Project Title: Development of a Low Input and Sustainable Switchgrass Feedstock Production System Utilizing Beneficial Bacterial Endophytes

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1. The DOE award number and name of the recipient:
Award Number: DE-SC0004951 (08/01/2010 to 07/31/2013, with one year no cost extension)
Recipient: Institute for Advanced Learning and Research

2. **Project objectives:**

- Global gene expression profiling during bacterial endophyte-switchgrass associations will be conducted using switchgrass EST microarrays to identify key genes involved in endophyte infection, colonization, and mutualism, allowing establishment of the complete gene networks governing plant growth promotion. Using responsive and non-responsive cultivars inoculated with the same strain of PsJN, we expect to discover specific plant genes involved in host plant-endophyte interaction.
- Once the key genes are identified by microarray chips, we will focus on genes involved in signaling pathways and in cell wall synthesis, including those encoding transcriptional factors, and verify the observed differences in gene expression levels using quantitative PCR. Transgenic plants will be generated for either over-expression or knockout of key genes to study their functions in bacterial endophyte-switchgrass associations.
- Gross cell wall composition and saccharification efficiency will be analyzed after bacterial endophyte inoculation to monitor any potential structural and extractability changes. Photosynthesis rate, water use efficiency, drought tolerance, and carbon sequestration will also be measured after bacterial endophyte inoculation. We expect that bacterial endophyte-inoculated plants will have higher photosynthesis rates, better water use efficiency, and more drought tolerance compared to non-inoculated control plants. These results will allow us to determine the relationship of any observed gene expression profile changes to overall plant physiology and lignocellulose accumulation.

3. Project report:

Growth promotion:

We have demonstrated that bacterial endophyte *Burkholderia phytofirmans* strain PsJN significantly promotes growth of switchgrass cv. Alamo in various conditions (particularly sub-optimal conditions); we published a paper in *Biotechnology for Biofuels* documenting this (Kim et al. 2012). Growth performance of PsJN-inoculated and control plants in low-fertility field soil was determined with glasshouse trials under ambient conditions in the fall 2010 and 2011 (Fig. 1). The results showed that the PsJN-inoculated plants produced significantly higher biomass compared to controls. This indicates potential benefits ofswitchgrass inoculated with PsJN for cultivation on marginal lands. Then two field trials were initiated in 2012 to determine efficacy of PsJN inoculation on different soil types. Trial 1 was located at Lynchburg Grows Urban Farm on USDA prime soil containing high organic matter and nutrients, and Trial 2 at Walden Farm, a former tobacco farm in Southern Virginia, on soil with a low content of organic matter and a poor nutrient profile. Using a paired t-test design, we tested the effects of PsJN bacterization of switchgrass seedlings on stand establishment, plant growth, and biomass yield in two field experiments conducted over two years on highly fertile prime land, and on low fertility soil. The results showed that PsJN bacterization improved growth and development of switchgrass seedlings, significantly stimulated plant root and shoot growth, and tiller number on the low fertility soil ($p<0.001$), and enhanced biomass accumulation on both poor ($p<0.001$) and rich ($p<0.05$) soils, with more effective stimulation of plant growth in low fertility soil. The study indicates the potential for the use of PsJN and/or other beneficial bacterial endophytes in the development of low-input switchgrass feedstock production systems. This study has been published in BioEnergy Research (Lowman et al. 2014).

