to students (Wandersee and Schussler, 2001).

Case Studies

Case One: The VT/IALR Molecular Plant Science Summer Camp - Hands-on Research and Residential Experiences for Rising High School Seniors from Southside Virginia

Background

The purpose of the VT/IALR Molecular Plant Science Summer Camp is to foster an enthusiasm for plant science, promote interest in plant science as a career while providing an on-campus experience to learn molecular plant biology through hands-on research experiences for students

from economically depressed Southside Virginia. The program is in its fourth year of development and execution and this paper focuses on impacts both in and outside of the lab as well as lessons learned. This model not only promotes a K-12 partnership with university research scientists in an on-campus experience, but it also emphasizes the possibility of bringing the experience of doing science with students from school systems in rural and economically depressed localities. While the long term impacts of short duration programs like these are hard to measure, survey outcomes and journal entries are compiled from the last two camps.

Introduction

In 2011, molecular plant science researchers and educators at Virginia Tech (VT) and The Institute for Advanced Learning and Research in Danville Virginia (IALR) teamed to create a week-long residential summer camp for rising seniors with the goal of increasing interest in molecular plant science research. During the week, students work side-by-side with researchers on campus at Virginia Tech utilizing various molecular biology techniques to express and visualize green fluorescent protein in different living tissues in tobacco. Surveys, questionnaires, and journal entries were utilized to measure interest in plant science and careers in plant biology research both before and after the camp. Long term evaluations of impacts of such programs are difficult to quantify as methodological limitations apply.

Materials and methods

Participant's first isolated plasmid DNA, transformed it into *Agrobacterium* and then inoculated it into tobacco plants. Students next observed green fluorescent protein (GFP) expressed in living plant cells. The transformed plant cells expressing GFP proteins were then treated with various chemical reagents and students observed the change/movement of GFP proteins under microscopes. In the lab, students learned how to use a microscope to observe green florescent proteins expressed in living plant cells. Outside of the lab, lecture sessions covered principles of microscopy, gene cloning, transient gene expression, plant tissue culture and genetic engineering with both gene gun and *Agrobacterium*. To accomplish these tasks, students worked with common tools in a molecular lab including pipetting (**Figure 5.1**), gel electrophoresis (**Figure 5.2**), calculating molarity, microscopy (**Figure 5.3**), and plasmid DNA isolation. Surveys, journal entries (**Table 5.1**), and interviews were primarily used for data collection regarding student interest in and content knowledge of plant science before and after the camp. A full

scholarship was provided for housing and meals to bring students from economically depressed Southside Virginia to participate in the hands-on research and on-campus experience centered on molecular plant science. Dr. Bingyu Zhao from the Department of Horticulture provided the lab supplies, developed curricula and lab protocols and three of his graduate students worked side by side with the students. Additionally, students toured other labs, research greenhouses, and field trials, where they were given presentations highlighting the importance of plant science research in the context of the grand challenges of the 21st Century (food, feed, fuel, and water) in the areas of bioenergy, molecular genetics, and bioinformatics. Tours included:

1) Virginia Tech's Kentland Farm is the location of Virginia Tech's field research where potential bioenergy crops including switchgrass, *miscanthus*, and giant reed are grown and evaluated. The switchgrass cultivars alone exemplified the variation observed in natural populations of native grasses. The contrasting plant characteristics were used to explain the regional nature of future bioenergy production.
2) The Virginia Bioinformatics Institute (VBI) houses both high speed genetic next generation sequencers as well as supercomputers capable of analyzing the large amounts of data generated from these machines.
3) The Institute for Advanced Learning and Research (IALR) was toured on the final day; students viewed the facilities at IALR in Danville to highlight advanced molecular plant research and commercial production of plants in their region.

