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STUDY OF DIFFERENTIAL PROTEINS AND METABOLITE PATTERN IN
ORGANIC AND CONVENTIONALLY GROWN TOMATOES IN SOIL AND
HYDROPONICS USING A PROTEOMICS AND METABOLOMICS APPROACH

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ABSTRACT

Tomato (*Lycopersicum esculentum*) belongs to the family *Solanaceae* and accounts for more than half of the vegetable consumption in the United States. Traditionally, tomatoes have always been cultivated on soil. Recently, attempts have been made to incorporate value added traits for early flowering, disease resistance, and high yield of tomatoes by growing them in soil-less media with mineral nutrient solutions such as hydroponics. This method is known to offer several advantages including conservation of water, reduction of pesticide use, increased root mass and efficient uptake of nitrogen and other minerals leading to high crop yields. Until now over 50 metabolic compounds are identified that differ in organic and conventionally grown tomatoes, but there is no study that highlights the differences in the morphological characteristics, their physiological responses and protein and metabolite profiles between organic and conventionally grown tomatoes in soil and hydroponic systems.

To understand how these two treatments would affect tomato's gene expression and metabolic pathways, we performed a pilot experiment to estimate the differences in their protein and metabolite profiles and physiological responses when they were grown in soil or hydroponics. Analysis of the morphological and physiological responses such as plant height, days to flowering, number of flowers, seed weight, and fruit yield was best displayed in the organic treatment groups. However, an increased root biomass was observed in the hydroponic grow systems. A preliminary analysis of total proteins using one dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis on tomato fruits indicated a differential expression of proteins among all treatments. In addition, a basic metabolic profile was obtained using high performance liquid chromatography.

These results also indicated that the hydroponic grow system yielded the best results as indicated by the metabolite concentrations.

This work, gave a better insight into the differences of the two different tomato types (organic and inorganic) grown in two different grow systems (soil and hydroponics) and helped in the identification of new proteins and metabolites. Further investigation would enable a better understanding of the plant pathways and possibly allow the identification of mechanisms targeted to increase the yield and produce nutritionally enhanced tomatoes, particularly rich in antioxidants, aroma and flavor.

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Chapter 1: Introduction

1.1 General introduction

Tomato (*Lycopersicon esculentum* Mill.) is a member of the family Solanaceae and is a hardy plant, which is diploid; $2n=24$ chromosomes and is widely cultivated in the United States. According to the Food and Agricultural Organization, in 2013, over 150 million metric tons of fruits are produced every year contributing to a \$35 billion industry. Tomatoes and its food derivatives are a great source of antioxidants, vitamins and minerals (Spencer et al. 2005). In addition, it also serves as a great plant research model species allowing us to explore several areas of plant biology such as physiology, genetics, and breeding. The entire tomato genome was sequenced in 2012 i.e. approximately 900 megabases (Mb), which can be found on the Sol Genomics Network. The knowledge gained from sequencing efforts have enabled the improvement of tomato crop quality (Bai et al. 2007; Fernie et al. 2006). Given the availability of the genome sequence, researchers are striving to successfully develop fast-growing, high-yielding tomatoes in addition to characteristics such as flavor, color, texture that satisfy the consumer's preference using modern tools of plant biotechnology such as proteomics, transcriptomics and metabolomics.

According to the USDA, there are six noticeable stages of ripeness (Figure 1). The six stages are characterized by the tomato's color, which are classified as green, color-breaker, turning, pink, light red, and red, respectively. During Stage 1, the tomato's surface is entirely green. Stage 2 is defined by a break in color from a shade of green to a tannish-yellowish, pink, or red on approximately 10% of the surface or less. In the Stage 3, approximately 10% to 30% of the tomato's surface is not green. In Stage 4, about 30%

to 60% of the tomato's surface is pinkish-red or red. Stage 4 is followed by Stage 5, which is characterized by 60% to 90% color change from green to pinkish-red or red. The final stage of ripening, Stage 6, is observed when more than 90% of the surface is a red color.



Figure 1. Different stages of tomato ripening defined by The U. S. Department of Agriculture

1.2 Benefits of Tomatoes

Tomatoes are widely cultivated all over the world and are adapted for growing in various climatic conditions. Their exotic germplasm makes it easy for the researchers to manipulate them genetically and develop high yielding, nutritious, disease and stress resistant plants. Tomatoes are very healthy vegetables since they are naturally low in calories and are rich in vitamins A, C, E, trace elements, phytosterols, dietary flavonoids, and carotenoids. Tomatoes are also excellent sources of folate and potassium. The predominant flavonoids found in tomatoes are phloretin, quercetin, chalconaringenin, and kaempferol. Chalconaringenin constitutes 35 to 75% of the total flavonoid content in tomatoes (Slimestad et al. 2011). Another essential nutrient which is abundant in tomatoes is carotenoid. Lycopene is the most prominent carotenoid followed by beta-carotene, gamma-carotene, and phytoene as well as minor carotenoids. Lycopene, gives red pigmentation to tomato fruit. The antioxidant activity of lycopene as well as several other carotenoids and their abundance in tomatoes makes these foods rich sources of antioxidant activity which helps to prevent cancer, heart disease, and premature aging.

1.3 Impact on Nutrition and Health

Oxidation of the circulating low density lipoprotein (LDL) is known to play a key role in the pathogenesis of Coronary heart disease (CHD). Dietary antioxidants found in tomatoes such as vitamin E, β -carotene, and lycopene have been shown to prevent the formation of oxidized LDL. Lycopene is used to inhibit the activity of an essential enzyme involved in cholesterol synthesis, LDL degradation, LDL particle size and composition, plaque rupture, and altered endothelial functions thus playing a predominant

role in controlling progression of coronary heart disease, cancer, and helping in bone growth.

Tomatoes have phytonutrients like esculeoside A, flavonoid like chalconaringenin, and another fatty-acid type molecule like called 9-oxo-octadecadienoic acid which help prevent blood coagulation and atherosclerosis leading to heart protective properties. Alpha-tomatine is a saponin phytonutrient found in tomatoes and has been shown to decrease prostate cancer (Renata et al. 2009; Friedman et al. 2009; Lippi et al. 2011).

Hydroponics is a subdivision of hydroculture method that grows plants in a liquid or soil-less medium that is supplemented with a mineral nutrient solution. Hydroponics offers several advantages, such as conservation of water, reduction of pesticide usage, and high crop yield. Prior experimentation has revealed hydroponic growth systems to have increased growth and higher production yields for various plants when compared to soil-grown medium. The high quality properties of plants grown in different conditions can be assessed by using methods of metabolomics and proteomics.

With the advent of “Omics” revolution coupled with availability of tomato genome sequence, there have been several advances in identifying proteins involved during tomato fruit ripening (Rose et al, 2000). Likewise, there are over 50 different metabolic compounds identified in organic and inorganic tomatoes or conventionally grown tomatoes (Dunn et al. 2012; Prinsloo et al.1999) through mass spectrometry. Literature review analysis in the present study indicated that there has not been a detailed study which has been reported comparing the effect of growth conditions on physiology, protein and metabolite profile in tomatoes grown in organic and inorganic soil mediums

and hydroponics. The present study aims to examine key morphological, physiological, biochemical and molecular differences that result from growing tomatoes under various growth conditions. The physiological parameters included in current study are plant height, days to flowering, number of flowers, and number of fruits, fruit weight, and root length. Biochemical and molecular studies included the analysis of proteins and metabolites through one dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (1D-SDS PAGE) and high performance liquid chromatography (HPLC).

Chapter 2: Literature Review

Solanum lycopersicum (tomato) is one of the most widely consumed fruits throughout the world.

2.1 Plant Physiology

Plant physiology is concerned with several elements that pertain to plant growth and development, as well as plant function. The study of plant physiology encompasses several fields of study that include photochemistry, environmental interactions, plant morphology, molecular and cellular biology, and cellular interactions. Plant physiology is concerned with how fundamental processes are affected. Some of the fundamental processes include plant respiration, plant nutrition, environmental stress physiology, seed germination, stomata, dormancy, and most importantly, photosynthesis.

It is important to observe and record key stages that mark a plant's growth and development. A plant's physiological development can be tracked by observing various aspects of plant development that include, but are not limited to, fruit number, fruit weight, seed number, plant height, days to flowering, numbers of flowers, and root length. Prior to making observations of plant development, it is critical to understand the variables that affect plant development. Plant growth and development is heavily dependent and regulated using light, temperature, water (humidity), and nutrition (Bradly et al. 1998).

The effect of light on plant growth and development is dependent on three primary characteristics, quantity, quality, and duration. The intensity of sunlight is an important factor to plant growth and has a positive correlation on photosynthesis. As the light quantity increases, to an extent, the photosynthetic ability of the plant increases.

Light quality, another important principal characteristic of light, has a great impact on plant growth (Bradly et al. 1998). The light emitted in our solar system by the sun can be fragmented into red, orange, yellow, green, blue, indigo, and violet. Studies have found that the colors blue and red had the most positive effect on plant growth, while green light is least effective (Bradly et al. 1998). Each fragmented color has different effects on the plant. A blue color will promote vegetative growth, such as leaf growth. The combination of red and blue lights stimulates flowering. Fluorescent light, which was used in the present study, is used to promote leafy growth, and is a good choice of lighting for the germination of new seedlings. Another key characteristic of light is duration. The amount of light exposure on a plant, a photoperiod, is important to the plant's development and physiology. Studies have found that the floral development is positively affected by the duration of uninterrupted dark periods (Bradly et al. 1998).

In addition to light, temperature is a critical component to the plant's development and growth. Often times, poor quality of vegetables or stunt growth is indicative of adverse temperatures during its early germination stages (Bradly et al. 1998). The classification of a plant as hardy or non-hardy is dependent on their ability to tolerate cold temperatures. It is important to note that extremely low temperature may induce cold stress on the plant and possible kill the plant (Bradly et al. 1998). Additionally, lower temperatures, the soil may solidify hindering the movement of water in the soil, thus impeding root development. Water is critical for the photosynthetic process that takes place in a plant (Bradly et al. 1998).

Water is one of the most important components to a plant's development. The appropriate amount of water maintains the turgor pressure and transports nutrients via

phloem, as well as water via xylem. The regulation of water takes place at the stomata of a plant, which are aperture structures situated on the epidermis (Bradly et al. 1998).

Additionally, water acts as a solvent and help transport minerals into the plant and carbohydrates for storage (starch). The relative humidity is very important to regulating transpiration and can be calculated by water in the air over the air saturated with water (at constant temperature and pressure) (Bradly et al. 1998). The movement of water will increase with a greater difference of relative water humidity. In addition to the water's contribution to plant growth, nutrition is a pivotal component to plant development.

Plant nutrition encompasses 18 different elements that are necessary for normal development. Six of the 18 elements are macronutrients, while the remaining 9 are micronutrients (Bradly et al. 1998). The macronutrients are nitrogen, phosphorus, potassium, magnesium, calcium, and sulfur, and the micronutrients are chlorine, iron, cobalt, zinc, copper, molybdenum, boron, manganese, and nickel (Bradly et al. 1998). The macromolecules and micronutrients combined are dissolved in water and are absorbed by the roots of the plant. The positively charged elements are paired with negatively charged elements resulting in a net zero charge (Bradly et al. 1998). Lastly, the nutrients in the water are transported from the root to the cells of the plant.

2.2 Proteomics

Proteomics is the study of structure and function of all the proteins in an organism. The term proteomics is also a generalized term used to explain protein techniques involved in protein purification, mass spectroscopy, and all the large scale experimental analysis of proteins.

2.2.1 An Introduction to Proteomics

Proteomics follows an organized approach in terms of methodology needed to analyze the proteins. The first step deals with techniques that isolate the whole set of proteins, or a particular single protein from any living organism. The following steps in proteomics deal with techniques involved in identification and characterization of resolved proteins or peptides, mostly by mass spectrometry.

Currently, three techniques are widely used for the separation of proteins from an organism or cell; (1) sodium dodecyl sulfate denaturing polyacrylamide gel electrophoresis (SDS-PAGE or 1 dimensional gel (1-D)); (2) two-dimensional (2-D) gel electrophoresis; and (3) liquid chromatography (LC). Other techniques for protein separation include preparative isoelectric focusing and native PAGE (Righetti et al. 2016).

2.2.1.1 Principle of the Bradford Assay

The Bradford protein assay, developed by Marion M. Bradford, is an analytical tool to measure the concentration of protein in a solution (Bradford et al. 1976). This spectroscopic analytical tool is based on the absorbance shift of the Coomassie Brilliant Blue G-250 as it binds to protein. Under acidic conditions, the red form of the dye is converted into its blue form when it binds to the protein. Thus, the amount of protein dye complex in solution determines the intensity of blue color and is a measure of the protein concentration in the solution. The absorbance of the protein (optical density or OD value) solution can be read at 595 nm. A standard graph (as depicted in Figure 2) is plotted with known concentrations of Bovine Serum Albumin (BSA) on X axis versus the absorbance of each protein concentration and a linear line is obtained from this plot. The slope of the

line and the intercept of the line on the Y axis is determined and the concentration of the protein solution is calculated by the formula $y=mx + b$ or $y=mx - b$. Depending on whether the intercept of line on Y axis is more (positive value of intercept) or less than zero (negative value of intercept) will determine whether the concentration is a positive value or negative value.

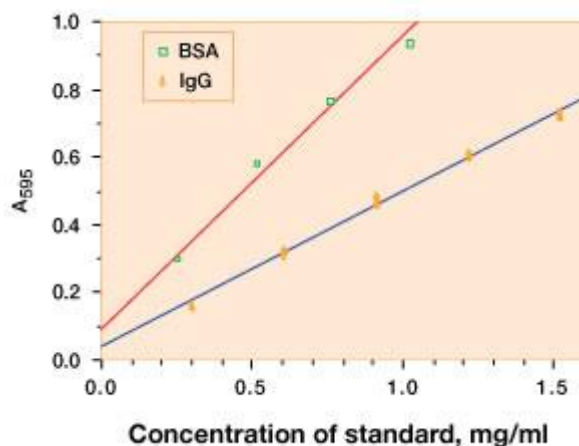


Figure 2. A standard curve showing the absorbance of different concentrations of proteins using Bradford Protein Assay

Where m = the slope of the line known as the gradient, b = intercept of the line on Y axis, and X is the unknown protein concentration. The coefficient of determination, R^2 , and it indicates how well the numerical data fit a line of best fit or curve. An R^2 equating to 1 indicates a positive or negative correlation, while R^2 equating to 0 indicates no correlation.

2.2.1.2 Electrophoresis-based Proteome Analysis

Gel electrophoresis is the most commonly and widely used technique in which charged molecules are separated according to physical properties such as charge or mass as they move through an acrylamide sieving gel matrix on the application of electrical current. This technique is mostly used to identify individual

proteins in complex samples or to examine multiple proteins within a single sample (Celis et al. 2012).