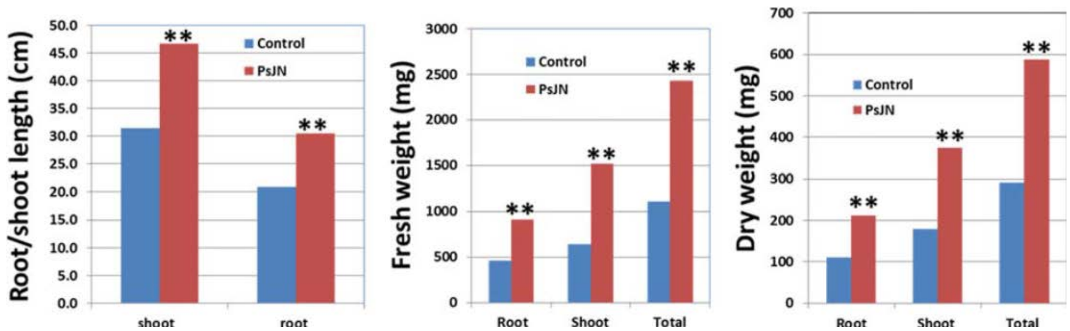

Figure 1. Growth promotion of Alamo by PsJN inoculation in sub-optimal conditions. The seedlings were inoculated with PsJN and grown *in vitro* for one month, then transferred to 4-gallon pots (5 plants/pot) with unfertilized field soil and grown in the glasshouse under ambient conditions for 2.5 months during the late fall of 2010. Dry weight was determined after samples were oven-dried at 65°C for one day. The sample number was 25; ** indicates a significant difference at the 0.01 level between PsJN and control using the student T-test.

Global gene expression profiling:

Using the DOE-funded switchgrass EST microarray, in a collaboration with the Genomics Core Facility at the Noble Foundation, we have determined gene expression profile changes in both responsive switchgrass cv. Alamo and non-responsive cv. Cave-in-Rock (CR) following PsJN bacterization. Microarray experimental results showed that there were approximately 30,000 switchgrass ID probe sets showing significant differences between switchgrass cultivars Alamo and CR after PsJN inoculation at 0.5, 2, 4, and 8 days after inoculation (DAI). Among them, about 27,000 switchgrass probes had rice hits (rice locus homologs), thus, the rice genome was used as a model for data analysis with MapMan software (Usadel et al. 2005. Plant Physiol 138:1195-204). The remaining switchgrass probes were not plotted because there were no matching rice identifiers. When more than one switchgrass ID probe had the same rice locus, the averages were used. If the rice locus had more than one identifier, the averaged value was used also. After these adjustments, a database of approximately 17,500 rice identifiers was plotted in MapMan to display the early responses of these two cultivars to the inoculation of PsJN.

Using the MapMan software's vein diagram tool at a threshold of log2 at ±0.693, i.e. a 2-fold increase and 2-fold decrease (Brotman et al. 2013. PLoS Pathog 9:e1003221. doi: 10.1371/journal.ppat.1003221), respectively, the number of up- and down-regulated probes were calculated (Figure 2). The number of up-regulated probes in Alamo was 26, 14, 14, and 12% at 0.5, 2, 4 and 8 DAI with PsJN, respectively while the corresponding number in CR was 24, 22, 21, and 19%, respectively (Figure 2). In both cultivars, the largest number of up-regulated probes occurred at 0.5 DAI. Noticeable differences throughout the timeframe between Alamo and CR were that the number was dramatically decreased to half (12%) in Alamo but remained high in CR (approximately 20%). The number of down regulated genes demonstrated different trends in Alamo and CR. Alamo had an increasing trend from 9% at 0.5 DAI to 11, 17, and 28% at 2, 4, and 8 DAI, respectively. However, CR had 13% at 0.5 and 2 DAI, and declined to 10% at 4 and 8 DAI.

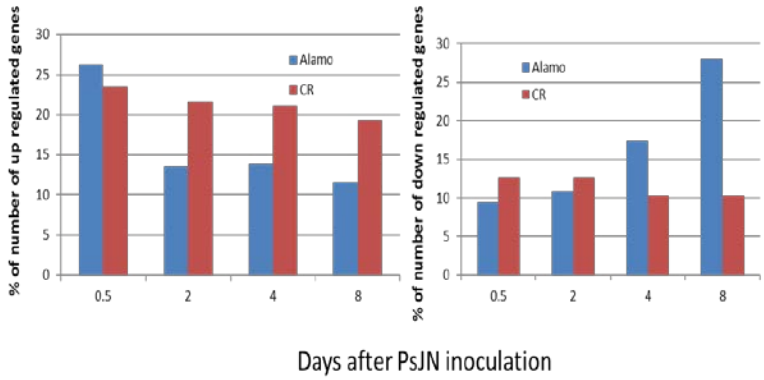

Figure 2. Number of probes up- and down-regulated in switchgrass cvs. Alamo and CR at threshold of log2 at ±0.693, which corresponded to 2-fold increase and 2-fold decrease, respectively, at 05, 2, 4 and 8 days after inoculation with Burkholderia phytofirmans strain PsJN, compared with the levels of control at 0 time under biotic stress category.