Results and discussion

In total, 20 rising high school seniors from economically depressed Southside Virginia participated in the camp in 2012 and 2013. Students came from a variety of schools including Public County and City Schools and a private Christian and girl's academy. After orientation and before students started lab work, they were given a quantitative questionnaire with answers ranked from 1 to 5, depending on the question. Students were also given journals which were reviewed at the end of the week for qualitative data and feedback. During the camp, emphasis was placed on increasing interest in plant science careers. When asked before camp if students were interested specifically in becoming a plant molecular biologist, only moderate interest was selected for the group (**Figure 5.4**) although one student that "they [plant scientists] do much more than mix compounds and grow plants, they can interpret their research and help improve

society". After the camp, a slight increase was detected in the surveys, although not significant for the 2013 camp, possible due to the smaller number of students participating. Also during the camp, the importance of plant science in the future was emphasized in the context of future needs of fuel, feed, and food. Question 2 was designed to measure students' perceptions of the importance of plant science discoveries to future challenges to mankind (**Figure 5.5**). Both the 2012 and 2013 camp surveys indicated that during the camp, participant awareness of opportunities to make important discoveries in plant molecular biology increased. One student commented, "There is plenty of opportunity for new discoveries in the field of plant sciences. This (the camp) shows that there could potentially be careers open to me in the future concerning practical, original, and interesting research". Another student recorded "I'm curious as to what the future holds for plant biology". The 2013 camp all rated this question with a 5 at the end of the camp, indicating they thought many opportunities were available for important discoveries in plant science. Question 3 gauged interest in plant molecular science before and after the camp (**Figure 5.6**). In both camps, there was an increase in interest and each camp result was consistent with the other. The highest increase comparing before and after the camp surveys was attained with question 4 in the 2013 camp, which students gauged their own knowledge of molecular plant science (**Figure 5.7**). **Figure 5.8** combines questions 5, 6, and 7. 80% of students in 2012 and only 50% of students in 2013 answered that the camp increased their interest in pursuing molecular plant sciences as a career. Question 6 asked if the camp increased their interest in plant biotechnology and 91% and 80 % answered yes in 2012 and 2013 respectively. A student recorded in their journal that they "were fascinated to see how technology was developing at such a fast pace". Finally, students asked if they would recommend the camp to their peers, 100% answered yes for both years. Importantly, after the camp, two students pursued more lab experience and both worked in the lab the remainder of the summer.

The student scientist connection was also observed and one student wrote in their journal "I enjoyed working with my mentor; he was a great teacher and worked well with our group". One of the mentors also commented on how important it was for him to "practice explaining my research to people outside of my field, I am accustomed to conversations with people who work in my lab and in the same research field; it [the camp] is an excellent chance to communicate with people who are not familiar with the field". Overall, results of quantitative and qualitative

surveys and journal entries were similar between the two years of camps. Participants all gave positive feedback regarding both what they learned and their experiences. After the camp was complete, two students pursued research and worked in the lab the remainder of the summer. Based on results from surveys, journals, interviews, and feedback, students' interest in plant science was increased and they were more likely to pursue research in the field. Students also gave positive feedback of their experiences working with graduate students in the Zhao Lab to complete their projects.

As camp funding was limited, affordable methods, such as surveys before and after camp, typically can only measure attitude changes. Whether these changes are long term, permanent, or lead to real change is not within the scope of this method of evaluation and such "ideal" evaluations are difficult to administer because of a number of reasons. It is also difficult to determine what truly leads to detected attitude changes because surveys given at the end of a camp happen when the students are excited may be more of a measurement of their enjoyment of the camp and not a measure of whether the larger camp objectives were met (Bogue, 2005). Indeed, even survey data that indicate attitudinal changes may not be sufficient to establish what caused those changes (Lott, 2003),