There are several types of gel electrophoresis and that can provide different types of information about the protein(s). The first type of gel electrophoresis is non-denaturing PAGE also called native PAGE and it separates proteins according to their mass-charge ratio. The second type of gel electrophoresis is the denaturing and reducing SDS-PAGE which separates proteins primarily by mass. The third type of gel electrophoresis is the two-dimensional (2D) PAGE which separates proteins by isoelectric point in the first dimension and by mass in the second direction. Isoelectric focusing and electrophoretic transfer (western blotting) are also included under protein electrophoresis. In western blotting, the expression of a particular protein is detected by using its antibody. After, the proteins are transferred onto a nitrocellulose membrane by electric transfer. Isoelectric focusing (IEF), also known as electro focusing, is a technique used for separating different proteins by differences in their isoelectric point (pI). IEF is based on the principle that overall charge on the molecule of interest is a function of the pH of its surroundings (Sambrook et al. 1989). Protein electrophoresis can be used for a variety of applications such as purifying proteins, estimating protein purity, determining the extent of protein expression, determining protein size, finding isoelectric point and enzymatic activity.

2.2.1.3 One-Dimensional Polyacrylamide Gel Electrophoresis

One dimensional polyacrylamide gel electrophoresis (1D PAGE), first reported by Laemmli in 1970, is the most common electrophoresis technique followed in proteomics. The gel is made by mixing acrylamide and bisacrylamide in a specific ratio

which determines the pore size and rigidity of the gel matrix. The addition of acrylamide to bisacrylamide forms a cross-linked polymer network when ammonium persulfate (APS), a polymerizing agent, is added. TEMED (N, N, N, N'-tetramethylethylenediamine) is used in the gel to catalyze the polymerization reaction by promoting the production of free radicals by APS (Figure 3). The pore size affects the range of proteins which can be separated by the gel (Dunn et al. 2014).

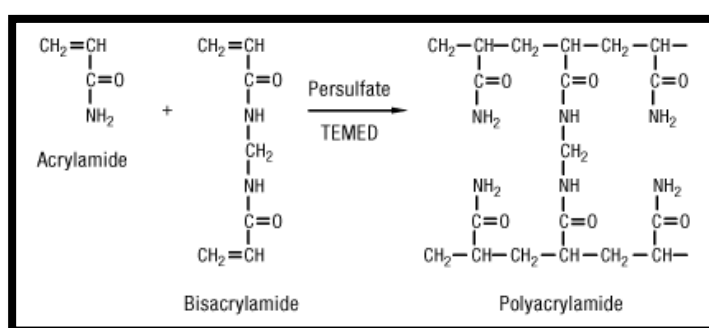


Figure 3. Polymerization and cross linking of acrylamide gel

Generally a 6% polyacrylamide gel has larger pores than a 10% polyacrylamide gel. Gels with a low percentage of acrylamide are typically used to resolve large molecular weight proteins, and high percentage gels are used to resolve low molecular weight proteins. Electrophoresis gels are run in tris-glycine electrophoresis buffer that provides ions for conduction of an electrical current through the matrix. The solution is poured into the thin space between two glass plates of an assembly called a "cassette." Once the gel polymerizes, the cassette is mounted (usually vertically) into an apparatus so that opposite edges (top and bottom) are placed in contact with buffer chambers containing cathode and anode electrodes, respectively. When proteins are added in wells at the top and current is applied, the proteins are drawn by the current through the matrix-

slab and separated in the gel. Generally the upper gel is called a “stacking” gel into which the comb is inserted for the formation of wells to load the protein samples. The lower gel is called resolving gel (pH 8.8) in which proteins get separated. The stacking gel has a lower concentration of acrylamide (5 to 7% for larger pore size), lower pH (6.8) and a different ionic content. This allows the proteins in a loaded sample to be concentrated into a tight band during the first few minutes of electrophoresis before entering the resolving portion of a gel (Gelfi et al. 1981).

Recently, use of precast gradient gels has become more widespread because of their ability to separate a broader range of protein sizes. These gradient gels have low percent-acrylamide at the top (beginning of sample path) and high percent-acrylamide at the bottom (end). A stacking gel is not necessary when using a gradient gel, as the gradient itself performs this function (Candiano et al. 2002).

Gel sizes range from a mini gel (8 x 10 cm) to a standard gel (15 x 18 cm). Standard gels provide a better resolution than mini-gels and are run when there is a need to separate similar proteins or more number of proteins. 1D gels are of two types - native and denaturing PAGE. In the denaturing PAGE, the proteins may be denatured by adding sodium dodecyl sulfate (SDS) in the gel and Dithiothreitol (DTT) or 2-mercaptoethanol in the protein loading dye (sample buffer). After the addition of sample buffer, the protein sample is denatured by heating at 95°C for 5 minutes. If native gels are run then, SDS is not included in the gel and the proteins are loaded in protein loading dye, which does not contain DTT or 2- mercaptoethanol. The protein sample is not boiled or denatured. DTT and 2-mercaptoethanol reduce disulfide bonds and thus separate the proteins into their individual subunits. The sample buffer contains glycerol and provides density to the

protein sample so that it can be loaded into the well properly and it will not float up. The sample buffer has bromophenol blue as the tracking dye. The tracking dye moves ahead of the proteins and serves as a relative mobility marker (Sambrook et al. 1989).

This technique is easy and reproducible and less expensive. However, it has one disadvantage, the resolving power of SDS-PAGE is limited. In SDS-PAGE, the proteins are separated based on their molecular weights so any single discrete band on SDS-PAGE can consist of up to 10 multiple proteins with the same molecular weight.

2.2.1.4 Two-Dimensional Polyacrylamide Gel Electrophoresis

The two-dimensional gel electrophoresis (2D PAGE) was developed by O'Farrell (O'Farrell et al. 1975). Two-dimensional electrophoresis separates proteins by isoelectric point in the first dimension and by mass in the second direction. First, isoelectric focusing (IEF) is applied to protein samples prior to SDS-PAGE. Two-dimensional electrophoresis provides a high resolution of protein analysis and thousands of proteins can be resolved on a single gel (Figure 4).

To perform IEF, a pH gradient is established in a tube or strip gel using an ampholyte mixture. During IEF, proteins migrate within the strip to their respective isoelectric points, which is the point where it has a net zero charge. The IEF strip is placed sideways across the top of a 1D gel. This orientation allows the proteins to be separated in the second dimension according to size (Bandow et al. 2008).

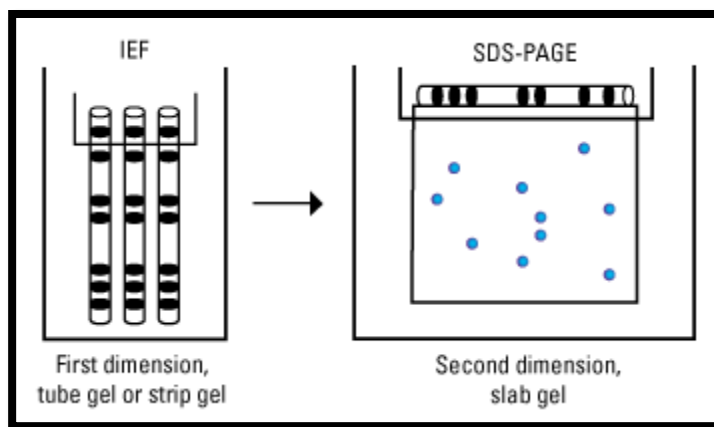


Figure 4. Overview of a 2-Dimensional Gel Electrophoresis

2.2.2 Applications of Proteomics

The one and two dimensional electrophoresis, western blotting, immunohistochemically staining, enzyme linked immunosorbent assay (ELISA), and mass spectroscopy techniques used in proteomics have been used profusely to separate and identify all proteins in many organisms, elucidate their amino acid sequence structure, and post translational modifications.

Further a proteomic approaches have helped identify the biomarkers (proteins) which are responsible for origin of diseases like cancer, Alzheimer's, diabetes etc. and this in turn unraveled novel biochemical pathways which could be targeted to control these diseases. With the aid of computer software, the three dimensional structure of disease causing proteins can be developed and can be screened for fitting or binding to many small molecules which inhibit the protein function (Wu et al. 2003). The computer rates the quality of the fit to various sites in the protein so that a potent inhibitor can be developed and tested in in-vitro and in vivo studies. This particular approach called “virtual ligand screening” in proteomics has helped in alleviating the spread of many

diseases. An excellent example of this approach is to control HIV progression by developing inhibitors against HIV-1 protease which is essential for the virus to form functional proteins and survive in humans. Moreover, proteomic techniques like liquid chromatography coupled to mass spectrometry (LC/MS) have been used to identify disease-associated membrane proteins and their modifications for development of antibodies or vaccines. The proteomic tools helped decipher the structure of HER2 (erbB2/neu) protein (which is over expressed on the breast cancer cells) and antibodies were developed against HER2. The treatment with HER2 antibodies lead to significant decrease in the progression of the disease (Slamon et al. 2001).

Proteomics is currently being used to improve the pharmaceutical drug development processes. Proteomics techniques are being used to monitor drug effectiveness in clinical trials. The protein profile might be changed with drug treatment and the extent of toxicity of drug can also be determined. Bodily fluids such as cerebrospinal fluid (CSF) and serum can be analyzed throughout the course of treatment. Changes in the protein composition of CSF may be indicative of altered protein expression in the central nervous system (CNS), which may be used for causative study or to diagnostic biomarkers (Steiner et al. 2000; Rohlf et al. 2000).

The latest trend in proteomics is the development of more sensitive and effective proteomic techniques to study low-copy number proteins (transcription factors) which cannot be detected on 2-D gels. This will help analyze the proteome in a real time/time-lapse manner which would enhance our knowledge on signaling pathways (Jarrett et al. 2012).

In tomato plants, many proteins involved in fruit ripening (e.g. carboxypeptidase), disease resistance, and stress resistance were identified using proteomic techniques (Mariapina et al. 2006; Roshni et al. 1996; Mireille et al. 2007; Yong-Qiang et al. 1996; Amjad et al. 2014; Noha et al. 2012; Corpillo et al. 2004).

2.3 Metabolomics

Metabolomics is the methodical study of the distinctive small chemical compounds (metabolites) that are formed as products or byproducts during cellular metabolic processes. Metabolic profiling helps enable the comprehension of the metabolic pathways in the cells.

2.3.1 An Introduction to Metabolomics

Metabolomics, an emerging field in science research, has tremendous potential in unraveling the biochemical pathways taking place in living organisms. The science of metabolomics started with paper chromatography experiments with urine and saliva samples which showed a particular pattern of compounds. This led to the concept that every living organism or cell can have a metabolic profile (Gika et al. 2007). Later metabolic studies were improved by nuclear magnetic resonance spectroscopy and most of the metabolites in the biochemical pathways of plant and animal cells were characterized.

METLIN, the first metabolomics web database, was developed for characterizing human metabolites and was created at the Scripps Research Institute; this database contains around 240,000 metabolites (Smith et al. 2005).

Gas chromatography, high pressure liquid chromatography, liquid chromatography, capillary electrophoresis, etc. are some of the analytical techniques used

to separate metabolites. Once they are separated, mass spectrometry (MS) is used to identify and quantify metabolites. There are libraries or databases which allow identification of a metabolite according to the fragmentation pattern.

In animals and humans, the metabolites are classified as endogenous or exogenous. Metabolites of foreign substances such as drugs are termed xenometabolites. The metabolites in plants are classified into primary and secondary metabolites. The primary metabolites (ex: amino acids, lactic acid) are involved in the normal growth, development, and reproduction. Secondary metabolites have important relational or species specific functions or may be produced at a particular stage of the life cycle. Some examples of secondary metabolites in bacteria, fungi, plants, and animals include alkaloids, antibiotics, naphthalene, nucleosides, steroids, lignin, tannins, essential oils, phenazine, terpenoids, and growth factors. Secondary metabolites are produced in lesser quantities than primary metabolites (*Bentley et al. 1999; Nordstrom et al. 2006; Michael et al. 2010*).

Plant secondary metabolites are of three types - flavonoids, allied phenolic and polyphenolic compounds, terpenoids and nitrogen-containing alkaloids, and sulfur-containing compounds (Michael et al. 2010). Metabolomic studies have improved the nutritional value of crops and crop yield. They have also helped in decreasing crop cost, determining best practices for food processing for enhancing food flavor, and nutrition (Macel et al. 2010; Beck et al. 2014; Resham et al. 2014; Khakimov et al. 2014; Lopez-Sanchez et al. 2015).

2.3.1.1 Liquid Chromatography-Mass Spectrometry (LC-MS)

Liquid chromatography–mass spectrometry (LC-MS) used in analytical chemistry is a technique that combines the physical separation capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry (MS). LC-MS has high sensitivity. It is generally used to separate and identify a particular compound having a specific mass from a mixture of compounds. Therefore, it is a valuable technique in separating and identifying the plant secondary metabolites. Furthermore, LC-MS has a vast application in agricultural and pharmaceutical industries (Moco et al. 2006).

In liquid chromatography, the liquid solvent containing the sample mixture (mobile phase) is passed under high pressure conditions through a column of solid adsorbent material (stationary phase). The mobile phase generally consists of combination of water with acetonitrile or methanol and the stationary phase consists of silica polymers. Each compound in the sample (mixture of compounds) interacts differently with the adsorbent material (hydrophobic or ionic interaction) and therefore, each compound flows through the column at different flow rates leading to the separation of the component as they flow out of the column. Each compound separated from the mixture can then be analyzed by a mass spectrometer (Wilson et al. 1993).

LC-MS is widely used in pharmacokinetics of drugs, metabolomics (profiling of secondary metabolites), drug screening, and drug development studies (Stobiecki et al. 2006; Lee et al. 1999; Arpino et al. 1992).

2.3.1.2 Gas Chromatography-Mass spectrometry (GC-MS)

Gas chromatography–mass spectrometry (GC-MS) is an analytical process that mirrors the features of gas chromatography and mass spectrometry to identify different substances within a test sample. In gas chromatography, the mobile phase consists of a carrier gas like helium or nitrogen and the stationary phase consists of liquid or solid polymer (5% phenyl polysiloxane). Gas chromatography is used to separate and analyze compounds that can be vaporized without decomposing (Wilson et al. 1993).

The gaseous compounds in the mixture being analyzed interact with the walls of the stationary phase column and each compound of the mixture elutes at a different time (called retention time). This retention time gives enough spacing for the mass spectrometer to capture, ionize, accelerate, and detect each of the ionized molecules separately (Wilson et al. 1993). GC-MS has a wide range of applications. It is used in environmental studies to identify the organic pollutants in the environment. GC-MS is used in forensic studies to identify particles from human bodies (especially fire accident victims). It is also used to identify narcotic drugs and anti-doping analysis. It is extensively used in defense activities such as explosives and chemical warfare detection (Eugene et al. 2004; *Eiceman et al. 2000*; Weber et al. 2011).

2.3.1.3 High Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography or high-pressure liquid chromatography is an analytical chemistry technique for separation, identification, and quantification of each component in a mixture. In this method, pumps pass a pressurized liquid solvent (mobile phase like water, acetonitrile, or methanol) that contains the sample mixture through a column-filled with a solid adsorbent material (stationary phase like

silica). Each of the different components in the sample differ with the adsorbent material used (by hydrophobic, dipole–dipole, and ionic interactions) causing different flow rates for the different components and leading to separation of individual components as they flow out the column (Karger et al. 1997).

The HPLC instrument consists of a sampler, pumps, and a detector. The sampler part of the instrument translates the sample mixture into the mobile phase stream, which is carried into the column. The pump regulates the flow rate of the mobile phase through the column. The pumps can also alter the composition of the mobile phase by mixing solvents together in a particular ratio to create a gradient. The detector will generate a signal that will enable the quantification of the individual samples in a mixture. The signal is proportional to the amount of sample emerging from the column. A digital microprocessor is used to control HPLC instrument, as well as provide data analysis.

