GELATIN, A BIOSTIMULANT SEED TREATMENT AND ITS IMPACT ON PLANT GROWTH, ABIOTIC STRESS, AND GENE REGULATION

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

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January 2015

GELATIN, A BIOSTIMULANT SEED TREATMENT AND ITS IMPACT ON PLANT GROWTH, ABIOTIC STRESS, AND GENE REGULATION

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Cornell University 2015

Biostimulants are chemicals that stimulate plant growth and increase plant protection from biotic and abiotic stress. Protein hydrolysates are classified as one type of biostimulant, and have been shown to have a positive effect on plant growth. The effect of gelatin, a kind of animal protein hydrolysate was evaluated on plant growth. Enhanced plant growth was measured in the aboveground portions of plants with the application of gelatin capsules applied at time of sowing adjacent to seeds. The crops tested (cucumber, tomato, broccoli, corn, arugula, pepper) showed increased plant growth as measured by leaf area, fresh and dry weight. The magnitude of plant growth enhancement was crop specific, and cucumber was used as the model crop for all further studies. Plants treated with gelatin capsules exhibited increased nitrogen content, and increased salinity tolerance compared to the non-treated control. Different types of hydrolyzed collagen, including granulated gelatin, gelatin hydrolysate, and amino acid mixtures containing amino acids present in gelatin were compared and revealed that granulated gelatin treatment had the greatest plant growth compared with other treatments. Plants were treated with two gelatin capsules and equivalent amounts of nitrogen in the form of urea, revealed that increased plant growth from the application of gelatin capsule was not solely due to the nitrogen.

RNA-seq results provided some insights on the mechanisms of the growth promotion from the gelatin capsule treatments. Genes were upregulated from the gelatin treatment involved in nitrogen transport including ammonium transporters and amino acid transporters, and MYB and WRKY family transcription factors that regulate diverse pathways including abiotic stress tolerance and other responses were upregulated in plants with gelatin treatment. Genes involved in detoxification such as Glutathione S-transferase exhibited a high positive correlation with increase leaf area and nitrogen content in plants treated with gelatin capsule.

BIOGRAPHICAL SKETCH

Hiromi Tasaki Wilson earned her Bachelor of Science degree in Horticulture from Virginia Polytechnic Institute and State University in 2007, and her Master of Science degree in Horticulture in 2008 from the same university. In 2010 she joined the Ph.D program in Horticulture at Cornell University.

Hiromi has been the recipient of the poster presentation award at International Seed Testing Association (ISTA) 30th Seed Conference at Antalya, Turkey.

While pursuing her degree, Hiromi has worked as a teaching assistant and research assistant for the department of Horticulture. She was involved as a presenter in a webinar hosted by the Organic Dairy Initiative.

Hiromi will be starting her career with Syngenta Seeds as a Quality Control Manager in Physiology Testing in Nampa, ID.

Dr. Alan Taylor supervised Hiromi's dissertation, *Gelain: a seed treatment biostimulant and its impact on plant growth, abiotic stress tolerance and gene regulation.*

ACKNOWLEDGMENTS

I would like to first thank my committee members, Dr. Alan Taylor, Dr. Neil Mattson, and Dr. Michael Mazourek for providing me with the guidance and helping me through my course of Ph.D. I would also like to extend my gratitude to Dr. Kenong Xu for his help on the project, especially the molecular biology work. Without his help this project would not have been completed. Dr. Yang Bai for teaching me all the molecular techniques used in this project.

I would also like to thank my sponsors, Dr. Josep Trias from Coating Supply Inc. and Mr. Livio Takahashi from Sakata Seed SDA, for awarding us with the grant, the seeds and coating material for the project and providing me with guidance through the project.

The lab members from Taylor lab: Michael Loos, Catharine Catranis, Masoume Amirkhani, and Wencheng Huang has been of great resource, assisting me with the experiments and allowing me to bounce-off experiment ideas with them.

Most of all, I would like to thank my family and friends for their support. My husband, Dr. Brent Wilson for tolerating me through the difficult times and celebrating my accomplishments with me.

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CHAPTER 1: Literature Review

Seed enhancement

Numerous techniques are used to improve seed handling, to improve seedling establishment and growth under a range of environments. These techniques are generally described as "seed enhancements" (Halmer 2004). Seed enhancement is a term widely used in the industry to describe beneficial techniques performed to seeds post-harvest, but prior to sowing (Taylor et al. 1998). Heydecker and Coolbear (1977) described the purpose of seed treatment and enhancement as following: "to select, improve hygiene and mechanical properties, break dormancy, advance and synchronize germination, apply nutrients, and impart stress tolerance" (Heydecker and Coolbear 1977, pg.353)

Seed treatment technologies can be grouped into three broad categories: (Halmer 2004) 1) *Physiological enhancements*: Germination enhancements in which physiological performance during germinating and seedling growth are modified (Ex. priming and pre-germination). Seedling growth and development are enhanced due to the application of growth enhancers to seeds (Halmer 2004).

2) *Seed treatment and coating:* Treatments and application of insecticides and fungicides to protect the seed against disease or pest (Ex. film coating, pelleting). Pelleting and coating can alter handling by changing the shape, weight, size of the seed, which benefits precision sowing systems, used in high-value crops (Halmer 2004).

3) *Encapsulation:* Placement of a single or multiple seeds that may be coated, treated or enhanced prior to encapsulation in a gelatin capsule (Takahashi and Trias 2012).

Physiological enhancements

Physiological enhancements can be divided into two categories: 1) seed treatments in which germination is enhanced by pre-sowing hydration treatments, such as priming and solid matrix priming (Taylor et al. 1998) and 2) dry applied seed treatments in which seedling growth and development is enhanced by the seed treatment.

Priming or osmoconditioning are techniques used to advance germination by controlled hydration to a level insufficient for the completion of germination (Taylor 2003). Osmotic solutions in which water potential has been adjusted with polyethylene glycol (PEG), inorganic salts, or mannitol are used to imbibe the seed to elevate seed moisture content (Taylor et al. 1998). Since seeds have not completed germination, they remain desiccation-tolerant and can be dried again for long-term storage (Taylor 2003). Solid matrix priming, unlike priming which uses liquid medium, uses solid particulate system to increase seed moisture in a controlled system (Taylor et al. 1998).

Seed priming allows for faster and uniform germination under unfavorable germination temperature conditions, which leads to a better crop stand, improved yield, and harvest quality under suboptimal growing conditions (Halmer 2004). Although there are differences in response from seed lot to seed lot, typically time to reach 50% of maximum emergence (T_{50}) can be decreased up to ⅓ by seed priming. Seed lots that exhibit slower germination benefit the most from priming treatment (Brocklehurst and Dearman 1983; Bradford, Steiner, and Trawatha

1990). One limitation in seed priming is the increased rate of deterioration in storage and accelerated aging (Halmer 2004). Priming of tomato and lettuce has shown to accelerate the rate of ageing in comparison with nonprimed seeds (Argerich, Bradford, and Tarquis 1989; Tarquis and Bradford 1992).

Seedling growth and development can be affected by biological seed treatments. *Trichoderma* spp. are endophytic plant symbionts that are applied as seed treatments to control disease and to enhance the plant growth and yield (Mastouri 2010). Mastouri (2010) reports that *Trichoderma* spp. not only enhanced plant growth and yield, but also aid the plants in alleviating abiotic stress such as osmotic stress, salt stress and suboptimal temperatures, by inducing protection against oxidative damage.

Seed treatment and coating

Seed coating technologies such as pelleting and film coating have been used to facilitate mechanical sowing to achieve uniformity of plant spacing by modifying the shape, weight, and size of the seed. Seed coating may act as a carrier for plant protectants, so materials can be applied in the target zone with minimal disruption to the soil ecology and environment (Taylor et al. 1998; Halmer 2004).

In general, pelleting refers to the deposition of layer of inert materials such as inorganic silica and diatomaceous earth, that may obscure the original shape and size of the seed, resulting in a substantial weight increase and improve handling of seeds. Film coating on the other hand, retains the shape and the general size of the nontreated seed with a minimal weight gain (Taylor et al. 1998; Butler 1993).

During seed pelleting, the seeds are coated with a combination of binder (adhesive) and filler (bulking agent). Plant protectants required at high loading rates can be applied during the coating process. Also known as, 'pellet loading', a small layers of coating materials are applied to the seeds, followed by active ingredients formulated as dry powers are applied to subsequent layers. The final coating application with filler material encapsulates the plant protectants and prevents worker exposure to the seed treatment (Hill 1998; Taylor et al. 1998). The physical separation between the plant protectant and the seed prevents phytotoxicity caused by high concentration of active materials in close proximity to the seed (Taylor and Eckenrode 1993; Taylor et al. 1998).

The negative factor in seed pelleting is that the pellet may act as a barrier to oxygen diffusion thus affecting germination (Sachs, Cantliffe, and Nell 1981). In order to address such issues, companies have added oxygen-liberating compounds such as, alkaline earth and peroxide-containing compounds in pellets to prevent issues with oxygen diffusion (Langan, Pendleton, and Oplinger 1986). Other companies have employed 'split-pellet' technology, in which pellets fracture during the early phases of water uptake (Taylor et al. 1998).

Seed encapsulation

Seed encapsulation is a technology developed by Alliance Seed Capsule, a consortium between Coating Supply and Sakata, in which raw and/or processed seeds are encapsulated in collagen-based gelatin capsule (Illustration 1.1 and 1.2). Seed encapsulation provides various advantages such as better handling and sowing, faster plant growth, precision planting, precise seed quantities per sowing unit, and provides capability of combining seed enhancement

technologies and other chemicals or biological additives such as pesticides, fertilizers, and Rhizobium (Takahashi and Trias 2012).

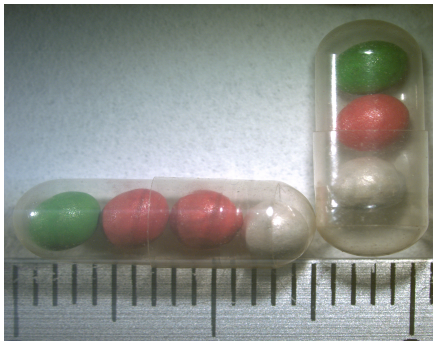

Illustration 1.1: Seed Encapsulation of pelleted seeds

Illustration 1.2: Seed Encapsulation of multiple seeds

Seed encapsulation serves as a multiple seed delivery system; however, the gelatin capsules also provide growth promotion effects as well as a carrier for plant protectants. Smaller sized capsules with plant protectants or fertilizer can be placed in a larger capsule with multiple seeds. Seeds can be primed, film coated or pelleted with plant protectant additive before being placed in the capsule (Takahashi and Trias 2012).

Biostimulant

Plant biostimulants are a broad class of substances and microorganisms that enhance plant growth. The European Biostimulants Industry Council (EBIC) has defined plant biostimulants as substance(s) and/or microorganisms whose function when applied to plants or the rhizosphere is to stimulate natural processes to enhance/benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and crop quality. Biostimulants have no direct action against pests, and therefore do not fall within the regulatory framework of pesticides (EBIC 2014; du Jardim 2012).

Calvo et al. (2014) conducted a thorough review on plant biostimulants and have provided a detailed review on four categories of biostimulants: microbial inoculants, humic acid, fulvic acid, protein hydrolysate and amino acids, and seaweed extracts. For the purpose of this review, only protein hydrolysate and amino acids will be discussed.

Protein hydrolysate and amino acids

There are two major categories of peptide-based products: protein hydrolysate that includes mixture of peptides and amino acids, and individual amino acids such as glutamate, glutamine, proline and glycine betaine (Calvo, Nelson, and Kloepper 2014).

Protein hydrolysates are produced by three methods of hydrolysis: enzymatic, chemical and thermal hydrolysis, and can be produced by various source of materials which include; animal epithelial tissue (Cavani et al. 2006; Kauffman, Kneivel, and Watschke 2007; Morales-Payan and Stall 2003; Maini 2006), animal collagen and elastine (Cavani et al. 2006), carob germ protein (Parrado et al. 2008), alfalfa residue (Ertani, Schiavon, et al. 2013), wheat-

condensed distiller soluble (Garcia-Martinez et al. 2010), and tannin residues (Ertani, Pizzeghello, et al. 2013).

The amino acid contents of the hydrolysates vary depending on the production method as well as the source material. Animal based hydrolysate contains high proportion of proline and glycine, while plant based hydrolysate contains high proportion of glutamine and arginine (Calvo, Nelson, and Kloepper 2014; Parrado et al. 2008; Ertani, Pizzeghello, et al. 2013). Other non-protein components present in hydrolysate are thought to contribute to the stimulatory effect in plants. Plant based hydrolysate contains fats, carbohydrates, macro and micronutrients and phytohormones such as auxin, gibberellins, and cytokines (Ertani, Schiavon, et al. 2013; Parrado et al. 2008; Schiavon, Ertani, and Nardi 2008). Animal based hydrolysate contains proteins, amino acids, fats, macro and micronutrients, but lacks carbohydrates and phytohormones (Parrado et al. 2008; Calvo, Nelson, and Kloepper 2014).

The second category of protein-based products is the individual amino acids, which includes all the structural amino acids involved in synthesis of proteins as well as non-protein amino acids that are found in plants (Vranova et al. 2011; Calvo, Nelson, and Kloepper 2014). Numerous authors have reported on exogenous application of amino acids such as glutamate, proline, and glycine betaine to provide abiotic stress protection (Vranova et al. 2011; Rhodes, Verslues, and Sharp 1999; Liang et al. 2013). These amino acids act as osmoprotectants, stabilizing proteins and membranes from denaturing effects of high salt concentration (Ashraf and Foolad 2007; Chen and Murata 2011; Ahmad, Lim, and Kwon 2013). Not only that, they act as reactive oxygen species scavenger and they induce expression of salt stress responsive genes

and genes involved transcription factors, membrane trafficking and antioxidants (Kinnersley and Turano 2000; Ashraf and Foolad 2007; Anjum et al. 2011; Liang et al. 2013). Accumulation of glycine betaine and proline is strongly correlated to stress tolerance in many crops, and exogenous application of these amino acids has been shown to enhance abiotic stress tolerance (Chen and Murata 2011; Reis, Lima, and Medeiros de Souza 2012; Ahmad, Lim, and Kwon 2013; Calvo, Nelson, and Kloepper 2014; Ashraf and Foolad 2007).

Protein hydrolysate and amino acid effects on plants

Protein hydrolysate and other protein-based product application have been reported to enhance plant growth and yield in field tomato (Parrado et al. 2008), greenhouse tomato (Koukounararas, Tsouvaltzis, and Siomos 2013), papaya (Morales-Payan and Stall 2003), maize seedling (Ertani, Schiavon, et al. 2013; Ertani, Pizzeghello, et al. 2013), and hydroponic lettuce (Colla et al. 2012). Positive effects of protein hydrolysate on abiotic stress have also been reported. Cucumber plants subjected to suboptimal pH level and temperature performed better with the application humic acid containing substance LACTOFOL™, with notable increase in leaf area, shoot and root mass (Boehme, Schevschenko, and Pinker 2008; Boehme, Schevtschenko, and Pinker 2005). Alfalfa plant-derived biostimulant increased maize plant biomass under salinity condition. It reduced Na^+ accumulation and enhanced K^+ accumulation in roots and leaves (Ertani, Schiavon, et al. 2013). Perennial ryegrass treated with Macro-Sorb Foliar (FOLIAR™), an animal membrane hydrolysate, subjected to high air temperature stress exhibited increased photochemical efficiency and membrane thermo-stability compared to plants without the treatment (Kauffman, Kneivel, and Watschke 2007).

Protein hydrolysate has been demonstrated to stimulate carbon and nitrogen metabolism and increase nitrogen assimilation. Increase in NAD-dependent glutamate dehydrogenase, nitrate reductase, and malate dehydrogenase in maize has been reported following application of animal epithelial hydrolysate (Maini 2006). Alfalfa protein hydrolysate applied to hydroponically grown maize increased the activity malate dehydrogenase, isocitrate dehydrogenase, and citrate synthase, which are enzymes found in TCA cycle. The hydrolysate application also increased the activity of nitrogen metabolism enzymes; nitrate reductase, nitrite reductase, glutamine synthetase, glutamate synthase, and aspartate aminotransferase (Schiavon, Ertani, and Nardi 2008). Ertani et al. (2009) reported that both alfalfa protein hydrolysate and animal connective tissue hydrolysate stimulated plant growth, and increased the nitrate conversion into organic nitrogen by inducing nitrate reductase and glutamine synthetase activities. The treatments enhanced especially glutamine synthetase 2 (GS2) isoform, which is responsible for assimilation of ammonia produced by nitrate reduction, confirms that protein hydrolysate enhances plant growth by up regulating nitrate assimilation (Ertani et al. 2009; Calvo, Nelson, and Kloepper 2014).

Properties of Gelatin

The main ingredient of collagen-based gelatin capsule used in seed encapsulation is hydrolyzed collagen. Collagen is a class of naturally occurring proteins found primarily in the flesh and connective tissues of animals (Balian and Bowes 1977; Schrieber and Gareis 2007). Collagen can be broken down through hydrolysis to form hydrolyzed collagen, which is known as gelatin. Gelatin is considered as a form of protein hydrolysate (Regenstein and Boran 2010; Balian and Bowes 1977). Gelatin is one of the most widely used biopolymers, with wide

application in food, pharmaceuticals, cosmetics, and photographic films, and other products including paints, matches, fertilizers, as a gelling agent foam stabilizer, and structure enhancer (Karim and Bahat 2009; Gudmundsson 2002; Yang et al. 2007; Hou and Regenstein 2004). Gelatin has a relatively low melting temperature at 35°C, and sets to a gel on cooling (Regenstein and Boran 2010; Schrieber and Gareis 2007). The chemical composition of gelatin is similar to that of collagen; however, unlike its parent molecule, gelatin is not composed of single sized peptide chain, but rather is a combination of many fractions of peptide chains varying in size, including the whole α-chain of the whole collagen molecule and hydrolytic fragments of parts of the α-chain of different lengths (Regenstein and Boran 2010; Eastoe and Leach 1977).

Composition of Gelatin

Gelatin contains 18 different amino acids with average of 1000 amino acid per/chain (Table 1.1). It has unique amino acid composition, with high content of proline and hydroxyproline. The amount of tryptophan and cystein are extremely low in gelatin. This means there are few disulfide bonds involved in gelatin structure (Belitz, Grosch, and Scieberle 2004).

Table 1.1: List of amino acid found in gelatin and the relative composition by weight of each amino acid per 100g of protein. (GMIA 2012)

Amino Acid	g of Amino Acid per 100g/prot
Alanine	11.3
Arginine	9
Aspartic acid	6.7
Glutamic acid	11.6
Glycine	27.2
Histidine	0.7
Proline	15.5
Hydroxyproline	13.3
Hydroxylysine	0.8
Isoleucine	1.6
Leucine	3.5
Lysine	4.4
Methionine	0.6
Phenylalanine	2.5
Serine	3.7
Threonine	2.4
Tryptophan	0
Tyrosine	0.2
Valine	2.8

Structure of Gelatin

Gelatin is mainly stabilized by hydrogen bonds between the backbone amino group of glycine and backbone carboxyl group of proline in neighboring chains (Veis 1964; Regenstein and Boran 2010). The hydroxyl group in hydroxyproline plays an important role in the formation of intra and inter molecular hydrogen bonds (Brinckmann 2005). The molecular structure for gelatin is a multiple repetition of glycine-X-Y sequence, where "X" is often a proline and "Y" a hydroxyproline. Proline and hydroxyproline residues are the only amino acids that permit sharp twists of the gelatin helix allowing for the relative low amino acid per turn in the peptide (Regenstein and Boran 2010; Schrieber and Gareis 2007).

Gelatin molecules are arranged head-to-tail, with a 35nm gap between molecules. Charged and uncharged residues are found clustered along the sequence of collagen at about every 230 residues. The molecules are aligned such that the maximum electrostatic and hydrophobic interactions occur between different molecules (Regenstein and Boran 2010). Each residue takes one 120° turns around the axis, thus three residues complete one turn. Three of the polypeptide chains are joined together as a left-hand spiral of the secondary structure, and the spiral winds and folds itself to a right-hand spiral (triple helix) of the tertiary structure of the protein, which is called tropocollagen (GMIA 2012; Regenstein and Boran 2010). Gelatin peptide has a rod-shape with a molecular weight of approximately 330 kDa with a length of 300nm and diameter of 1.5nm (Belitz, Grosch, and Scieberle 2004).

When hydrolyzed, the collagen molecules are fractionated into different peptide fragments, which differ in molecular weight. Independent α-chains, a β-chain (two α-chains

linked to each other by covalent bonds) and a γ-chain (three α-chains linked to one another by covalent bonds) (Regenstein and Boran 2010).

Gelling Process

Hydrogen bonds play an important role in the gelling process (John and Courts 1977). Gelation is the recovery of cross-linkages by gelatin molecules, which allows for its structural integrity (Regenstein and Boran 2010; Schrieber and Gareis 2007). It is theorized that small sections of a number of gelatin molecules unite to form crystallites, which forms a structure of highly ramified three-dimensional network capable of immobilizing liquid (Djagny, Wang, and Xu 2001). Ferry (1948), suggested that both hydrogen bonds and van der Waals forces acts in the binding of gelatin molecules to form a fragile architectures of the gel (Ferry 1948). The concentration of α-chains and the cooling rate are the most important factors affecting the final gelation. At high gelatin concentration, intermolecular bond formation typically occurs with multiple strands, while the same process is more likely to occur as intra molecular bonds within a single strand at low concentration. Slow rates of cooling allow more intra and inter-molecular cross-link formation, while rapid cooling does not (Belitz, Grosch, and Scieberle 2004).