Case Two: Graduate Student Lead Research Experiences for High School Juniors

In 2010, a research partnership between a graduate student studying science in the Center for Peace Studies and Violence Prevention at Virginia Tech and Central Virginia Governors School for Science and Technology (CVGST) for students to become co-investigators of the potential bioenergy crop switchgrass. A presentation was given to all students in the research class at CVGST to gauge interest in plant biotechnology and two students chose to participate in the semester long project. During the project, students and the graduate student mentor constructed two hydroponic tables (**Figure 5.9**) to investigate growth in low nitrogen, and high salt hydroponic media. Students designed the study with the help of the mentor, collected data weekly on shoot growth in various growth media by measuring heights. At the end of the research period, the students, with the help of their scientist mentor, compiled the data and presented it at a state wide Governors School conference. Students also gave feedback regarding their experiences with the research and plant science in general;

Research- “This project was extremely interesting and enjoyable. After putting in so much hard work on background knowledge, it was fun to watch my plants grow and make conclusions. This project also showed me how important a large sample size can be, especially with plants”.

Plant science- “I enjoyed plant science. Plants do not make their own decisions so it seems like a more controlled field. The hydroponic tables definitely made studying plants easier and more fun”.

Additional comments – “Thank you so much for helping me! I can’t imagine how this whole class would have been without this project. It has been really interesting and I am excited to present it at the science fairs”.

Case Three: Horticultural Science for At Risk Students “Young Champions” 2010

During the fall and spring semester of 2009-2010, the young champion program was developed with the help of a graduate student from Virginia Tech’s Center for Peace Studies and Violence Prevention and Pat Price, M.Ed and interim director from Lynchburg College’s Center for Community Development and Social Justice. The group of 12 students from Rivermont Alternative School for Troubled Students visited the farm each week for an entire semester and learned plant science and business development skills from the graduate student and Lynchburg Grows Staff which also consisted of people with disabilities. Students, as co-investigators and entrepreneurs, first created a business plan and then developed their products including grafted roses and micro-greens. At the end of the semester, the students took pictures of the farm and displayed them in an art show at Lynchburg City Lofts.

Pat Price ’95, ’05 M.Ed., an interim director of LC’s Center for Community Development and Social Justice (ccdsj) reported “The kids loved it and became more interested in science than they could have ever imagined,”

Case Four: The Department of Energy - Bioenergy Short Duration Outreach to the Community

Lynchburg Grows Education Center (www.lynchburggrows.org) programs were developed to include outreach to K-12 students, college students, and the general public. The partnership was developed with the Executive Director, Michael Van Ness and feature a 20 foot by 40 foot switchgrass research field trial, and plantings of other common bioenergy crops such as

Miscanthus as a bioenergy demonstration site and highlighted to the more than 1200 students and adults that visit the farm each year. Developed programs emphasize sustainable bioenergy, DOE research, and plant science as a career and range from week-long farm camps with disadvantaged school children to the recent "Urban Agriculture Day" where over 500 city school fifth graders visited the farm to learn about renewable resources. Each K-12 program emphasizes SOL based, grade appropriate lessons featuring renewable energy and resources. In 2010 and 2011 alone, over 120 at-risk third through fifth grade students from the Jubilee Center and Bass Elementary School participated in a week long farm camp where a scientist in training have the students participate in hands-on activities and the bioenergy test site is highlighted along with beneficial bacteria and renewable resources. Students who attend these camps are often from diverse inner city households and most say it is the first time they have talked to a scientist. Last year, over 80% of the students responded that they "knew more about natural resources from attending this day camp".

Another field trial demonstration site is located at Chatham Hall Girls Academy where over 50 students learn and biology classes participate in switch grass research. Other outreach partners include the Central Virginia Governors School for Science and Technology and Lynchburg College and assessments for various programs have been developed with help from Virginia Tech's School of Teaching and Learning. Together these programs reach more than 5000 students and adults yearly (**Table 5.2**).