With the aid of MapMan (Usadel et al. 2005) and PageMan (Usadel et al. 2006 BMC Bioinformatics 7:535), we mapped the response of the ID probes to the observed major gene regulatory network and major biosynthetic pathway changes associated with the beneficial bacterial endophyte infection, colonization, and early growth promotion process. We found significant differences in gene expression patterns between responsive and non-responsive cultivars in many pathways, including redox state regulation, signaling, transcription factors, as well as hormone-associated pathways (SA, JA). The representative pathways were showed in Fig. 3.

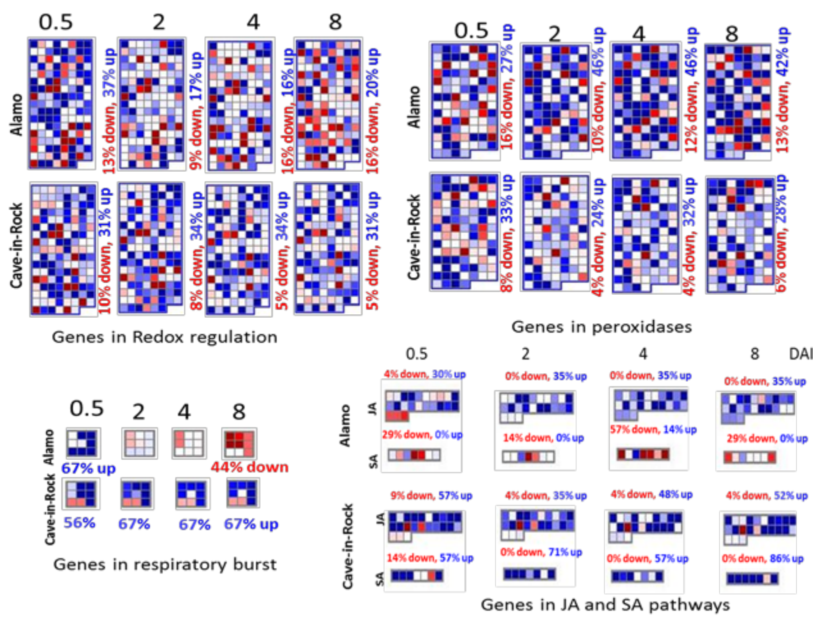

Figure 3. Comparison of genes in redox regulation, peroxidases and respiratory burst, and JA and SA pathways between responsive Alamo and non-responsive Cave-in-Rock following PsJN inoculation. MapMan software was used to analyze data from microarray chip. Data was compared with control at 0 d, and up and down regulation were set at 2 times increase and decrease, respectively.

In order to further confirm if hydrogen peroxide plays an important role in the response of switchgrass to endophyte PsJN, we determined H_2O_2 content in switchgrass after PsJN inoculation using the OxiSelectTM Hydrogen Peroxide Assay Kit (Cell Biolabs, Inc.) The preliminary results showed that responsive cv. Alamo had a lower level of H_2O_2 at 0 time and this remained at a low level after PsJN inoculation. In contrast, nonresponsive cv. CR had a higher level of H_2O_2 at 0 time (41% more than that of Alamo), which increased to 58% after endophyte inoculation at 1 DAI. The different patterns may imply CR could be more resistant to PsJN. This manuscript will be submitted to the Journal of Experimental Botany for publication. The microarray data generated during this project were incorporated into the Switchgrass Functional Genomics Server, currently hosted at the Noble Foundation (http://switchgrassgenomics.noble.org/index.php). This database contains switchgrass (*Panicum virgatum*

L.) unique transcript sequences (PviUTs), a gene expression atlas (PviGEA), GBrowser views of ESTs mapped to the PviUTs, and other data that will be made available to the public once completed.