Gelatin Extraction

Gelatin is extracted from collagen by series of processing steps. In the pretreatment step, non-collagen impurities are removed and the source material is prepared for collagen extraction. The source material is treated with alkali and/or acid to weaken the collagen structure by breaking intra molecular cross linkages including covalent and hydrogen bonds. In the water extraction step, collagen is converted into gelatin. Multiple extractions are performed with

gradually increasing temperatures. Lower temperature fractions have minimal degradation and the resulting gelatin has higher molecular weight, higher viscosity and gel strength. As the temperature increases, the extracted fractions have more variable molecular weights and the gel strength is lower (Hinterwaldner 1977; GMIA 2012; Schrieber and Gareis 2007). The last step is a series of refinement and recovery processes, such as filtration, evaporation and deionization to get a highly purified dried gelatin.

Gelatins are classified according to the pretreatment step (alkali or acid). Type A gelatin are obtained when collagen has been treated with acid, they tend to have a higher isoelectric point compared to Type B gelatin (alkali process). The mild acid process does not remove the amide nitrogen of Glutamine and Asparagine, resulting in a higher isoelectric point. However, in a severe acid treatment, some of the amine groups are hydrolyzed and the isoelectric point is lower. Isoelectric point for Type B gelatin is significantly lower then Type A as the alkali process results in the loss of amide groups (Eastoe and Leach 1977). Another important measurement of gelatin quality is the gel strength, which is measured in bloom. Bloom is the empirical gel strength measure, which the measures the firmness of gel, the weight in gram required to depress a plunger to a fixed distance. The more rigid the gel, the higher the bloom; gelatin with high bloom is regarded as of a higher grade (GMIA 2012; Schrieber and Gareis 2007).

Amino Acid uptake in plants

Most plants acquire nitrogen as nitrate and ammonium but also as organic nitrogen forms such as amino acids and protein from the soil (Nasholm, Kielland, and Ganeteg 2009). Transport

studies with isolated membrane vesicle and plant tissue uncovered presence of multiple transport systems for amino acids (Tegeder and Rentsch 2010). Amino acid transporters have been reported to have a variety of roles in plant growth and development. They function in long-distance transport, acquisition by import-dependent cells, and intracellular partitioning between different compartments in the cell (Liu and Bush 2006).

Two superfamilies of amino acid transporters have been identified; amino acid, polyamine and choline transporters superfamily (APC) and amino acid transporter family (ATF) (Fischer 1998; Ortiz-Lopez, Chang, and Bush 2000; Su, Frommer, and Ludewig 2004). There are 14 APC transporters in the *Arabidopsis* genome, among these transporters cationic amino acid transporters (CAT), and high-affinity basic amino acid transporters, are well characterized (Su, Frommer, and Ludewig 2004). The ATF superfamily contains five subclasses of transporter gene families: amino acid permeases (AAPs) (Frommer, Hummel, and Riesmeier 1993), lysine/histidine transporters (LHTs) (Chen and Bush 1997), proline transporters (ProTs) (Rentsch et al. 1996), aromatic and neutral amino acid transporters (ANTs) (Chen et al. 2001), and putative auxin transporters (AUXs) (Bennett et al. 1996). The functional significance of these amino acid transporters are not only defined by the substrate specificity, but also by expression pattern controlled by developmental stages and differential responses to environmental cues (Liu and Bush 2006). For the purpose of this review, only ProTs are discussed in relation to salinity stress.

Three proline transporter genes (*ProT1*, *ProT2* and *ProT3*) have been identified in *Arabidopsis*. *ProT1* is highly expressed in roots, stems and flowers, mainly in the stalk phloem

that enters the carpels of flowers (Rentsch et al. 1996). *ProT2* is expressed in the epidermis and cortex cells in roots and its expression is regulated by salt-stress (Rentsch et al. 1996; Grallath et al. 2005). *ProT3* expression was only detected in the aboveground organs such as leaves, flower and siliques (Grallath et al. 2005).

Amino acid transport is highly regulated by environmental signals such as light (Guo 2004), osmotic changes (Rentsch et al. 1996; Popova, Dietz, and Golldack 2003) and pathogen attack (Carginale et al. 2004). Plants synthesize and accumulate osmoprotectants such as proline, glycinebetaine and organic sugar under salt-stress. Up regulation of proline transporter gene expression in response to water and salt stress has been reported in many plant species, such as *AtProT2* in *Arabidopsis* (Rentsch et al. 1996), *HyProT* in barley (Ueda et al. 2001), and *McAAT1* in *Mesembryanthemum crystallinum* (Popova, Dietz, and Golldack 2003). *Arabidopsis* over expressing *AhProT*, proline transporter from halophyte *Atriplex hortensis* L., had increased proline content in root tips and exhibited salt tolerance (Shen et al. 2002).

Expression of other amino acid transporters is regulated by osmotic stress. *AtAAP4* and *AtAAP6* expression are down regulated by water and salt stress in *Arabidopsis* (Rentsch et al. 1996). The induction of proline transport and depression of other amino acid transport is thought to be important for plants to overcome stress conditions. Popova et al. (2003) has reported that proline concentration increased in both root and leaf tissues in response to high salt in *Mesembryanthemum crystallinum*. In the same study, differential regulation of expression of *McAAT1* (ProT subfamily) and *McAAT2* (LHT subfamily) amino transporters were observed under salt stress. *McAAT1* was expressed only in leaf tissues; however, *McAAT2* was specifically

expressed in root tissues. When the plants were subjected to salt-stress for 6 hours, *McAAT1* expression was induced in leaves, whereas *McAAT2* expression was down regulated.

Role of Hydroxyproline in plants

The main ingredient of gelatin capsules used in seed encapsulation is hydrolyzed collagen. A unique property of collagen protein is its high proportion of amino acids such as glycine, proline, and hydroxyproline. For the purpose of this review, hydroxyproline and proline are discussed.

Hydroxyproline is especially important for its primary role in the structure and maintenance of gelatin and its believed to be confined exclusively to the cell wall proteins in plants (Lamport 1965). Hydroxyproline may account for up to 10% of the amino acid content of purified primary cell wall and is derived from the hydroxyproline rich glycoprotein present in primary cell wall (Kieliszewski and Shpak 2001).

Vaughan and Cusens (1973) revealed that hydroxyproline enhanced the extension growth of pea root segments, without affecting their protein synthesis or respiration. They proposed that externally supplied hydroxyproline enhanced extension growth in root by interfering with some aspect of cell wall protein synthesis involving hydroxyproline rich protein (HRP) (Vaughan 1973; Vaughan and Cusens 1973).

Chemical analysis of cell walls has shown that the protein component is rich in hydroxyproline, proline, serine, threonine, and glycine (Lamport 1965). They are components of hydroxyproline rich glycoprotein (HRGPs), which includes Extensin, Arabinogalactan-proteins (AGPs) and Proline rich proteins (PRPs), and Glycine rich proteins (GRPs) (Showalter 1993;

Cassab and Varner 1988). HPGP backbones are highly glycosylated with sugars such as arabinofuranosyl and galactopyranosyl residues (Lamport 1969; Clarke, Anderson, and Stone 1979; Fincher, Stone, and Clarke 1983).

Salt stress in cucumber

Approximately 20% of the world's cultivated land and half of all irrigated lands are affected by salinity (Zhu 2001). Cucumber (*Cucumis sativus* L.) is one of the most popular vegetables in the world, and it is highly sensitive to salinity (Huang et al. 2009). Salinity is one of the most significant factors limiting crop productivity in cucumber (Zhu 2001).

Salinity imposes two constrains on plants: an osmotic effect resulting from the lower soil water potential and ionic effect, which results from the direct toxicity of saline ions and the ion imbalance in the plants (Munns and Tester 2008). The water status in plants is highly sensitive to salinity, which is the cause of drought response in plants when subjected to salt stress (Yeo, Capron, and Flowers 1985). Plants must take up inorganic solutes such as Na^+, Cl^- and K^+ and synthesize compatible solutes such as proline, sorbitol, trehalose and glycine betaine to maintain relative water content under high salt condition (Munns and Tester 2008; Turkan and Demiral 2009). These compatible solutes, or osmolytes, do not interfere with normal biochemical reactions and act as osmoprotectants during osmotic stress (McCur and Hanson 1990; Delauney and Verma 1993; Nounjan, Nghis, and Theerakulpisut 2012). At high level of salt exposure, plants build up high levels of Na^+ and Cl^- within the cell, this in turn inhibits K^+ uptake. This can severely inhibit several enzymes that require K^+ as a cofactor, which leads to a whole range of metabolic impairment by the plant (Munns and Tester 2008).

The primary site of salt injury is thought to be the plasma membrane, part of the cytoplasm that first encounters the salt. Cell membrane stability has been widely used to differentiate stress tolerant and susceptible cultivars. Higher membrane stability is correlated with abiotic stress tolerance (Zhu et al. 2008; Zhu, Bie, and Li 2008; Meloni et al. 2003; Sudhakar, Lakshmi, and Giridarakumar 2001). Lipid peroxidation has been associated with damage caused by various abiotic stress, and is often used as an indicator of salt induced oxidative damage (Elkahoui et al. 2005). Malondialdehyde (MDA) is the decomposition product of polyunsaturated fatty acid of membrane, and it is used as an indicator of lipid peroxidation. MDA tends to accumulate to greater extent in plant under salt stress (Meloni et al. 2003; Sudhakar, Lakshmi, and Giridarakumar 2001).

The exposure of plants to salt stress can increase the production of reactive oxygen species (ROS), such as superoxide radicals and hydrogen peroxide (Apel and Hirt 2004). These ROS are strongly reactive and they can interact with essential macromolecules and metabolites causing cellular damage through oxidation of membrane lipids, proteins and nucleic acid (Nounjan, Nghis, and Theerakulpisut 2012; Apel and Hirt 2004). In general, plants posses antioxidant systems that include antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX), to protect their cells against ROS (Apel and Hirt 2004). Huang et al. (2009) reported that exogenous application of proline on cucumber plants subjected to salt stress, significantly alleviated the growth inhibition of plants induced by salt stress, by improving water status and increase peroxidase enzyme activity in the leaf (Huang et al. 2009).

Role of proline in plants under salt stress

Proline is the most common osmolyte accumulating in plants in response to various stress conditions such as drought stress, salinity stress, heat stress and cold stress. It offers a wide range of protective roles including, osmotic adjustment, stabilization of cellular structure, and reduction of damage to the photosynthetic apparatus (Nounjan, Nghis, and Theerakulpisut 2012). The function of proline as an osmoprotectant was first reported by Christian (1966), who demonstrated that exogenous proline could alleviate the inhibition of growth of *Salmonella oranienburg* from osmotic stress. It is understood that a wide range of organisms accumulates proline under stress: eubacteria, protozoa, marine invertebrates, algae, plants (halophytes, tobacco, spinach, potato, tomato, *Arabidopsis*, alfalfa, field bean, soybean, wheat, barley and rice) (McCur and Hanson 1990; Delauney and Verma 1993; Yoshiba, Kiyosue, and Nakashima 1997).

Handa (1986) reported that tomato cells cultured under water stress rapidly accumulated 300 times more proline than non water stressed cells, and they adapted to osmotic stress. This observation suggests that some plants have the ability to adapt to water stress at the cellular level, and proline is involved in osmotic stress tolerance by acting as a compatible osmolyte by accumulating in cytosol (Munns and Tester 2008; Yoshiba, Kiyosue, and Nakashima 1997), and 34% of total intracellular proline was accumulated in vacuoles in non water-stressed cultured cells of potato. However, when subjected to salt stress, total amount of proline in the cell increased, but the amount of proline in vacuoles decreased (Fricke and Pahlich 1990).

In dehydrated plants, the accumulation of proline occurs as the result of both the activations of its biosynthesis and the inactivation of its degradation (Yoshiba, Kiyosue, and Nakashima 1997). L-proline is produced from L-glutamine via Δ^1-pyrroline-5-caboxylate (P5C) in a reaction catalyzed by enzymes Δ^1-pyrroline-5-caboxylate synthetase (P5CS) and Δ^1-pyrroline-5-caboxylate reductase (P5CR) (Yoshiba, Kiyosue, and Nakashima 1997). Expression of genes for P5CS and P5CR has been analyzed under dehydration in *Arabidopsis* (Savoure et al. 1995; Savoure 2009; Verbruggen, Villarrole, and Van Montagu 1993) Levels of P5CS protein increased after the initiation of dehydration treatment in direct proportion to the level of P5CS mRNA accumulation. The expression of P5CR was not enhanced by dehydration or salinity (Yoshiba et al. 1995). These results taken together suggest that P5CS plays a more important role than P5CR in the accumulation of proline under osmotic stress (Yoshiba, Kiyosue, and Nakashima 1997).

Exogenous application of osmoprotectants have been reported to have some effect protecting osmotic status of the plant in abiotic stress and have been suggested as an alternative approach to improve crop productivity under saline conditions (Nakayama et al. 2005). Exogenous application of proline improved crop tolerance in groundnut (Jain et al. 2001), *Pancratium maritimum* (Khedr et al. 2003), melon (Kaya et al. 2007), bell pepper (Kaya and Higgs 2003), tobacco (Hoque et al. 2007; Ashraf and Foolad 2007), and canola (Athar et al. 2009).

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CHAPTER 2: Gelatin applied at sowing to selected vegetable crops enhances plant growth

ABSTRACT

Biostimulants are chemicals that stimulate plant growth and enhance plant protection from biotic and abiotic stress. Protein hydrolysates are classified as one type of biostimulant, and have been shown to have a positive effect on plant growth. The effect of gelatin, a kind of protein hydrolysate was evaluated on plant growth. Enhanced plant growth was measured in the aboveground portions of plants with the application of gelatin capsules applied at time of sowing adjacent to seeds. Crops tested (cucumber, tomato, broccoli, corn, arugula, pepper) showed increased plant growth; the magnitude of plant growth enhancement was crop specific. Cucumber plants treated with three gelatin capsules exhibited 356% increase in total leaf area compared to the control; however, arugula plants with the same treatment had 88% increase in total leaf area. In general, increased gelatin capsules applied resulted in increased plant growth; however, some crops in particular tomato, exhibited phytotoxicity at the highest dosage of three capsules. Amino acids in the gelatin capsule provided a source of nitrogen for plant growth, and a strong positive linear correlation existed between nitrogen supplied by the gelatin treatment and nitrogen content of cucumber leaf tissue.

INTRODUCTION

Numerous techniques are currently used to improve seed handling and to improve seedling establishment and growth under a range of environments. These techniques are generally described as “seed enhancements” (Halmer 2004). Seed enhancement is a term widely used in the industry to describe beneficial techniques performed to seeds post-harvest, but prior to sowing (Taylor et al. 1998). Heydecker and Coolbear (1977) described the purpose of seed treatment and enhancement to select, improve hygiene and mechanical properties, break dormancy, advance and synchronize germination, apply nutrients, and impart stress tolerance.

Seed encapsulation is a technology developed by Alliance Seed Capsule, a consortium between Coating Supply and Sakata, in which raw and or processed seed are encapsulated in gelatin capsule (Illustration 1.1 and Illustration 1.2). The encapsulation provides various advantages such as improved handling and sowing, faster plant growth, precision planting, precise seed quantities per sowing unit, and also provides capability of combining seed enhancement technologies and other chemicals or biological additives such as pesticides, fertilizers, and Rhizobium (Takahashi and Trias 2012).

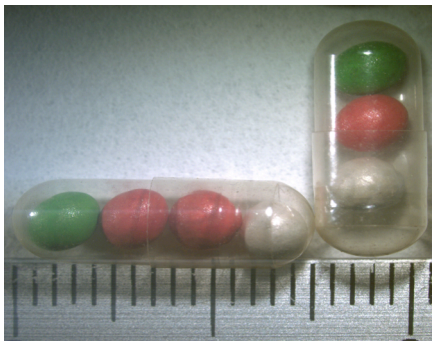

Illustration 2.1: Seed Encapsulation of pelleted seeds

Illustration 2.2: Seed Encapsulation of multiple seeds

Seed encapsulation may be combined with physiological enhancements, seed treatments and can serve as a multiple seed delivery system. It provides growth promotion effects as well as plant protectants. Smaller sized capsules with plant protectants or fertilizer can be placed in a larger capsule with multiple seeds. Seeds can be primed, film coated or pelleted with plant protectants added before being placed in the capsule (Takahashi and Trias 2012).

Seed encapsulation belongs in the category of plant biostimulants, as it uses animal based protein hydrolysate as the main component. Plant biostimulants are a broad class of substances and microorganisms that enhance plant growth. The European biostimulants industry council (EBIC) has defined plant biostimulants as "substance(s) and/or microorganisms whose function when applied to plants or the rhizosphere is to stimulate natural processes to enhance/benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and crop quality. Biostimulants have no direct action against pests, and therefore do not fall within the regulatory framework of pesticides" (du Jardim, 2012; EBIC, 2014).

Protein hydrolysate and other protein-based product application were reported to enhance plant growth and yield in field tomato (Parrado et al. 2008), greenhouse tomato (Koukounararas et al. 2013), papaya (Morales-Payan and Stall 2003), maize seedlings (Ertani, Schiavon, et al. 2013; Ertani, Pizzeghello, et al. 2013), and hydroponic lettuce (Colla et al. 2012). The positive effect of protein hydrolysate on ameliorating the effects of abiotic stress has also been reported. Cucumber plants subjected to suboptimal pH level and temperature performed better with the application humic acid containing substance LACTOFOL™, with notable increase in leaf area, shoot and root mass (Boehme et al. 2008; Boehme et al. 2005). Alfalfa plant-derived

biostimulant increased maize plant biomass under salinity condition, and enhanced K+ accumulation and reduced Na+ accumulation in roots and leaves (Ertani, Schiavon, et al. 2013). Perennial ryegrass treated with Macro-Sorb Foliar (FOLIAR™), an animal membrane hydrolysate, subjected to high air temperature stress exhibited increased photochemical efficiency and membrane thermostability compared to plants without the treatment (Kauffman et al. 2007).

Seed encapsulation using gelatin capsules is a novel approach to seed treatment (Takahashi and Trias 2012), and thus the effect of gelatin chemistry on plant growth has not been fully characterized. The objective of this study was to physiologically characterize the growth promotional effect of the capsule treatment on plant growth and determine if the effect of gelatin chemistry was consistent across several crop species.

MATERIALS & METHODS

Effect of capsule treatment on different growth parameters

Cucumber seeds 'Vlaspik' (Seminis, Oxnard, CA) were planted in a controlled greenhouse maintained at 24°C/21°C temperature with 14/10 hours photoperiod at New York State Agricultural Experiment Station in Geneva, NY in the winter of 2011. Size #3 gelatin capsules (Capsule line, Pompano Beach, FL) (Table 2.1) were placed adjacent to each seed in a 4-inch pot (Illustration 2.3). Cucumber seeds were placed in the center of the pot with capsules surrounding the seed but not touching the seed to prevent a decrease in germination rate, and for controlling the amount of gelatin used in each treatment. Four rates of capsule were compared;

control (no capsule), half capsule (0.5), one capsule (1.0), and two capsules (2.0). Five replication of each treatment was placed in random block design in the greenhouse, with 15 samples per replication. The plants were harvested 28 days after emergence and seven physiological parameters were measured (plant height, root length, leaf area, shoot fresh/dry weight, and root fresh/dry weight). Total leaf area was measured using CI-202 Leaf Area Meter (CID Bio-Science, Camas, WA). Data were then statistically analyzed by ANOVA ($\alpha = 0.05$) and Tukey's HSD test was then used to determine significance.

Table 2.1: Size 3 gelatin capsule, dimension, weight (mg), nitrogen content (mg), protein content (mg).

	Half capsule (0.5)	One capsule (1.0)	Two capsules (2.0)	Three capsules (3.0)
Dimension (mm)		17.6 x 5.5		
Weight (mg)	23.4	46.8	93.7	140.5
Nitrogen (mg)	3.6	7.1	14.2	21.3
Protein (mg)	20	40	80	120

Illustration 2.3: Two gelatin capsules (four halves)

placed adjacent to the seeds in 4-inch pot.

Effect of capsule treatment levels on various vegetable crops

Six crops: cucumber 'Vlaspik'(Seminis, Oxnard, CA), arugula 'Astro' (Sakata, Morgan Hill, CA), broccoli 'Centura' (Rogers, Boise, ID), tomato 'Talladega' (Syngenta, Greensboro, NC), pepper 'Boynton Bell' (Harris Moran, Modesto, CA), corn 'Cornell D2901' (Cornell University, Ithaca, NY) were grown in a controlled greenhouse maintained at 24°C/21°C temperature with 14/10 hours photoperiod at New York State Agricultural Experiment Station in Geneva, NY in the summer of 2013. Five different rates of gelatin capsules (Capsule line, Pompano Beach, FL) were placed adjacent to each seed in a 4-inch pot: control (no capsule), half capsule (0.5), whole capsule (1.0), two capsules (2.0), and three capsules (3.0). Five replications of each treatment were placed in a random block design in the greenhouse. Plants were harvested 28 days after emergence and plant height, total leaf area, fresh weight, and dry weight were measured. Total leaf area was measured using CI-202 Leaf Area Meter (CID Bio-Science, Camas, WA). Data were then statistically analyzed by ANOVA ($\alpha = 0.05$) and Tukey's HSD test was then used to determine significance.