Conclusions

From the national news to grant funding agencies, there are reports highlighting and promoting the importance STEM education. However, while presented as an urgent necessity to increase STEM teaching and learning in the classroom, the US K-12 education system does not tend to move quickly and abrupt changes may be out of the question. The multiple reasons that underlie the difficulty of change in the system include; teachers must be prepared in STEM content delivery, agreed upon curriculum must be developed, and time during the school day must be allotted. On the other hand, promotion of the STEM acronym and funding of related activities have moved to the forefront of education, but measurable improvements in the integrative approach to teaching and learning are still in question. Adoption of new curricula has momentum as the Enhancing Science, Technology, Engineering, and Mathematics Education Act of 2008

(eSTEM Act, H.R. 6104) was passed that institutes STEM education related governmental entities, a consortium, a clearing house to disseminate creative programs and ideas in teaching and learning, and perhaps most importantly, content standards for K-12 STEM. Furthermore, funding and initiatives at the local, state, federal, and private foundation levels support the growth in and emphasis of STEM education and college faculty are being encouraged to engage and connect with K-12 education.

Emphasized in the chapter are short term duration activities, not fundamental changes like national curriculum or school schedule adjustments, instead they provide the basis and support for both K-12 educators and researchers to combine forces, with mutual benefits for both. These short duration intervention strategies are primarily based on the change model (Seymour, 2002) under the assumption that developing interest in and enthusiasm around science may include short hands-on experiences with science, interacting with scientists as role models, and learning about scientific careers and opportunities to make a difference in the world. Together, these experiences may contribute later to pursuing a career in science. Also, these programs may benefit school systems from economically depressed areas, often with lower resources, the most. Regardless of the student's background, "The learning experiences provided for students should engage them with fundamental questions about the world and with how scientists have investigated and found answers to those questions. Throughout grades K-12, students should have the opportunity to carry out scientific investigations and engineering design projects related to the disciplinary core ideas" (NAS, 2011, p.9).

Scientists have the ability to help improve teacher development and student understanding of the practice of science and with the delivery of quality K-12 evidence-based, designed-based and integrative STEM outreach programs all parties can benefit from. For teachers, research experience may improve confidence in the scientific process and scientists can help explain what they do every day, all of which may improve delivery of designed based lessons in the K-12 classroom. For students, the enthusiasm and presence of a scientist in the classroom as well as participation in or "doing" science may have may inspire and increase interest in science as a career. For the scientist, these quality programs create an avenue to share their research and even acquire new data utilizing the classroom teachers and students as "co-investigators" with new and fresh perspectives. Together, "understanding science and the extraordinary insights it has

produced can be meaningful and relevant on a personal level, opening new worlds to explore and offering lifelong opportunities for enriching people's lives" (NAS, 2011 p.7). This document supports and encourages scientists' engagement in the K-12 community.

Figures and Tables

Figure 5.1 2012 Molecular Biology Summer Camp students learning how to pipette.

Figure 5.2 Student learning the principles of gel electrophoresis.

Figure 5.3 Student learning microscopy.