HPLC has been used for manufacturing drugs in pharmaceutical industries. It has been used in clinical research and medical science (e.g. to detect the performance of enhancement drugs in urine, detecting vitamin D levels in blood serum). It is extensively used to separate and study the properties of organic compounds in industries (Pesce et al. 2010; Zahedi et al. 2015).

2.3.2 Applications of Metabolomics

Metabolomics has a wide range of applications in medicine, pharmaceutical industry, agriculture, and functional genomics (Gupta et al. 2014). In pharmaceutical research and clinical medicine, metabolic profiling of saliva, urine or blood plasma can detect the physiological changes after administration of drugs and assess the toxic effects of drugs (Tohge et al. 2014). Apart from that, metabolic profiling has led to development

of biomarkers (metabolites) which help in the diagnosis of diseases like cancer, diabetes, and heart disease. Metabolic profiling has been used to develop biochemical methods for screening for metabolic diseases (e.g. measuring blood glucose concentration).

In functional genomic studies, metabolomics can be used to determine the change in metabolic profile of a cell or organism due to genetic manipulation, such as gene deletion or insertion. Metabolic profiling is used to identify novel substrates for enzymes (e.g. N-acyltaurines were found to be uncharacterized endogenous substrates for the enzyme fatty acid amide hydrolase). Metabolomics has been applied in nutrigenomics to explore the metabolic pathways in plants in order to improve the food quality and quantity and also develop disease, drought, insecticide resistant, fast growing plants (Griffin et al. 2003; Beckonert et al. 2007; Nicholson et al. 2002; Kathleen et al. 2013). The metabolomic approach may allow the discovery of bio-protective foods which contain more vitamins or antioxidants or cancer suppressive nutrients (e.g. development of golden rice which is rich in beta carotene).

2.4 Metabolomics of Tomatoes

Tomato (*Solanum lycopersicum*) is a delicious, nutritious, vegetable or fruit and belongs to the *Solanaceae* family. It originated from Andean, South America. The first tomatoes grown were similar in size to modern day cherry tomatoes. In 1600's, the conquistadors shipped them to Europe. Today, tomato is the leading vegetable crop (Vincent et al. 2013; Bai et al. 2007).

There is a wide range of diversity in the type of tomato plants, color, and size of tomatoes as well as the climatic conditions under which they grow. Researchers have taken advantage of this exotic diversity in the genetic make up to introduce high yield

and stress resistance genes into tomatoes (Frary et al. 2010; Frary et al. 2000). Further, genes were also introduced to increase the nutritive value and develop fleshy fruits (Klee et al. 2011; Klee et al. 2013).

Tomato was the first plant to be analyzed by metabolomics. The first metabolic studies in tomato used GC-MS, NMR, and LC-MS to analyze a number of secondary metabolites as well as metabolites of flavonoid pathway (Burns et al. 2003; Le Gall et al. 2003; Roessner-Tunali et al. 2003).

Later metabolic studies identified the metabolites involved in tomato fruit ripening. It was found that the metabolites of tricarboxylic acid (TCA) cycle determine the tomato fruit ripening (Centeno et al. 2011; Osorio et al. 2013). Further studies were done to correlate the metabolites to the corresponding genes and it was found that regulatory genes like bZIP and MYB were involved in metabolic shifts during the tomato fruit development. A study by Klie et al., who identified conserved and non-conserved patterns of tomato fruit ripening in both normal and mutant (non-ripening, never ripening, with ripening inhibitor) tomatoes. They concluded that the levels of malate, serine, threonine, and aspartate metabolites discriminated the climacteric from the non-climacteric fruits (Mounet et al. 2009).

Lately, ethylene mediated regulatory pathways have been implicated in tomato ripening (Lee et al. 2012). Series of metabolic profiling studies focused on identifying metabolites which confer stress tolerance in tomatoes in different species of tomatoes. Their study concluded that proline and hexoses were involved in giving resistance to tomato plant to grow in stressful conditions. Further, essential amino acids and vitamins were involved in determining the nutritional content of tomatoes. Thus, these studies

gave an idea about the genetic pathways which could be exploited for obtaining nutritionally rich stress resistant tomato plants without compromising in yield (Schauer et al. 2005; Sauvage et al. 2014).

In Tikunov et al (2005), metabolic studies were carried out with 400 volatile organic compounds and out of them sixteen compounds derived from phenylalanine, leucine, linolenic acid, or isoleucine and were shown to increase the flavor of tomatoes. Furthermore, the phenylpropanoid metabolic pathway was discovered to be responsible for plant stress responses and methyl salicylate pathway was responsible for pathogen resistance (Dixon et al. 1995; Tieman et al. 2010; Shulaev et al. 1997). Studies were also done to analyze the non-volatile secondary metabolites like phenylpropanoids, flavonoids, glycoalkaloids, and carotenoids. It was found that the conversion of tomatine to esculeoside glycoalkaloids is regulated by ethylene dependent fruit maturation and is the main factor responsible for ripening of tomatoes (Itkin et al. 2009; Friedman et al. 2000).

In Lee et al (2012) and Pan et al (2012), the researchers performed a HPLC profiling of carotenoid and lycopene contents and attempted to identify transcription factors responsible for fruit color in tomatoes. They concluded that two genes SIERF6 and APRR2 were involved in carotenoid pathway and ripening of tomatoes.

2.5 Project Rationale

Tomato plants were grown in organic and inorganic conditions in two different types of media – soil and hydroponics (Figure 5), which resulted in four groups of tomato plants for analysis (organic hydroponics, inorganic hydroponics, organic soil and inorganic soil). The tomatoes were harvested at specific stages following USDA

specifications (Stage 1, Stage 3 and Stage 6). The protein and metabolite profiling was performed to assess the difference in phenotypic characteristics and yield (quality and quantity) of tomatoes grown under the different conditions. The morphological studies done in the present study included statistical analysis of characteristics, such as plant height, days to flowering, number of flowers, seeds, and fruits, as well as seed weight and fruit size. The genetic isolation study involved analyzing the metabolites through high performance liquid chromatography (HPLC). This was followed by a protein profiling analysis, which was performed by isolating the proteins using a modified phenol extraction method and followed by a One Dimensional Sodium Dodecyl Sulfate Gel Electrophoresis (1D SDS-PAGE) analysis. The analysis of the protein and metabolite profiles between the tomatoes grown in different conditions might unravel new metabolic pathways, which could be targeted to produce fast growing, nutritionally rich tomatoes, and the metabolites could be identified and tested for their anti-cancer properties.

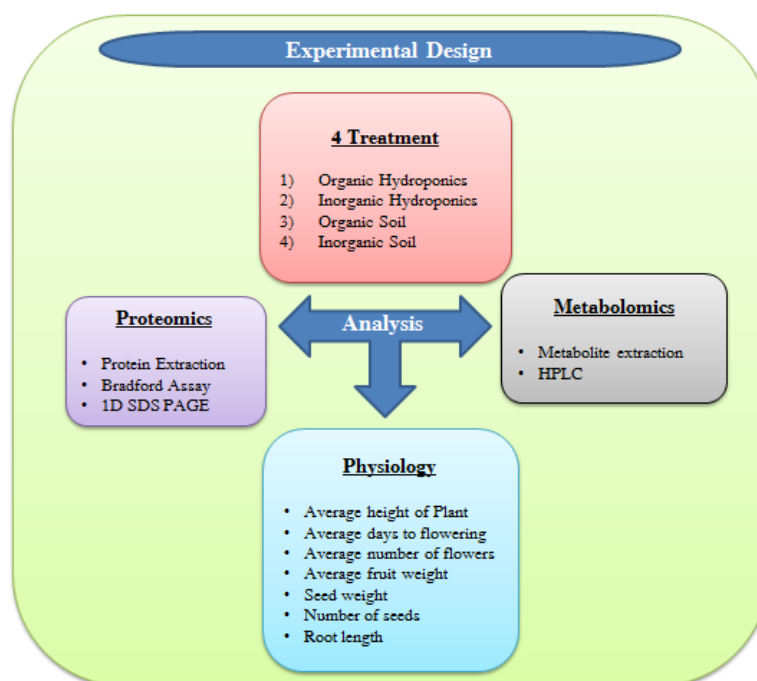


Figure 5. The experimental design work flow

Chapter 3: Material and Methods

3.1 Experimental Design

The experiment conducted during the fall of 2015, consisted of four separate treatments to examine the growth of inorganic and organic Roma tomato plants using two different grow systems: soil and hydroponics. The parameters were researched in the greenhouse of Penn State Harrisburg's Central Pennsylvania Research and Teaching Laboratory for Biofuels (CPRTL B). Tomato seeds of the Roma Organic and Roma Verticillium and Fusarium type were used to perform all experiments. The National Institutes of Health certified and USDA Level 2 facility is climate controlled around-the-clock for parameters such as temperature, light, and humidity. During the course of this experiment, the greenhouse was maintained at 26°C with a 14-hour light photoperiod at 25-40 $\mu\text{Em}^{-2}\text{s}^{-1}$. Twelve separate 18 Liter bucket systems were set up using tap water and hydroponics nutrient solution to grow the Roma tomato plants originating either from an organic source (OH) or from conventionally grown (IH) tomatoes. Twelve nine inch pots were used to grow the tomato plants, six of which were set up using a high porosity soil, HP Mycorrhizae from Pro-Mix (Rivière-du-Loop, Québec, Canada) and the remaining six pots were set up using an organic soil (OS), Michigan Peat® BACCTO® Veggie Mix (Houston, TX, USA), certified by the Organic Materials Review Institute (OMRITM). One tomato seed (variety Roma) was sown per individual pot and were watered every day at the same time of the day to maintain consistency of the plant watering regime.

Tomato plants from all the four treatments (OH, IH, OS, IS) were grown to maturity and tomato fruits at stage 1, 3 and 6, according to the USDA "Tomato Ripening

Stages” rubric, were collected randomly from each individual plant and used for further proteomic and metabolomic analysis. Physiological phenomena such as plant height, number of seeds, weight of seeds, number of fruits, number of flowers, days to flowering, weight of fruits, and root length were monitored at specific times during the course of the experiment.

The growth of the tomato seedlings (organic hydroponics, inorganic hydroponics, organic soil, inorganic soil) after 11 days of germination in the greenhouse are depicted in Figure 6. The growth at 31 days is shown in Figure 7 and Figure 8 shows growth at 100 days.

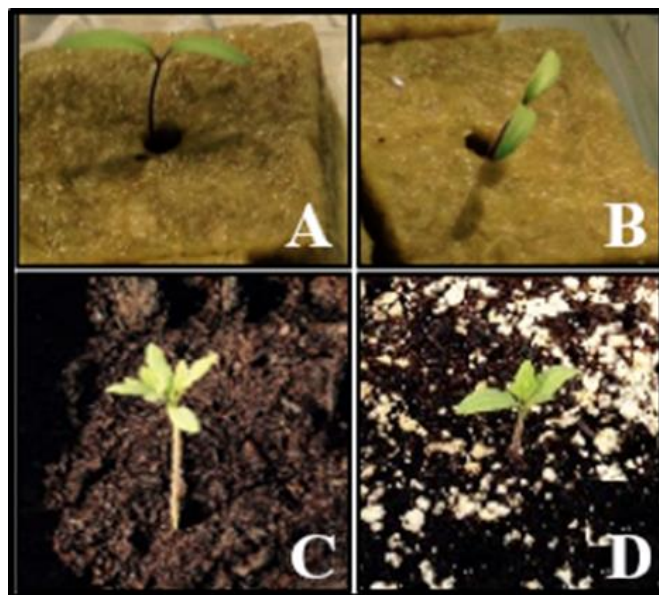


Figure 6. Eleven day old Roma seedlings: (A) organic hydroponics, (B) inorganic hydroponics, (C) organic soil, (D) inorganic soil

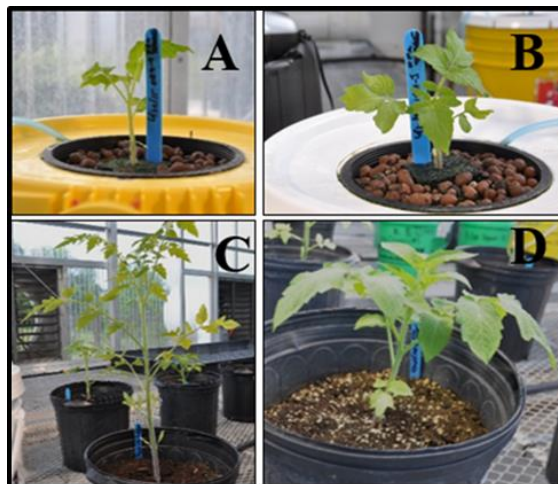


Figure 7. Thirty-one day old Roma plants: (A) organic hydroponics, (B) inorganic hydroponics, (C) organic plants, (D) inorganic soil



Figure 8. One-hundred day old Roma tomato plants: (A) organic hydroponics, (B) inorganic hydroponics, (C) organic plants, (D) inorganic soil

All the physical parameters of the greenhouse such as the photoperiod, light intensity, humidity, and the amount of water the plants received were carefully monitored to maintain uniformity. The location of the soil pots and hydroponic systems were placed strategically in order to allow for equal amounts of sunlight. The amount of water the treatments received was carefully monitored to determine the water efficiency and conservation of the four treatment groups.

3.2 Soil System Setup

The experimental soil growth system consisted of six pots containing high porosity soil, HP Mycorrhizae from Pro-Mix (Rivière-du-Loop, Québec, Canada), and six pots with organic soil, Michigan Peat[®] BACCTO[®] Veggie Mix (Houston, TX, USA), certified by the Organic Materials Review Institute (OMRI[™]). The soil was dampened and one seed of Roma VF and Roma organic were sown per individual pot in the respective soils. The plants were watered every day with 315 mL of water at the same time of the day at 10 am to maintain consistency of the plant watering regime. The fertilization regime for the soil media occurred once a week using Miracle Gro[®] Water Soluble All Purpose Plant Food, 24-8-16, (Marysville, OH, USA) for the inorganic treatment group and Dr. Earth[®] Home Grown Tomato, Vegetable, and Herb fertilizer, 4-6-2, (Winters, CA, USA) for the organic treatment group.

3.3 Hydroponic System Setup

The hydroponic grow systems used to grow the tomato plantlets for this research consisted of twelve buckets each with a 18 liter capacity. Each of the twelve hydroponics systems were set up using one Groden rockwool mini-block, PLANT!T clay pebbles, a Gro Pro 6 inch net pot, 18 liter bucket and with lid, a Top Fin[®] air stone, Top Fin[®]

aquarium silicone airline tubing, and a Top Fin[®] air pump. One Roma tomato seed was placed in each of the sixteen rockwool cubes for germination, eight for Roma organic and eight for Roma VF. The seedlings were transplanted after ten days to a net pot and surrounded by clay pebbles. The pot was then placed on the bucket's lid. Each bucket's lid has a triangular cut for the net pot to rest on while having the bottom of the pot completely submerged in the water within the bucket. This cut also had enough room to allow for an aquarium tubing that was connected to an air pump and air stone. The air pump and air stones provided oxygen to the roots of the plant and created a constant movement of water. Water for the hydroponic systems was replenished once a week till day 60, followed by twice to three times a week until the conclusion of the experiment. The hydroponic nutrients included FloraBloom (0-5-4), FloraGrow (2-1-6), and FloraMicro (5-0-1) all from General Hydroponics (Sebastopol, CA, USA) were added to the hydroponic systems through the entire experiment. The two hydroponic systems intended to grow tomato plants from organic or inorganic seed origin were fertilized once a week. The recommended fertilization stages used were as follows: cuttings and seedlings stages (330 ppm of each), mild vegetative stages (1320 ppm of each), aggressive vegetative stages (3960 ppm of each), transition to bloom stages (2640 ppm of each), and blooming and ripening stages (1320 ppm of each).

