

BIOAG PROJECT FINAL REPORT

TITLE: UNDERSTANDING THE MOLECULAR BASIS OF PLANT RESPONSE TO ORGANIC VERSUS CONVENTIONAL FERTILIZER USING A METATRANSCRIPTOMIC APPROACH

PRINCIPAL INVESTIGATOR(S) AND COOPERATOR(S): AMIT DHINGRA (PI) AND PRESTON ANDREWS (CO-PI)

ABSTRACT: This project addresses breeding, varietal selection, management practices, processing, or marketing practices to increase food quality, nutrition, safety or access. In a previous study, we demonstrated that tomato fruit grown under an organic fertilizer regime had higher phytonutrient content. Thereafter, using a comprehensive transcriptome analysis we tested the following hypotheses: 1. Growth under organic fertilizer regime will result in differential expression of the tomato genome and 2. Genes and pathways associated with phytonutrients that were observed to be significantly higher under organic fertilizer regime will demonstrate higher expression. Both the hypotheses tested true, indicating an adjustment of the plants' genomic activity in response to a different nitrogen regime. We have identified genes and associated pathways --among them, lycopene, ascorbate, soluble solids, and salvage pathways --which are expressed at higher levels under organic conditions. Next, investigation of the genomic response of the roots immediately after exposure to organic vs conventional fertilizer will provide understanding of how the two different forms of nitrogen are metabolized. We propose to test the following hypotheses: 1. Different fertilizer regimes will elicit differential expression of nitrogen metabolism genes, and 2. A different microbiome will be fostered under organic nitrogen conditions. This investigation will entail a time course root and microbiome-targeted transcriptome analysis in which we will focus specifically on genes and pathways associated with nitrogen metabolism and on the microbial symbionts whose activity is favored under the different fertilizer conditions. The gene-based knowledge generated through our investigation will facilitate identification of genotypes that are able to utilize organic fertilizer efficiently.

PROJECT DESCRIPTION

OUTPUTS

Summary - The aim of the project was to perform metatranscriptome analysis of the rhizosphere and the roots of tomato plants grown under organic and conventional fertilizer regime.

- Overview of Work Completed and in Progress:
 - A. Fertilizer and potting soil chemical testing: The first aspect of this work required that all chemical components were of identical concentration in the organic and conventional fertilizer. The chemical composition of organic and conventional fertilizer, greenhouse water, and potting soil were tested. BioLink 3-3-3 (ORG) and Jack's Pro 20-10-20 (CON) fertilizers were chosen for the analysis. The concentrations of each component were normalized for total N. The analysis showed that ORG fertilizer has 21 mg/L of available NH₄ in the BioLink 3-3-3 and the rest of the nitrogen in that fertilizer is in organic form (i.e. proteins and amino acids). It was also determined that P and K are much higher in the 20-10-20 fertilizer, and S, Ca, and Na are higher in the 3-3-3 fertilizer. In order to balance the concentration of these chemical components, Mg sulfate and chelates of Fe, Zn, and Mn were added in the CON fertilizer to balance those mineral elements. Further, analysis of the potting media revealed a C:N ratio of 37:1 which is a somewhat higher than the recommended 24:1 for optimal soil microbial activity. However, with the addition of N fertilizers the ratio adjusted itself to the favorable range (Table 1).

Table 1: Final adjusted macro and micro elements in the two fertilizers (ORG and CON) used in this study.

Concentration (mg/L)	BioLink 3-3-3 (Phosphorus balance + additions S-T)	Jack's Pro (1.02mL/L + additions)
Total Nitrogen	200.00	200.22
Nitrate-N + Nitrite-N	0.00	94.76
Available NH4-N	21.54	105.46
Phosphorus (P)	29.78	29.78
P2O5	67.69	67.82
Potassium (K)	226.67	226.63
K2O5	271.79	167.63
	0.00	0.00
Magnesium (Mg)	0.00	0.00
Sulfur (S)	81.31	81.21
Calcium (Ca)	71.79	71.68
	0.00	0.00
Iron (Fe)	10.36	10.42
Zinc (Zn)	0.39	0.39
Boron (B)	1.23	1.23
Copper (Cu)	0.39	0.39
Manganese (Mn)	0.00	0.00
Sodium (Na)	76.00	75.99

- B. Soil RNA isolation protocol: Isolation of total RNA from soil is a tedious task. Several protocols were evaluated and one particular method enabled successful isolation of RNA from soil samples. This is critical for successful total RNAseq step. Soil RNA extraction was conducted using a modified CTAB buffer and nucleic acid precipitation solution prepared according to Griffiths et al. 2000. (Griffiths, R. I., Whiteley, A. S., O'Donnell, A. G., & Bailey, M. J. (2000). Rapid method for co-extraction of DNA and RNA from natural environments for analysis of ribosomal DNA-and rRNA-based microbial community composition. *Applied and environmental microbiology*, 66(12), 5488-5491.). However the quality of sequence data generated from RNA isolated with this protocol was not good.