Effect of capsule treatment levels on nitrogen content in cucumber plant tissue

Dry cucumber plant tissue was ground with a Wiley mill (Thomas Scientific Swedesboro, NJ) to a particle size of 2 mm. 100 mg of sample was sent to Cornell University Stable Isotope Laboratory (Ithaca, NY) for elemental analysis of nitrogen percent. Data were then statistically analyzed by ANOVA ($\alpha = 0.05$) and Tukey's HSD test was then used to determine significance.

RESULTS

Effect of capsule treatment on different growth parameters

The effect of capsule treatment on cucumber plant was investigated by measuring seven different growth parameters (plant height, petiole length, total leaf area, shoot/root fresh weight, and shoot/root dry weight) 28 days after emergence. The total leaf area was measured with CI-202 Leaf Area Meter.

The half capsule treatment had the least effect on plant growth, and the two capsule treatments had the greatest effect on the overall cucumber plant growth compared to the non-treated control. An additive effect of the hard-gel capsule treatment was measured in plant height, petiole length, total leaf area, fresh and dry shoot weight (Figure 2.1). The effects of the capsule treatments were not consistent for all plant parts. The above ground parts of the plant exhibited a significant increase ($p<0.05$) with the addition of two capsule treatments. Petiole length increased 37%, total leaf area had a 48% increase, shoot fresh weight had a 45% increase (Figure 2.1). The below ground part of the plant did not exhibit the growth increase as measured for the above ground parts of the plant. The two capsules treatment had root fresh weight increase of 4% and root dry weight decrease of 10%. Results from the study indicates that above ground part of the plant exhibited more pronounced growth increase compared to the below ground part of the plant. It suggests that the above ground part of the plant benefited from the addition of the capsule treatments. There was no significant difference in growth for the below ground part of the cucumber plant, which suggests that gelatin capsule did not affect the growth of below ground parts.

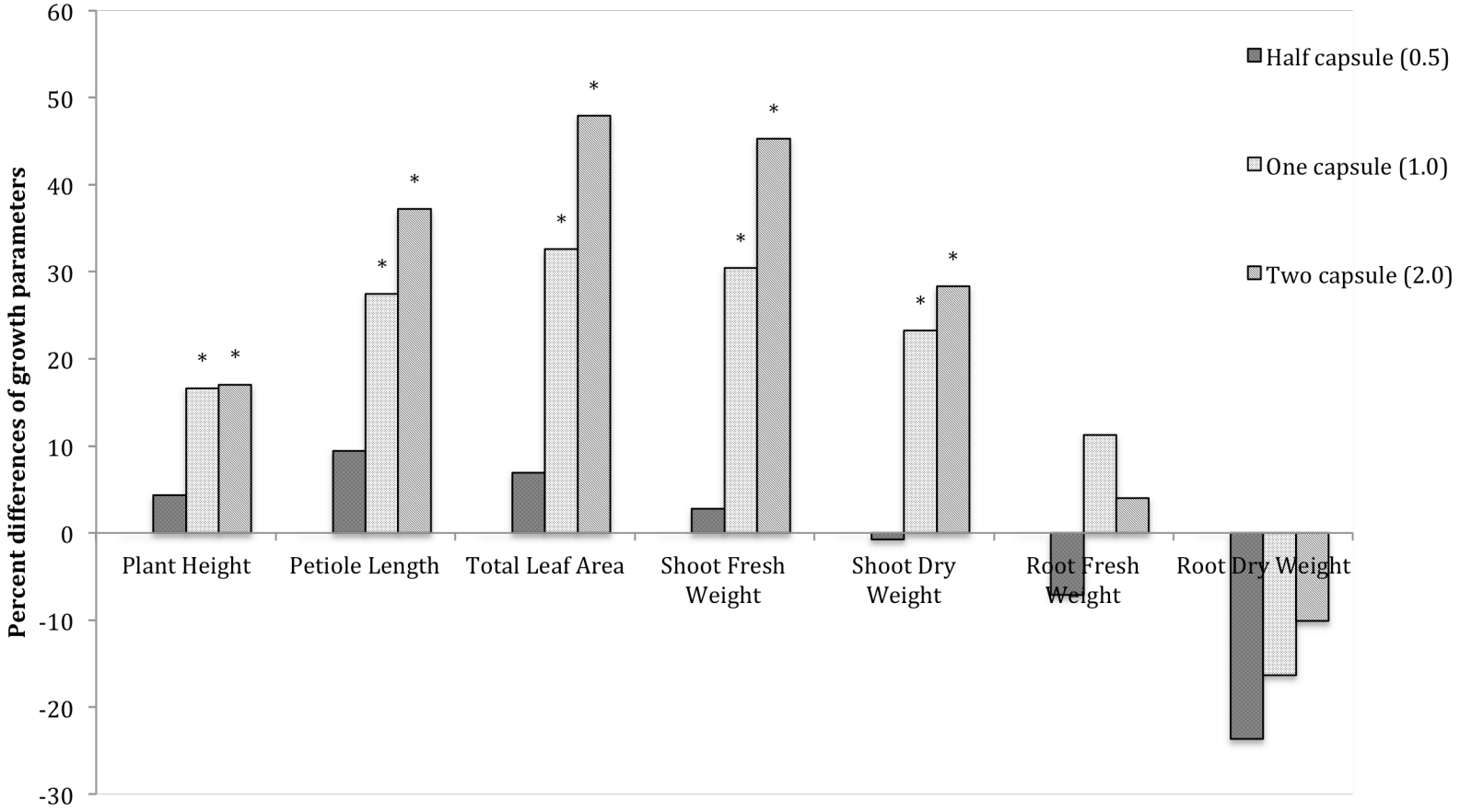

Figure 2.1: Percent difference of seven growth parameters (plant height, petiole length, total leaf area, shoot fresh weight, shoot dry weight, root fresh weight and root dry weight) 28 days after emergence of cucumber ('Vlaspik') with three treatments: half capsule (0.5), whole capsule (1.0), and two capsules (2.0) compared to control as a base line. * denotes significance ($p<0.05$)

Effect of capsule treatment levels on selected vegetable crops and field corn

All results for total leaf area, fresh weight, dry weight and nitrogen content are described as the percent change with respect to the control.

All of the crops exhibited significant increase in total leaf area with the capsule treatment. The magnitude of the effect of the capsule treatment on the total leaf area was specific to each crop (Figure 2.2). Cucumber had a significant increase in total leaf area ($p<0.0001$) with a 39% increase with half capsule treatment, a 102% increase with one capsule treatment, and a 193% increase with two capsules treatment (Figure 2.2). The maximum total leaf area in cucumber was achieved with three capsules treatment with a 356% increase (Figure 2.2). Arugula exhibited a similar increase in total leaf area with capsule treatments to cucumber, with significant increase in total leaf area ($p<0.0001$) with increasing amount of gelatin capsule treatment. Half capsule treatment had a 40% increase, a 53% increase with one capsule, a 77% increase with two capsules, and the maximum increase was achieved with the three capsule treatment with an 88% increase (Figure 2.2). Broccoli also exhibited significant increase in total leaf area ($p<0.0001$) with increasing amount of gelatin capsule treatment, with a 43% increase with half capsule treatment, a 75% increase with one capsule, a 124% increase with two capsule and a 149% increase with the three capsule treatment (Figure 2.2). Tomato had significant increase in total leaf area with the addition of capsule treatment ($p<0.0001$). There was a 61% increase with half capsule treatment, a 123% increase with one capsule, and maximum increase of a 126% with two capsule treatments (Figure 2.2). There was a slight decrease in total leaf area with the three capsule treatments with a 116% increase (Figure 2.2). Pepper plants exhibited a similar trend as tomato, a significant increase in total leaf area was measured with the increasing amount of

capsule treatment ($p<0.0001$), with a 44% increase with the half capsule treatment, a 92% increase with one capsule, and maximum increase of a 144% with the two capsule treatment (Figure 2.2). Three capsule treatments were not significantly different from the two capsules treatment with a 149% increase (Figure 2.2). Corn did not respond as well to the capsule treatment and had the least increase in total leaf area of the six crops used in the study; however, there was significant increase in total leaf area ($p<0.0006$). There was a 7% increase with half capsule, a 15% increase with one capsule, and maximum increase of 20% with two capsules treatment (Figure 2.2). Three capsule treatments had a significant decrease in total leaf area compared to the two capsule treatments, with 10% increase (Figure 2.2).

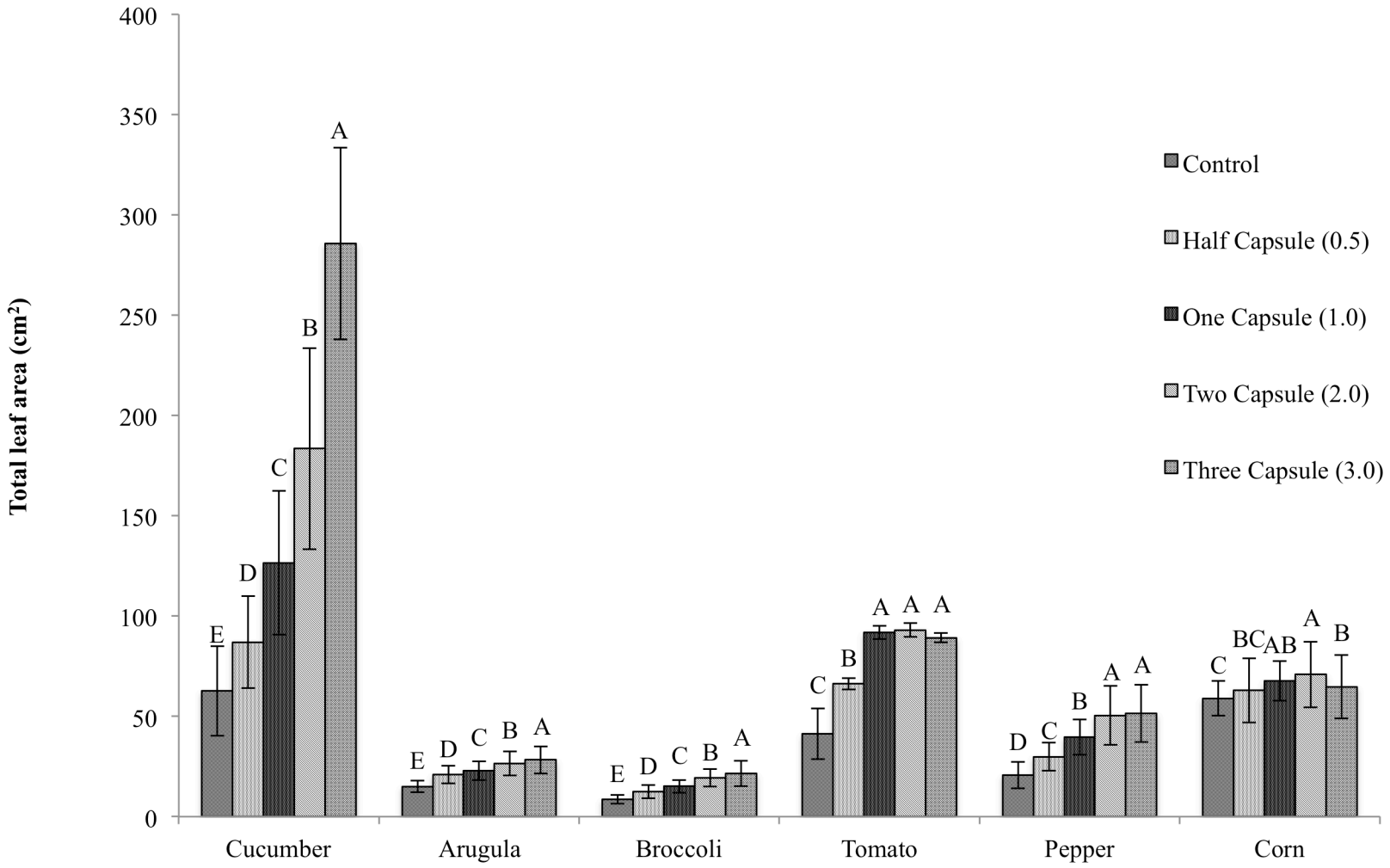

Figure 2.2: Mean total leaf area 28 days after emergence of six crops: cucumber ('Vlaspik'), arugula ('Astro'), broccoli ('Centura'), tomato ('Talladega'), pepper ('Boyton Bell') , and corn ('Cornell D2901') with five treatments: control, half capsule (0.5), whole capsule (1.0), two capsules (2.0), and three capsules (3.0). Data analyzed by ANOVA ($\alpha = 0.05$) and Tukey's HSD test was used to conduct mean separation comparison. Letters not associated with same letter are significantly different. Bars represent standard deviation

Similar to total leaf area, all of the crops exhibited significant increases in fresh weight with the capsule treatments ($p<0.0001$). The magnitude of the effect of the capsule treatment on fresh weight was, as in the case with total leaf area, specific to each crop (Table 2.3). Cucumber exhibited the greatest increase in fresh weight by the capsule treatment ($p<0.0001$). There was a significant difference between the treatments ($p<0.0001$) of 62% increase with half capsule, a 228% increase with one capsule, a 371% increase with two capsule treatment, and maximum fresh weight increase of 718% with a three capsule treatment (Figure 2.3). Arugula exhibited significant differences between treatments ($p<0.0001$) with a 50% increase with half capsule treatment, a 63% increase with one capsule, a 100% increase with two capsule, and maximum increase was achieved with three capsule treatment with 114% increase (Figure 2.3). Broccoli had a similar trend in fresh weight increase as arugula. There was significant difference between the treatments ($p<0.0001$) with a 39% increase with half capsule treatment, a 63% increase with one capsule, a 112% increase with two capsule and a 121% increase with three capsule treatment (Figure 2.3). Tomato also exhibited a large increase in fresh weight with the addition of the capsule treatments. There were significant differences between the treatments ($p<0.0001$) with a 70% increase with half capsule treatment, a 148% increase with one capsule, and maximum increase of 157% with two capsule treatments (Figure 2.3). There was a significant decrease in fresh weight with three capsule treatment compared to two capsule treatment ($p<0.0001$), with 131% increase (Figure 2.3). Pepper plants exhibited a similar trend as tomato a significant difference between the treatments ($p>0.0001$) with a 54% increase with half capsule treatment, 105% increase with one capsule, and greatest increase of 156% with the two capsule treatment (Figure 2.3). There was no significant difference between the fresh weight of two capsule

treatments and three capsule treatments with 148% increase (Figure 2.3). Corn did not respond and had the least increase in fresh weight out of the six crops used in the study. There was significant difference between the treatments ($p<0.016$), a 5% increase with half capsule, an 18% increase with one capsule, and maximum increase of 22% with two capsules treatment (Figure 2.3). Three capsule treatments were not significantly different from the control with only 11% increase (Figure 2.3).

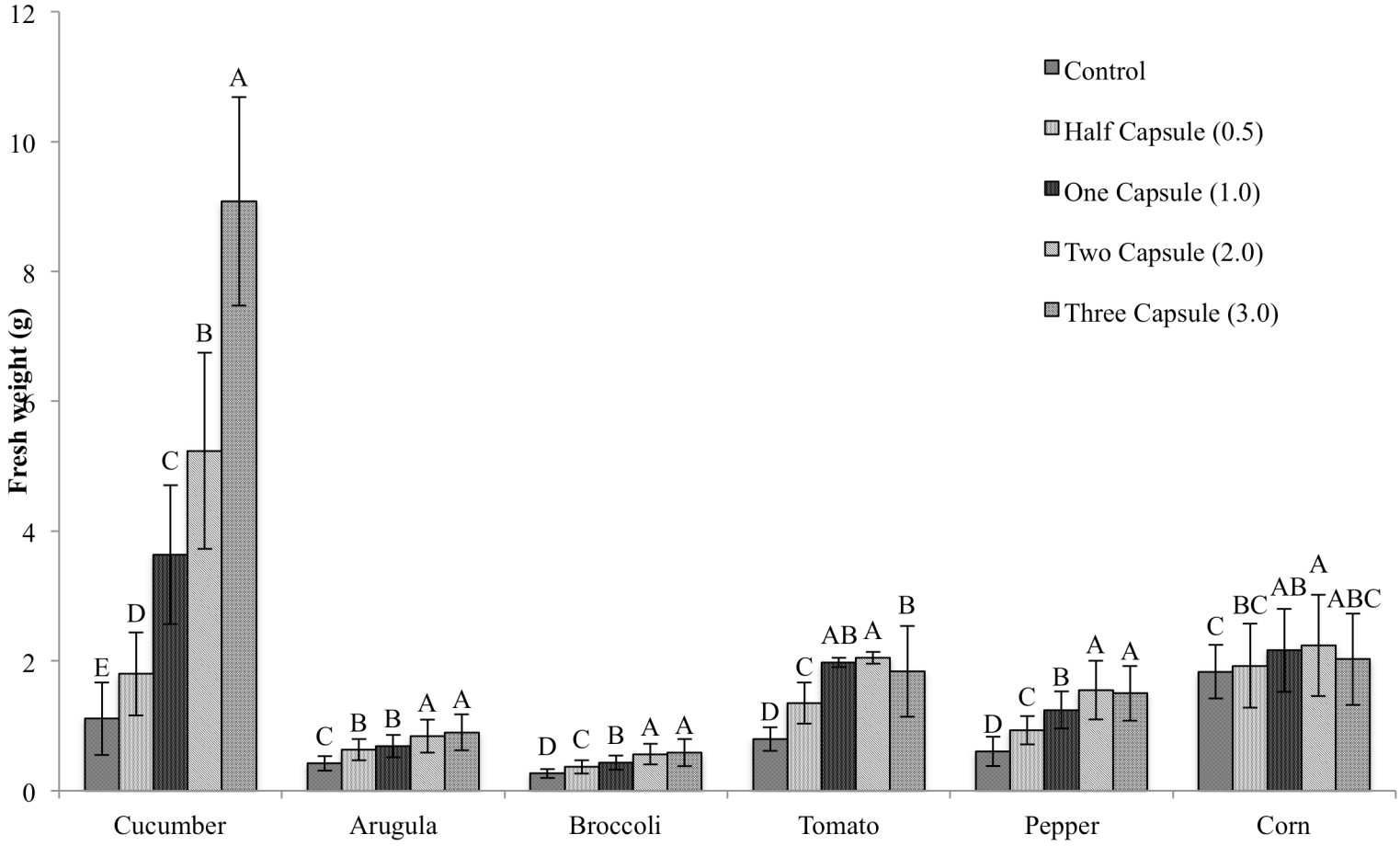

Figure 2.3: Mean fresh weight 28 days after emergence of six crops: cucumber ('Vlaspik'), arugula ('Astro'), broccoli ('Centura'), tomato ('Talladega'), pepper ('Boyton Bell') , and corn ('Cornell D2901') with five treatments: control, half capsule (0.5), whole capsule (1.0), two capsules (2.0), three capsules (3.0). Data analyzed by ANOVA ($\alpha = 0.05$) and Tukey's HSD test was used to conduct mean separation. Letters not associated with same letter are significantly different. Bars represent standard deviation.

Similar to total leaf area and fresh weight, all of the crops exhibited significant increase in dry weight with the capsule treatments ($p<0.005$). The magnitude of the effect of the capsule treatment on dry weight was similar to those in total leaf area and fresh weight and again was specific to each crop (Table 2.4). Cucumber plant exhibited significant difference between the treatments ($p<0.0001$), a 29% increase with half capsule, an 84% increase with one capsule, a 138% increase was achieved with two capsule treatment, and a maximum increase of 251% with three capsule treatment (Figure 2.4). Arugula exhibited significant difference between the treatments ($p<0.0001$), a 29% increase with half capsule treatment, a 35% increase with one capsule, a 142% increase with two capsules, and the maximum increase was achieved with three capsule treatment with 48% increase (Figure 2.4). Broccoli had a similar trend in dry weight increase as arugula, with significant difference between the treatments ($p<0.0001$). There was a 26% increase with half capsule treatment, a 44% increase with one capsule, a 49% increase with two capsules and a 57% increase with three capsules treatment (Figure 2.4). Tomato also exhibited a large increase in dry weight with the addition of capsules. There were significant differences between the treatments ($p<0.0001$), a 37% increase with half capsule treatment, a 56% increase with one capsule, a 45% increase with the two capsules treatment (Figure 2.4). There was a slight decrease in dry weight with three compared to the two capsule treatments (Figure 2.4). Pepper plants exhibited significant differences between the treatments ($p<0.0001$), a 67% increase with half capsule treatment, a 114% increase with one capsule, and a maximum increase of 243% with the two capsules treatment (Figure 2.4). There was no significant difference in fresh weight between two capsules treatment and three capsules treatment with 228% increase (Figure 2.4). Corn showed little response to the capsule treatment and had the

least increase in dry weight out of the six crops used in the study. There were significant differences between treatments ($p<0.0198$), a 5% increase with half capsule, a 14% increase with one capsule, and a maximum increase of 18% increase with two capsule treatment (Figure 2.4). Three capsule treatments had no significant difference from the control with an 11% increase. The dry weight percent increase for corn was similar to the fresh weight percent increase (Figure 2.3 and 2.4).

The growth response to capsule treatments can be grouped according the plant growth response. Cucumber, arugula and broccoli exhibited significant linear trend ($p<0.0001$) in total leaf area, fresh weight, and dry weight in relation to gelatin capsule application rate. Tomato, and pepper exhibited significant quadratic trend ($p<0.0001$) in total leaf area, fresh weight, and dry weight in relative to the gelatin capsule rate. Corn exhibited significant quadratic trend in total leaf area ($p<0.0003$), fresh weight ($p<0.0054$) and dry weight ($p<0.0126$) in relative to the gelatin capsule rate (Table 2.2).