Key gene function analysis:

A total of 50 key genes have been verified using qPCR. Ten of these genes were chosen for further functional study via either overexpression and/or RNAi knockout technologies. These genes were calmodulin-related calcium sensor protein (CAM), glutathione S-transferase (GST), histidine-containing phosphotransfer protein (H-221), 3 different zinc finger proteins (ZF-371, ZF131 and ZF242), EF hand transcription factor (EF-622), peroxidase, cellulose synthase catalytic submit A2 (CESA2), and Aux/IAA family. A total of 8 overexpression and 5 RNAi transgenic plants have been regenerated, and their gene expression levels determined using qPCR. Consequently high, medium and low expression lines were propagated *in vitro* for gene function study. When adequate numbers of individual transgenic lines were obtained, they were challenged with PsJN to see if PsJN promotes or inhibits growth of transgenic plants. Our results demonstrated that EF-622 overexpression, ZF-371, GST, H-221 and CAM RNAi transgenic lines lost responses to PsJN, i.e. PsJN had no growth promotive effects on these transgenic plants. Further study needs to be done to characterize this loss of responsiveness to PsJN.

Plant physiology changes and drought test:

Our study demonstrated that PsJN significantly increased both aboveground and underground dry biomass as early as 17 days after inoculation. Photosynthetic rates also quickly increased in response to inoculation and were significantly higher in PsJN inoculated seedlings after 17 days (54%, p=0.0016), which was attributable to both higher water use efficiency (p=0.0467) and higher stomatal conductance (p=0.0326). PsJN inoculation increased photosynthetic rates of upper canopy leaves by 13.6%. Nevertheless photosynthetic rates of lower canopy leaves were decreased by 8.2%. In summary, this study verified the advanced development of switchgrass due to PsJN inoculation and suggested that increased photosynthesis of newly developing foliage is related to enhanced productivity.

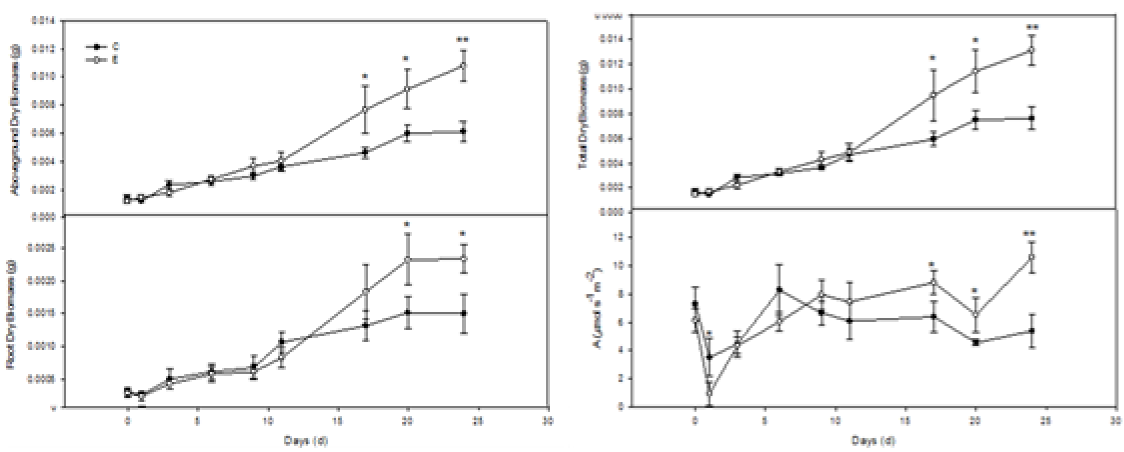

Figure 4. Effect of *Burkholderia phytofirmans* strain PsJN on aboveground biomass, root biomass, total biomass and photosynthetic rates (A) of switchgrass cv. Alamo. C refers to non-inoculated plants, E refers to PsJN inoculated plants. Each value is the mean of six plants. Error bars are ±SE of the means. * means significant difference at α=0.1 level and ** means significant difference at α=0.01 level between PsJN and control using student T-test.