At this point, additional RNA protocols were identified and discussed with soil scientists at WSU to overcome the issues with RNA quality. A new RNA isolation protocol that produces higher quality RNA was established as shown in (Figure 1). The RNA from this method will be utilized for generating sequencing data.

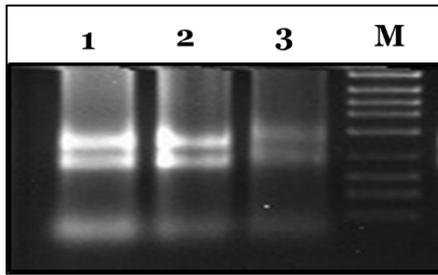


Figure 1: RNA from potting soil. Lanes 1-3 total RNA (without DNase treatment) from 3 different samples. The intact ribosomal bands indicate RNA of good integrity.

- C. Plant growth standardization and collection of samples: Prior to performing the experiment, the plant growth process was standardized. Seeds were germinated on $\frac{1}{2}$ MS Agar media. To determine the most efficient process, plants were transferred to small pony pack pots with perlite at various developmental stages starting from cotyledonary emergence stage to 3rd leaf stage. From different trials it was determined that it was best to transfer plants to pony pack pots once the cotyledons had emerged and prior to emergence of the first leaf. At this point the roots were small enough to perform the transfers without damaging the roots. The standardization of this process was critical to enable work with plants at comparable developmental stage. Thereafter, the plants were fertilized with liquid $\frac{1}{2}$ MS solution as needed. Once the plants reached the 3rd leaf stage, they were transferred to new pony packs with potting soil. The perlite was washed away. The potting soil was first saturated with the respective fertilizer by allowing the pony pack pots to stand in a tray filled with liquid fertilizer for 4 hours. Thereafter, the plants were transferred to the potting soil and samples harvested at 0, 6, 12, 24 and 48 hour time points. A sample in the context of this experiment represents the roots of the plants along with the potting soil in the immediate vicinity of the root system. Four technical replicates (4 plants) per treatment were sampled and pooled for each sampling time point. RNA was isolated from the pooled potting soil/plant roots constituting one RNAseq sample. The experiment was repeated 3 times (3 biological replicates).
- D. Sample collection and total RNAseq: Samples (as described above) from three biological replicates of the experiment were collected and the material was processed for isolating total RNA. Samples were submitted to for sequencing. The dataset received was not of high quality. We are continuing to improve RNA isolation process and consulting with other soil scientists at WSU to ensure isolation of best quality RNA to generate good quality reliable data.
- E. Data simulation and workflow optimization: To establish a data analysis workflow, simulated data, where tomato transcriptome data was combined with microbial RNAseq data available from public data repositories, was developed. These simulated data were processed through two different analysis workflows which involve separation of plant and microbial reads followed by clustering of reads to determine frequency of each read. Finally the data were processed through annotation and mapping to different metabolic pathways. Through this process the analysis workflows were established.
- F. Data analysis: The raw data did not meet quality standards. Quality of sequence reads is denoted by Phred quality score, which represents the accuracy of the base call in percentage. For example a Phred score of 30 means that 1 in a 1000 bases will be called wrong or the accuracy of the base call is 99.9%. Majority of the data failed to meet a Phred score of 30. In the meantime,

we have established better protocols for soil RNA analysis in consultation with WSU soil scientists. Graduate student involved in the project has been able to win a W-SARE grant to support the generation of high quality data in spring 2018.

- G. Problems encountered: At the start of the project we found out that the BioLink 5:5:5 fertilizer, which had been used in a previous study, had been discontinued. We had some delays in obtaining a substitute organic fertilizer with comparable chemical composition. BioLink 3:3:3 was chosen for this project however the availability of this product was limited at the start of the project. We were eventually able to acquire the product and proceed with the chemical analysis.

RNAseq data was not of high quality for proper analysis. This was perhaps due to RNase contamination in the soil RNA samples. In discussion with soil scientists at WSU, higher quality RNA isolation protocol has been established and RNAseq data will be generated using this protocol for subsequent analysis.

- H. Discussion:
The sampling at short time intervals after transplant into organic and conventional treatments is not expected to impact plant growth and development significantly. The focus is to quantify changes in the microbial communities and root responses at the transcriptomic level. Although the macro and micro nutrients between the two types of treatments were balanced, differences are still expected in the overall composition given the source of the nutrients. In that regard, it is important to consider the conventional treatment as a treatment unto itself and not a control treatment. To reiterate, the goal of this project is not to identify which nutrients contribute to differences at the transcriptome level. We aim to test the following hypotheses: 1. Different fertilizer regimes will elicit differential expression of nitrogen metabolism genes, and 2. A different microbiome will be fostered under organic nitrogen conditions. Given the fundamental difference between the two treatments, it is only feasible to record the impact of the treatments. To identify how each nutrient impacts the differences in the transcriptome would require a much different experimental design which quantifies the responses in the presence, absence or substitution of individual nutrients. That goal is outside the purview of this project.