Table 2.2: Trend analysis by crop for each growth parameter (total leaf area, fresh weight and dry weight)

Crops	**Total leaf area**	**Fresh weight**	**Dry weight**
Cucumber	Linear ($p<0.0001$)	Linear ($p<0.0001$)	Linear ($p<0.0001$)
Arugula	Linear ($p<0.0001$)	Linear ($p<0.0001$)	Linear ($p<0.0001$)
Broccoli	Linear ($p<0.0001$)	Linear ($p<0.0001$)	Linear ($p<0.0001$)
Tomato	Quadratic ($p<0.0001$)	Quadratic ($p<0.0001$)	Quadratic ($p<0.0001$)
Pepper	Quadratic ($p<0.0001$)	Quadratic ($p<0.0001$)	Quadratic ($p<0.0001$)
Corn	Quadratic ($p<0.0003$)	Quadratic ($p<0.0054$)	Quadratic ($p<0.0126$)

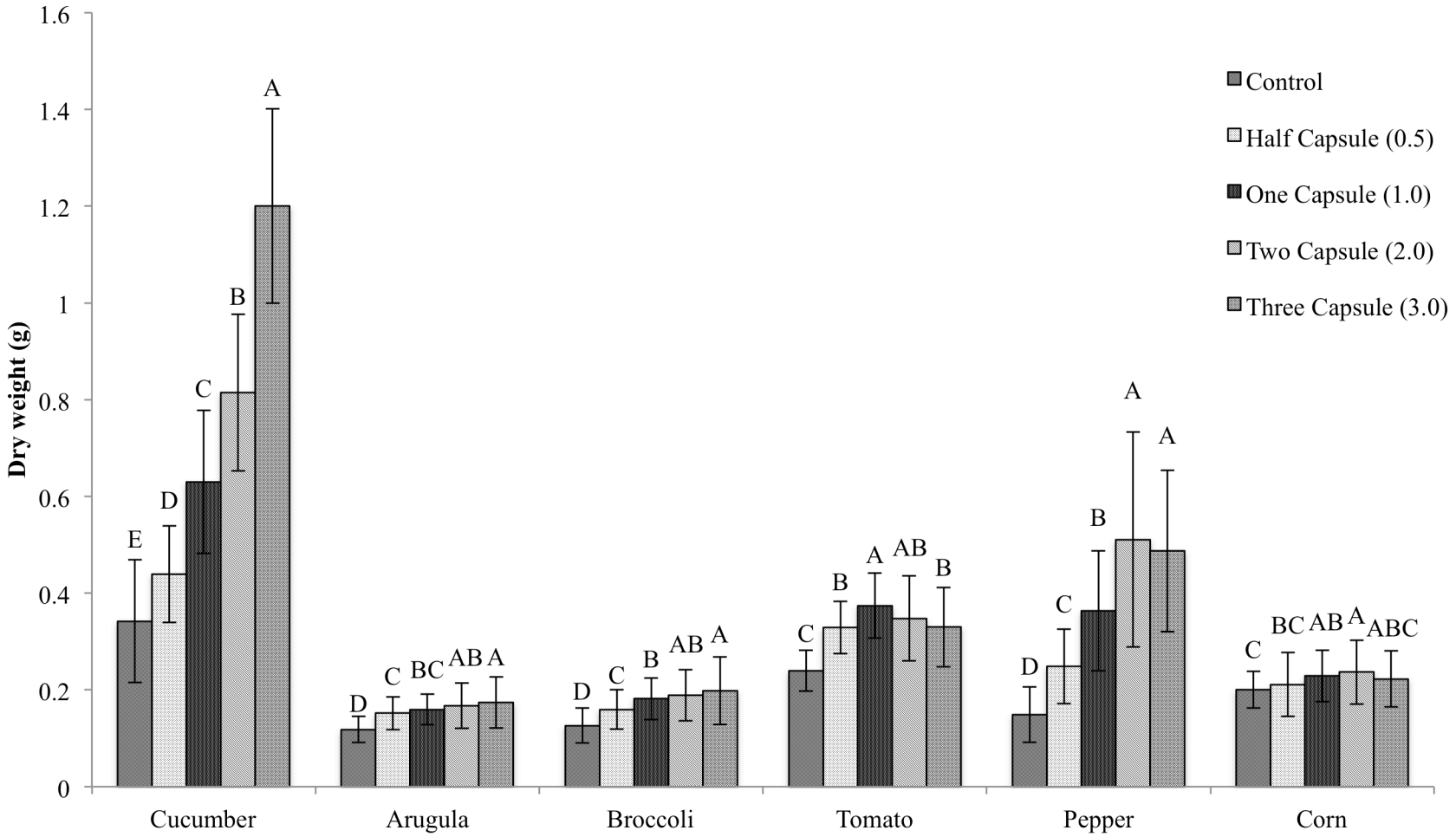

Figure 2.4: Mean dry weight 28 days after emergence of six crops: cucumber ('Vlaspik'), arugula ('Astro'), broccoli ('Centura'), tomato ('Talladega'), pepper ('Boyton Bell') , and corn ('Cornell D2901') with five treatments: control, half capsule (0.5), whole capsule (1.0), two capsules (2.0), and three capsules (3.0). Data analyzed by ANOVA ($\alpha = 0.05$) and Tukey's HSD test was used to conduct mean separation comparison. Letters not associated with same letter are significantly different. Bars represent standard deviation

Effect of capsule treatment levels on nitrogen content in cucumber plant tissue

The results from the physiological parameters indicate a significant increase in total leaf area, fresh weight, and dry weight of the plants with the capsule treatment, which lead to the question if the capsule treatment is acting as a nitrogen source for plant growth. In order to address this question, total nitrogen of the plant tissue was measured in response to supplemental application of nitrogen from the capsules.

All the cucumber plants that were treated with gelatin capsules exhibited an increase in total percent nitrogen compared to the control (Figure 2.5) There were significant differences in total percent nitrogen between the treatments ($p<0.0001$). Three capsules treatment exhibited the greatest increase in total percent nitrogen with 104%, followed by two capsules treatment with 77%, one capsule treatment with 51%, and half capsule treatment with 17%. The control had the lowest total nitrogen percent with 17%. The trend exhibited by the total nitrogen percent is similar to the one observed in the total leaf area, fresh weight and dry weight (Figure 2.2, 2.3, 2.4).

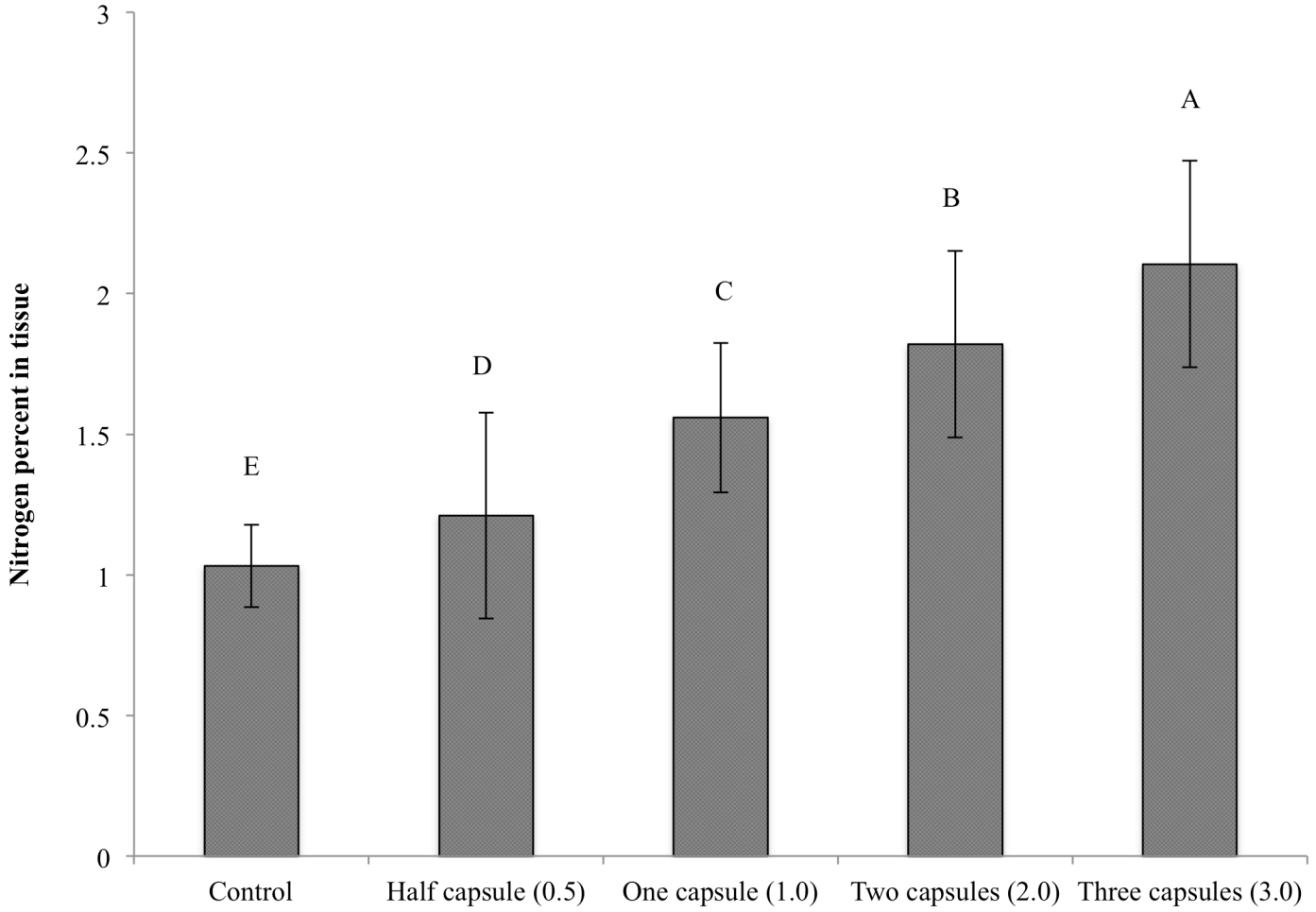

Figure 2.5: Mean nitrogen percent in tissue 28 days after emergence of cucumber ('Vlaspik') measured for five treatments: control (0.0), half capsule (0.5), one capsule (1.0), two capsules (2.0), and three capsules (3.0). Data analyzed by ANOVA ($\alpha = 0.05$) and Tukey's HSD test was used to conduct mean separation. Letters not associated with same letter are significantly different. Bars represent standard deviation.

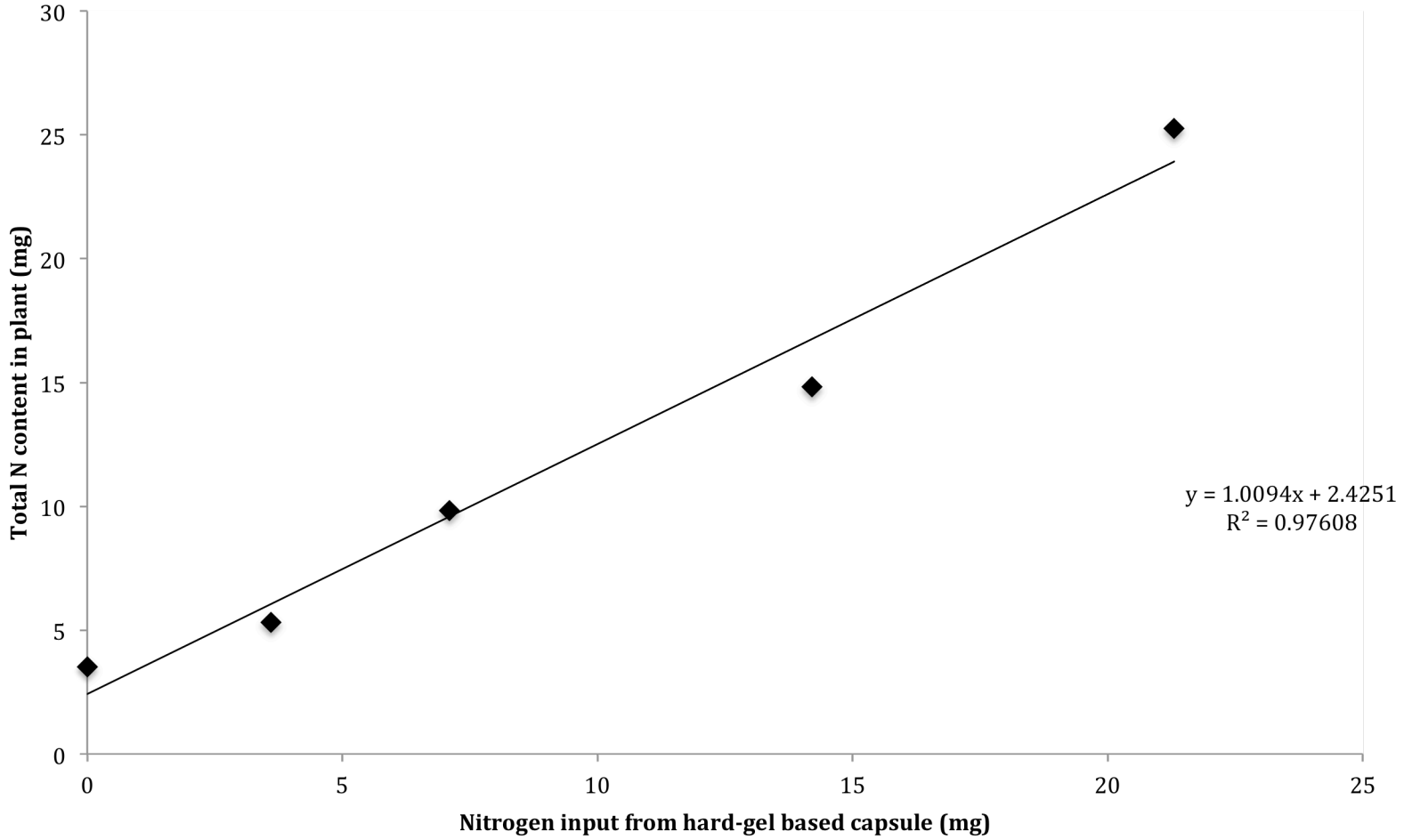

Figure 2.6: Correlation graph between nitrogen input from gelatin capsules (mg) and nitrogen recovered from plant tissue (mg)

DISCUSSION

The capsule treatment applied adjacent to the cucumber seed affected the subsequent growth of the cucumber plant. The capsule treatment exhibited an additive effect, with half capsule exhibiting least increase in plant growth, and two capsules exhibiting the greatest effect (Figure 2.1). The effect of the capsule treatment on cucumber plant was found only in the above ground part of the plant. There were significant increases in plant height (17%), petiole length (37%), total leaf area (48%), shoot fresh weight (45%), and shoot dry weight (28%) with two capsules treatment compared with the control (Figure 2.1). However, the belowground part of the plant did not benefit from the capsule treatment, and root growth was reduced by the same treatment that contributed to the increased shoot growth. There was a decrease of 10% in root dry weight with two capsules treatment (Figure 2.1). The results illustrate that addition of gelatin capsules around the seed had a positive effect on the subsequent plant growth that was limited to the above ground part of the cucumber plant.

The six crops examined in the study (cucumber, arugula, broccoli, tomato, pepper, and corn) exhibited increased total leaf area, fresh weight, and dry weight with the addition of capsule treatments (Figure 2.2, 2.3 and 2.4). However, the crops exhibited different level of response to each treatment and thus exhibited a range of growth to the different treatment levels.

The magnitude or the percent increase compared to the control plants differed from crop to crop. Cucumber plants exhibited a 356% increase in total leaf area with three capsules treatment, whereas corn only had a 10% increase in total leaf area with the same level of treatment. Likewise, tomato had 157% increase in fresh weight with two capsules treatment, but corn only

had a 22% increase in fresh weight with the same treatment. Trend analysis revealed a liner trend in the three growth parameters for cucumber, arugula and broccoli, while a quadratic response was revealed for tomato and pepper (Table 2.2). A quadratic trend was measured for dry weight increase in corn, but was only significant at the ($p<0.0126$). The quantity and form of nitrogen nutrition have been known to affect plant growth and ultimately affect the yield and quality of crop (Gunes, Inal, and Aktas 1996). The magnitude difference of the effect of gelatin capsule in this study is thought to be due to difference in the uptake mechanism of hydrolyzed collagen that is the main ingredient in gelatin capsule. Hydrolyzed collagen, a polypeptide, is comprised of amino acids that are composed of amine and carboxylic acid functional groups. The amine group contains nitrogen and can be used as a nitrogen source in plant fertilizers (Schrieber and Gareis 2007). Most plants acquire nitrogen not as nitrate and ammonium but also as organic nitrogen forms such as amino acids and protein from the soil (Nasholm, Kielland, and Ganeteg 2009; Tegeder and Rentsch 2010). Transport studies with isolated membrane vesicle and plant tissue uncovered presence of multiple transport systems for amino acids (Tegeder and Rentsch 2010). Amino acid transporters have been reported to play a variety of roles in growth and development in plants. They function in long-distance transport, acquisition by import-dependent cells, and intracellular partitioning between different compartments in the cell (Liu and Bush 2006).

Two subfamilies of amino acid transporters have been identified; the amino acid, polyamine and choline transporters superfamily (APC) and amino acid transporter family (ATF) (Fischer 1998; Ortiz-Lopez et al. 2000; Su et al. 2004). There are 14 APC transporters in the *Arabidopsis* genome, among these transporters cationic amino acid transporters (CAT), high-affinity basic amino acid transporters, are well characterized (Su et al. 2004). The ATF superfamily contains

five subclasses of transporter gene families: amino acid permeases (AAPs) (Frommer et al. 1993), the lysine/histidine transporters (LHTs) (Chen and Bush 1997), the proline transporters (ProTs) (Rentsch et al. 1996), the aromatic and neutral amino acid transporters (ANTs) (Chen et al. 2001), and the putative auxin transporters (AUXs) (Bennett et al. 1996). The functional significance of these amino acid transporters are not only defined by the substrate specificity, but also by expression pattern controlled by developmental stages and differential responses to environmental cues (Liu and Bush 2006).

Uptake of amino acid by roots is an energy-driven process, where H^+ ATPase generates the proton motive gradient to drive the amino acid and H^+ cotransport (Fischer 1998; Badalucco and Nannipieri 2007). Once the amino acid is taken up into the root cytoplasm, they are assimilated by GS/GOGAT for production of new cell biomass and to generate energy through deamination, which will then be used as a substrate (keto acid) to be introduced into the TCA cycle. Following uptake, amino acids can also be translocated to the shoot via the xylem where some amino acids may also return to the root via the phloem (Fischer 1998; Badalucco and Nannipieri 2007; Schenk 1996). It is speculated that the variation in nitrogen uptake (transporters) and utilization mechanism (efficiency of GS/GOGAT) is the probable cause for the differences in plant growth between crops from gelatin capsule application.

The increase in nitrogen availability in the soil is reflected in the nitrogen content of plants, thus increase in total nitrogen in the plant tissue in response to the capsule treatment indicates that the amino acid in the gelatin capsule are being used as nitrogen source for the plant or that the treatment is promoting nitrogen metabolism in the plant (Schenk 1998). The nitrogen

utilization efficiency of the plants was the same across all treatments (~5%; data not shown) and thus the increase in nitrogen percent in the plant tissue can be attributed to increased nitrogen supplied by the gelatin capsule treatments. The linear correlation of the nitrogen input to the nitrogen recovered from the plant tissue further demonstrates that the nitrogen in the plant tissue was directly correlated to the nitrogen supplied by the gelatin treatment (Figure 2.6).

Protein hydrolysate has been shown to stimulate carbon and nitrogen metabolism and increase nitrogen assimilation in plants (Schiavon et al. 2008; Ertani et al. 2009). NAD-dependent glutamate dehydrogenase, nitrate reductase and malate dehydrogenase in maize has been reported following application of animal epithelial hydrolysate (Maini 2006). Alfalfa protein hydrolysate applied to hydroponically-grown maize increased the activity malate dehydrogenase, isocitrate dehydrogenase, and citrate synthase, which are enzymes found in TCA cycle; as well as nitrogen metabolism enzymes, nitrate reductase, nitrite reductase, glutamine GS, GOGAT, and aspartate aminotransferase (Schiavon et al. 2008). Ertani et al. (2009) reported that both alfalfa protein hydrolysate and animal connective tissue hydrolysate stimulated plant growth, and also increase the nitrate conversion into organic nitrogen by inducing nitrate reductase and GS activities. The treatments enhanced especially GS2 isoform, which is responsible for assimilation of ammonia produced by nitrate reduction, suggests that protein hydrolysate enhances plant growth by up regulating nitrate assimilation (Ertani et al. 2009; Calvo et al. 2014).

Further investigation is required to determine 1) if the amino acid composition of the gelatin capsule contributes to the growth promotion, and 2) the mechanism in which gelatin capsules contributes to plant growth promotion.

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CHAPTER 3: Hydrolyzed collagens applied at sowing to cucumber enhances plant growth

ABSTRACT

The growth promotional effect of the gelatin capsule was partially attributed to an increase in nitrogen content in the plant tissue in the previous study (Chapter 2). The effect of types of gelatin (hydrolyzed collagen) and the proportions of amino acids found in gelatin was studied on enhancing cucumber plant growth. Treatment with hydrolyzed collagen with small molecular weight, such as gelatin hydrolysate and a treatment containing an amino acid mixture of the same proportion as found in gelatin capsule had a lower leaf area compared to plants treated with two gelatin capsules. However, all treatments had a larger biomass compared to the nontreated control. Growth comparison between two gelatin capsules and urea with equivalent amount of nitrogen revealed that although there was no significant difference in nitrogen content between the treatments that the gelatin treatment had a greater leaf area. These results imply that nitrogen fertility alone could not account for the increased plant growth. Gelatin is rich in the amino acids proline and hydroxyproline. However, amino acid mixtures lacking each amino acid singly or both amino acids did not affect plant growth that indicated that these amino acids were not responsible for the growth enhancement measured in plants treated with gelatin capsules.