Drought experiments, consisting of two successive drought-rehydration cycles, were conducted to examine drought tolerance of PsJN-inoculated switchgrass. The results showed that PsJN inoculation increased height by 40% (p<0.0001) and tiller number by 110% (p<0.0001) during early drought. Based on leaf gas exchange, PsJN inoculated switchgrass showed better tolerance to low leaf water potential (<-1MPa). Regardless of the leaf water potential, pre-stressed PsJN seedlings always had significantly higher photosynthetic rates than control (no prior water stress) seedlings during the second drought period. In summary, the growth and drought tolerance of PsJN inoculated plant was enhanced by mild drought stress.

Osmotic adjustments of PsJN inoculated switchgrass was also examined over the course of 3 drought cycles. At the end of the second drought cycle, drought stressed endophyte inoculated switchgrass significantly lowered the osmotic potential at full turgor and the osmotic potential at turgor loss point by 0.49 MPa ($p=0.006$) and 0.62 MPa ($p=0.0098$), respectively. However, PsJN inoculation and drought stress alone did not cause significant osmotic adjustments in switchgrass at the end of 2^{nd} drought cycle. Similar to osmotic adjustments, PsJN inoculation significantly reduced the modulus of elasticity in response to drought stress but PsJN inoculation or drought stress alone did not significantly reduce modulus elasticity. Both PsJN inoculation and drought stress decreased apoplastic water percentage at the end of 2^{nd} drought cycle. At the end of 3^{rd} drought cycle (when each individual plants was stressed to near zero Ps), there were no difference in terms of osmotic potential and apoplastic water percentage among different treatments.

In summary, PsJN inoculation rapidly results in an increase in photosynthetic rates which contributes to the advanced growth and development of inoculated switchgrass cv. Alamo. Some evidence suggests that this initial growth advantage decreases with time when resources are not limited such as in greenhouse studies. Additionally, better drought resistance and drought hardening were observed in PsJN inoculated switchgrass.

Lignin concentration and saccharification:

Lignin is an important component of the cell wall, but also acts as a barrier to saccharification during cell wall hydrolysis. We have determined lignin concentration in PsJN-inoculated switchgrass and assessed how the PsJN endophyte affects hydrolysis efficiency. Using the acetyl bromide method for lignin quantification (Hatfield et al. 1999. J Agric Food Chem 47:628-632), we found no significant differences between PsJN-inoculated and control plants in the stem samples taken from field-grown mature plants. However, PsJN-inoculated samples had more sugars released after cellulase enzymatic treatment, as determined using the phenol-sulfuric acid assay (Dubois et al. 1956. Anal Chem 28:350-356). The sugar amount was 19.1% and 25.9% higher in the top and bottom stem parts of PsJN-inoculated plants than that of control plants, respectively. The increasing saccharification efficiency may be due to the fact that strain PsJN secretes endoglucanase and endopolygalacturonase enzymes (Compant et al. 2005. Appl Environ Microbiol 71:1685-1693) or may be due to cell wall structural changes following PsJN inoculation. These areas need further study to fully understand the causal effects.

During this funding period, we have done more work related to this funded project and established collaborations with other institutions and obtained some interesting results, building a foundation for further research projects:

A novel bacterial endophyte PaKM: we isolated a naturally-occurring bacterium from surface-sterilized switchgrass seeds, identified as a unique *Panteoa agglomerans* species, and named strain PaKM. Our experiments have demonstrated that PaKM significantly boosts switchgrass growth/vigor and root system development, and increases biomass yield in in vitro (Figure 5), growth chamber, greenhouse, as well as field conditions. Not only does PaKM promote growth of switchgrass cv. Alamo, but also 8 other switchgrass cultivars tested. When compared with PsJN in vitro, PaKM enhances switchgrass growth to a greater extent (Figure 5). PaKM also enhanced salt and drought stress tolerance in vitro. PaKM produces high levels of IAA and has a different mechanism of growth promotion compared to PsJN has high ACC deaminase activity. In summary, PaKM is an efficient growth promoter of switchgrass over a broad spectrum of genotypes and has potential in applications with low input and sustainable production systems on marginal lands. This work has been submitted to ISME Journal for publication.

