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14. ABSTRACT The emphasis of this project is to procure essential equipments required to establish new capacity to conduct research and education in bioenergy and environmental biotechnology at West Virginia State University (WVSU). Bioenergy and environmental biotechnology research and education affected by the acquisition of this new equipment and instrumentations includes development of new technology to produce biofuels, bioremediation for environmental issues, to attract students to pursue studies leading STEM careers and train future workforce in the Appalachian region. The experiences with modern instrumentations will broaden WVSU undergraduate and					
15. SUBJECT TERMS Biofuels, STEM education, environmental biotechnology, transgenic plants, algae					
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				19b. TELEPHONE NUMBER 304-414-4062	

Report Title

Final Report: Engineering Photosynthetic Organisms for the Production of Renewable Energy Products and Environmental Remediation

ABSTRACT

The emphasis of this project is to procure essential equipments required to establish new capacity to conduct research and education in bioenergy and environmental biotechnology at West Virginia State University (WVSU). Bioenergy and environmental biotechnology research and education affected by the acquisition of this new equipment and instrumentations includes development of new technology to produce biofuels, bioremediation for environmental issues, to attract students to pursue studies leading STEM careers and train future workforce in the Appalachian region. The experiences with modern instrumentations will broaden WVSU undergraduate and graduate students skill sets and make them more attractive to employers and graduate programs. Since a vast majority of our students are first-generation college students, having an opportunity to understand and apply molecular biology techniques will encourage them to pursue a career in STEM education. Additionally, enhancement of oils in vegetative tissues of plants and algae is synergistic with efforts to develop lignocellulosic feedstock for biofuel production. These oils can be used directly as a fuel in many applications, including drop-in-fuels.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

<u>Received</u>	<u>Paper</u>
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TOTAL:

Number of Papers published in peer-reviewed journals:

(b) Papers published in non-peer-reviewed journals (N/A for none)

<u>Received</u>	<u>Paper</u>
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TOTAL:

Number of Papers published in non peer-reviewed journals:

(c) Presentations

Participated in the Biodiesel Education Program along with the Mid-Atlantic Technology, Research and Innovation Center, South Charleston, WV.

Attended the Gordon Research Conference on Plant Lipids: Structure and, Metabolism and Function, Galveston, TX, February 2015.

Number of Presentations: 1.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):

(d) Manuscripts

Received Paper

TOTAL:

Number of Manuscripts:

Books

Received Book

TOTAL:

Received

Book Chapter

TOTAL:

Patents Submitted

Nothing to Report

Patents Awarded

Nothing to Report

Awards

Nothing to Report

Graduate Students

<u>NAME</u>	<u>PERCENT SUPPORTED</u>	Discipline
Graduate Students	0.00	
FTE Equivalent:	0.00	
Total Number:	1	

Names of Post Doctorates

<u>NAME</u>	<u>PERCENT SUPPORTED</u>	
Post Doctorates	0.00	
FTE Equivalent:	0.00	
Total Number:	1	

Names of Faculty Supported

<u>NAME</u>	<u>PERCENT SUPPORTED</u>	National Academy Member
Dr. Sanjaya	0.00	
FTE Equivalent:	0.00	
Total Number:	1	

Names of Under Graduate students supported

<u>NAME</u>	<u>PERCENT SUPPORTED</u>	Discipline
Under Graduate Student	0.00	
FTE Equivalent:	0.00	
Total Number:	1	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period: 18.00

The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:..... 12.00

The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:..... 6.00

Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):..... 0.00

Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:..... 0.00

The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense 5.00

The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields:..... 0.00

Names of Personnel receiving masters degrees

<u>NAME</u>	
N/A	
Total Number:	1

Names of personnel receiving PHDs

<u>NAME</u>	
Doctorate Degrees Awarded	
Total Number:	1

Names of other research staff

<u>NAME</u>	<u>PERCENT SUPPORTED</u>
Other Staff	0.00
FTE Equivalent:	0.00
Total Number:	1

Sub Contractors (DD882)

Inventions (DD882)

Scientific Progress

Funding from this grant was used to establish plant and green algae transformation facility at West Virginia State University (WVSU). We have procured multiple equipment's requested in the grant application such as GC with flame ionization detector (GC-FID), hydrogen and air generation system, plant growth chambers, plant tissue culture chambers, floor model centrifuge, bench top centrifuge, Helios gene gun system, gene pulser, qPCR system, bomb calorimeter system, autoclave, orbit shaker, biosafety cabinet, freeze drier, Nanodrop 2000, Milli-Q Direct 8 water purification system, and flake icemaker. Currently, we are using these equipment's to conduct research and education in bioenergy and environmental biotechnology (Figure 1). In addition, WVSU Gus R. Douglass Land-Grant Institute scientists (and associated undergraduates), postdoctoral fellows, and visiting scientists are using these equipment/instrumentations for research and educational purpose.