3.4 Plant Material Collection and Tissue Storage

3.4.1 Fruit Collection

Tomato fruits from all the four different treatments (OH, IH, OS, IS) were collected at stage 1, 3, and 6 as depicted in Figure 9 according to the USDA "Tomato Ripening Stages", flash frozen in liquid nitrogen, and stored at -80°C. In summary, one

biological replicate was considered as an individual fruit harvested from any of the six plants (grown in identical greenhouse conditions) that belonged to any one of the four individual treatments of the experiment. This random sampling of the fruits was done to reduce the variability of fruits collected from three individual plants of every treatment.

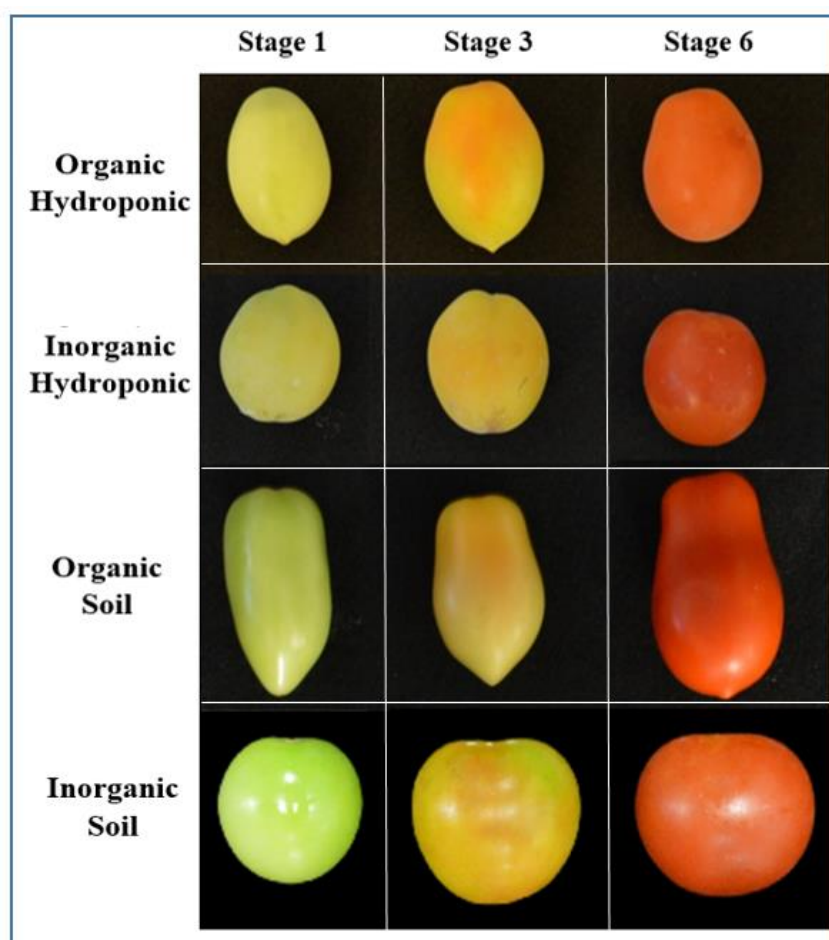


Figure 9. Tomato fruits collected at stages 1, 3, and 6 for organic hydroponic, inorganic hydroponic, organic soil, and inorganic soil

3.4.2 Grinding and Lyophilization

For any given treatment, three biological replicates i.e. three individual tomato fruits from three separate plants were pooled into one sample. The resulting four samples represented the four treatments of the experiment (OH, IH, OS, IS). Stage 6 tomatoes from each of the treatments were retrieved from -80°C and ground very carefully into a

very fine powder using a mortar and pestle (Figure 10). A copious amount of liquid nitrogen was used to keep the samples in a powder form and thus not allow any thawing of the sample to keep the proteins and metabolites intact without any degradation.



Figure 10. Grinding of stage 6 tomato fruits for proteomic and metabolomic analyses

These powdered samples were aliquoted and stored at -80°C and subsequently used for protein extractions. The remaining powdered samples were lyophilized at the Hershey Center for Applied Research and the resulting dry powder was subsequently used for metabolite analysis using high-performance liquid chromatography (HPLC) in the pharmacology department at the College of Medicine at Hershey Medical Center.

3.5 Phenol Extraction

Protein extraction was performed on all four individual treatments using 6.0 mg of finely ground tomato samples using a modified phenol chloroform extraction method as described below. Only freshly prepared solutions and buffers were used for extracting proteins from the tomato fruits (Table 1).

Solutions and Buffers	Composition
Sucrose Extraction Buffer	1% v/v polyvinylpyrrolidone (PVPP); 0.7 M Sucrose; 0.1 M potassium chloride (KCl), 0.5 M Tris; 500 mM ethylenediaminetetraacetic acid (EDTA); 1 mM phenylmethane sulfonyl fluoride (PMSF) and 2% (v/v) 2-mercaptoethanol. pH 7.5
Buffered Phenol Sigma (St. Louis, MO, USA)	pH: 8
Methanol and Ammonium acetate wash	100 mM Ammonium Acetate in 100% methanol
Urea Rehydration Buffer	7 M Urea; 2 M Thiourea; 4% w/v CHAPS; 65 mM Dithiothreitol (DTT); 0.5% v/v Triton X-100

Table 1. Solutions and buffers used for total protein extraction using a modified phenol chloroform protocol

Total proteins were extracted over the course of four days and all procedures were followed according to the optimized protocol. Ground tomato powder was homogenized with 15 mL of sucrose extraction buffer and 300 μ l of 2-mercaptoethanol. The samples were shaken on ice at 150 revolutions per minute (rpm) for 15 minutes. Buffered phenol (15 mL) was added to each sample and placed on the shaker for 30 minutes at 150 rpm. Samples were centrifuged for 30 minutes at 6000 rpm at 4°C and the supernatant was pipetted into a new tube. An equal volume of sucrose buffer was added and homogenized on the shaker for 30 minutes at 150 rpm followed by centrifugation for 30 min at 6000 rpm at 4°C. The phenol layer was transferred to a new tube. Methanol and ammonium acetate wash were added to all the tubes, mixed, and stored at -20°C overnight to let the methanol aggregate the proteins into a pellet. The following day, the samples were centrifuged for 30 minutes at 6000xg at 4°C and the supernatant was discarded. The pellets were washed with 10 mL of methanol and ammonium acetate wash and vortexed for 30 seconds. Samples were then centrifuged for 20 minutes at 9000xg at 4°C to wash the pellet. After the centrifugation, the supernatant was discarded and the prior wash

steps were repeated five times to obtain a clean protein pellet without other cellular contamination. Following the removal of the supernatant, the tubes were air dried in the chemical fume hood on ice for 15-20 minutes for the protein pellet to dry. After drying each pellet, they were re-suspended in 100 to 500 μ L of urea rehydration buffer (depending on the size of the pellet) by vortexing and the samples were kept at -20°C for overnight to 48 hours for the pellet to rehydrate. After 48 hours, the samples were removed from -20°C and thawed on ice at room temperature for 20 minutes. After the pellets were thawed, they were vortexed for 30 sec. If the pellet was still solid then 100 μ L of urea rehydration buffer was added and vortexed again. Once the pellets completely dissolved, the entire solution was transferred to 1.5 mL Eppendorf tubes and centrifuged for 10 minutes at 14,000 rpm. After centrifugation, the supernatant was transferred to fresh Eppendorf tubes and pellet was discarded. This supernatant was the total protein extract and was preserved in -80°C until further use.

3.5.1 Estimation of total protein content by Bradford method

The total protein concentration was estimated for each of the samples using the protocol established by Bradford (1976) and bovine serum albumin was used as the standard. The formula $y = mx + b$, was used to calculate the concentration of the protein samples.

3.5.2 Preparation of SDS running buffer and protein loading dye

A 500 mL of 10X SDS running buffer (250mM Tris, 1.92 M glycine, 1% SDS) was prepared and after the constituents were completely dissolved, the pH was adjusted to 8.3.

A 10 mL of protein loading dye (200 mM Tris.HCl (pH 6.8), 400 mM DTT, 8%

SDS, 0.4% bromophenol blue and 40% glycerol) was prepared with 4 mL of 0.5 M Tris.HCl, pH 6.8 and 4 mL of 100% glycerol. After the constituents completely dissolved, the volume was made up to 100 mL with double distilled water (DDW). Then 600 µl of 4X sample buffer was taken and 400 µl of 1 M DTT solution (made by dissolving 154 mg of DTT in 1 mL of DDW) was added to make a 1 mL working stock of 4X protein loading dye just before use, since it is a denaturing agent and it should be added fresh to the working stock. The remaining 9 mL of 4X sample was stored at room temperature without the addition of DTT. The empty wells were loaded with 1X protein loading dye without DTT (250µl of 4X protein loading dye without DTT+ 750 µl of DDW) for the gel to run evenly.

3.5.2.1 Preparation of Coomassie Brilliant Blue stain solution and destain solution

A 100 mL of the staining solution was prepared by dissolving 0.25 g Coomassie Brilliant Blue R- 250 in 90 mL 1:1 methanol: DDW (45 mL 100% methanol and 45 mL DDW) and 10 mL of Glacial Acetic Acid.

A 100 mL of destain solution was prepared by adding 90 mL 1:1 methanol: DDW (45 mL 100% methanol and 45 mL DDW) and 10 mL of Glacial Acetic Acid.

3.6 One Dimensional Sodium Dodecyl-Polyacrylamide Gel Electrophoresis

(1D SDS-PAGE)

A 1D SDS-PAGE was performed using 5, 10, 20 and 40 µg protein to optimize the protein concentration at which single protein bands differences between the different tomato samples could be visualized clearly on the gel. The miniprotean TGX™ precast gradient gels (4- 20%) from BioRad (Hercules, CA, USA) were used. They were supplied with a 10 well comb and each comb could accommodate a total volume of 30 µL. The

wells were properly cleaned by rinsing with the 1X SDS running buffer with the help of syringe before loading the sample. The required amount of protein sample was taken and DDW were added to equalize the volume in all samples. Then 4X protein loading dye was added to a final concentration of 1X of the dye in the prepared protein samples. All the components in the sample were mixed by vortexing, spun down and denatured by heating at 95°C for 5 minutes. The samples were then spun down at 6000 rpm for 5 minutes and then loaded carefully on the gel. The BioRad precision plus protein markers were also loaded on the gel to enable the determination of molecular weights of the proteins. The empty wells were loaded with 1X protein loading dye. The sample quantities for running the 1D SDS-PAGE is. Five μL each of the protein samples was taken from all the four treatments, diluted with DDW to a final concentration of 2 $\mu\text{g}/\mu\text{L}$. Subsequently, 2.5 μL , 5 μL , 10 μL and 20 μL were pipette out to run proteins gels with 5, 10, 20 and 40 μg proteins respectively. For loading 20 and 40 μg double the amount of 2 $\mu\text{g}/\mu\text{L}$ sample was prepared.

After the gels were run, the gel cassettes were dismantled carefully and the gel was removed taking adequate precautions to not damage the gel. The gel was stained with the Coomassie stain for 4 to 8 hours at room temperature. After 4 to 8 hours of staining, the staining solution was removed and destain solution was added. Then the gel was put on rocker with gentle shaking and the destain solution was changed every half an hour to remove the excess stain. The total destaining took 3 to 4 hours and was performed till the gel background was clear (wherever proteins were not present) and only protein bands and markers could be visualized clearly.

3.6.1 Denistometric scanning of proteins profiles

The 1D SDS-PAGE gel was scanned at 570 nm (reference, 730 nm) for protein quantification using the GS-900 scanner (BioRad) with a scanning speed of 10 mm per minute and a short speed of 20 mm per minute peak areas were measured by planimetry. Standard curves of each protein were determined according to internal standard method.

3.7 High Performance Liquid Chromatography (HPLC)

All HPLC procedures were carried out using the HP Agilent 1100. The analysis was performed using HpCore software. Lyophilized tomato fruit powder was used to extract the metabolites using methanol and tetrahydrofuran (THF) as solvents. The extraction was performed by measuring out 0.5 grams of the lyophilized tomato fruit powder into an Erlenmeyer flask. Exactly 10 mL of methanol was added and the mixture was sonicated for 3-5 minutes. Briefly after, the preceding mixture was separated via vacuum filtration using a Buchner funnel, which held a 0.5 micron filter paper attached to a vacuum flask. The extract was placed in a 20 mL vial for filtration. Any solids remaining were dried thoroughly, re-weighed, and extracted with THF. The flask was sonicated and vacuum filtered in a manner similar to the methanol, and then placed in a 20 mL vial for filtration.

The filtrates were further filtered to ensure a smooth passage through the HPLC machine with no obstruction by any residual particulates. Filtration occurred by fixing a 0.2 micron syringe filter to the end of a 1 mL syringe. One mL of the extract was pipetted into the syringe and filtered into a separate 5 mL vial. The filtration process was repeated until 3 mL of extract was filtered into each of the vials. The vials were stored in at -4°C until all samples were ready to be carried out with HPLC.

Solvents used for the HPLC machine were HPLC-grade methanol and deionized water. Filtered extracts were brought to room temperature before injection and the UV detector was set to 254 nanometers prior to beginning the next step. Using a 25 μL syringe, 15 μL was pipetted and injected into the machine. The HPLC pump setup is depicted in Table 2. The HPLC process was carried out using Vydac C₁₈ reverse-phase HPLC analytical columns. The columns consist of octadecyl aliphatic groups fused to the surface of 300 Å pore diameter silica, which was manufactured to give highly pure synthetic silica.

Time (min)	Water %	Methanol %	Flow (mL/min)	Maximum Pressure (bar)
0	100 %	0 %	1.0	400
30	0 %	100 %	1.0	400
40	0 %	100 %	1.0	400
50	100 %	0 %	1.0	400
65	100 %	0 %	1.0	400

Table 2. The pump setup for HPLC analysis

Chapter 4: Results and Discussion

Various grow systems have been researched to develop crops with increased agricultural production, disease resistance, and improved nutritional profiles to meet the food demands of the inevitably growing population. The goal of this research work was to identify new metabolite and protein candidates, which could lead to the advancement of the current understanding of signaling pathways involved in the biochemistry of the plant.

4.1 Plant Growth and Assessment of Morphological and Physiological Responses

The experiment was started in the CPRTL B greenhouse using four separate treatments, organic or inorganic seeds grown in hydroponics and soil systems. The conditions for the plants were monitored carefully to maintain uniformity of the physical parameters such as the photoperiod, light intensity, humidity, and the amount of water the plants received. A high porosity soil, HP Mycorrhizae and BACCTO Veggie Mix was used respectively for the inorganic and organic treatments to ensure the seedlings maintained adequate water adsorption. The hydroponic grow systems used to grow the tomato plantlets for this research consisted of rockwool mini-blocks, clay pebbles, net pots, 18 liter buckets and with lids, air stones, aquarium silicone airline tubing, and an air pump.