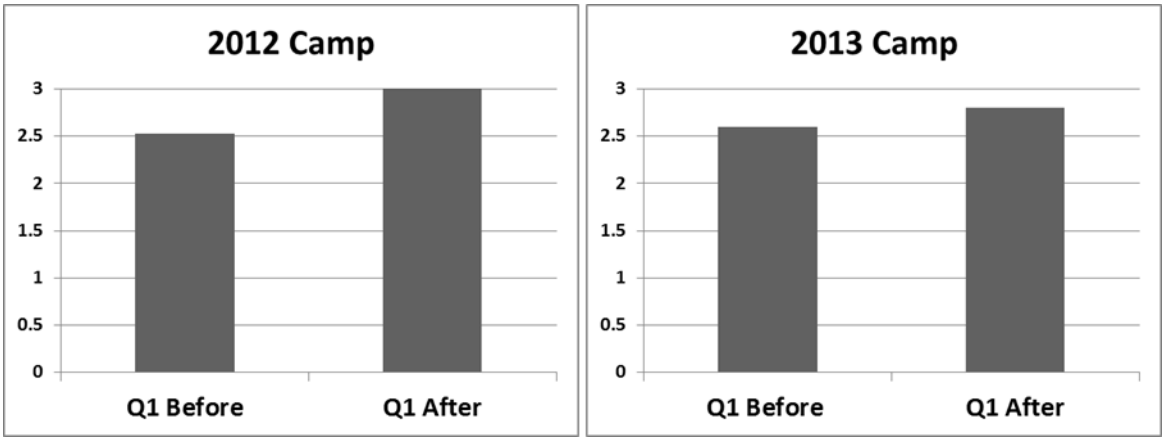

Figure 5.4 Question 1 (Q1) to measure interest in becoming a plant molecular biologist. Using a Wilcoxon Signed Ranks Test, there was a statistically significant increase in students' interest in becoming a plant molecular biologist during the 2012 camp ($Z = 1.896$; $p = .058$) (No interest = 1, Very interested = 5).

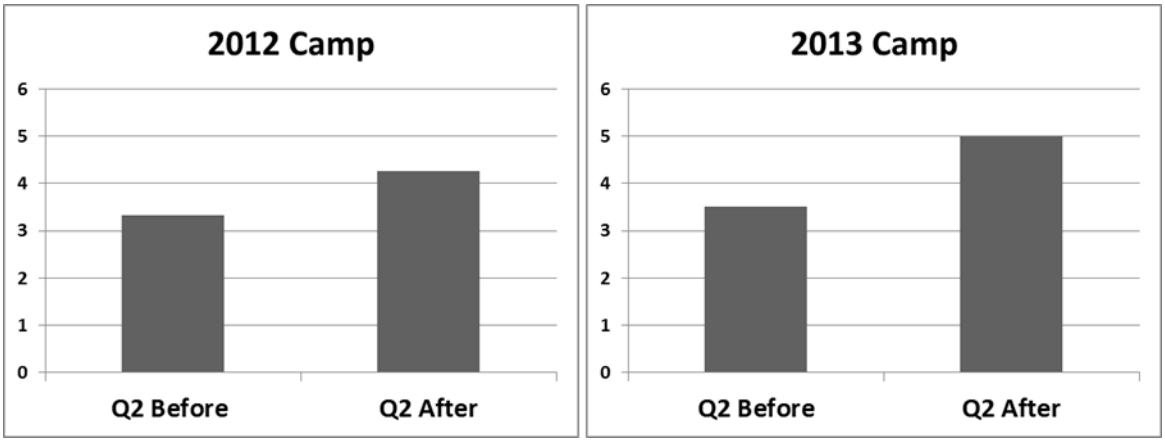

Figure 5.5 Question 2 (Q2) to measure awareness of opportunities to make important discoveries. The belief that there are many opportunities to make important discoveries in plant sciences significantly increased among student participants ($Z = 2.54$; $p = .011$) (No opportunities = 1, Many opportunities = 5).