Scientific Progress:

Research Project 1: Increasing the energy density in the plant biomass

Plant oils (triacylglycerols, TAGs) derive from oilseed crops are used as feedstock for the production of biodiesel. TAGs possess higher energy content and are compatible with existing fuel technology. However, low oilseed crop yield, competition for resources, and interference with world food demand limit the current supply for the production of biodiesel. One biotechnological approach is to increase the availability of feedstock, for the production of biodiesel, is to increase oil content in vegetative tissues/biomass, where oil does not normally accumulate. The proposed research is to focus on developing tools and methods to engineer energy crop switchgrass to accumulate oil in the biomass for the production of bioenergy. We introduce Arabidopsis transcription factor WRINKLED1 (WRI1) involved in oil biosynthesis pathway in to switchgrass producing oil in vegetative tissues. Agrobacterium-mediated transformation methods was developed to introduce Arabidopsis gene into suitable switchgrass varieties. Putative transgenic plants were selected on stringent antibiotic containing medium and the integration of foreign genes were checked by different techniques such as qRT-PCR and Southern blotting. Using biochemical analysis changes in metabolites such as oils, starch, free sugars, glucose and proteins were measured in the transgenic plants (Sanjaya et al., 2013). All the methods and techniques developed in this project will allow us engineer other oil biosynthesis genes alone or in combination in switchgrass.

The WRI1 cDNA from Arabidopsis thaliana was amplified with specific primers as previously described by Sanjaya et al., 2011. A fragment of 1463 bp containing the complete open reading frame was subjected to codon optimization for monocot and then cloned into a suitable primary vector and sequenced before sub cloning using the maize ubiquitin promoter (ZmUbi1) in a monocot specific transformation binary vector containing the hygromycin selection marker gene under the control of CAMV 35S promoter. This construct will be used in Agrobacterium-mediated transformation of switchgrass. Seeds of switchgrass, *Panicum virgatum* L. were first tested for the ability to produce callus. Optimized the seed surface sterilization procedure for dry seeds. Tested different concentrations of plant growth hormones in Murashige and Skoog (MS) medium with 0.75% (w/v) Agar on callus induction and differentiation of plant lets. Combination of cytokinin and auxin with 3% sucrose resulted in prolific callus initiation, further these cultures were maintained on medium with low concentrations of growth regulators by sub culturing onto fresh plates. Additionally, embryonic callus were induced from inflorescence to maintain as an alternative supply of callus for both Agrobacterium and gene gun-mediated transformation in collaboration with Dr. Christian Tobias, Research Molecular Biologist, USDA Western Regional Research Center – Crop Improvement and Genetics Research Unit, Albany, CA. Agrobacterium-mediated transformation using callus derived from different explant source was tested. Putative transgenic plants were selected on stringent antibiotic containing medium and the integration of foreign genes were checked by qRT-PCR, Southern blotting and Western blotting (Figure 2). Transgenic plants were maintained in the tissue culture chambers for a month and subsequently transformed into pots containing soil and maintained in the larger size plant growth chambers to collect progeny. Using biochemical analysis changes in metabolites such as oils, starch, free sugars, glucose and proteins were measured in the transgenic plants by use of GC-FID, spectrophotometer, nanno drop, q-RT-PCR, ChemiDoc etc.,. Bomb calorimeter analysis were employed to determine changes in the energy content of biomass.

Research Project 2: Bioengineering of energy corps for the development of vegetable oil-based advanced biofuels

The goal of this project is to understand how plants and algae respond to acid mine soils/water, as well as how the toxic elements in acid mine drainage influence lipid metabolism, TAGs accumulation, growth, and physiology. The findings from such a detailed study will help to design an improved biofuel crop that can grow in the extreme environmental condition such as those found on surface coal mine land. Thus, to gain insights into the mechanisms that lead to understanding lipid metabolism in plants in response to acid mine soil/water in Arabidopsis (C16 plant), Seteria (C4 grass) and microalgae were chosen since their primary metabolism and its regulation differ in several aspects.