Physical parameters such as plant height, number of seeds, weight of seeds, number of fruits, number of flowers, days to flowering, weight of fruits, and root length were monitored at specific times throughout the course of the experiment.

Plant heights were determined measuring from the base of the plant above the ground (soil treatments) and above the pebbles (hydroponics treatments) to the last expanded leaf of the growing tip and expressed in centimeters (Table 3). The average height at Day 44 was measured to be the highest in the organic soil treatment at 66.13 cm followed by inorganic soil (53.68 cm), and inorganic hydroponics (49.58 cm). The treatment group with the least average plant height was observed in organic hydroponic treatment (47.25 cm).

Average Plant Height	OH	IH	OS	IS
Day 30	13.97 cm	12.32 cm	37.13 cm	20.40 cm
Day 44	47.25 cm	49.58 cm	66.13 cm	53.68 cm

Table 3. Plant height at day 30 and 44 for A) organic hydroponic B) inorganic hydroponics C) organic soil D) inorganic soil

It was observed that the least average time to flowering (34.83 days) was in the organic soil treatment. Anthesis was observed first in the organic soil treatment whereas the other treatments flowered later. The longest amount of time to flower was observed in the plants belonging to the inorganic soil treatment (50.67 days: 15 more days than the organic soil treatment). The average days to flowering in both organic and inorganic hydroponic treatments was within the range of these two time points which around 44 days. The organic and inorganic treatments made a difference with respect to days to flowering when they were grown in soil, however both these treatments grown in hydroponics did not show any significant difference (Table 4).

	OH	IH	OS	IS
Average Days to Flowering	43.83	45.17	34.17	48.50
Average Number of Flowers on Day 60	20	14.75	8	10

Table 4. Flowering response for A) organic hydroponic B) inorganic hydroponics C) organic soil D) inorganic soil

The highest average number of flowers on Day 95 were observed for the organic hydroponic treatment group (57) followed by inorganic hydroponics (38) and inorganic soil (23.6). The least number of flowers were found in the organic soil treatment (9).

These numbers adjusted during the course of the plants' reproductive phases.

Data for number of fruits was collected at several points of time. In general, it was observed that the organic hydroponics treatment produced the maximum number of fruits followed by the inorganic hydroponics and inorganic soil. The least number of fruits was observed in organic soil treatment.

The highest fruit weights were observed in both the inorganic treatments and both the organic treatments had lesser fruit weights. One of the possible reasons for reduced fruit weights in organic hydroponics could be contributed to the production of the maximum number of fruits (up to four times) when compared to other treatments. This indicates that the plants in organic hydroponics distributed their resources to more number of fruits thereby reducing the individual fruit weight the plant produced.

The highest average number of seeds was found in the inorganic soil treatment (17.44) and the least number was found in the organic soil treatment (9.81) as shown in

Table 5. Generally higher seed numbers were found in both the inorganic treatments when compared to both the organic treatments.

	OH	IH	OS	IS
Average Fruit Weight of one Stage 6 Fruit	19.23 g	27.12 g	25.46 g	36.90 g
Average Seed Number per one Stage 6 Fruit	11.31	16.88	9.81	17.44
Fresh Seed Weight/Seed Number * 100	0.64 g	0.38 g	0.55 g	0.33 g
Dry Seed Weight/Seed Number * 100	0.34 g	0.22 g	0.31 g	0.27 g

Table 5. Fruit weight, seed number and seed weight of A) organic hydroponic B) inorganic hydroponics C) organic soil D) inorganic soil

Average seed weight was observed to be higher in both the organic treatments when compared to both the inorganic treatments. It was observed that soil or hydroponic treatments did not contribute to any difference in seed weight.

The roots systems as shown in Figure 11, in both the hydroponic systems, had more biomass resulting from more available space and efficient uptake of the nutrients when compared to the roots in the soil treatments. This result could be due to the hydroponic systems providing more room for root growth, more oxygenation, and a uniform amount of minerals and salts at all points of plant growth (no depletion in the level of nutrients). The high root biomass thus resulted in increased number of flowers and fruit production.



Figure 11. Comparison of root growth in tomato plants representing A) organic hydroponic B) inorganic hydroponic C) organic soil D) inorganic soil

4.2 Proteomics

4.2.1 Extraction of Total Proteins

The phenol extraction method has been routinely used for the extraction of total proteins from plants. During this process, the plant cell wall is broken, all the proteins are extracted into the upper phenol layer and the non-protein components (polysaccharides, DNA, lipids, and phenolic compounds) form a pellet in the lower sucrose layer. After phenol extraction, proteins are precipitated with ammonium acetate in methanol. The pelleted proteins were resuspended in rehydration buffer and preserved at -80°C till further use. The most important steps during the protein extraction procedure was to

(1) keep the samples at the required low temperatures (4°C or -20°C) during the resuspension of the tomato samples in sucrose buffer (2) to carefully recover the upper phenolic phase after the centrifugation steps (another crucial step in this protocol) and (3) all the steps needed to be performed on ice and centrifugations conducted at 4°C.

4.2.2 Generation of a Standard Protein Curve using Bradford Assay

In this experiment commercial Bradford solution from Sigma Aldrich (St. Louis, MO) was used. Bovine Serum Albumin (BSA) stock solution (1 mg/mL) was prepared by dissolving 30 mg of BSA in 30 mL of autoclaved double distilled water (DDW). A 0.1 and 1 mg/mL of BSA stocks was prepared for the protein assay. The range of BSA concentrations taken to plot the standard graph were 0 (blank), 0.2, 0.4, 0.6, 0.8, 1, 2, 4, 6, 8, 10 and 20 (µg) and the absorbance of the samples was noted at 595 nm (Table 6). The samples were made up to 100 µL in all the Eppendorf microfuge tubes with DDW and then 1 mL of Bradford reagent was added.

Sample	Volume from 0.1 µg/µL BSA	Volume of DDW	Volume of Bradford	Assay 1 OD	Assay 2 OD	Average OD
Blank	0 µL	100 µL	1 mL	0	0	0
0.2 µg	2 µL	98 µL	1 mL	0.005	0.006	0.0055
0.4 µg	4 µL	96 µL	1 mL	0.015	0.01	0.0125
0.6 µg	6 µL	94 µL	1 mL	0.019	0.019	0.019
0.8 µg	8 µL	92 µL	1 mL	0.028	0.029	0.0285
1 µg	1 µL	99 µL	1 mL	0.057	0.038	0.0475
2 µg	2 µL	98 µL	1 mL	0.069	0.081	0.075
4 µg	4 µL	96 µL	1 mL	0.135	0.134	0.1345
6 µg	6 µL	94 µL	1 mL	0.172	0.17	0.171
8 µg	8 µL	92 µL	1 mL	0.212	0.213	0.2125
10 µg	10 µL	90 µL	1 mL	0.257	0.255	0.256
20 µg	20 µL	80 µL	1 mL	0.487	0.479	0.483

Table 6. Sample preparation for a Bradford assay and calculation of absorbance values to calibrate a standard curve

The absorbance values were plotted on the Y axis and the corresponding BSA concentration were plotted on X axis to obtain the standard graph. The standard graph is a linear line and the unknown protein concentration was calculated by the formula $y=mx+b$. Eleven concentrations of BSA were assayed and the absorbance was plotted and the best fit line drawn through the points. Concentrations of unknown protein samples had been tested and their concentrations were determined using the standard curve (Figure 12) and represented in Table 7.

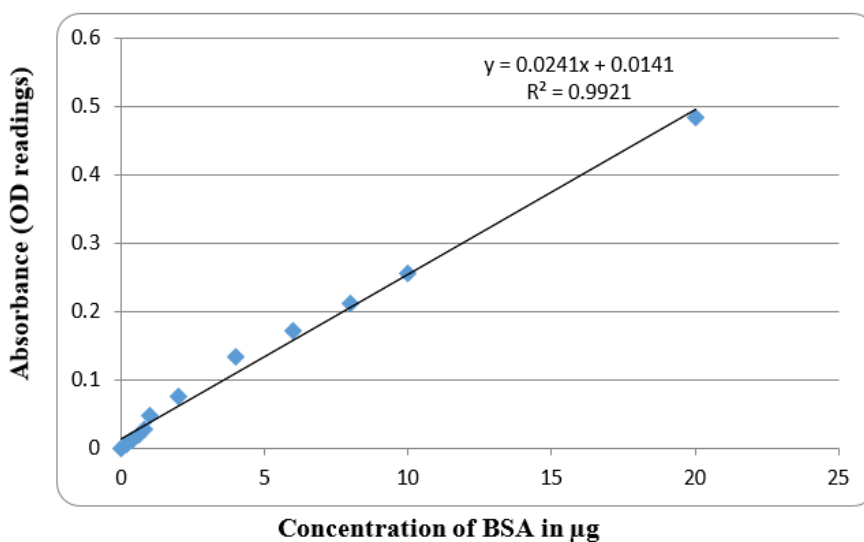


Figure 12. Standard curve showing the absorbance values of known concentrations of protein (Bovine Serum Albumin; 2 trials for each measurement)

Treatment	Assay 1 OD	Assay 2 OD	Assay 3 OD	Average OD	Protein concentration (µg/µL)
OH	0.66	0.62	0.63	0.63	12.87
IH	0.56	0.46	0.53	0.52	10.43
OS	0.45	0.47	0.36	0.43	8.56
IS	0.62	0.54	0.69	0.62	12.49

Table 7. Estimation of total protein concentrations of organic hydroponic, inorganic hydroponics, organic soil and inorganic soil treatments using Bradford assay

It is important to understand how organic and conventionally grown tomato plants are functioning while they are grown in two different grow systems. One way of studying this is to compare and understand their differential gene expression profiles (Kong, Ngern et al. 2005). Proteins with differential expression under the four different conditions can be used as molecular markers to develop the best growing strategies for tomato plants which would yield nutritionally enhanced fruits particularly rich in antioxidants, aroma and flavor.

4.2.3 Sodium Dodecyl Sulfate (SDS)

A 1D SDS-PAGE was used to study the different protein expression profiles of the four treatments (OH, IH, OS, IS) that were grown in the greenhouse. Table 8 describes the tomato fruit tissue preparation for the protein and metabolite analysis.

Treatment	Fresh Weight	Ground Weight	Weight before Lyophilization	Weight after Lyophilization	Percent Yield
OH	18.50 g	99.33 g	60.38 g	4.89 g	8.10%
	19.38 g				
	19.80 g				
IH	27.15 g	68.36 g	29.79 g	1.67 g	5.61%
	35.14 g				
	19.06 g				
OS	27.67 g	53.74 g	16.46 g	1.25 g	7.60%
	25.93 g				
	22.78 g				
IS	43.43 g	87.57 g	49.24 g	3.49 g	7.09%
	37.58 g				
	29.68 g				

Table 8. Tomato samples used for protein and metabolite extraction and for 1D SDS-PAGE and HPLC analysis, respectively

Stock proteins were diluted according to required concentrations as depicted in Table 9. Proteins were separated on a 4-20% gradient gel in the presence of sodium dodecyl sulfate.

Treatment	Protein concentration ($\mu\text{g}/\mu\text{L}$)	Volume of DDW (μL)	Total volume of sample (μL)	Protein concentration ($\mu\text{g}/\mu\text{L}$)
OH	12.87	16.29	19.29	2
IH	10.43	12.63	15.63	2
OS	8.56	9.82	12.82	2
IS	12.49	15.6	18.6	2

Table 9. Preparation of diluted proteins ($2 \mu\text{g}/\mu\text{L}$) of organic hydroponic, inorganic hydroponics, organic soil and inorganic soil treatments from original total protein extractions

A total amount of either 40, 20, 10, or 5 μg of total protein (Table 10) were loaded into separate gels to determine the optimal concentration of proteins that would give the best resolution of protein band separation for future densitometric analysis (Figures 13, 14, 15 and 16).

Protein amount	Volume of diluted protein ($2 \mu\text{g}/\mu\text{L}$)	Volume of DDW (μL)	Volume of 4X protein loading dye (μL)	Total volume of sample (μL)
5 μg	2.5 μL	9.5 μL	4 μL	16
10 μg	5 μL	7 μL	4 μL	16
20 μg	10 μL	2 μL	4 μL	16
40 μg	20 μL	7 μL	1 μL	28

Table 10. Preparation of protein samples for loading 5, 10, 20 and 40 μg of total protein. This is to run a 1D SDS-PAGE gel with good separation of bands for future densitometric analysis

The SDS-PAGE gel that was run using 10 μg of total protein showed the best resolution of band separation and therefore this gel was repeated one more time to conduct the densitometric analysis (Figure 17). The molecular weight of separate protein

bands were calibrated against a 10-250 kDa protein marker. A GS-900 calibrated densitometer with Image Lab software version 5.1 from BioRad was used to analyze the differentially expressed proteins using the differences in band intensities.

It is very important to remember that each unique protein can exist in many different copy numbers. Therefore we may see the actual bands to appear equal in size but the proteins within each band could be made up of different sizes. SDS-PAGE typically separates proteins based on their primary structure of size (molecular weight), but does not reveal the amino acid sequence. Therefore, it is possible to have many copies of two different proteins that are up made up of the same number of amino acids that would travel together through the gel as a mixed band. Thus, we would not be able to use the SDS-PAGE to separate these two proteins of the same molecular weight. The SDS-PAGE analysis is not conclusive and powerful enough to understand independent protein function and therefore further in-depth protein analysis including protein quantification and sequencing may be required.

4.2.4 Analysis of the 1D Gel Electrophoresis gel

Four SDS-PAGE gels were run using 40, 20, 10 and 5 μg proteins are shown in Figures 13, 14, 15, and 16. Ten micrograms of protein were found to be the optimum concentration of protein which could be run to differentiate discrete protein bands. There is a clear differential profile of proteins in tomatoes that were grown in the different grow systems. This study highlighted some proteins which are produced in more quantities (over expressed) in specific conditions. These protein bands will be further analyzed by mass spectrometry which would reveal the identity and help in recognizing the genes and the biochemical pathways involved.