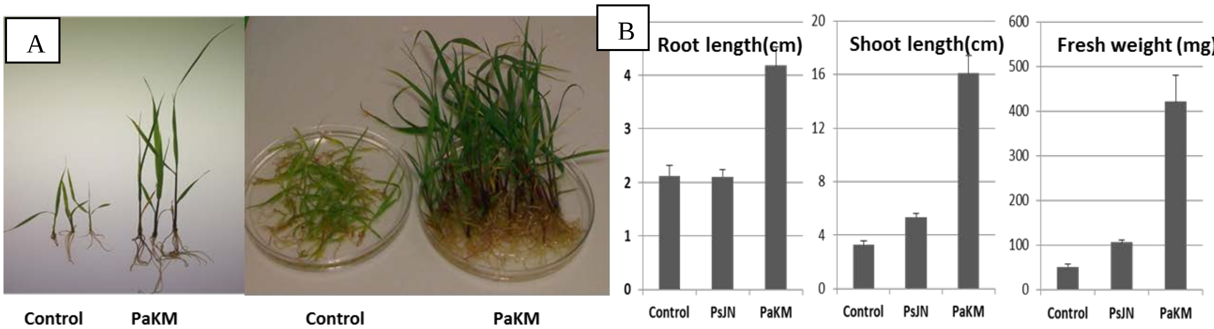

Figure 5. A: Effect of PaKM on growth of switchgrass cv. Alamo one month after inoculation under in vitro conditions. B: Comparison of PaKM with PsJN in growth promotion of Alamo grown under in vitro conditions for one month.

Free-living Nitrogen Fixing Experiments: As PsJN belongs to the Burkholderia taxa, we identified another member of the same taxa with the proven ability to fix atmospheric nitrogen in a free-living state. This bacterium, *Burkholderia phymatum,* was acquired, and the genomic DNA was isolated. This genomic DNA was then introduced into PsJN utilizing the freeze-thaw method, and bacteria subsequently screened for the ability to fix nitrogen on Norris nitrogen free selection media. The best growing colonies were confirmed to contain the *nifH* gene, required for nitrogen fixation, by PCR amplification with primers designed for the *B. phymatum nifH* gene. A growth curve was developed for switchgrass cv. Alamo utilizing differing levels of ammonium sulfate as a nitrogen source and from this, deficient, minimal, and adequate levels of nitrogen was established. Switchgrass cv. Alamo was then inoculated with either PsJN, PsJN + nifH (the ability to grow on N-free selection media), and buffer alone for a control. Interestingly, PsJN+ nifH promoted growth significantly at deficient levels while PsJN promoted growth significantly over controls at adequate levels. This was published as Chapter 4 of Scott Lowman's Ph.D. dissertation and will be the subject of a manuscript.

MicroRNA detection: Recent research also indicates that 20-24 bp microRNAs (miRNAs) are critical regulators of gene expression in plant growth, development, and stress adaptation (Matts et al. 2010. J Plant Physiol 167:896-904). In switchgrass, several important miRNAs have been identified and functionally studied. For example, miR156 targets squamosal promoter binding protein-like transcripts and is involved in shoot development and juvenile-to-adult transition. Moderate expression of miR156 in switchgrass led to increased biomass without flowering, improved saccharification, and forage digestibility (Fu et al. 2012. Plant Biotechnol J 10:443-452). MicroRNAs are likely also involved in plant growth promotion by beneficial bacterial endophytes. In collaboration with Dr. Lei Li (University of Virginia) we studied the functions of switchgrass miR528 and miR408 via overexpression in switchgrass. The result showed miR408 transgenic plants increased 27% of photosynthesis rate compared with vector control transgenic plants in greenhouse. Also, a collaboration with the Genewiz Company to sequence microRNA libraries from Alamo and Cave-in-Rock at 7 days after PsJN inoculation found that two thirds of conserved miRNA in Alamo-endophyte was down-regulated, compared to Alamo-control, and a similar pattern occurred in nonresponsive cv. CR, but with much less significance.