INTRODUCTION

The main ingredient of collagen-based gelatin capsules used in seed encapsulation is hydrolyzed collagen. Collagen is a class of naturally occurring proteins found primarily in the flesh and connective tissues of animals (Balian and Bowes 1977; Schrieber and Gareis 2007). Collagen can be broken down through hydrolysis to form hydrolyzed collagen, which is known as gelatin (Balian and Bowes 1977; Regenstein and Boran 2010). Gelatin is one of the most widely used biopolymers with wide application in foods, pharmaceuticals, cosmetics, and photographic films, and other products including paints, matches, fertilizers, as a gelling agent foam stabilizer, and structure enhancer (Karim and Bahat 2009; Gudmundsson 2002; Yang et al. 2007; Hou and Regenstein 2004; Takahashi and Trias 2012). The chemical composition of gelatin is similar to that of collagen; however, unlike its parent molecule, gelatin is not composed of a single sized peptide chain, but rather many fractions of peptide chains varying in size, including the whole α-chain of the whole collagen molecule and hydrolytic fragments of parts of the α-chain of different lengths (Regenstein and Boran 2010; Eastoe and Leach 1977).

Hydrolyzed collagen is also a part of a class of substances called plant biostimulants that enhance plant growth. The European biostimulants industry council (EBIC) has defined plant biostimulants as "substance(s) and/or microorganisms whose function when applied to plants or the rhizosphere is to stimulate natural processes to enhance/benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and crop quality. Biostimulants have no direct action against pests, and therefore do not fall within the regulatory framework of pesticides" (EBIC 2014; du Jardim 2012).

The amino acid contents of protein hydrolysates vary depending on the production method as well as the source of the material. Animal based hydrolysates, such as gelatin, contains a high proportion of proline and glycine, while plant based hydrolysates contain a high proportion of glutamine and arginine (Calvo et al. 2014; Parrado et al. 2008; Ertani, Pizzeghello, et al. 2013). Gelatin contains 18 different amino acids with an average of 1000 amino acid per/chain (Table 3.1). Gelatin has unique amino acid composition with a high content of proline and hydroxyproline. The amount of tryptophan and cystein are extremely low in gelatin. This means there are few disulfide bonds involved in gelatin structure (Belitz et al. 2004). Hydroxyproline is especially important for its primary role in the structure and maintenance of gelatin. Hydroxyproline is confined exclusively to the cell wall proteins in plants (Lamport 1965), and may account for up to 10% of the amino acid content of purified primary cell walls and is derived from the hydroxyproline rich glycoprotein present in the primary cell walls (Kieliszewski and Shpak 2001). Vaughan and Cusens (1973) revealed that hydroxyproline enhanced the extension growth of pea root segments without affecting their protein synthesis or respiration. They proposed that externally supplied hydroxyproline enhanced extension growth in root by interfering with some aspect of cell wall protein synthesis involving hydroxyproline rich protein (HRP) (Vaughan 1973; Vaughan and Cusens 1973).

Other non-protein components present in hydrolysate are also thought to contribute to the stimulatory effect in plants. Plant based hydrolysates contain fats, carbohydrates, macro and micronutrients and phytohormones such as auxin, gibberellins and cytokines (Ertani, Schiavon, et al. 2013; Parrado et al. 2008; Schiavon et al. 2008). Animal based hydrolysates, on the other

hand, have a similar content of proteins, amino acids, fats, macro and micronutrients, but lacked carbohydrates and phytohormones (Parrado et al. 2008; Calvo et al. 2014).

Exogenous application of amino acids such as glutamate, proline, and glycine betaine provide abiotic stress protection (Vranova et al. 2011; Rhodes, Verslues, and Sharp 1999; Liang et al. 2013). These amino acids act as osmoprotectants, stabilizing proteins and membranes from denaturing effects of high salt concentration (Ashraf and Foolad 2007; Chen and Murata 2011; Ahmad, Lim, and Kwon 2013). They also act as reactive oxygen species scavengers, to induce expression of salt stress responsive genes and genes involved transcription factors, in membrane trafficking (Kinnersley and Turano 2000; Ashraf and Foolad 2007; Anjum et al. 2011; Liang et al. 2013). Accumulation of glycine betaine and proline was strongly correlated with stress tolerance in many crops, and exogenous application of these amino acids has been shown to enhance abiotic stress tolerance (Chen and Murata 2011; dos Reis, Lima, and Medeiros de Souza 2012; Ahmad, Lim, and Kwon 2013; Calvo, Nelson, and Kloepper 2014; Ashraf and Foolad 2007; El-Samad, Shaddad, and Barakat 2010).

The effect of gelatin applied at time of sowing on plant growth was physiologically characterized in the previous chapter (Wilson 2014, chap. 2). The growth promotional effect of the gelatin capsule was partially attributed to the increase in N content in the plant tissue in the previous study (Wilson 2014, chap. 2). The effect of different hydrolyzed collagen type on cucumber plant growth has not been previously determined. The object of this study is to characterize different types of hydrolyzed collagen and determine if the type of hydrolyzed collagen affects cucumber plant growth. Urea was used to determine if the growth promotion by

the gelatin capsules was caused by the N fertilizer effect from the amino acids. The effect of amino acid proline and hydroxyproline on plant growth was also investigated, as it is hypothesized that these amino acids may promote plant growth.

MATERIALS AND METHODS

Analysis of different hydrolyzed collagen types on molecular weight distribution

Ten gram samples of three different hydrolyzed collagen types: granulated 220 bloom gelatin, granulated gelatin capsule, and gelatin hydrolysate were submitted to Eastman Gelatin Co. (Peabody, MA) for molecular weight distribution analysis. Molecular weight distribution (MWD) of hydrolyzed collagen was performed by high-performance liquid chromatography (HPLC) in the aqueous size exclusion mode (AEC). Gelatin samples were dissolved in the chromatographic eluent, a phosphate buffer containing sodium dodecyl sulfate (SDS). Different molecular weight fractions were separated on a Toso Haas TSK Gel size exclusion column and the effluent was monitored with a UV detector set at 220 nm. Known molecular weight standards were run to prepare a calibration curve, which was constructed by plotting the log of these molecular weights versus the retention time. The molecular weight distributions of unknown gelatin samples were then determined from the linear portion of the calibration curve.

Results were reported in terms of relative area percent of 5 molecular weight regions that had been designated: the high molecular weight fraction (greater than 250,000 g mol^{-1}), the beta fraction (between 150,000 g mol^{-1} and 250,000 g mol^{-1}), the alpha fraction (between 50,000 g mol^{-1} and 150,000 g mol^{-1}), the sub-alpha fraction (between 20,000 g mol^{-1}, and 50,000 g mol^{-1}),

and the low molecular weight fraction (between 4,000 g mol^{-1} and 20,000 g mol^{-1}) of the size exclusion elution profile (Table 3.2).

Table 3.2: Five molecular weight regions that have been designated by the size exclusion elution profile and the corresponding molecular weight region of each fraction (Farrugia et al. 1998)

Fraction	Molecular Weight Region
High Molecular Weight Fraction (HMW)	> 250,000
Beta Fraction	150,000 – 250,000
Alpha Fraction	50,000 – 150,000
Sub Alpha Fraction	20,000 – 50,000
Low Molecular Weight Fraction	4,000 – 20,000

Effect of different hydrolyzed collagens on plant growth

Cucumber seeds 'Vlaspik' (Seminis) were planted in a controlled greenhouse maintained at 24°C/21°C temperature with 14/10 hours photoperiod at New York State Agricultural Experiment Station in Geneva, NY in the winter of 2013. Six treatments were compared; control, two #3 gelatin capsules (Capsule line, Pompano Beach, FL), hydrolyzed gelatin with bloom value of 220 (bloom 220), gelatin hydrolysate (Eastman Gelatin Co, Peabody, MA), urea (Sigma Aldrich, St. Louis, MO) and an amino acid mixture (Sigma Aldrich, St. Louis, MO) (Table 3.1). Two gelatin capsules were placed adjacent to each seed in a four-inch pot (Illustration 3.1) to prevent a decrease in the germination rate, and for controlling the amount of gelatin used in the treatment. Bloom 220 gelatin was chosen as it has the same bloom value as the gelatin capsule. Gelatin hydrolysate was chosen for its small molecular size and its unique characteristic of lack

of gelling capability. To determine if the growth promotion by the gelatin capsules was caused by the N fertilizer effect from the amino acids in the gelatin, urea was included as a treatment. Amino acid mixture was chosen for its small molecular size (300kDa) and to determine if the amino acids alone could affect plant growth. The bloom 220 gelatin, gelatin hydrolysate, urea and amino acid mixture was weighed to the equivalent weight of two gelatin capsule (90 mg) and applied to the seed as powders in the pot. Each treatment had a total of 75 samples arranged in 5 replications of 15 samples per block, and the treatments were placed in a random block design on the greenhouse bench. The plants were harvested 28 days after emergence and leaf area, fresh weight and dry weight were measured. Total leaf area was measured using a CI-202 Leaf Area Meter (CID Bio-Science, Camas, WA). Data was then statistically analyzed by ANOVA ($\alpha = 0.05$) and Tukey's HSD test was used to conduct mean separation comparison.

Table 3.1: List of amino acid and its relative amount in 100g of gelatin protein and equivalent amount in two gelatin capsule (Gelatin Manufacturers Institute of America 2012)

Amino Acid	Amino acid (g) per 100 g of gelatin protein	Amino Acid equivalent of two capsule (mg)
Alanine	11.3	9.00
Arginine	9	7.20
Aspartic acid	6.7	5.30
Glutamic acid	11.6	9.20
Glycine	27.2	21.7
Histidine	0.7	0.60
Proline	15.5	1.30
Hydroxyproline	13.3	10.6
Hydroxylysine	0.8	0.60
Isoleucine	1.6	2.80
Leucine	3.5	3.50
Lysine	4.4	0.50
Methionine	0.6	2.00
Phenylalanine	2.5	12.2
Serine	3.7	2.90
Threonine	2.4	1.60
Tryptophan	0	0.00
Tyrosine	0.2	0.20
Valine	2.8	0.22

Illustration 3.1: Two gelatin capsules (four halves) placed adjacent to the seeds in 4-inch pot.

Effect of different hydrolyzed collagens on nitrogen content in cucumber plant tissue

Dry cucumber plant tissue was ground with a Wiley mill (Thomas Scientific Swedesboro, NJ) to a particle size of 2 mm. One hundred mg samples were sent to Cornell University Stable Isotope Laboratory (Ithaca, NY) for elemental analysis of N by combustion method. Data was then statistically analyzed by ANOVA ($\alpha = 0.05$) and Tukey's HSD test was then used to conduct mean separation comparison.

Seed coating with different amino acid mixtures

A laboratory-scale rotary pan, R-6 (Universal Coating Systems, Independence, OR) in the Seed Science and Technology program at Cornell University (Geneva, NY) was used in this project. The six-inch (15 cm) diameter rotary pan was used to coat 50 g of cucumber seeds 'Vlaspik' (Seminis, St Lois, MO) with coating powder CS-OC-03 (Coating Supply Inc., Palm Beach, FL) and 15% SELVOL™ Polyvinyl Alcohol (prepared according to manufactures specification) (Sekisui Specialty Chemicals America, Dallas, TX) as a binder. The polyvinyl alcohol was selected as a binder due to its relatively low molecular weight distribution of 50,000

g mol^{-1} to 31,000 g mol^{-1} (Sekisui Specialty Chemicals America, SELVOL™ Polyvinyl Alcohol product description), but also as it does not contain any N that could affect plant growth. Amino acid mixtures (Table 3.3) with different proportions of proline, hydroxyproline and glycine were mixed in the coating powder and coated on the cucumber seeds. The amino acid mixtures were designed to assess the effect of amino acids, proline and hydroxyproline on the plant growth. They were selected as a unique candidate as hydrolyzed collagen contains high concentration of these two amino acids.

Five different amino acid mixtures were used for seed coating: an amino acid mixture containing all the amino acids found in hydrolyzed collagen (All Amino Acid), all amino acids found in hydrolyzed collagen with proline replaced by glycine (No Proline), all amino acids found in hydrolyzed collagen with hydroxyproline replaced by glycine (No Hydroxyproline), and all amino acids found in hydrolyzed collagen with proline and hydroxyproline replaced by glycine (No Proline/Hydroxyproline) (Table 3.3). A control (No Amino Acid) contained no amino acid, but was coated with 15% PVA and coating powder CS-OC-03. Seed coating was on average 50% weight buildup, 25 ml of 15% PVA and 25 g of CS-OC-03 coating powder for 50 g of seed (1770 seeds). Due to the effect of increasing seed coating build up on seed germination, only 10 mg of amino acid per seed was applied to the seeds.

Effect of different amino acid mixture ratios on plant growth

Cucumber seeds 'Vlaspik' (Seminis) coated with 25 ml of 15% PVA, 25 g of coating powder CS-OC-03, and different amino acid mixture were planted in controlled greenhouse maintained at 24°C/21°C temperature with 14/10 hours photoperiod at New York State

Agricultural Experiment Station in Geneva, NY in the spring of 2013. Six treatments were compared; control (No Amino Acid), amino acid mixture containing all amino acids found in hydrolyzed collagen (All Amino Acid), all amino acids found in hydrolyzed collagen with proline replaced by glycine (No Proline), all amino acids found in hydrolyzed collagen with hydroxyproline replaced by glycine (No Hydroxyproline), and all amino acids found in hydrolyzed collagen with proline and hydroxyproline replaced by glycine (No Proline/Hydroxyproline). Each treatment had a total of 75 samples arranged in 5 replications of 15 samples per block, and blocks were arranged in randomized block design? on the greenhouse bench. The plants were harvested 28 days after emergence and leaf area, fresh weight and dry weight were measured. Total leaf area was measured using CI-202 Leaf Area Meter (CID Bio-Science, Camas, WA). Data was then statistically analyzed by ANOVA ($\alpha = 0.05$) and Tukey's HSD test was then used to conduct mean separation comparison.

Table 3.3: Amino acid composition of seed coating for 1000 seeds (~50 g of seeds) containing all amino acid found in hydrolyzed collagen (All Amino Acid), all amino acid found in hydrolyzed collagen with proline replaced by glycine (No Proline), all amino acid found in hydrolyzed collagen with hydroxyproline replaced by glycine (No Hydroxyproline), and all amino acid found in hydrolyzed collagen with proline and hydroxyproline replaced by glycine (No Proline/Hydroxyproline).

	All Amino acid (g)	No Proline (g)	No Hydroxyproline (g)	No Proline/Hydroxyproline (g)
Alanine	1.706	1.706	1.706	1.706
Arginine	1.364	1.364	1.364	1.364
Asparagine	1.004	1.004	1.004	1.004
Glutamine	1.744	1.744	1.744	1.744
Glycine	4.112	6.424	6.121	8.433
Histidine	0.114	0.114	0.114	0.114
Isoleucine	0.246	0.246	0.246	0.246
Hydroxyproline	2.009	2.009	0	0
Hydroxylysine	0.114	0.114	0.114	0.114
Leucine	0.531	0.531	0.531	0.531
Lysine	0.663	0.663	0.663	0.663
Methionine	0.095	0.095	0.095	0.095
Phenylalanine	0.379	0.379	0.379	0.379
Proline	2.312	0	2.312	0
Serine	0.55	0.55	0.55	0.55
Threonine	0.303	0.303	0.303	0.303
Tyrosine	0.038	0.038	0.038	0.038
Valine	0.417	0.417	0.417	0.417

RESULTS

Analysis of different hydrolyzed collagen types on molecular weight distribution

The molecular weight distribution (MWD) of hydrolyzed collagen was determined by high-performance liquid chromatography (HPLC) in the aqueous size exclusion mode (AEC). According to the size exclusion elution profile of the different hydrolyzed collagen, gelatin hydrolysate had the smallest molecular weight, and 100% of its fraction was in the low molecular weight fraction, which has a molecular weight region of < 20,000 g mol^{-1} (Figure 3.1). The granulated gelatin capsule, which has the same bloom value as the gelatin capsule used in previous study (Wilson 2014, chap. 2), had an elution profile similar to bloom 220 gelatin. The granulated gelatin capsule and bloom 220 gelatin both had HMW, molecular weight region above 250,000 g mol^{-1}, of 25.3% and 19.9% respectfully. The beta fraction, molecular weight region between 250,000 g mol^{-1} and 150,000 g mol^{-1}, was 15.9% for granulated gelatin capsule, and 14.3% for bloom 220 gelatin. The alpha fraction, molecular weight region between 150,000 g mol^{-1} and 50,000 g mol^{-1}, was 34.9% for granulated gelatin capsule, and 33.4% for bloom 220 gelatin. The sub alpha fraction, molecular weight region between 50,000 g mol^{-1} and 20,000 g mol^{-1}, was 16.5% for granulated gelatin capsule, and 21.1% for bloom 220 gelatin. The low molecular weight fraction, molecular weight region < 20,000 g mol^{-1}, was 7.5% for granulated gelatin capsule, and 11.33% for bloom 220 gelatin. Data was analyzed by ANOVA ($\alpha = 0.05$) and Tukey's HSD test and there were significant differences ($p<0.001$ in the molecular distribution of the three hydrolyzed collagen. No significant difference was detected between granulated gelatin capsule and bloom 220 gelatin, while there were significant differences ($p<0.001$) between granulated gelatin capsule, bloom 220 gelatin and gelatin hydrolysate.

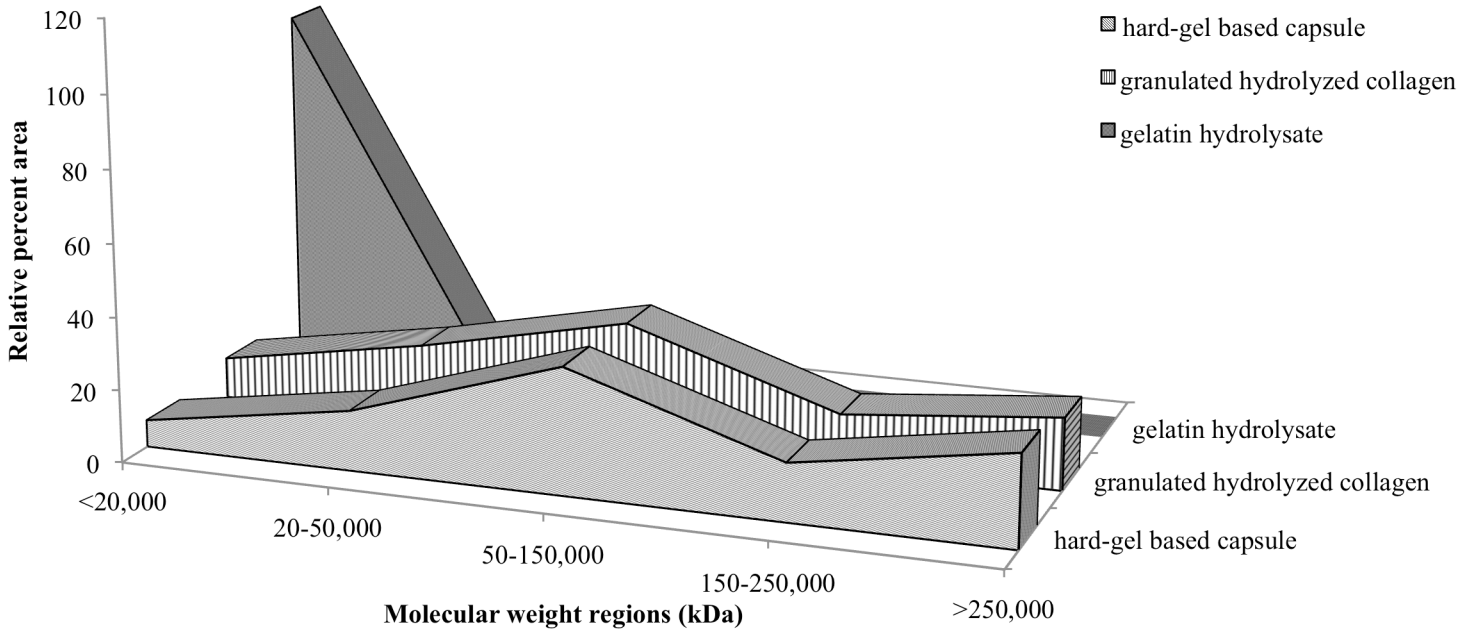

Figure 3.1: Molecular weight distribution of hydrolyzed collagen by type, reported in relative area percent of the five molecular weight regions the high molecular weight fraction, the beta fraction, the alpha fraction, the sub-alpha fraction, and the low molecular weight fraction of the size exclusion elution profile.

Effect of different hydrolyzed collagens on plant growth

The effect of four types of hydrolyzed collagen on plant growth was investigated by measuring total leaf area, fresh weight and dry weight, 28 days after emergence. Results for total leaf area, fresh weight, dry weight and nitrogen content are described as the percent change with respect to the control. There were significant differences ($p<0.0001$) in total leaf area between the treatments and the largest total leaf area was observed in the bloom 220 and 2 capsule treatment with a 135% and 124% increase, respectively, compared to the control. The gelatin hydrolysate and amino acid mixture followed with a 101% and 91% increase, respectively, compared to the control. Urea had the least increase compared to the control at 79% (Figure 3.2). There was no significant difference in total leaf area between 2 capsules and bloom 220, and between gelatin hydrolysate and amino acid mix; however, there was a significant difference ($p<0.0001$) between the 2 capsules and gelatin hydrolysate treatments.