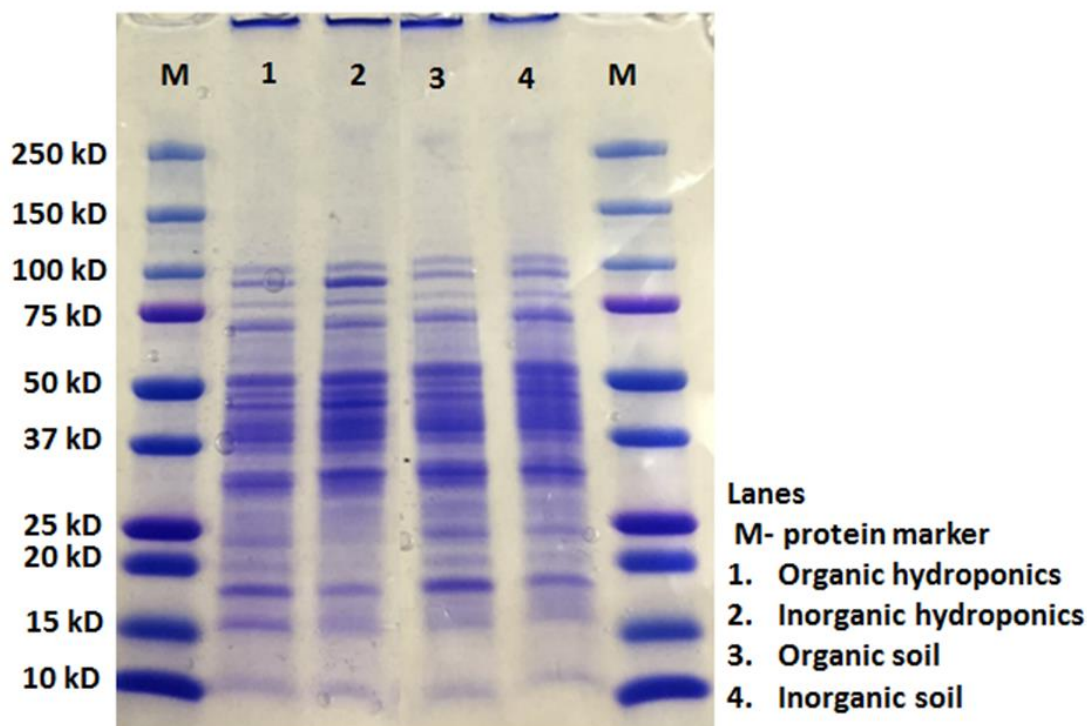


Figure 13. Optimization of tomato fruit proteins from organic hydroponic, inorganic hydroponics, organic soil and inorganic soil treatments. Total of 40 μ g was used to run a 1D SDS-PAGE gel to determine the right concentration for further desitometric analysis

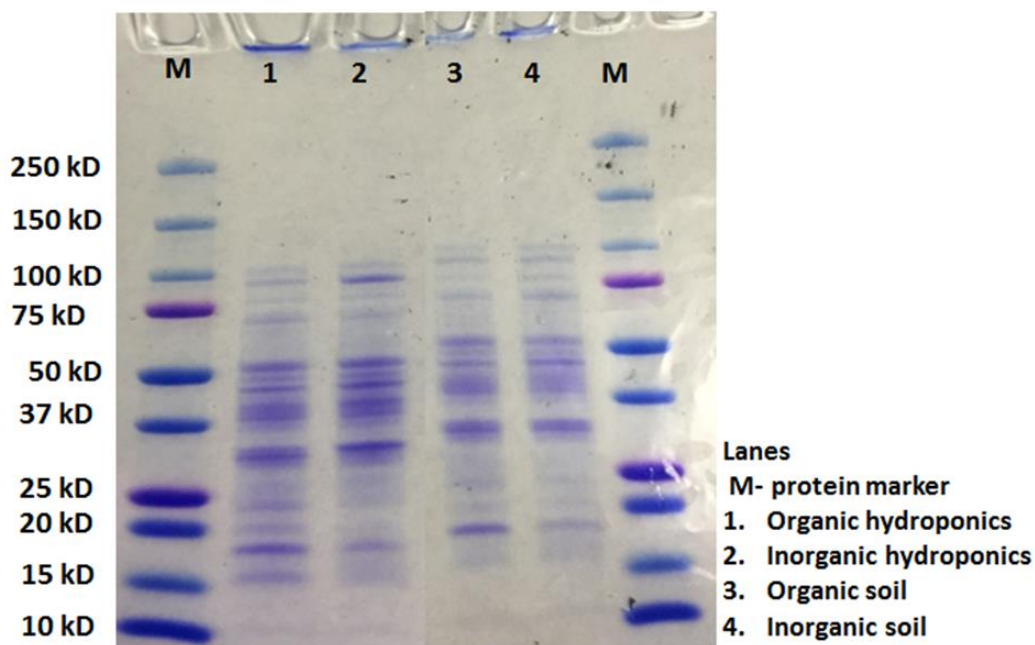


Figure 14. Optimization of tomato fruit proteins from organic hydroponic, inorganic hydroponics, organic soil and inorganic soil treatments. Total of 20 μ g was used to run a 1D SDS-PAGE gel to determine the right concentration for further desitometric analysis

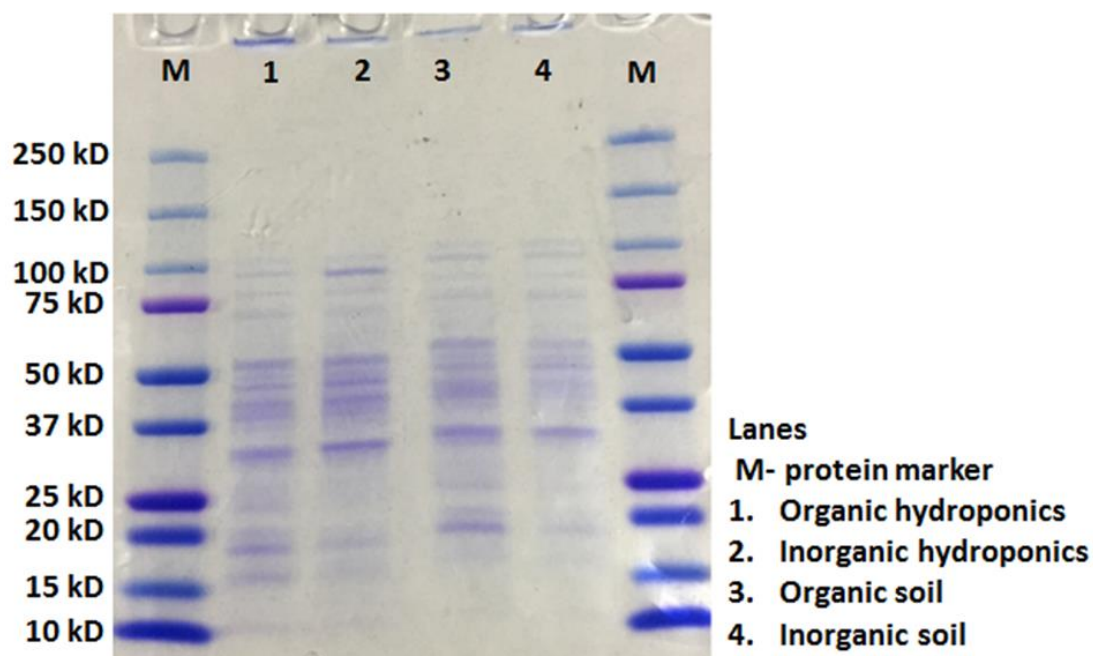


Figure 15. Optimization of tomato fruit proteins from organic hydroponic, inorganic hydroponics, organic soil and inorganic soil treatments. Total of 10 μ g was used to run a 1D SDS-PAGE gel to determine the right concentration for further desitometric analysis

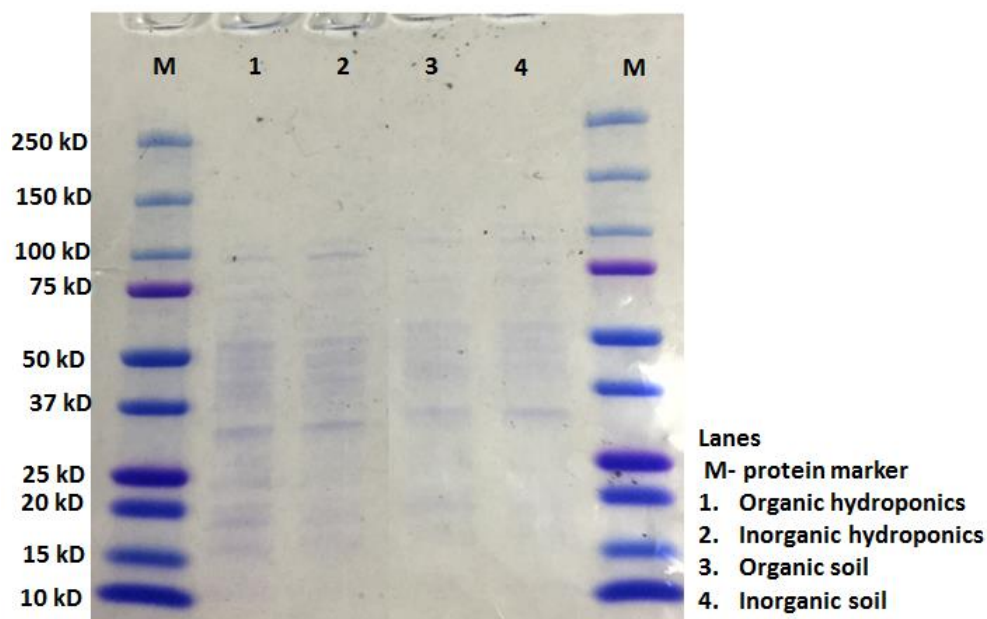


Figure 16. Optimization of tomato fruit proteins from organic hydroponic, inorganic hydroponics, organic soil and inorganic soil treatments. Total of 5 μ g was used to run a 1D SDS-PAGE gel to determine the right concentration for future desitometric analysis

4.2.4.1 Densitometric Analysis

The experiment was performed to compare the protein expression profile of the tomato plants using two different growth systems (soil and hydroponics). Densitometric analysis of 1D SDS-PAGE gel indicated differences between the protein banding patterns for all of the four treatments (organic hydroponics, inorganic hydroponics, organic soil, inorganic soil) (Figures 17-22).

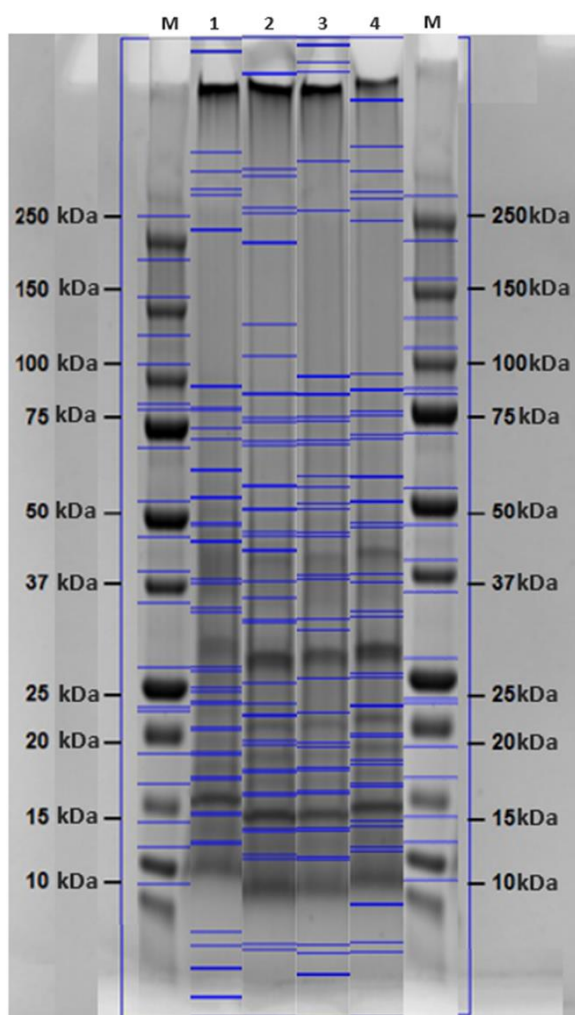


Figure 17. Differentially expressed protein banding patterns on a 1D SDS-PAGE gel of the total tomato fruit protein extracted from four different treatments. A 4-20% gradient gel was used and 18 μ g of BioRad precision plus dual standard marker protein was loaded (M- protein marker, 1- Organic hydroponics, 2- Inorganic hydroponics, 3- Organic Soil, 4- Inorganic soil, M- protein marker)

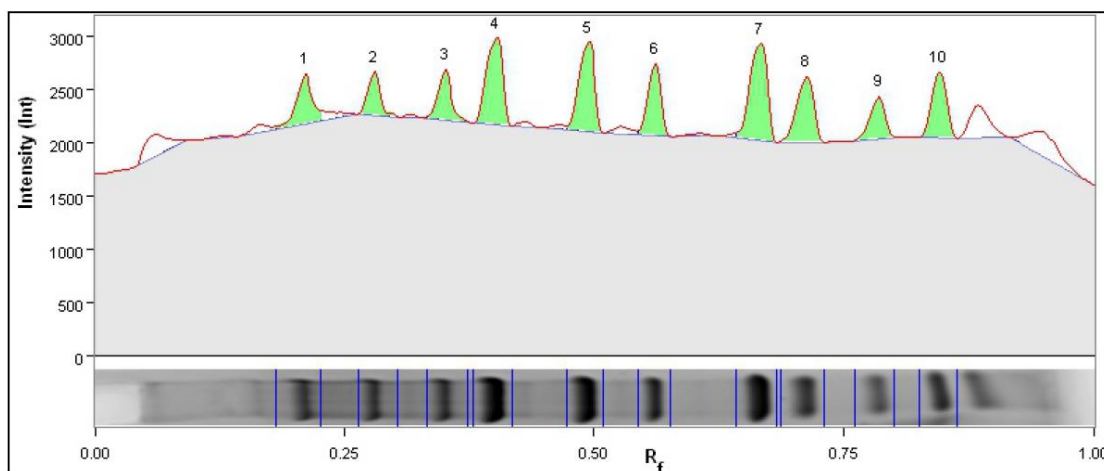


Figure 18. Densitometer scan of the dual precision plus protein marker using a GS-900 calibrated densitometer (BioRad)

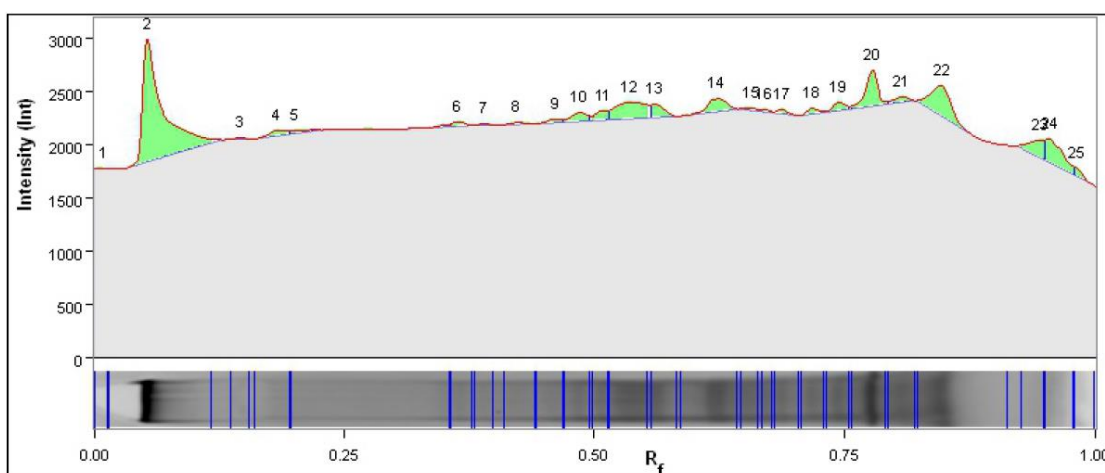


Figure 19. Densitometer scan of the organic hydroponics (OH) using a GS-900 calibrated densitometer (BioRad)