N and C cycling and AMF: In collaboration with Dr. Shuijin Hu (North Carolina State University), we conducted experiments on how endophyte-inoculated switchgrass affects soil N and P availability and the number of AMF in roots. Our preliminary results showed that PsJN increased AMF infection of switchgrass roots, and enhanced soil N availability and soil N mineralization on a low nutrient field. Further study of this phenomenon on different soils, over longer time periods, is needed to assess its potential impact on the productivity and longevity of switchgrass stands.

<u>Soil microbial community change:</u> In collaboration with Dr. Patrick Gillevet, Professor at the Environmental Biocomplexity and Ecology group at George Mason University, we developed an ongoing project to monitor soil microbial community changes after growing switchgrass inoculated with PsJN in different fertility fields over two years.

4. **Broad Impacts**:
Our microarray data have been incorporated into the switchgrass GeneAtlas database at the BESC (the DOE BioEnergy Science Center) through Genomics Core Facility at the Noble Foundation, which will benefit the entire switchgrass research community. Our project has been involved in K-12 and undergraduate education programs locally, primarily involving research internships for regional Governor's high school students, local Averett University seniors, and undergraduate summer interns to promote a greater public understanding of the use of beneficial endophytes to improve bioenergy crop yield and sustainability. The project has also collaborated with regional partners, including Lynchburg Grows (Lynchburg VA), a non-profit urban farm and environmental educational center that provides job training and education for over 2,000 youth-at-risk annually, as well as mentally- and physically-handicapped individuals in the field of agriculture (www. lynchburggrows.org).

5. Deliverables

<u>Publications:</u>
- Switchgrass field performance on two soils as affected by bacterization of seedlings with Burkholderia phytofirmans strain PsJN. BioEnergy Research 2014, DOI: 10.1007/s12155-014-9536-3.
- Growth promotion and colonization of switchgrass (*Panicum virgatum*) cv. Alamo by bacterial endophyte *Burkholderia phytofirmans* strain PsJN. Biotechnol Biofules 2012, 5:37.
- Scott Lowman, Ph.D. thesis defended in the Department of Horticulture, Virginia Tech, January 2014
- The use of endophytes and mycorrhizae in switchgrass for biomass production. 2014. In: Compendium of Bioenergy Plants: Switchgrass, edited by Luo H., Wu Y. and Kole C. The Science publishers, Inc. (New Hampshire) jointly with CRC Press of Taylor and Francis Group. pp67-108.
- Developing a low input and sustainable switchgrass feedstock production system harnessing beneficial bacterial endophytes. Science for Biomass Feedstock Production and Utilization. In: Proceedings of the 2012 Sun Grant National Conference, Vol. 1 pp86-90.
- *Panteoa agglomerans* strain PaKM, a novel and efficient switchgrass growth promoting bacterial endophyte isolated from seeds has been submitted to ISME Journal.
- Global gene expression profiling of switchgrass following inoculation with *Burkholderia Phytofirmans* strain PsJN will be submitted to Journal of Experimental Botany.
- Early growth promotion and leaf level physiology changes in *Burkholderia phytofirmans* PsJN inoculated switchgrass has been submitted to Environmental and Experimental Botany.
- *Burkholderia phytofirmans* strain PsJN advanced development and altered leaf level physiology of switchgrass is being prepared for publication.
- *Burkholderia phytofirmans* strain PsJN affects growth, leaf level physiology and response to water stress in switchgrass will be submitted to BioEnergy Research for publication.

<u>Oral Presentations:</u>
- Beneficial bacterial endophytes enhance switchgrass performance in fields with different fertility and their molecular mechanisms. Plants and BioEnergy 2014 Congress to be held in Conference Center of University of Guelph, Canada, June 4 -7, 2014.
- Developing a low input and sustainable switchgrass feedstock production system harnessing beneficial bacterial endophytes. Symbiosis Conference: Expanding Commercialization of Mutualistic

Microbes to Increase Bioenergy Crop Production supported by the U.S. Department of Energy, Bioenergy Technologies Office (BETO) held at Cornell University from June 20-21, 2013.