There were significant differences ($p<0.0001$) in fresh weight (FW) between treatments (Figure 3.3). The bloom 220 treatments had the highest fresh weight (FW) increase at 158% compared to the control, followed by the gelatin hydrolysate at 102% increase and the amino acid mixture with an 111% increase. Two capsules and urea had a 95% and 97% increase, respectively compared to the control. Only the bloom 220 had a significantly greater fresh weight compared to the gelatin hydrolysate, amino acid mixture, urea and 2 capsules treatments. Dry weights (DW) followed a similar trend as total leaf area, with significant differences ($p<0.0001$) in DW between the treatments. The bloom 220 treatments had the highest DW, 114% increase compared to the control, followed by 2 capsules at 102% increase compared to the control. Gelatin hydrolysate had an 86% increase, amino acid mix had a 78% increase, and urea

had the lowest DW increase at 66% compared to the control (Figure 3.4). There were no significant differences between the 2 capsules and bloom 220 treatment, between gelatin hydrolysate and amino acid mix, and between amino acid mix and urea. However, there were significant differences ($p<0.0001$) between gelatin hydrolysate and bloom 220 and between 2 capsules and urea.

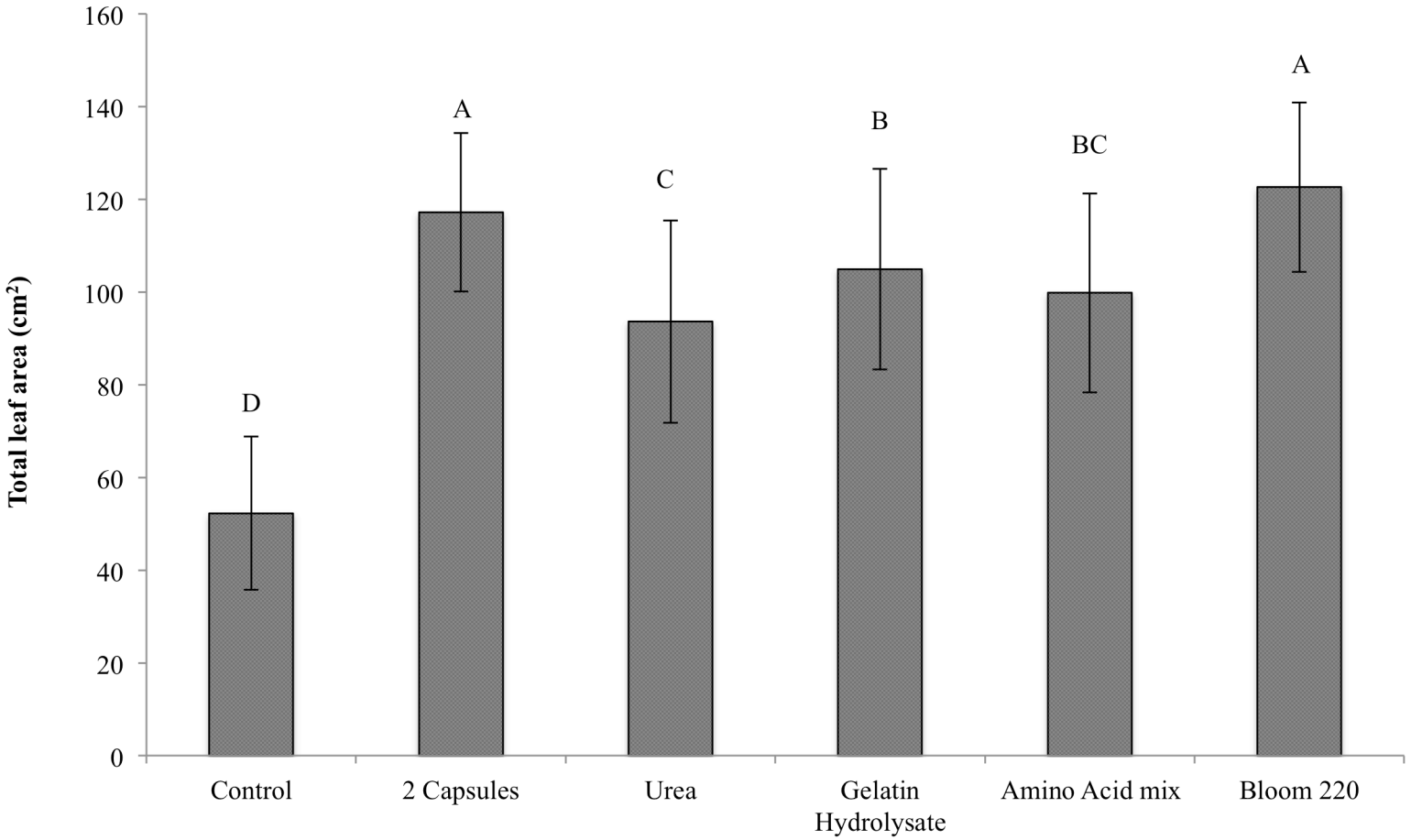

Figure 3.2: Mean total leaf area 28 days after emergence of cucumber ('Vlaspik') with six treatments: control, two gelatin capsule (2 Capsules), urea, gelatin hydrolysate, amino acid mixture (Amino acid mix), and bloom 220 gelatin (Bloom 220). Data analyzed by ANOVA ($\alpha = 0.05$) and Tukey's HSD test was used to conduct mean separation. Letters not associated with same letter are significantly different. Bar represent standard deviation.

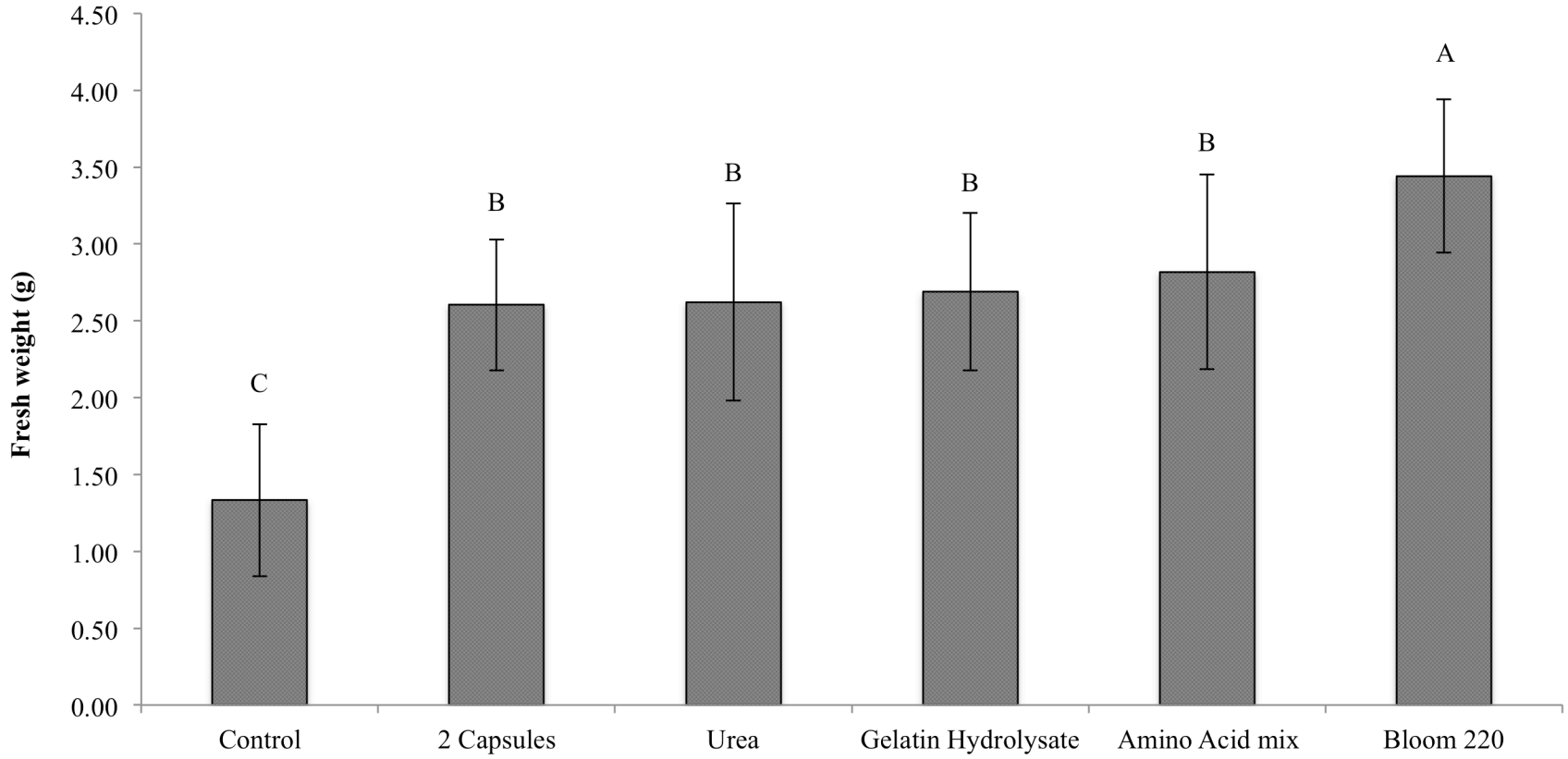

Figure 3.3: Mean fresh weight 28 days after emergence of cucumber ('Vlaspik') with six treatments: control, two gelatin capsule (2 Capsules), urea, gelatin hydrolysate, amino acid mixture (Amino acid mix), and bloom 220 gelatin (Bloom 220). Data analyzed by ANOVA ($\alpha = 0.05$) and Tukey's HSD test was used to conduct mean separation. Letters not associated with same letter are significantly different. Bar represent standard deviation.

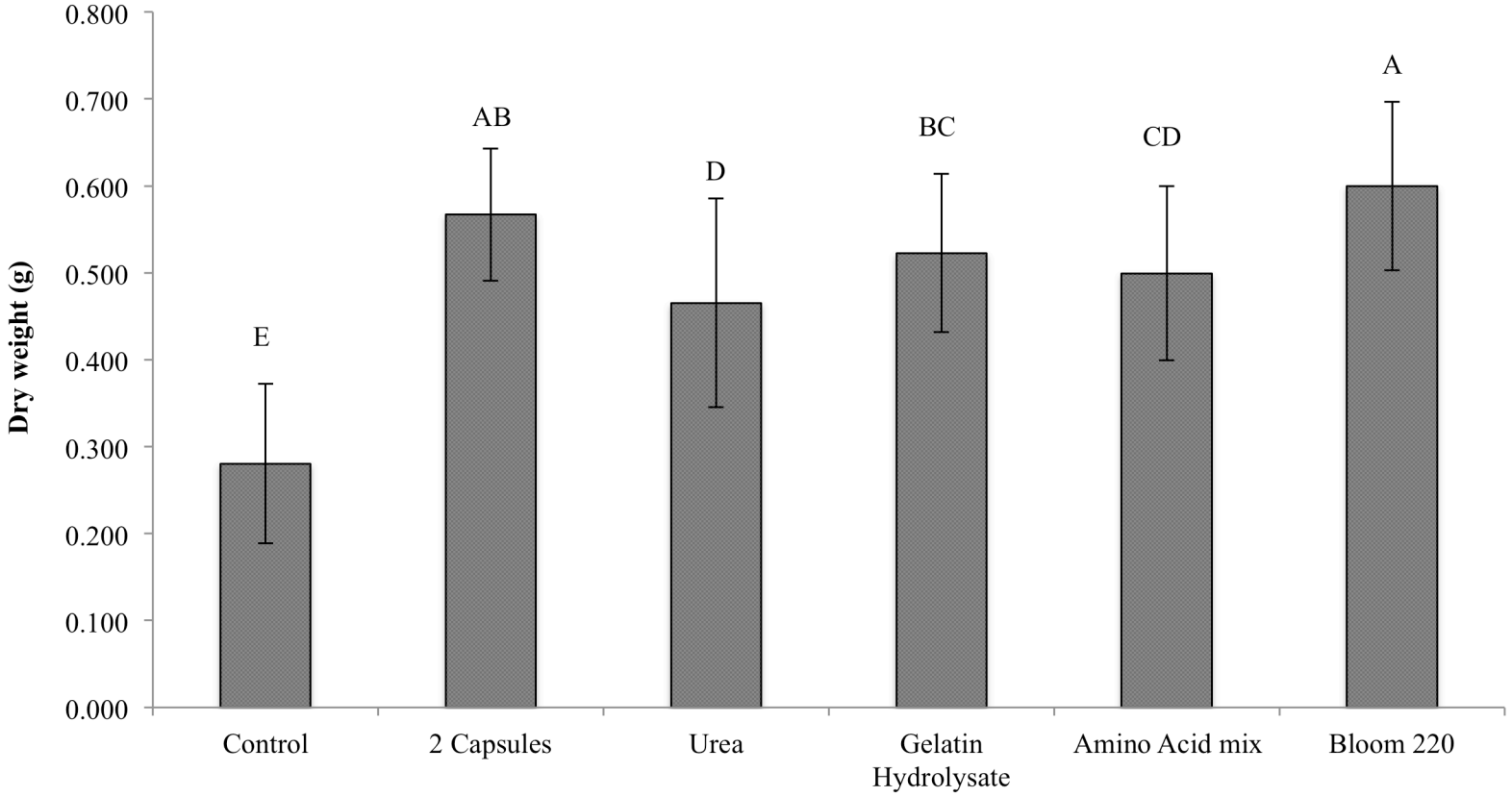

Figure 3.4: Mean dry weight 28 days after emergence of cucumber ('Vlaspik') with six treatments: control, two gelatin capsule (2 Capsules), urea, gelatin hydrolysate, amino acid mixture (Amino acid mix), and bloom 220 gelatin (Bloom 220). Data analyzed by ANOVA ($\alpha = 0.05$) and Tukey's HSD test was used to conduct mean separation. Letters not associated with same letter are significantly different. Bar represent standard deviation.

Effect of different hydrolyzed collagens on nitrogen content in cucumber plant tissue

The effect of different types of hydrolyzed collagen on total N content in the plant was investigated. Results nitrogen content is described as the percent change with respect to the control. Similar to the results of total leaf area, fresh weight, and dry weight, there were significant differences ($p<0.0001$) in N content between the treatments. The bloom 220 had the highest N content per plant sample at 0.77 mg, a 128% increase from the control. The two gelatin capsules and gelatin hydrolysate treatments had the next highest N content with a 106% increase from the control, followed by the amino acid mix treatment with a 91% increase. Urea had the lowest percent increase in N content at 89%. There was a significant difference ($p<0.0001$) in N content between bloom 220 and urea and amino acid; however, no significant difference was measured between bloom 220 and gelatin hydrolysate and 2 capsules (Figure 3.5)

Effect of different amino acid mixture ratios on plant growth

The effect of amino acid composition in the treatment was studied by coating cucumber seeds ('Vlaspik') with amino acid mixtures containing different ratios of proline and hydroxyproline and analyzing the physiological parameters (total leaf area, FW, and DW). There were significant differences ($p<0.0017$) in total leaf area between the treatments. The all amino acid treatment had the largest total leaf area at 175 cm^2, followed by no proline (157 cm^2), no hydroxyproline (155cm^2), and no proline/hydroxyproline (154 cm^2) treatments. The no amino acid treatment had the lowest total leaf area (118 cm^2). There were significant differences ($p<0.0001$) between no amino acid treatment and all other treatments. However, there were no significant differences in total leaf area between all amino acid, no proline, no hydroxyproline, and no proline/hydroxyproline (Figure 3.6).

The same trend was measured in both fresh weight (FW) and dry weight (DW) data. The amino acid treatment had the greatest FW (2.02 g) and DW (0.299 g), followed by, no proline (FW 1.79 g, DW 0.299 g), no hydroxyproline (FW 1.70 g, DW 0.293 g), and no proline/hydroxyproline (FW 1.68 g, DW 0.289 g). No amino acid treatment had the lowest FW (1.09 g) and DW (0.224 g) (Figure 3.7 and 3.8). Significant differences ($p<0.0001$) were measured in both FW and DW between the no amino acid and all other treatments. There was no significant different in FW and DW between the all amino acid, no proline, no hydroxyproline, and no proline/hydroxyproline treatments.

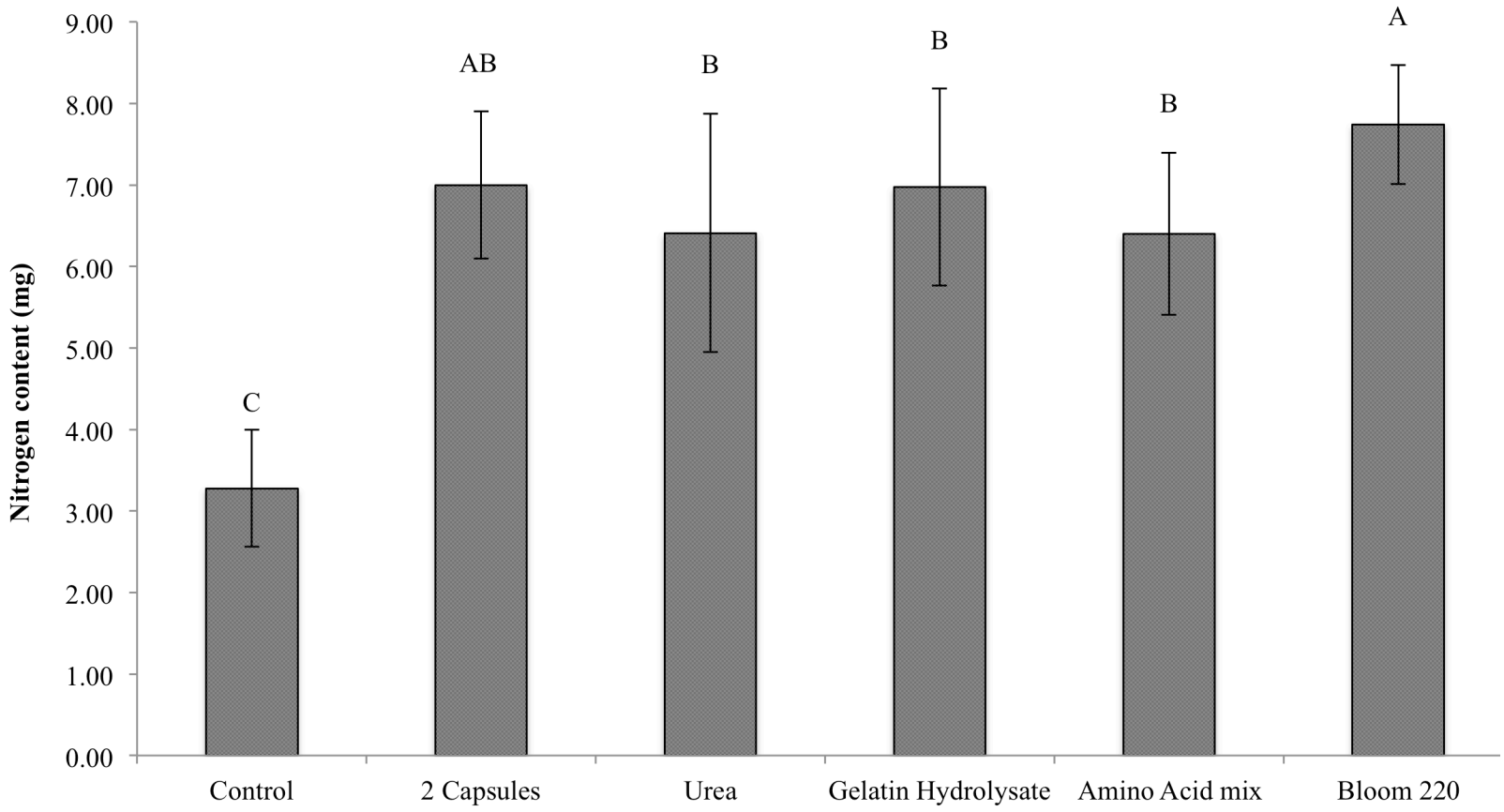

Figure 3.5: Mean nitrogen content (mg) 28 days after emergence of cucumber ('Vlaspik') with six treatments: control, two gelatin capsule (2 Capsules), urea, gelatin hydrolysate, amino acid mixture (Amino acid mix), and bloom 220 gelatin (Bloom 220). Data analyzed by ANOVA ($\alpha = 0.05$) and Tukey's HSD test was used to conduct mean separation. Letters not associated with same letter are significantly different. Bar represent standard deviation.