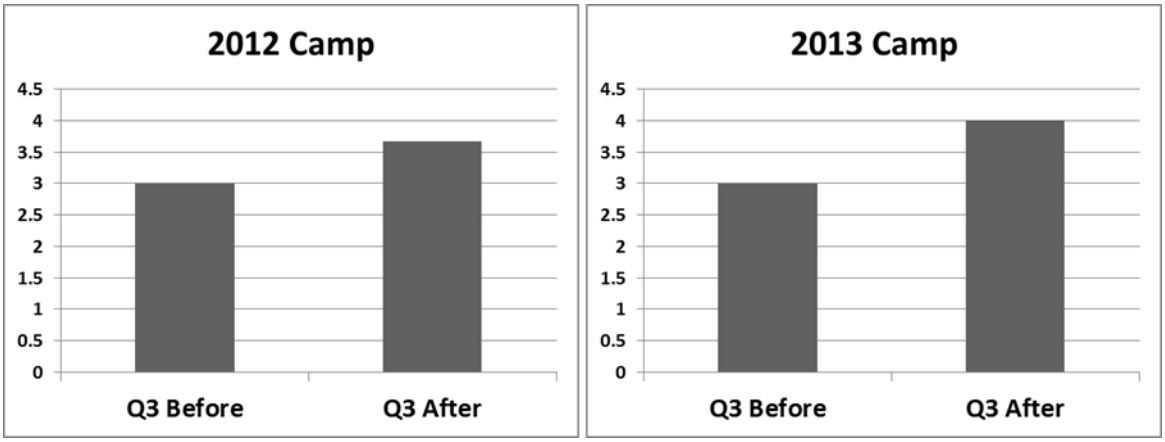

Figure 5.6 Question 3 (Q3) to measure how interesting participants find plant molecular science. Student's interest in plant molecular science increased significantly ($Z = 1.92$; $p = .054$) (Not interesting = 1, Very interesting = 5).

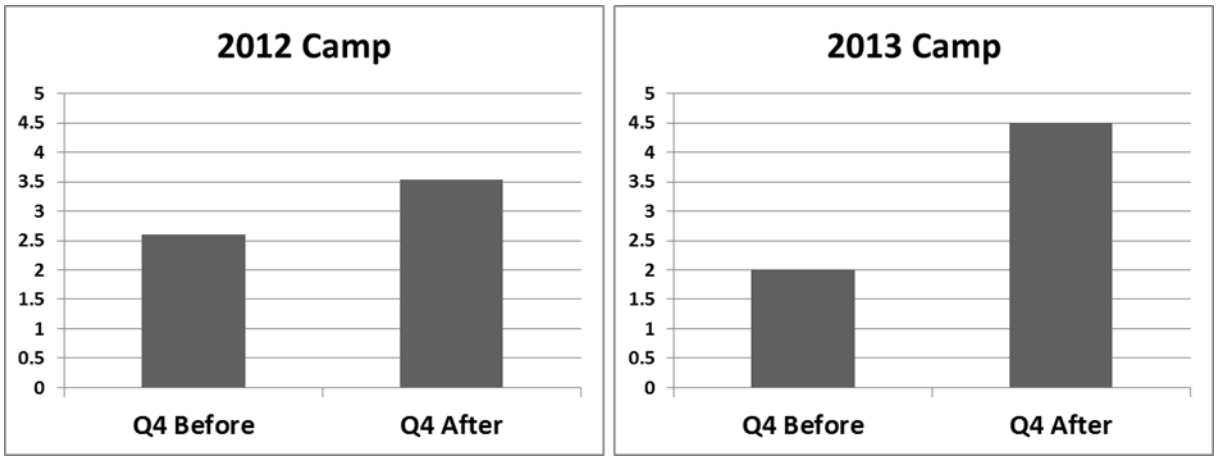

Figure 5.7 Question 4 (Q4) to measure knowledge of plant molecular biology. (Little Knowledge = 1, Very Knowledgeable = 5)