Publications, Handouts, Other Text & Web Products:

Richard M Sharpe, Luke Gustafson, Seanna Hewitt, Benjamin Kilian, James Crabb, Christopher Hendrickson, Derick Jiwan, Preston Andrews and Amit Dhingra. Understanding the molecular basis of phytonutrient composition differences in organic tomatoes via quantitative transcriptome analysis. Manuscript has been prepared to be submitted to PNAS in spring 2018.

- Outreach & Education Activities:
Invited Presentations:
 1. RM Sharpe - Why Does Organic Fertilizer Reduce Yields and Produce Healthful Products? Annual Washington State Tree Fruit Association meeting, Kennewick, WA, Dec 4-6, 2017
 2. RM Sharpe, S Hewitt and A Dhingra. Genomic Approaches to Understanding the Enhancement of Phytonutrients in Plants Grown under an Organic Nitrogen Regime Concurrent Symposium: Concurrent Symposium 8: Whole Plant Ecology/Ecophysiology. Annual ASPB Meeting, Honolulu, HI, June 23 – 28, 2017.
 3. RM Sharpe and A Dhingra. Transcriptomic Differences in *Solanum lycopersicum* Grown under Organic and Conventional Production Systems. Leibniz Universität Hannover, Germany, March 16, 2016.

4. S Hewitt, R M Sharpe, L Gustafson, B Kilian, J Crabb, C Hendrickson, D Jiwan, P Andrews and A Dhingra: Genomic approaches to understanding the enhancement of phytonutrients in plants grown under an organic nitrogen regime. Western ASPB, Pullman, WA. June 26-28, 2015
5. S Hewitt, RM Sharpe, L Gustafson, B Kilian, J Crabb, C Hendrickson, D Jiwan, P Andrews and A Dhingra. Understanding the Molecular Basis of Enhanced Phytonutrient Content of Plants Grown Under an Organic Fertilizer Regime. American Council for Medicinally Active Plants Conference, Spokane, WA. June 9-13, 2015

Outreach

- Shared the concept of the research with scientists from Tech Accel Inc – a venture technology development company.
- Established a collaboration with Cool Planet LLC, a biochar production company from Colorado.
- Shared the concept of the research with several tree fruit and forestry industry members.
- Shared the approach and methods with principals from AgEnergy Systems Inc., Spokane, WA
- Shared the approach and methods with principals from Inland Empire Paper Company, Spokane, WA

IMPACTS

- Short-Term: The project will increase our knowledge of the relationship between observed phytonutrient changes and underlying gene expression changes in plants when grown under organic soil fertility management in a model crop plant (tomato). Such an interdisciplinary and comprehensive analysis remains to be done in the field of crop production. The results of this study will be used to apply for grant funding in USDA's AFRI program in order to conduct a more comprehensive study, which includes field research.
- Intermediate-Term: The knowledge generated from this BioAg and subsequent federal projects will assist organic and sustainable producers to develop management plans for soil fertility and pest control that help them optimize the nutritional quality and yields of their products. The understanding of gene function and of markers associated with those genes will help plant breeders in the breeding and selection of more efficient crop cultivars that optimize both nutritional quality and yields of crops grown using more sustainable farming practices.
- Long-Term: Increased consumption of foods with better nutritional quality should contribute to the health of American children and adults and reduce health care costs. Using more sustainable farming practices, especially of soil fertility and pest management, will enhance the quality of U.S. agroecosystems. Besides improving agricultural sustainability and public health, this project could also contribute to the long-term economic viability of American farmers.

ADDITIONAL FUNDING APPLIED FOR / SECURED

- W-SARE Grant awarded to Seanna Hewitt in 2017
- Support for a MS graduate student secured from WSU Graduate School.
- Obtained funding from the Cowles Company, Stump Farmer Farm, and Cool Planet Inc. to evaluate the impact of organic fertilizer in conjunction with biochar.

- USDA OREI grant in progress for submission in 2018 based on the results obtained from this and additional complementary projects. Awaiting publication of the first research article prior to grant submission.
- I plan to apply to the NSF Integrated Ecological Physiology panel in 2018

GRADUATE STUDENTS FUNDED

No graduate student was directly funded with support from this project.

RECOMMENDATIONS FOR FUTURE RESEARCH

Since the data analysis still needs to be completed, no recommendations have been provided.