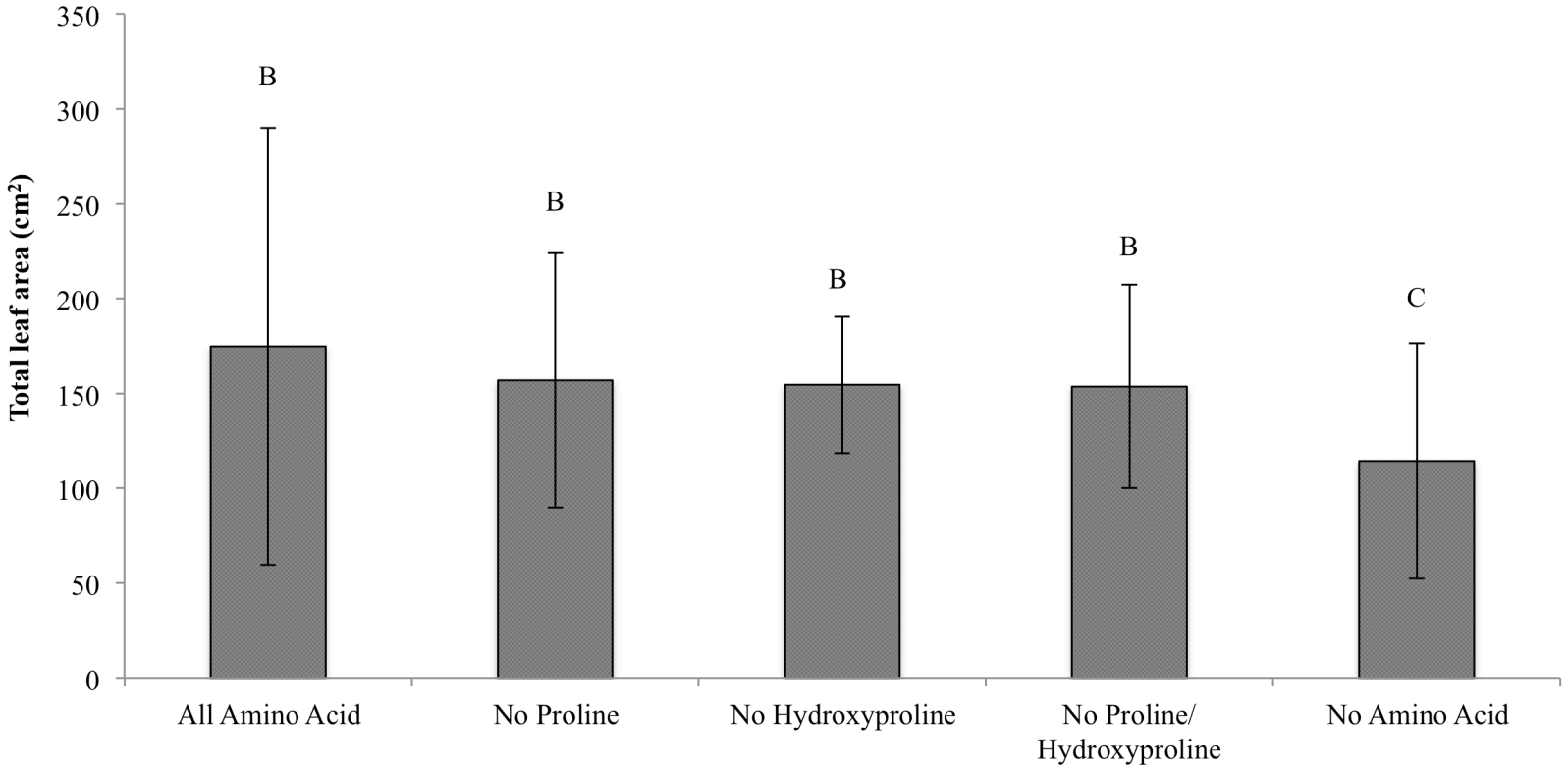

Figure 3.6: Mean total leaf area 28 days after emergence of cucumber ('Vlaspik') with six treatments: two gelatin capsules (2 capsules), control (No Amino Acid), amino acid mixture containing all amino acid found in hydrolyzed collagen (All Amino Acid), all amino acid found in hydrolyzed collagen but proline replaced by glycine (No Proline), all amino acid found in hydrolyzed collagen but hydroxyproline replaced by glycine (No Hydroxyproline), and all amino acid found in hydrolyzed collagen but proline and hydroxyproline replaced by glycine (No Proline/Hydroxyproline). Data was then statistically analyzed by ANOVA ($\alpha = 0.05$) and Tukey's HSD test was used to conduct mean separation. Bars represent standard deviation.

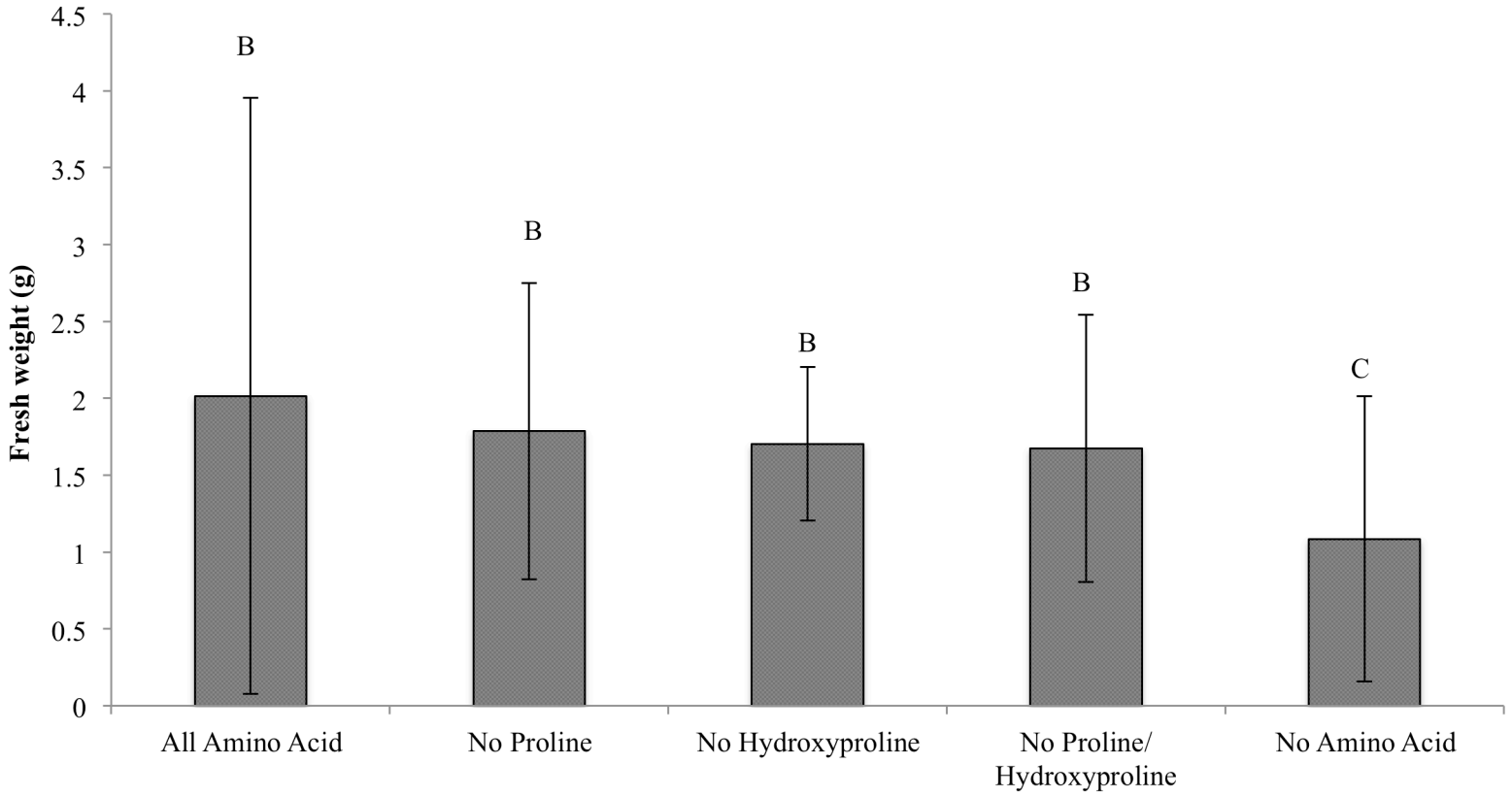

Figure 3.7: Mean fresh weight 28 days after emergence of cucumber ('Vlaspik') with six treatments: two gelatin capsules (2 Capsules) control (No Amino Acid), amino acid mixture containing all amino acid found in hydrolyzed collagen (All Amino Acid), all amino acid found in hydrolyzed collagen but proline replaced by glycine (No Proline), all amino acid found in hydrolyzed collagen but hydroxyproline replaced by glycine (No Hydroxyproline), and all amino acid found in hydrolyzed collagen but proline and hydroxyproline replaced by glycine (No Proline/Hydroxyproline). Data was then statistically analyzed by ANOVA ($\alpha = 0.05$) and Tukey's HSD test was conducted for mean separation. Bars represents standard deviation.

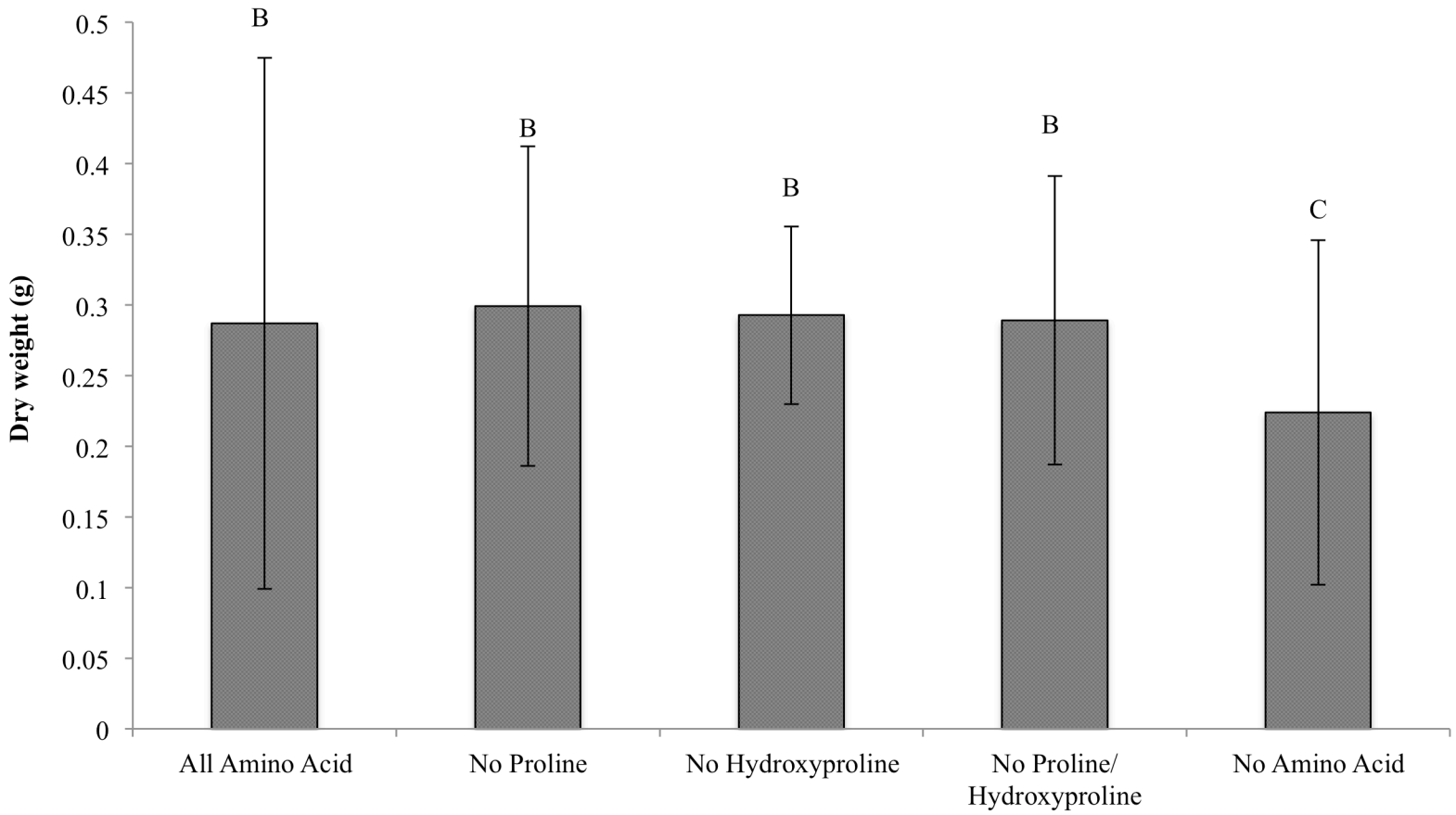

Figure 3.8: Mean dry weight 28 days after emergence of cucumber ('Vlaspik') with six treatments: two gelatin capsules (2 Capsules) control (No Amino Acid), amino acid mixture containing all amino acids found in hydrolyzed collagen (All Amino Acid), all amino acids found in hydrolyzed collagen but proline replaced by glycine (No Proline), all amino acids found in hydrolyzed collagen but hydroxyproline replaced by glycine (No Hydroxyproline), and all amino acids found in hydrolyzed collagen but proline and hydroxyproline replaced by glycine (No Proline/Hydroxyproline). Data was then statistically analyzed by ANOVA ($\alpha = 0.05$) and Tukey's HSD test was conducted for mean separation. Bars represent standard deviation.

DISCUSSION

The hydrolyzed gelatin characterization by HPLC revealed that molecular weight distribution differs between different gelatin types. Gelatin hydrolysate had the smallest molecular weight distribution with 100% of the elusion in the low molecular weight fraction, which has molecular weight regions between 4,000 g mol^{-1} and 20,000 g mol^{-1} (Figure 3.1). The gelatin capsule had an elusion profile similar to granulated hydrolyzed collagen, which suggests that structurally they are similar. This speculation was further supported by the total leaf area results in which the effect of different hydrolyzed collagens was studied. There was no significant difference in total leaf area between gelatin capsule (2 capsules), and granulated hydrolyzed collagen (bloom 220) (Figure 3.2), while the total leaf area for gelatin hydrolysate was significantly less than the 2 capsule and bloom 220 treatments. According to Schrieber and Gareis (2007) the molecular size for amino acids present in hydrolyzed gelatin (Table 3.1) is between 89.1 g mol^{-1} to 204.2 g mol^{-1}. Therefore, the lower molecular weight of the gelatin hydrolysate had a similar affect on plant growth as the low molecular weight amino acids in the mixture. (Figure 3.1).

Another explanation for the difference in total leaf area between the treatments is the solubility of the different hydrolyzed collagen. Both gelatin hydrolysate and amino acid mix has a high water solubility (data not shown), which can be explained by their low molecular weight distribution. Granulated hydrolyzed gelatin and gelatin capsules both have a much higher molecular weight distribution, compared to gelatin hydrolysate and amino acid mix, thus have lower solubility. As the plants were watered in the greenhouse during its growing time, the treatments, which were applied to the seeds in dry powder form, may have dissolved in the water

and leached from the containers. Thus the treatments with lower solubility may have remained in the container for a longer period of time and thus available for plant growth resulting a higher total leaf area at the end of the experiment.

To determine if the growth promotion by the gelatin capsules are caused by the fertilizer effect from the amino acids in the gelatin, urea was added to the seeds at sowing at equivalent level of N found in two gelatin capsules (14 mg). There were significant differences in total leaf area and DW between 2 capsules and urea, 2 capsule treatments had a significant improvement in plant biomass compared to urea alone. The growth promotion for urea was comparable to that of amino acid mix treatment (Figure 3.2 and 3.4). The results suggest that growth promotion induced by addition of gelatin capsules was not caused by the N fertility alone. As the treatments were all equivalent in the amount of N found in gelatin capsules, if the growth promotion was caused by N fertility, there would have been no significant difference in the plant growth parameters. In fact, the N content results showed no significant differences between all hydrolyzed collagen treatments: 2 capsules, gelatin hydrolysate and bloom 220 (Figure 3.5). Urea applied at the same N amount as the hydrolyzed collagen treatments had significantly lower total leaf area, and DW, compared to the hydrolyzed collagen treatments (2 capsules, bloom 220 and gelatin hydrolysate) (Figure 3.2, 3.4, 3.5). The results indicate that growth promotion by the hydrolyzed collagen treatments does not act by the same mechanism as that of urea, and that N fertility alone cannot explain the dramatic growth promotion by the hydrolyzed collagen. However, another explanation maybe that the urea leached out of the container faster compared to gelatin capsule treatment and bloom 220, due to the increased solubility from the smaller molecular size of urea molecule.

To determine if the amino acid mixture ratio had an effect on plant growth, seed coating (encrustment) technology was used to bind the amino acids to the coating and mimic the low solubility of the gelatin capsule. Seed coating with the amino acid mixture was not able to deliver the high amount of amino acids as the gelatin capsule, due to the negative effect of seed coating build up on germination. In order to maintain high germination percentage, only 10 mg of amino acid mixture per seed was applied to the seed coating compared to 94 mg of hydrolyzed collagen per seed as two capsules. The amount of N was maintained in the amino acids mixture by replacing proline and hydroxyproline with glycine, another abundant amino acid in hydrolyzed collagen (Schrieber and Gareis 2007). However, the effect of the amino acid mixture in the seed coating still had a significant effect on plant growth as there were significant increases in total leaf area, fresh weight and dry weight between all amino acids treatment compared to the no amino acid treatment (Figure 3.7, 3.8, and 3.9). This result suggests that the proportion of amino acids does not have an effect on the plant growth promotion. This result is further supported by the fact that there were no significant differences in total leaf area and fresh weight between the treatments with different amino acid mixture ratio: no proline, no hydroxyproline, and no proline/hydroxyproline (Figure 3.7). These results combined, suggests that amount of proline and hydroxyproline in the amino acid mixture did not affect plant growth, and thus these two amino acids are not primarily responsible for the plant growth promotion measured with gelatin capsule treatment.

The result of the study provides evidence that amino acids and the relative solubility of hydrolyzed collagen in the gelatin capsule provided a sustained source of N for enhancing plant growth. The plant tissue N content result suggests that hydrolyzed collagen could supplement

initial N fertility for plant growth; however, the mechanism in which it improves plant growth is not clear. These results provides evidence that hydrolyzed collagen does not act by the same mechanism as a N fertilizer such as urea with high water solubility, and that N fertility alone cannot explain the dramatic growth promotion by the hydrolyzed collagen.

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CHAPTER 4: Gelatin applied at sowing ameliorates salinity stress on cucumber plant growth

ABSTRACT

Growth enhancement of gelatin capsule treatment on cucumber plants was characterized in previous studies (Chapter 2 and Chapter 3); however, these cucumber plants were grown under ideal conditions without environmental stress. A greenhouse extended was conducted by sowing nontreated control seed and two gelatin treated capsules applied at time of sowing adjacent to seeds. Four salinity levels were developed by watering with 0, 25, 50 or 100 mM NaCl.. Plants biomass in both control and plants treated with two gelatin capsules decreased with increased NaCl concentration. However, within each NaCl concentration level, both plant biomass and nitrogen content in plants treated with gelatin capsule was greater than the control. The linear regression analysis revealed that leaf area decreased as salinity increased at the same rates for the control and gelatin treatments. The plants treated with gelatin capsules had a decreased sodium uptake rate compared to the control as salinity increased. The result suggests that the gelatin capsules may have acted or have induced salt stress protectants. Collectively, the enhanced growth from gelatin treated plants was shown at all levels of salinity and several mechanisms may be responsible for this amelioration of salinity stress.

INTRODUCTION

Approximately 20% of the world's cultivated land and half of all irrigated lands are affected by salinity (Zhu 2001). Salinity is one of the significant factors limiting crop productivity (Zhu 2001). Cucumber (*Cucumis sativus* L.) is one of the most popular vegetables in the world, and is highly sensitive to salinity (Huang et al. 2009).

Water status in plants is highly sensitive to salinity, which is the cause of physiological drought response in plants when subjected to salt stress (Yeo, Capron, and Flowers 1985). Salinity imposes two constraints on plants: an osmotic effect, which creates unfavorable water relations to sustain cellular turgor, and ionic effect, which results from the direct toxicity of specific ions and an ion imbalance in plants (Munns and Tester 2008). Under high salt conditions, in order to maintain a favorable relative water content within the cells, plants must take up inorganic solutes such as Na^+, Cl^- and K^+ and synthesize compatible solutes such as proline, sorbitol, trehalose and glycine betaine (Munns and Tester 2008; Turkan and Demiral 2009). These compatible solutes and osmolytes do not interfere with normal biochemical reactions and act as osmoprotectants during osmotic stress (McCur and Hanson 1990; Delauney and Verma 1993; Nounjan, Nghis, and Theerakulpisut 2012). At a high level of salt exposure, plants build up high levels of Na^+ and Cl^- within the cell, this in turn inhibits K^+ uptake. This can severely inhibit several enzymes that require K^+ as a cofactor, which leads to metabolic impairment in plants (Munns and Tester 2008).

The primary site of salt injury is thought to be the plasma membrane, part of the cytoplasm that first encounters the salt. Cell membrane stability has been widely used to

differentiate stress tolerant and susceptible cultivars. Greater membrane stability is correlated with abiotic stress tolerance (Zhu et al. 2008; Meloni et al. 2003; Sudhakar, Lakshmi, and Giridarakumar 2001). Lipid peroxidation has been associated with damage caused by various abiotic stress, and is often used as an indicator of salt-induced oxidative damage (Elkahoui et al. 2005). Malondialdehyde (MDA) is the decomposition product of polyunsaturated fatty acid of membrane, and it is used as an indicator of lipid peroxidation. MDA tends to accumulate to a greater extent in plant under salt stress (Meloni et al. 2003; Sudhakar, Lakshmi, and Giridarakumar 2001).

Exposure of plants to salt stress can increase the production of reactive oxygen species (ROS), such as superoxide radicals and hydrogen peroxide (Apel and Hirt 2004). These ROS are highly reactive and can interact with essential macromolecules and metabolites causing cellular damage through oxidation of membrane lipids, proteins and nucleic acid (Nounjan, Nghis, and Theerakulpisut 2012; Apel and Hirt 2004). In general, plants possess antioxidant systems that include antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX), to protect their cells against ROS (Apel and Hirt 2004).