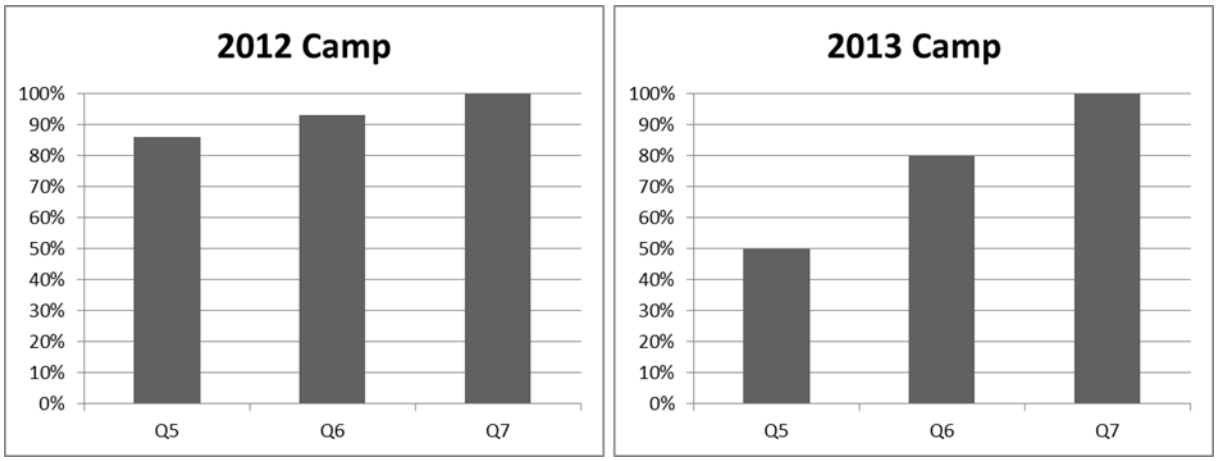

Figure 5.8 Answers to remaining questions. Question 5 (Q5) Did the camp increase your interest in pursuing Molecular Plant Sciences as a Career? Question 6 (Q6) Did the camp increase your interest in plant biotechnology? Question 7 (Q7) Would you recommend the camp to your peers?

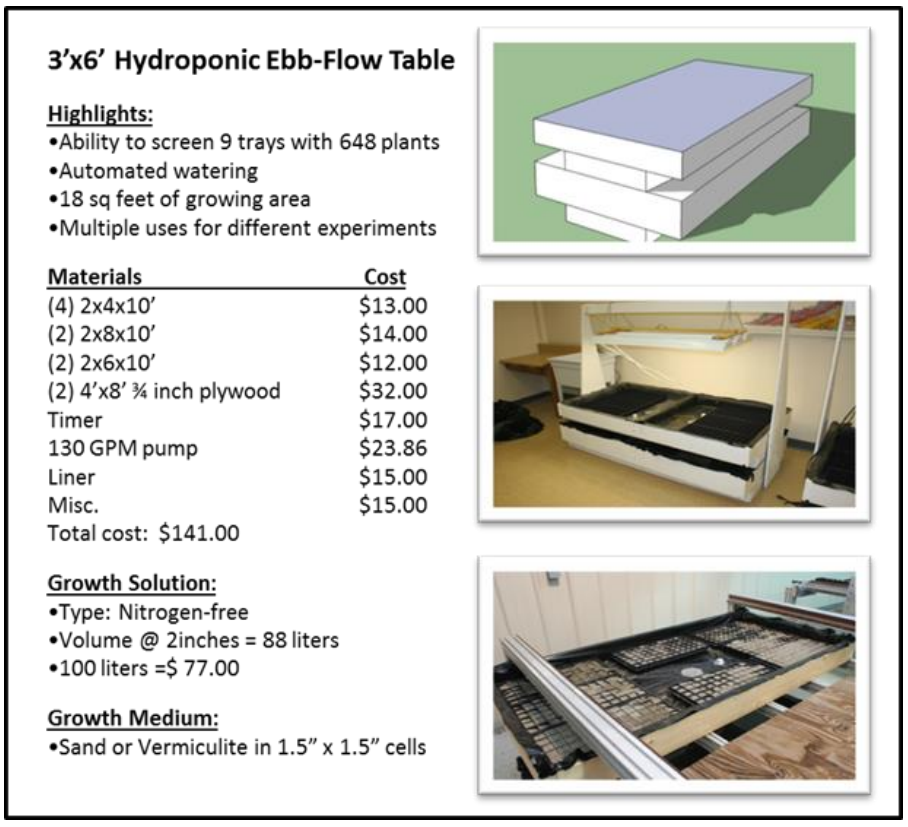

Figure 5.9 Hydroponic table descriptions. Tables were located at Central Virginia Governors School for Science and Technology and the Institute for Advanced Learning and Research.

Table 5.1 A summary of journal quotes from the 2011 and 2012 science camps.