We collected acid main soil samples at a depth of approximately 15-cm and acid mine water from a mine site in West Virginia; samples were analyzed for percent fines, pH, electrical conductivity, heavy metals, and extractable elements. Wild type Arabidopsis (C16 plant) and Seteria (C4 grass) were grown in pots filled with Arabidopsis soil mix and will serve as a control. Pots were filled with acid mine soil with three biological replication under standard growth conditions. Plants were irrigated with no additional nutrients and grown for 8–10 weeks. WT microalgae Chlamydomonas (*Chlamydomonas reinhardtii*) or Chlorella (*Chlorella protothecoides*) was cultivated in standard culture medium and in medium with acid mine water for up to 2–3 weeks

under standard culture conditions. Leaves and root samples were collected from 8-week-old plants; similarly, *Chlamydomonas* grown under control and experimental conditions were harvested for RNA extraction, metabolite analysis, and elemental analysis as previously described. The mRNA were used to construct cDNA libraries using the mRNA-Seq Sample Preparation Kit™ (Illumina, San Diego, CA) following standard protocols. The DNA yield and fragment insert size distribution of the library were determined on an Agilent Bioanalyzer. Library quantifications were performed by qPCR using the KAPA Library Quant Kit™ following the manufacturer's instructions. Approximately 24 constructed libraries were sequenced.

We collected T-DNA gene knockout or knock down mutant *Arabidopsis* lines for at least the top 10 candidate genes involved in fatty acids and TAGs biosynthesis from the *Arabidopsis* Biological Resource Center (ABRC; The Ohio State University) to use in the mutant screening experiments. The homozygous mutant seeds were grown on half-strength Murashige and Skoog (1/2 MS) medium with or without sucrose and early seedling growth of the WT and mutant plants were compared. *Arabidopsis* seeds rely on stored compound reserves to support post-germinative growth until photosynthetic competence was achieved; therefore, we used agar plates with or without sucrose (1%) in the medium to screen the mutants. WT and mutant surface-sterilized seeds were inoculated on agar plates and after two days of dark stratification (incubation at 4°C to induce uniform germination) the plates were moved to a growth chamber set at 22°C. Three replicates were used per mutant line in a randomized block design. The seeds were then exposed to a 16-hour light/8-hour dark photoperiod cycle. The number of seeds germinated, number of normal and defective seedlings, color of seedlings, morphology of root and hypocotyl and cotyledon/leaves were recorded from Day 1 through Day 7 for the mutant and WT seeds. The data was analyzed using one-way ANOVA. We used 1% sucrose plates to rescue mutant seedlings for subsequent growth and progeny collection.

We selected at least two candidate genes for complementation studies based on the previous experiments. We generated complementation lines using genomic DNA clones containing putative candidate gene loci along with the respective promoter sequence (~3.5-4 kb), as described previously. Complementation lines that display equal gene expression to WT plants with reduced expression on the mutant background were considered for further analysis. Our research team was isolated the clone using PCR-based techniques and then subclone into a suitable primary vector for sequencing. This fragment was excised from the cloning vector, inserted into a suitable binary vector, introduced into the *A. tumefaciens* strain GV3101, and subsequently transformed into homozygous respective T-DNA mutant plants. Transgenic *Arabidopsis* plants were selected by kanamycin/hygromycin resistance. Segregation analysis of the endogenous loci was performed by PCR using suitable primer combinations determined using the SALK database. The expression of candidate genes in a complementation, mutant, and WT line was analyzed using quantitative q-RT-PCR. *Agrobacterium*-mediated transformation of *Camelina* was attempted using oil biosynthesis genes.

We generated transgenic *Camelina* plants using *Arabidopsis* transformation methods for fatty acid and TAG metabolic pathway enzymes. Storage compounds such as TAGs, fatty acid composition, and protein content in the acid mine soil/water treated plants, *Chlamydomonas*, mutant plants, and respective controls was analyzed using methods we have published previously, including thin layer chromatography (TLC), and using a GC-FID. The research team was also record the size and shape of overexpression and mutant leaves and seeds.

Research Project 3: Genetic engineering of Microalgae for the production of bioenergy, bioproducts and bioremediation