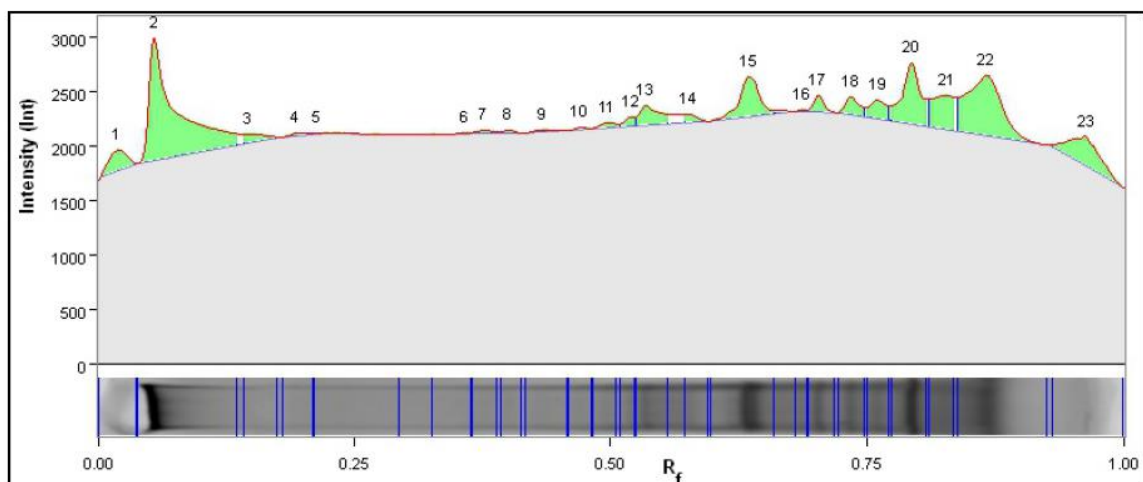


Figure 20. Densitometer scan of the inorganic hydroponics (IH) using a GS-900 calibrated densitometer (BioRad)

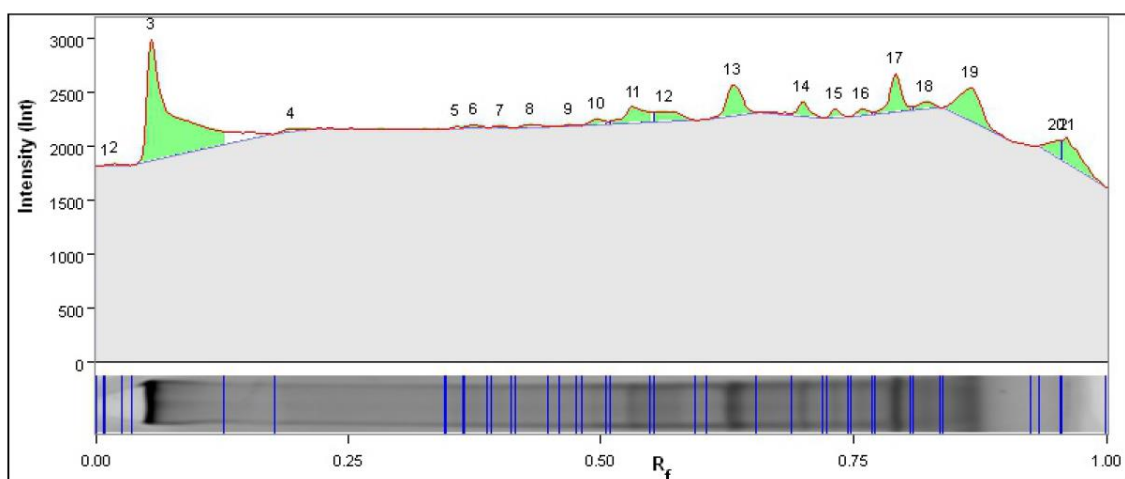


Figure 21. Densitometer scan of the organic soil (OS) using a GS-900 calibrated densitometer (BioRad)

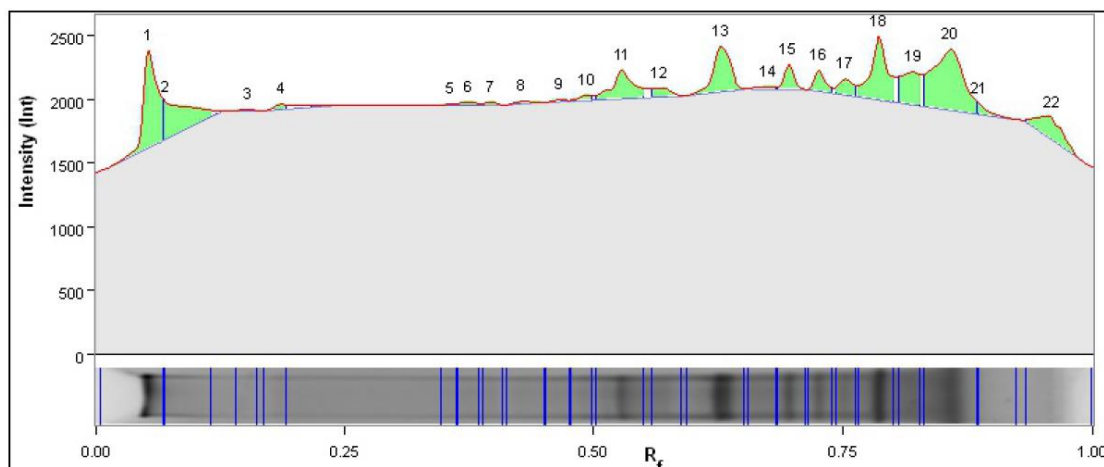


Figure 22. Densitometer scan of the inorganic soil (IS) using a GS-900 calibrated densitometer (BioRad)

	Inorganic Soil	Organic Soil	Inorganic Hydroponic	Organic Hydroponic
Number of Bands 250 kDa and up	4	4	4	5
Sum of bands 250 kDa and up	1,152,880	1,585,408	2,013,952	1,424,320
Number of Bands between 150-250 kDa	-	-	1	-
Sum of Bands between 150-250 kDa	-	-	12,672	-
Number of Bands between 100-150 kDa	-	-	-	-
Sum of Bands between 100-150 kDa	-	-	-	-
Number of Bands between 75-100 kDa	3	3	3	2
Sum of Bands between 75-100 kDa	52,928	43,840	51,904	38,976
Number of Bands between 50-75 kDa	3	2	2	3
Sum of Bands between 50-75 kDa	64,576	35,520	29,312	115,712
Number of Bands between 37-50 kDa	1	2	3	3
Sum of Bands between 37-50 kDa	292,160	218,368	284,992	415,104
Number of Bands between 25-37 kDa	2	2	2	2
Sum of Bands	472,640	394,048	473,856	150,336

between 25-37 kDa				
Number of Bands between 20-25 kDa	2	1	2	2
Sum of Bands between 20-25 kDa	148,096	90,368	95,680	36,672
Number of Bands between 15-20 kDa	2	2	2	3
Sum of Bands between 15-20 kDa	120,800	79,680	280,576	330,048
Number of Bands between 10-15 kDa	2	2	2	1
Sum of Bands between 10-15 kDa	803,968	302,144	927,104	42,560
Number of Bands 10 kDa and down	3	3	2	3
Sum of bands 10 kDa and down	1,238,144	740,352	1,517,184	726,336

Table 11. Breakdown of molecular marker weights of organic hydroponics (OH), inorganic hydroponic (IH), organic soil (OS) and inorganic soil (IS) treatment

Gel analysis showed a range of 21-25 bands differencing in expression levels between all the four treatments (Table 11). Specifically, the most number of bands were found in the organic hydroponic treatment (25), followed by inorganic hydroponics (23), inorganic soil (22), and organic soil (21). The densitometric scan showed the highest intensity for inorganic hydroponics followed by inorganic soil, organic soil and lastly organic hydroponics. Across the four treatment groups, 54 of the total 62 bands were unique which accounted for a total of 87% of the total bands found. Regression method (.2 semi-log) was used to analyze the molecular weights of individual bands.

The two treatment groups of inorganic soil and organic soil shared two similar molecular weights bands that were not present in neither of the hydroponic treatments. This indicates that unique proteins were expressed due to the effect of the different grow systems (soil v. hydroponics). The band molecular weight that were the same 96.2 kDa

and 86.9 kDa. Organic soil had a higher intensity (11,008 pixels; px) than inorganic soil (9,152 px) for the molecular weight at 96.2 kDa and Inorganic had a higher intensity (25,920 px) than organic soil (20,928 px) at the molecular weight of 86.9 kDa.

At the molecular weight of 12.8, all the treatments had expression but at different intensities. Inorganic hydroponics at the highest intensity (357,952 px) followed by inorganic soil (283,712 px), organic soil (55,680 px), and organic hydroponics (42,560 px).

Inorganic hydroponics had the most intense 250 kDa band (2,013,952 px), followed by organic soil (1,585,408 px), organic hydroponics (1,424,320 px), inorganic soil, while the least intense was inorganic soil (1,152,880 px). Definitely the treatment did effect the production of different kinds of proteins.

The inorganic hydroponics had the highest intensity at 10 kDa band (1,517,184 px), followed by Inorganic soil (1,238,144 px), organic soil (740,352 px), while organic hydroponics (726,336 px) had the least intensity.

These experiments need to be repeated and verified, and are presented here in the thesis as preliminary results about some of the mechanisms that are taking place in these various treatments.

4.3 HPLC Results and Discussion

Metabolites were extracted using methanol and THF followed by water. Independent extracts were examined using HP Agilent 1100 and later analyzed using the HpCore software. The extract using water turned out to be very viscous and incompatible to go through the capillary system of the HPLC machine and therefore the analysis was

only restricted to methanol and THF. The HPLC graphs for the four treatments using methanol or THF as a solvent are depicted in Figure 23 and 24.

Graph analysis was done by comparing the graphs that had been produced with graphs from other research that used similar parameters. This was only an educated guess, final confirmation can be made once the NMR and mass spectrometry results are obtained. As such the analysis portion was done to give a general idea as to what sort of metabolites are expected from these tomatoes. Each treatment had a different number of peaks with a different area underneath each peak, the different areas correspond to the different concentrations.

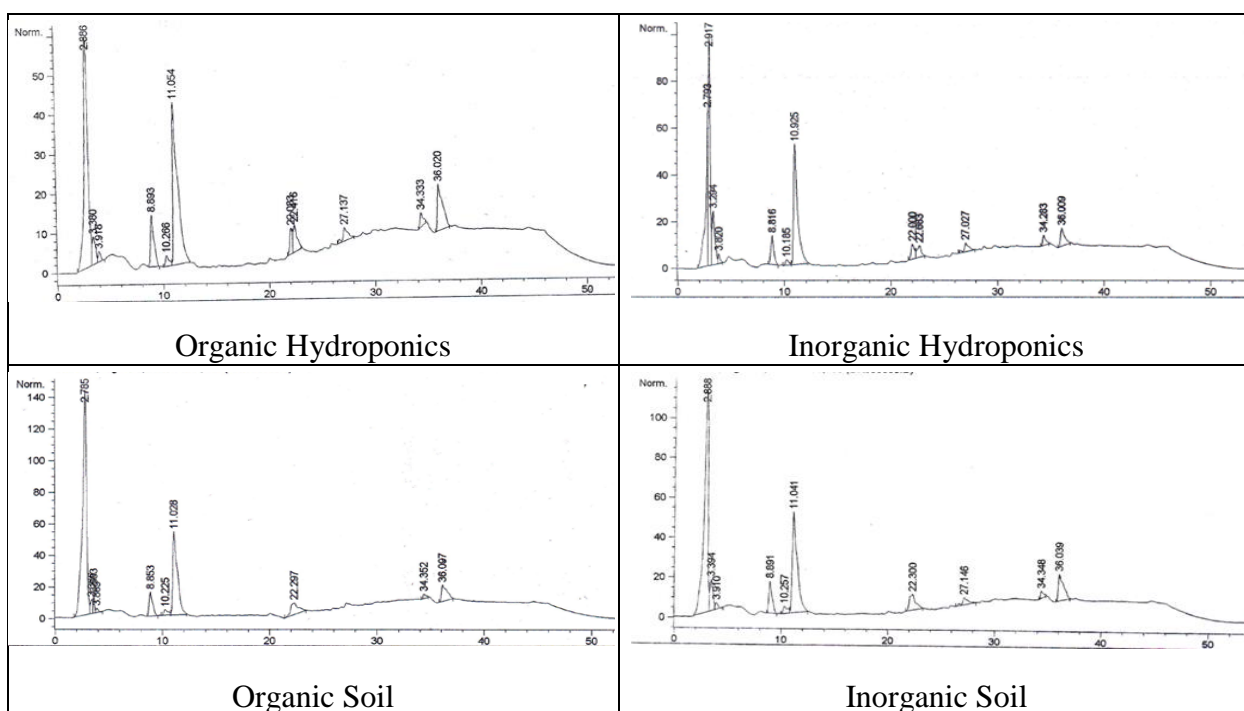


Figure 23. HPLC graphs for methanol solvent for the four treatments

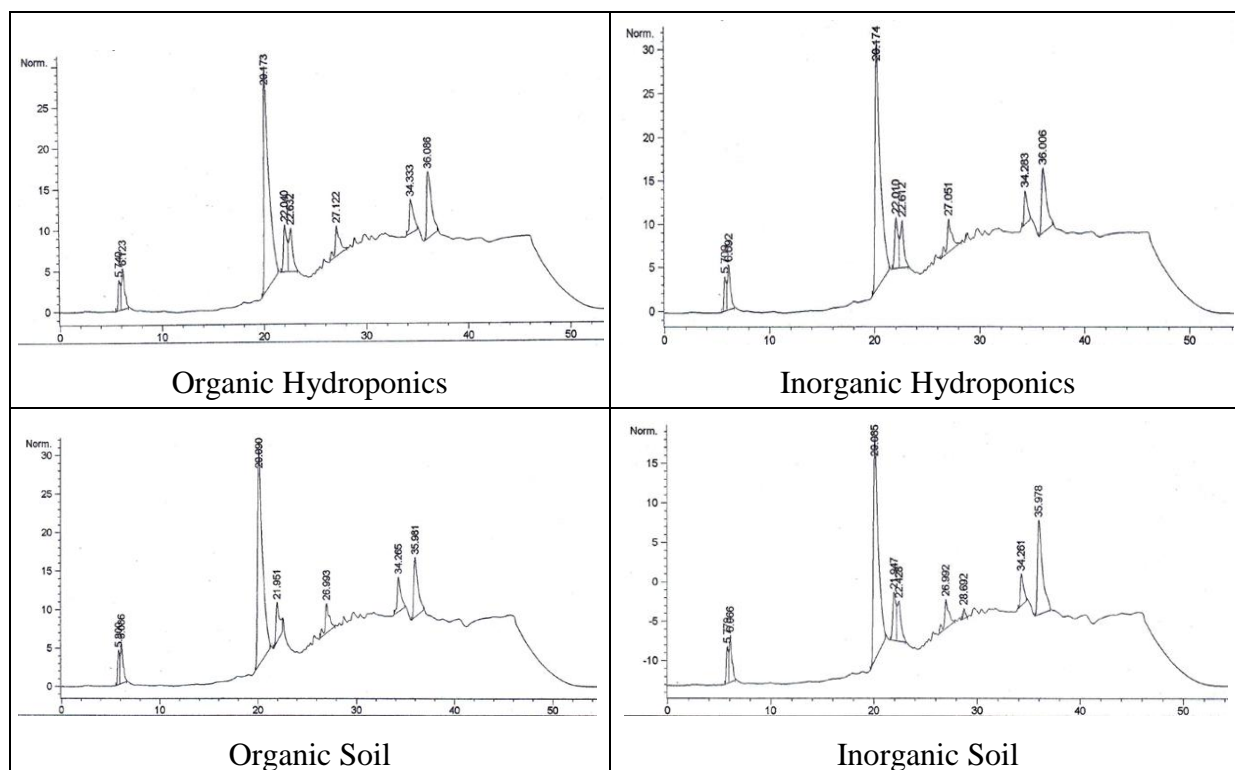


Figure 24. HPLC graphs for THF solvent for the four treatments

Seven compounds were identified in three out of the four treatments (OH, IH, IS) when methanol was extracted (Table 12). In organic soil, one compound was not found to be present from results, dicaffeoylquinic acid I. In inorganic hydroponics, the first peak at a concentration of 20.4, was not identified as methanol unlike in the other three treatments (OH, OS, IS). Inorganic hydroponics had the highest total concentration of all four treatments (OH, IH, OS, IS).