2012 Journal quotes from the week:

"I was impressed that researchers from various places employ VBI to use the advanced machines"

"Learning that switchgrass could be used as a biofuel was the most interesting thing I learned this week. It makes me feel better about the future knowing we have new sources of fuel available"

"I enjoyed working with my mentor, he was a great teacher and worked well with our group"

"I'm curious as to what the future holds for plant biology"

"I was fascinated to see how technology was developing at such a fast pace"

"I loved to see and learn about how the agrobacterium works as a middle man to placing desired proteins into plants"

2013 Journal quotes from the week:

"Learning how by studying plants we can learn about ourselves"

"I appreciate now the time and research that goes into making the world around us suitable for living conditions. I also loved being in the lab and learning how to use the equipment"

"There is plenty of opportunity for new discoveries in the field of plant sciences. This shows that there could potentially be careers open to me in the future concerning practical, original, and interesting research "

"That they do much more than mix compounds and grow plants, they [scientist] can interpret their research and help improve society"

"How plants and their diseases are similar to humans. If we find a scientific breakthrough in plants, then we may be able to solve similar problems in humans."

Table 5.2 Overview of short duration outreach performed for the Department of Energy Plant Feedstock Genomics grant.

	Outreach Activity	Target Audience	Duration	Frequency	Impacted	Description
K-12 Outreach	Career Day at Rustburg Elementary	All 3 grade	45 minutes	1 per year for 3 years	375 students	Presented what it is like to be a scientist, highlighted bioenergy and the importance of renewable energy, and included a hands-on activity of isolating DNA from a Strawberry
	Chatham Hall Girls Academy field trial	11th grade biology class	45 minutes	1 per year for 3 years	44 students	Presented grade level appropriate material related to genomics and bioenergy research, students participated as co-researchers with hands-on experiment in sustainable agriculture using beneficial endophytes
	Field trial site at Lynchburg Grows	K-12	20 minutes	1 class per week for 3 years	3600 students	During field trips, tours, and open houses, students were given a presentation on bioenergy feedstock, beneficial bacteria, and sustainable agriculture. Switchgrass and Miscanthus was grown on-site at the Urban Farm and Environmental Education Center
	Bass Elementary Farm Camps	3rd – 5th grade	1 week	1 per year for 3 years	65 students	Yearly farm camps were given tours and participated in hands-on activities related to bioenergy and other forms of renewable energy. Students from Bass Elementary are defined as at-risk (97% qualify for free lunch)
	Jubilee Family Center Farm Camp	3rd – 8th grade	1 week	1 per year for 3 years	89 students	Yearly farm camps were given tours and participated in hands-on activities related to bioenergy and other forms of renewable energy. Students that attend the program at Jubilee are generally considered at-risk because of family income
	Young Champions	9th grade	2 hours per week	9 weeks for 2 years	30 students	Students attending Rivermont School, an alternative school for troubled students, visited the farm once per week for 2 hours and participated in hands-on activities centered on sustainable agriculture in partnership with Lynchburg College and The Center for Peace Studies and Violence Prevention at Virginia Tech
	Girl Power	6th grade	2 hours	2 years	22 students	At-risk girls attending a summer camp at Lynchburg College visited the bioenergy outreach site where they were given a presentation on bioenergy and other forms of renewable energy as well as science as a career
Total Number of K-12 Students Impacted by Outreach:					**4225**	

College Students	Lynchburg College Environmental Chemistry Class	Juniors and Seniors	1 hour	3 years	45 students	Visited Lynchburg College and gave presentations on bioenergy and science as a career.
	Alternative Spring Break Group Educational Activity	Freshman to Seniors	2 hours	3 years / 6 groups	85 students	Students participating in college alternative spring breaks and visiting Lynchburg Grows participated in activities involving bioenergy feedstock and lingo-cellulosic energy production
	Field trial demonstration site	Freshman to Seniors	20 minutes	1 per month	340 students	Students toured bioenergy demonstration site to learn about feedstocks and renewable energy

Total Number of College Students Impacted by Outreach: 470

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