Recently, NREL successfully developed a novel lipid extraction technique for algal biomass using dilute acid pretreatment process that deconstructs algal cells, hydrolyzing carbohydrates and making the lipids accessible for hexane extraction of the wet slurry. Further refinement of the process allows for fermentation of algal sugars and higher recovery of lipids. We are testing a similar conversion process on the oil-rich switchgrass biomass and green algae biomass to break down hemicellulose and other cell wall barriers, facilitate lipid extraction, and use the cellulose for enzyme hydrolysis. We are taking advantage of already available switchgrass and microalgae biomass feedstocks in the lab for optimization experiments. Freeze dried/fresh biomass was used for pretreatment in a small size ZipperClave (ZC) reactor using direct steam injection. We tested the effect of different deconstruction conditions on the release of fermentable monosaccharides and lipids. We assessed the enzymatic saccharification and fermentation on the production of level ethanol, lipids, carbohydrates, and total proteins from oil vegetative biomass, as previously described. We used biomass extract containing lipids for single/multi-step hydrotreatment to produce high quality diesel-ranged alkanes. The effect of Ni/Zeolite on beta zeolites on catalysis, release of products were studied. The effect of metal support on zeolites, the effect of the metal loading amount and influence of the reaction temperature was studied. We tested the production of bio-oil and biochar using oil-rich biomass using pyrolysis, as well as the effect of different pretreatments on palletization. Due to the chemical complexity of vegetative biomass, different analytical methods were used to assess the efficacy of pyrolysis and catalysis including GC-FID, ultimate analysis, bomb calorimetry and ash analysis.

Number of samples analyzed using GC-FID: 5,500 (both plant and algal samples).

Number of plants grown in growth chambers: *Arabidopsis* 10,000, *Brachypodium*: 4,500, *Camelina*: 3,400, *Seteria*: 2,300.

Bibliography:

Sanjaya; Durrett, T.P.; Weise, S.E.; Benning, C. Increasing the energy density of vegetative tissues by diverting carbon from

starch to oil biosynthesis in transgenic Arabidopsis. *Plant Biotechnology Journal*. 9(8): 874–883, 2011.

Sanjaya; Miller, R.; Durrett, T.; Kosma, D.; Lydic, T.; Muthan, B.; Koo, A.; Bukhman, Y.; Reid, G.; Howe, G.; Ohlrogge, J.; Benning, C. Altered lipid composition and enhanced nutritional value of Arabidopsis leaves following introduction of an algal diacylglycerol acyltransferase 2. *The Plant Cell*. 25(2): 677–693, 2013.

Technology Transfer

Team taught following classes; Fall 2014 and 2015- Energy and the Environment; Spring 2015- Biotechniques-I Gene transformation; Fall 2016- Biotechniques II; Genetic engineering, Plant Tissue Culture and Gene Transformation. Hosted a group of undergraduate students from Princeton University visit through the “Breakout” program to learn about the effects of recent carbon regulations on West Virginian coal communities.

Initiated collaboration with MATRIC, Liberty Hydro, South Charleston WV, the USDA Western Regional Research Center – Crop Improvement and Genetics Research Unit, Albany, CA on switchgrass biotechnology, established collaboration with researchers from University of Nebraska. Visited Mexican Universities along with WVSU faculty members to establish collaborations related to research and academic endeavors in the Biotechnology areas, including environmental, agricultural, and water quality.

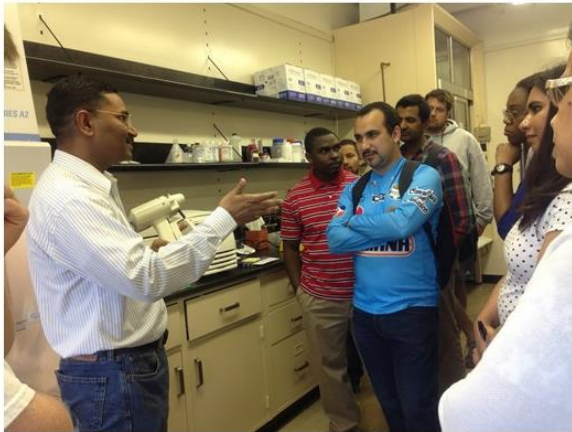


Figure 1: The PI (Dr. Sanjaya) is demonstrating the use of gene gun in plant transformation to BT 571 Techniques in Biotechnology I class

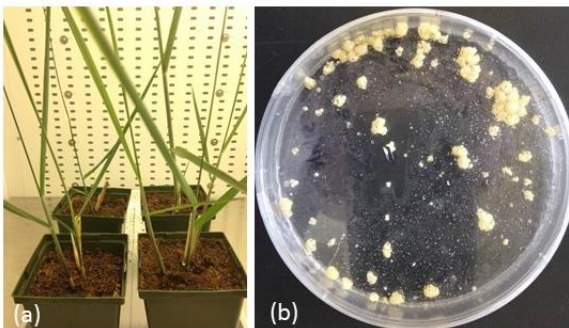


Figure 2: Switchgrass callus culture establishment: (a). Morphology of switchgrass plants; (b). switchgrass Callus culture initiated from seeds/inflorescence