- Developing a low input and sustainable switchgrass feedstock production system harnessing beneficial bacterial endophytes. BIT's 3rd Annual World Congress of Bioenergy held in Nanjing, China, April 25-27, 2013.
- Switchgrass-beneficial bacterial endophyte interactions to improve soil remediation. 24th Annual Environment Virginia Symposium held in Virginia Military Institute, Lexington, VA, April 9-11, 2013.
- Growth promotion of switchgrass by bacterial endophyte Burkholderia phytofirmans strain PsJN and its mechanisms. Genomic Science contractors-Grantees Meeting XI/USDA-DOE Plant Feedstock Genomics for Bioenergy Awardee Meeting 2013. Bethesda, MD. February 24-27, 2013.
- Developing a low input and sustainable switchgrass feedstock production system harnessing beneficial bacterial endophytes. The Sun Grant Initiative's Science for Biomass Feedstock Production and Utilization National Conference. New Orleans on October 2-5, 2012.
- Mechanisms of switchgrass Alamo growth promotion by beneficial bacterial endophyte Burkholderia phytofirmans strain PsJN. Plant and Animal Genomics XX held in San Diego, CA, January 14-18, 2012.
- Development of a low input switchgrass production system harnessing beneficial bacterial endophytes. International Biomass Conference & Expo, a biorefinery and biomass power & thermal event to be held in St. Louis, May 2-5, 2011.
- Development of switchgrass (Panicum virgatum L.) for marginal lands based on genotypic compatibility with beneficial bacteria in Track 2-1: Feedstock Genomics and Plant Biotechnology Development during the First Annual World Congress of Bioenergy held in Dalian, China, April 25-29, 2011.
- A selected lunchtime speaker by 2011 Genomic Sciences Meeting Student Travel Grant: The Beneficial Bacterial Endophyte *Burkholderia phytofirmans* Strain PsJN Significantly Promotes Switchgrass Alamo Growth in the 2011 Genomic Science Annual Contractor-Grantee Meeting/USDA-DOE Plant Feedstock Genomics for Bioenergy Program Meeting, April 10-13, 2010, Crystal City, VA .

Training (List the names of undergraduate, graduate, postdoctoral and technical personnel trained under this project and highlight their contributions as appropriate to items in the "Deliverables" section above.):

Alejandra Lara-Chavez, Postdoctoral Scientist was trained for molecular biology and analytical chemistry.

Scott Lowman, Ph.D. student was trained in molecular biology, switchgrass tissue culture and transformation, and plant-endophyte interaction and received Ph.D. in January 2014.

Bingxue Wang, Ph.D. student is being trained in plant physiology, especially in photosynthesis and drought tolerance and will finish her Ph.D. study in December 2014.

***Chrysa Hopper*,** lab technician**,** was trained in switchgrass-endophyte interaction and tissue culture.

Sandra Johansson, *undergraduate* student, was trained in switchgrass-endophyte interaction and cell wall hydrolysis and sugar quantification.

Daniel Reddy, Undergraduate Summer Intern, was trained in endophyte inoculation and H_2O_2 quantification.

Mira Tissari, undergraduate student senior, was trained in switchgrass-endophyte interaction and switchgrass tissue culture.

Bethany Gregory, undergraduate student, was trained in how to use Li-COR Photosynthesis System for the measurements of photosynthesis rate, leaf transpiration, and leaf conductance, etc.

Maggie MacLeish, Piedmont Governor's School for Mathematics, Science & Technology, was trained in switchgrass-endophyte interaction.

Collaborations (Please list new interactions made possible from project support.):

- ***Dr. Shuijin Hu,*** Professor in Department of Plant Pathology, North Carolina State Univeristy
- ***Dr. Patrick Gillevet,*** Professor at the Environmental Biocomplexity and Ecology group, George Mason University
- ***Dr. Yuhong Tang,*** Manager, Genomics Core Facility, Samuel Roberts Noble Foundation
- ***Dr. Lei Li,*** Assistant Professor in Department of Biology, University of Virginia
- ***Dr. Guichuan Hou,*** Director of the Dewel Microscopy Facility at the College of Arts and Sciences, Appalachian State University
- ***Michael G. Van Ness, Esq.,*** the Executive Director at Lynchburg Grows
- ***Ken Walden,*** local tobacco farmer