Adenosine had the highest concentration (Gómez-Romero et al., 2010) in inorganic hydroponics (8.4337) and the least concentration in organic soil (1.7924). Adenosine freely combines with phosphate to form adenosine monophosphate (AMP) and adenosine triphosphate (ATP). These two chemicals are existent in all cells within the human body but can also be taken in a form of medication. AMP can be taken orally

to treat shingles and porphyria cutanea tarda, a blood disorder. If taken, ATP can treat numerous medical conditions, including kidney failure, multiple organ failure, high blood pressure, cystic fibrosis, lung cancer, weight loss associated with cancer, and to control blood pressure during anesthesia and surgery. It can be given intravenously or taken to increase physical energy orally (Li et al, 2016).

Identified Compounds	Treatment	Peak Number	Area %
Methanol	OH	1	35.5806
	IH	2	22.2028
	OS	1	54.9246
	IS	1	46.1344
Adenosine	OH	2	3.3659
	IH	3	8.4337
	OS	2	1.7924
	IS	2	4.9241
Caffeic Acid Hexose II	OH	5	1.5625
	IH	6	1.0533
	OS	6	1.4364
	IS	5	1.5944
Lycopene	OH	6	31.0151
	IH	7	27.5122
	OS	7	22.9135
	IS	6	25.3121
Absciscic Acid Hexose Ester	OH	8	4.0688
	IH	9	2.9274
	OS	8	5.5083
	IS	7	5.191
DicaFFEoylquinic Acid I	OH	9	2.7582
	IH	10	2.3651
	OS	-	-
	IS	8	2.108
Hydroxyl-Octadecanedioic Acid II	OH	10	1.6352
	IH	11	1.7824
	OS	9	0.8231
	IS	9	1.1351

Table 12. Summary of compounds identified by HPLC graphs from methanol extraction

Caffeic acid hexose II had the highest concentration (Moco, Sofia et al., 2006) in inorganic soil (1.5944) and the least concentration in inorganic hydroponics (1.0533). Caffeic acid has been found to affect the body as an antioxidant and anti-inflammatory. It is also an important intermediate step in the biosynthesis of lignin, which protects against breast cancer in women. The body transforms lignans into chemical with estrogen-like effects (Mozzetti et al, 2015). Caffeic acid possesses cancer preventing and anti-tumour properties, while also reducing body weight, lipid metabolism and obesity-related hormones levels. It can be used as a supplement for exercise related fatigue and to enhance athletic performance and help with the treatment of HIV/AIDS, herpes, and other conditions virus infections (Rizzo et al, 2016).

Lycopene had the highest concentration (Cucu, Tatiana et al., 2011) in organic hydroponics (31.0151) and the least concentration in organic soil (22.9135). Lycopene is a naturally occurring chemical that gives tomatoes their red color. Even though it is not an essential vitamin, lycopene is a powerful antioxidant. It has been found to prevent heart disease, atherosclerosis, and cancer of the prostate, breast, lung, bladder, ovaries, colon and pancreas (Holzapfel et al, 2016).

Abscisic acid hexose ester had the highest concentration (Gómez-Romero et al., 2010) in inorganic soil (5.191) and the least concentration of inorganic hydroponics (2.9274). Hydroxyl-octadecanedioic acid II had the highest concentration (Gómez-Romero et al., 2010) in organic hydroponics (1.6352) and the least concentration in organic soil (0.8231). Diacaffeoylquinic acid I, commonly known as chlorogenic acid (CGA), had the highest concentration (Gómez-Romero et al., 2010) in organic hydroponics (2.7582) and the least concentration in inorganic soil (2.108). CGA was not

found in organic soil. CGA also is an important step in the biosynthesis of lignin (Mozzetti et al, 2015). Also, CGA is an antioxidant and has been found to slow the release of glucose into the bloodstream after the digestion of tomatoes (Rizzo et al, 2016).

Three compounds were identified in all four treatments (OH, IH, OS, IS) when THF was extracted (Table 13). Inorganic soil had the highest total concentration of all four treatments. Organic soil had the highest concentration of the compounds identified.

Lutein had the highest concentration (D'Evoli, Laura et al., 2013) in organic soil (3.667) and organic hydroponics had the least concentration (3.2527). Lutein is found naturally in plant pigments of dark green leaves and known to be related to β -carotene and vitamin A. It has been found to prevent eye diseases, colon cancer, breast cancer, type II diabetes and heart disease and supports healthy skin, tissue, blood and the immune system (Maurer et al, 2014).

Identified Compounds	Treatment	Peak Number	Area %
THF	OH	1	50.0569
	IH	1	52.3553
	OS	1	55.6109
	IS	1	46.3941
Lutein	OH	3	3.2527
	IH	3	3.3842
	OS	3	3.667
	IS	3	3.3084
β -carotene	OH	7	6.4685
	IH	7	4.5869
	OS	6	6.7568
	IS	8	4.375

Table 13. Summary of compounds identified from HPLC graphs from THF extraction

β -Carotene had the highest concentration (D'Evoli, Laura et al., 2013) in organic soil (6.7568) and inorganic soil had the least concentration (4.3375). β -Carotene can be found naturally in fruits, vegetables and whole grains but can also be made in a laboratory. β -Carotene is also known as the pro-vitamin A, being an essential component in inhibiting the growth of many pre-cancerous tumors. This compound is used to decrease asthma symptoms caused by exercise, prevent certain cancers, heart disease, cataracts age related macular degeneration and to treat AIDS, alcoholism, Alzheimer's disease, depression, epilepsy, headaches, heartburn, high blood pressure, infertility, Parkinson's disease, rheumatoid arthritis, schizophrenia, and skin disorders including psoriasis (Haftel et al, 2015).

To get a better understanding of protein and metabolite production occurring in the tomato fruits, powerful tools like proteomics and metabolomics were employed to get a preliminary understanding of the biochemical state of the tomato plants in this experiment. The total protein analysis using 1D SDS-PAGE revealed that there was a clear differential profile of proteins in the four different treatments. The highest number of bands was observed in organic tomatoes that were grown in a hydroponic system. However, the most intense bands were noticed in the inorganic hydroponic treatment group. In addition, there were two unique bands that were only present in both organic and inorganic tomatoes grown in the soil system. This indicates that some unique proteins were expressed due to the effect of different grow systems. This study has highlighted both unique as well as proteins which were over expressed and these protein bands will require further analysis by mass spectrometry. The mass spectrometric studies would reveal the identity of the over expressed proteins and thus help identify the genes whose

expression could be exploited for growing nutritionally rich tomatoes.

The numbers of metabolites in plants generally are expected to be very low when compared to the number of genes, messenger RNA species or proteins. However, their composition is known to change during different physiological states of plants. The HPLC analysis showed some similar as well as different peaks for all four treatments of this experiment. Overall, higher concentration of metabolite compounds was observed in the inorganic hydroponic treatment group. Our results indicated higher levels of lycopene in both the organic treatment groups. Some compounds such as adenosine was present in very large quantities in inorganic hydroponic treatment. Adenosine is known to help with numerous medical conditions including lung cancer, high blood pressure and cystic fibrosis. Some compounds like decaffeoylquinic acid-1 was present in all three treatments except organic soil for reasons that needs further investigation. Beta carotene is known to inhibit the growth of pre-cancerous tumors, decrease asthma symptoms, help in the treatment of heart diseases, aids, Alzheimer's, depression, Parkinsons etc. and was higher in both organic treatments when compared to both inorganic treatments. Therefore, a detailed mass spectrometry and NMR studies would help in further understanding of the individual metabolites and their specific involvement in plant functioning.

These above observations could potentially help us develop an efficient growing system that will be sustainable and produce nutritionally enhanced produce.

Chapter 5: Conclusion

In general, fruits and vegetables are a very rich source of antioxidants, phytonutrients, essential amino acids, fiber and provide a wide array of benefits for the improvement of human health. The metabolic profile of tomatoes presents a collection of nutritionally significant vitamins, minerals, antioxidants, glycosides, flavonoids, and fatty acids. The present research was focused on studying the growth of organic and inorganic tomatoes in two different grow systems (soil and hydroponics) using a multifaceted approach through studying the changes in plant development, protein and metabolite profiles.

Physiological studies during the vegetative growth phase of the tomato plants indicated an exponential increase in plant height for all the treatments by approximately 35 cm, however the organic soil treatment showed the highest. Anthesis was observed first in the organic soil treatment. The preceding results suggest that the biochemical pathways operating in organic mode of development may have genes and proteins that are activated earlier when compared to inorganic development methods.

The overall representation of flower quantity and fruit production was highest in organic tomatoes that were grown in the hydroponic systems, whereas the lowest number of flowers and fruits was observed in organic soil treatment. The efficacy of the nutrient accessibility was observed to be greater in the hydroponic systems due to the high root biomass which resulted in the increased number of flowers and fruit production. Although fruit mass and seed number was higher in both inorganic treatments and lower in both organic treatments, it was observed that dry mass of the seeds was higher in both the organic groups. Therefore we saw that the treatments that produced the most number

of seed did not necessarily produce seeds with more mass. The root length data indicated that the hydroponic grow systems contributed large differences in root mass when compared to both organic and inorganic tomatoes soil treatments.

This investigative study revealed a clear differential profile of proteins in the four different treatments using 1D SDS-PAGE. The highest intensity protein bands were observed in the inorganic hydroponic treatment group. The two unique bands, present in both organic and inorganic tomatoes grown in the soil system, indicated that some unique proteins were expressed due to the effect of different agronomic environments.

The HPLC analysis resulted in an overall higher concentration of metabolite compounds from the inorganic hydroponic treatment group. The analysis also showed higher levels of lycopene and beta-carotene in both the organic treatment groups, suggesting the presence of possible signaling molecules in organic plants that induced the production of more lycopene. This study in general, indicated the production of a more diverse group of protein and metabolites in both organic treatment groups and the presence of higher quantities of proteins and metabolites with the hydroponic treatments. Some of the unique proteins and metabolites revealed in this study could be functioning as signaling molecules that trigger the biochemical spectrum of the plants.

A detailed mass spectrometry and NMR studies would therefore be helpful in further understanding of the individual metabolites and their specific involvement in plant functioning. More advanced proteomic and metabolomic studies could potentially uncover individual genes, proteins and metabolites and their specific roles involved in understanding the biochemical profiles of plants of organic and inorganic origin and the difference when grown in different grow systems. Future in depth studies would help in

developing efficient growing systems that could be sustainable and produce nutritionally enhanced tomatoes.

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Academic Vita

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EDUCATION

B.S. Science (Life Sciences Option)
Schreyer Honors College Student

August 2012-present

Pennsylvania State University at Harrisburg
Middletown, PA 17057

High School Diploma
Honor Student

June 2012

Hershey High School
Hershey, PA 17033

EXTRACURRICULAR ACTIVITIES

Penn State University at Harrisburg

August 2012-present

- Penn State Harrisburg women soccer team. (Captain in 2015)
- Completed NSF- REU Program, summer 2012.
- Teacher Assistant for Microbiology 106 and Biology 120.
- Co-Founder, President and Vice President, Penn State Harrisburg Health Science Club.
- Penn State Harrisburg women tennis team.
- Traveled to Guatemala and Seville, Spain on a study tour/service trip.
- The National Society of Leadership and Success (Sigma Alpha Pi).

COMMUNITY SERVICE/VOLUNTEERING

Pennsylvania Chapter of the American Chestnut Association, Harrisburg, PA

January 2016

- Served as a volunteer to educate others about the restoration of the American Chestnut which is in the brink of extinction.

Children's Hospital, Pennsylvania State University Milton S. Hershey Medical Center, Hershey, PA

June 2014-January 2015

- Volunteered in the family room at the Children's Hospital.
- Helped the patient's families to be comfortable and brought them any necessities that they needed.

Hershey Invitational Soccer Tournament, Hershey, PA

June 2011-June 2012

- Provided information about the soccer tournament.

- Worked as a field Marshall for the different games.

Kicks for Kids, Hershey, PA

June 2008-June 2012

- Coached children to learn the sport of soccer.
- Cared for the children.

Olmsted Regional Soccer Association, Middletown, PA

April 2008-April 2011

- Evaluated girls during soccer tryouts for selection to play on Under 9 through Under 15 soccer travel teams.
- Directed training girls playing soccer in Under 12 and Under 13 travel teams.

WORK EXPERIENCE

Honors Program/International Program Office at Penn State Harrisburg,

Middletown, PA

- Worked as an office assistant for Ms. Stephanie Ponnett February 2014-May 2014
- Assisted in honors and international program events.

INTREPID, Pennsylvania State University Milton S. Hershey Medical Center

College of Medicine, Hershey, PA

May 2013-August 2014

- Worked under supervision of Dr. Christopher A. Siedlecki on bioengineering and surgery.
- Attended numerous medical and research seminars.
- Attended meeting with Dr. Michael Chorney to discuss different medical and research topics.
- Shadowed under supervision of Dr. Timothy Craig and other doctors (Asthma, Allergy, and Immunology).

Central Pennsylvania Laboratory for Biofuels, Middletown, PA July 2010-present

- Worked under supervision of Dr. Sairam Rudrabhatla and Dr. Shobha Potlakayala on plant tissue culture and molecular biology.
- Attended numerous seminars and meetings about plant biology.
- Attended the Science of In vitro Biology conference in Providence, Rhode Island to present research.

AWARDS

- Awarded Second Team All Conference in the North Eastern Athletic Conference. November 2012
- Honor Student, Penn State Harrisburg August 2012-present
- Dean's List, Penn State Harrisburg Fall 2012-Spring 2014
- Awarded Scholar Athlete Award for Fall 2012 October 2013
- The Capital College Honors Program Certificate of Achievement April 2014
- The Capital Athletic Conference All-Academic Team July 2014

RESEARCH POSTERS

- *Camelina sativa* Protoplast Isolation, Purification, and Culture (NSF-REU, Penn State University-Harrisburg)
- Physiological and Molecular Studies of Salt Stress on *Camelina sativa* (SIVB Conference in Providence, Rhode Island)

- Evaluating Hemocompatibility Measurements of Human, Bovine, and Ovine blood Using Three Different Techniques in order to Improve Ventricular Assistant Device (VAD) testing (INTREPID, Penn State Hershey)
- Evaluating the use of Icatibant injections (FIRAZYR®) with C1 esterase inhibitors (CINRYZE® and BERINERT®) during pregnancy of a type III hereditary angioedema patient (INTREPID, Penn State Hershey and in Atlanta, Georgia for the ACAAI conference)
- Successful graded challenge to adalimumab following infliximab anaphylaxis refractory to desensitization in a 15 year old girl with severe Crohn's disease (Asthma, Allergy, and Immunology Penn State Hershey and in San Antonio, Texas for the ACAAI conference)

SPECIAL SKILLS

Speak Spanish, Farsi, Kurdish, and English