Proline is the most common osmolyte accumulating in plants in response to various stress conditions such as drought stress, salinity stress, heat stress, and cold stress. It offers a wide range of protective roles including but not limited to, osmotic adjustment, stabilization of cellular structure, and reduction of damage to the photosynthetic apparatus (Nounjan, Nghis, and Theerakulpisut 2012). The role of proline as an osmoprotectant was first reported by Christian (1966), who demonstrated that exogenous proline could alleviate the inhibition of growth of

Salmonella oranienburg due to osmotic stress. It is now understood that a wide range of organisms accumulate proline under stress: eubacteria, protozoa, marine invertebrates, algae, plants (halophytes, tobacco, spinach, potato, tomato, Arabidopsis, alfalfa, field bean, soybean, wheat, barley and rice) (McCur and Hanson 1990; Delauney and Verma 1993; Yoshiba, Kiyosue, and Nakashima 1997).

Handa (1986) reported that tomato cells cultured under water stress rapidly accumulated 300 times more proline than non-water stressed cells, and the cells adapted to osmotic stress. This observation suggests that plants have the ability to adapt to water stress at the cellular level, and proline is involved in osmotic stress tolerance by acting as a compatible osmolyte by accumulating in the cytosol (Yoshiba, Kiyosue, and Nakashima 1997; Munns and Tester 2008), and 34% of total intracellular proline was accumulated in vacuoles in non-water-stressed cultured cells of potato. However, when subjected to salt stress, the total amount of proline in the cell increased, but the amount of proline in vacuoles decreased (Fricke and Pahlich 1990).

Exogenous application of osmoprotectants were reported to have some effect protecting osmotic status of the plant in abiotic stress and were suggested as an alternative approach to improve crop productivity under saline conditions (Nakayama et al. 2005). Huang et al. (2009) reported that exogenous application of proline to cucumber plants subjected to salt stress significantly alleviated the growth inhibition of plants induced by salt-stress by improving water status and increase peroxidase enzyme activity in the leaf. Exogenous application of proline improved crop tolerance in groundnut (Jain et al. 2001), *Pancratium maritimum* (Khedr et al. 2003), melon (Kaya et al. 2007), tobacco (Hoque et al. 2007; Ashraf and Foolad 2007), and

canola (Athar et al. 2009). Protein hydrolysate, a class of plant biostimulants was reported to have positive effects on plants under abiotic stress. Cucumber plants subjected to suboptimal pH level and temperature performed better with the application of humic acid containing substance LACTOFOL™, with notable increase in leaf area, shoot and root mass (Boehme, Schevschenko, and Pinker 2008; Boehme, Schevtschenko, and Pinker 2005). Alfalfa plant-derived biostimulant increased maize plant biomass under salinity condition, and enhanced Na^+ accumulation and reduced K^+ accumulation in roots and leaves (Ertani et al. 2013). Perennial ryegrass treated with Macro-Sorb Foliar (FOLIAR™), an animal membrane hydrolysate, subjected to high air temperature stress exhibited an increased photochemical efficiency and membrane thermostability compared to plants without the treatment (Kauffman, Kneivel, and Watschke 2007).

In dehydrated plants, the accumulation of proline occurs as the result of both the activations of its biosynthesis and the inactivation of its degradation (Yoshiba, Kiyosue, and Nakashima 1997). L-proline is produced from L-glutamine via Δ^1-pyrroline-5-caboxylate (P5C) in a reaction catalyzed by enzymes Δ^1-pyrroline-5-caboxylate synthetase (P5CS) and Δ^1-pyrroline-5-caboxylate reductase (P5CR) (Yoshiba et al. 1995; Yoshiba, Kiyosue, and Nakashima 1997). Expression of genes for P5CS and P5CR have been analyzed under dehydration in *Arabidopsis* (Savoure et al. 1995; Verbruggen, Villarrole, and Van Montagu 1993). Levels of P5CS protein increased after the initiation of dehydration treatment in direct proportion to the level of P5CS mRNA accumulation, while the expression of P5CR was not enhanced by dehydration or salinity (Yoshiba et al. 1995). Taken together these results suggest

that P5CS plays a more important role than P5CR in the accumulation of proline under osmotic stress (Yoshiba, Kiyosue, and Nakashima 1997).

Seed encapsulation using gelatin capsules is a novel approach as a seed treatment to enhance plant growth (Takahashi and Trias 2012). The effect of gelatin applied at time of sowing on cucumber plant growth was physiologically characterized (Wilson 2015, chap. 2, 3). The gelatin treatment increased leaf area, fresh and dry weight compared to the non-treated control. However, cucumber plants were grown under ideal conditions without abiotic stress. The objective of this study is to determine if gelatin treatment has a positive effect on cucumber plants under salinity stress.

MATERIALS AND METHODS

Effect of gelatin capsule treatment on cucumber plants under salt stress

Cucumber seeds 'Vlaspik' (Seminis, Oxnard, CA) were planted in a controlled greenhouse maintained at 24°C/21°C temperature with 14/10 hours photoperiod at New York State Agricultural Experiment Station in Geneva, NY in the spring of 2014. Two size #3 gelatin capsules (Capsule line, Pompano Beach, FL) were placed adjacent to each seed in a 4-inch pot (Illustration 4.1). Gelatin capsules were placed adjacent to the seeds to prevent a decrease in germination rate, and for controlling the amount of gelatin used in the treatment. Two gelatin capsules had a weight of 93.7 mg, nitrogen content of 14.2 mg and protein content of 80.0 mg.

Ninety pots per treatment were grouped into six replications of 15 samples per treatment and were placed in a randomized block design on the greenhouse bench. Plants were watered with regular tap water for one week after sowing to allow uniform emergence of the seeds. Once all the seeds have emerged, 150 ml of four different concentrations of NaCl were applied: 0 mM, 25 mM, 50 mM, and 100 mM. NaCl solution was applied daily to prevent the media from drying out, and saturated with NaCl solution so any growth change would be due to salt stress and not drought stress. To prevent acute sodium toxicity by high NaCL concentration in 50 mM and 100 mM, plants received 150 ml of the NaCl solution daily with increments of 25 mM NaCl concentration per day over a week until the target NaCl concentration was reached. Plants were harvested 28 days after emergence and total leaf area and fresh weight were measured at time of harvest. Total leaf area was measured using a CI-202 Leaf Area Meter (CID Bio-Science, Camas, WA). Data were then statistically analyzed by ANOVA ($\alpha = 0.05$) and Tukey's HSD test was then used to compare means separation, and linear regression was used to determine correlation.

Illustration 4.1: Two gelatin capsules (four half) placed adjacent to the seeds in 4-inch pot.

Effect of gelatin capsule treatment on total nitrogen percent and sodium in cucumber plant tissue under salt stress

Cucumber plants ground in liquid nitrogen were dried in a 100 °C oven overnight. The dry plant tissue was sent to Iowa State University Soil and Plant Analysis Laboratory (Ames, IO) for total elemental analysis of Na^+ via nitric acid microwave digestion, and total nitrogen (N) via combustion elemental analysis. Data were then statistically analyzed by ANOVA ($\alpha = 0.05$) and Tukey's HSD test was then used to compare means separation, and linear regression was used to determine correlation.

Effect of gelatin capsule treatment on proline content in cucumber plant tissue under salt stress

Cucumber plants were ground in liquid nitrogen, and approximately 100 mg was used for proline analysis, using methods developed by Bates et al. (1973). Proline was extracted with 1 ml of 3% sulfosalicylic acid (3g 5-sulfosalicylic acid in 100 ml distilled water). 200 µl of the plant extract

was reacted with a reaction mixture of 200 µl glacial acetic acid and 200 µl acidic ninhydrin (1.25 g ninhydin, 30 ml glacial acetic acid, 20 ml 6M orthophosphoric acid), incubated in 96°C for 90 minutes, and reaction was terminated by placing in ice. Proline was then extracted by adding 1 ml toluene. Absorbance was measured at 520 nm using toluene as reference. Proline concentration was determined using a standard curve using analytical grade proline (Sigma Aldrich, St, Louis, MO) and calculated on a fresh weight basis, where 115.13 is the molar mass of proline.

$$Proline\ \left({}^{ug}/_{g}\right) = \frac{Proline\ ({}^{ug}/_{ml})}{115.13} \times \frac{Toluene\ (ml)}{Sample\ weight\ (g)}$$

RESULTS

Effect of gelatin capsule treatment on cucumber plants under salt stress

Cucumber plant biomass was negatively effected by salt stress. The negative effect was particularly evident in total leaf area, and there was a significant decrease ($p<0.0396$) in growth as NaCl concentration increased. Results for total leaf area, fresh weight, dry weight and nitrogen content are described as the percent change for the control or gelatin treatment with increasing salinity stress. In addition, the percent change between gelatin treatment and control is described at each stress level. The total leaf area in control plants decreased by 17%, 24% and 46% with 25, 50 and 100 mM NaCl treatment, respectively compared to plants that were not subjected to salt stress (Figure 4.1). The decrease in total leaf area was similar in plants that

received the gelatin treatment. The total leaf area in gelatin treated plants decreased by 16%, 28% and 35% with 25, 50 and 100 mM NaCl treatment, respectively compared to plants that were not subjected to salt stress. However, the gelatin treatment had a positive effect on plant growth, and there was a significant difference ($p<0.0001$) in total leaf area between control plants and plants treated with gelatin. In the plants that were not subjected to any salt stress, the gelatin treatment had 42% greater total leaf area compared to the control. Gelatin treated plants had 44%, 34% and 72% greater leaf area than the control plants at 25, 50 and 100 mM NaCl, respectively (Figure 4.1).

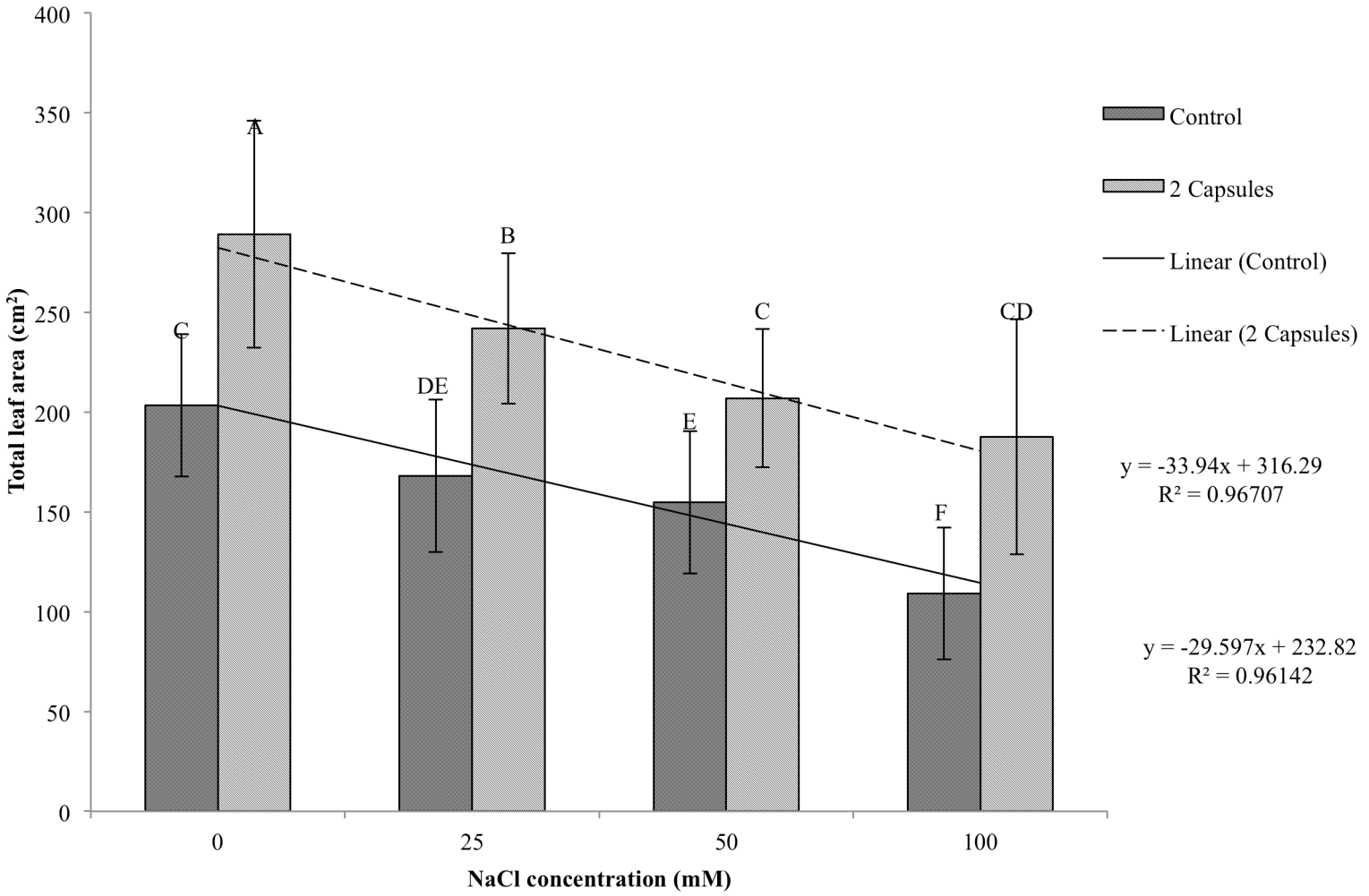

Figure 4.1: Mean total leaf area comparison of cucumber ('Vlaspik') 28 days after emergence between control and two gelatin capsule treatment under four levels of salt stress: 0 mM, 25 mM, 50 mM, and 100 mM. Data analyzed by ANOVA ($\alpha = 0.05$) and Tukey's HSD test was used to compare mean separation. Letters not associated with same letter are significantly different. Bars represent standard deviation. Linear regression was used to determine correlation.

Although the increased salinity treatment had a detrimental effect on plant growth characterized by a dramatic decrease in total leaf area due to the salt stress, the gelatin capsules were able to provide some protection of the plants from the negative effect caused by the salt stress. Moreover, there was no significant difference in leaf area between control plants at 0 mM NaCl and gelatin treatment at 50 mM or 100 mM NaCl (Figure 4.1). The regression analysis revealed that the treated plants had a significantly higher ($p<0.0001$) y-intercept (316), compared to the control (233), which indicates that the treated plants had a significantly larger leaf area at 0mM. There was no significant difference in the slope of the regression for the control and the treated plants, which indicates that the leaf area for both decreased at the same rate with increased NaCl concentration (Figure 4.1).

Similar trends were observed with fresh weight and dry weight. In the control plants there were no significant differences in fresh weight in plants that were subjected to salt stress (0 mM), 25 mM NaCl, and 50 mM NaCl. There was a significant difference ($p<0.0001$) in fresh weight in plants subjected to 100 mM with a 29% decrease compared to the plants with no salt stress (Figure 4.2). Plants treated with gelatin capsules exhibited a significant difference ($p<0.0001$) in fresh weight with increased salt stress. An 18% decrease in fresh weight at 25 mM NaCl or 50 mM NaCl and a 35% decrease at 100 mM NaCl compared to plants not subjected to salt stress (Figure 4.2). There were no significant differences in dry weight in control plants between 0 mM NaCl, 25 mM NaCl, and 50 mM NaCl. The only significant difference ($p<0.0011$) in dry weight was measured between 0 mM and 100 mM NaCl with a 22% decrease in dry weight compared to plants not subjected to salt stress (Figure 4.3). There were significant differences ($p<0.0011$) in dry weight of plants treated with gelatin with a 10%, 15% and 29% decrease at 25, 50 and 100 mM NaCl, respectively (Figure 4.3)

Significant differences ($p<0.0001$) in fresh weight between control plants and gelatin capsules treated plants were measured at each salt level and the plants treated with gelatin capsules had greater fresh weight compared to the control plants. At 0 mM, gelatin capsules treated plants had a 52% increase in fresh weight compared to control plants. Plants treated with gelatin capsules had a 22% increase at 25 mM NaCl, a 28% increase at 50 mM NaCl, and a 39% increase in fresh weight at 100 mM NaCl (Figure 4.2).). The regression analysis revealed that the treated plants had a significantly higher ($p<0.0001$) y-intercept (12.1), compared to the control (8.50), which indicates that the treated plants had a significantly higher fresh weight at 0mM. There was a significant difference ($p<0.0022$) in the slope of the regression for the control

and the treated plants, which indicates that the fresh weight of the treated plant decreased at a higher rate compared to the control with increased NaCl concentration (Figure 4.2).

Significant differences ($p<0.0001$) in dry weight between control plants and plants treated with gelatin capsules were measured at 0 mM NaCl level with a 40% increase in dry weight treated with gelatin compared to the control. A 26% increase in dry weight in plants treated with gelatin at 25 mM NaCl, a 21% increase at 50 mM NaCl, and a 27% increase at 100 mM (Figure 4.3). There was no significant difference between control plants not subjected to salt stress and gelatin treated plants at 100 mM NaCl (Figure 4.3). The regression analysis revealed that the treated plants had a significantly higher ($p<0.0001$) y-intercept (1.57), compared to the control (1.14), which indicates that the treated plants had a significantly higher dry weight at 0mM. There was a significant difference ($p<0.0022$) in the slope of the regression for the control and the treated plants, which indicates that the dry weight of the treated plant decreased at a higher rate compared to the control with increased NaCl concentration (Figure 4.3).

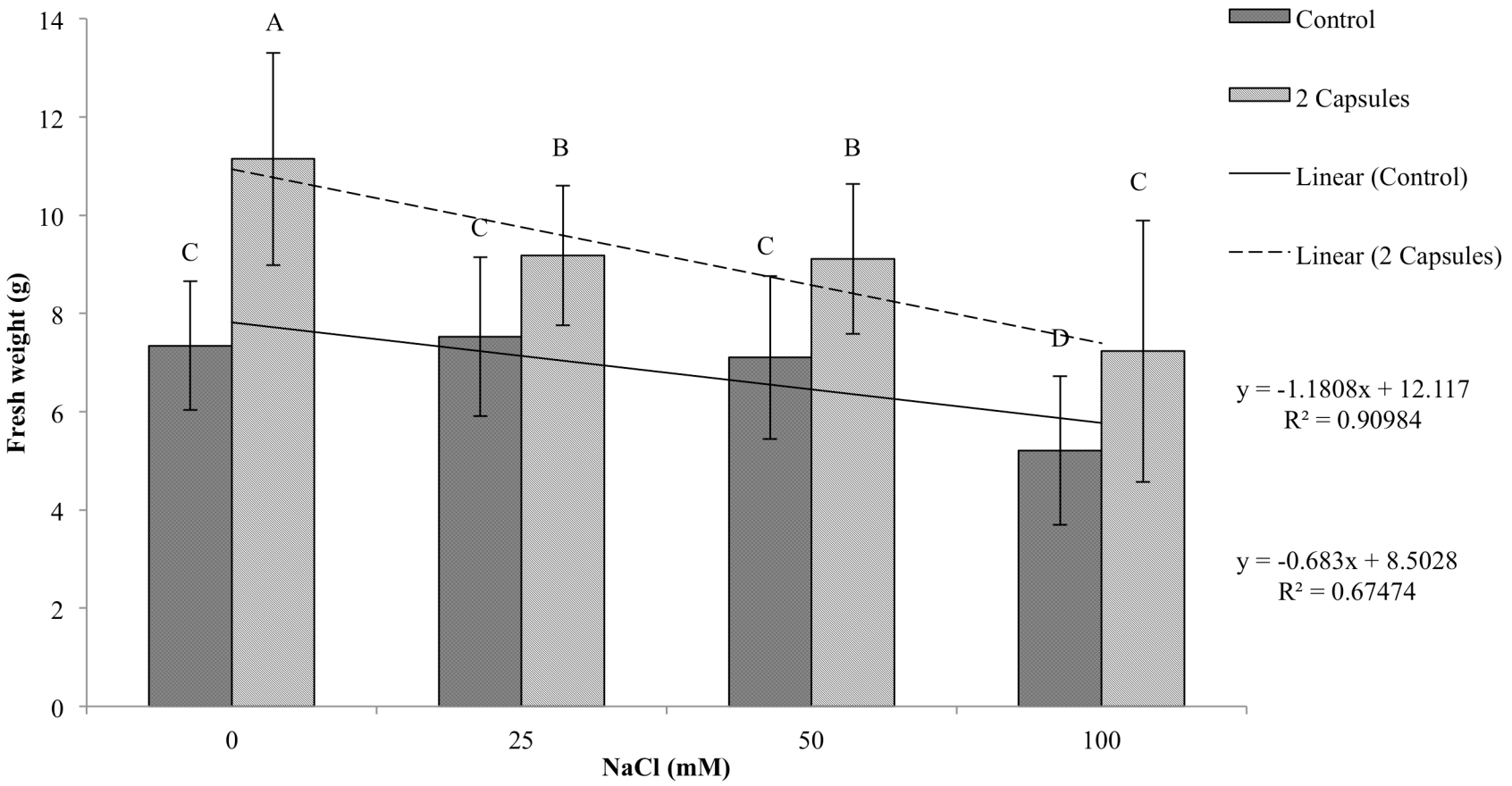

Figure 4.2: Mean fresh weight comparison of cucumber ('Vlaspik') 28 days after emergence between control and two gelatin capsule treatment under four levels of salt stress: 0 mM, 25 mM, 50 mM, and 100 mM. Data analyzed by ANOVA ($\alpha = 0.05$) and Tukey's HSD test was used to compare mean separation. Letters not associated with same letter are significantly different. Bars represent standard deviation. Linear regression was used to determine correlation.

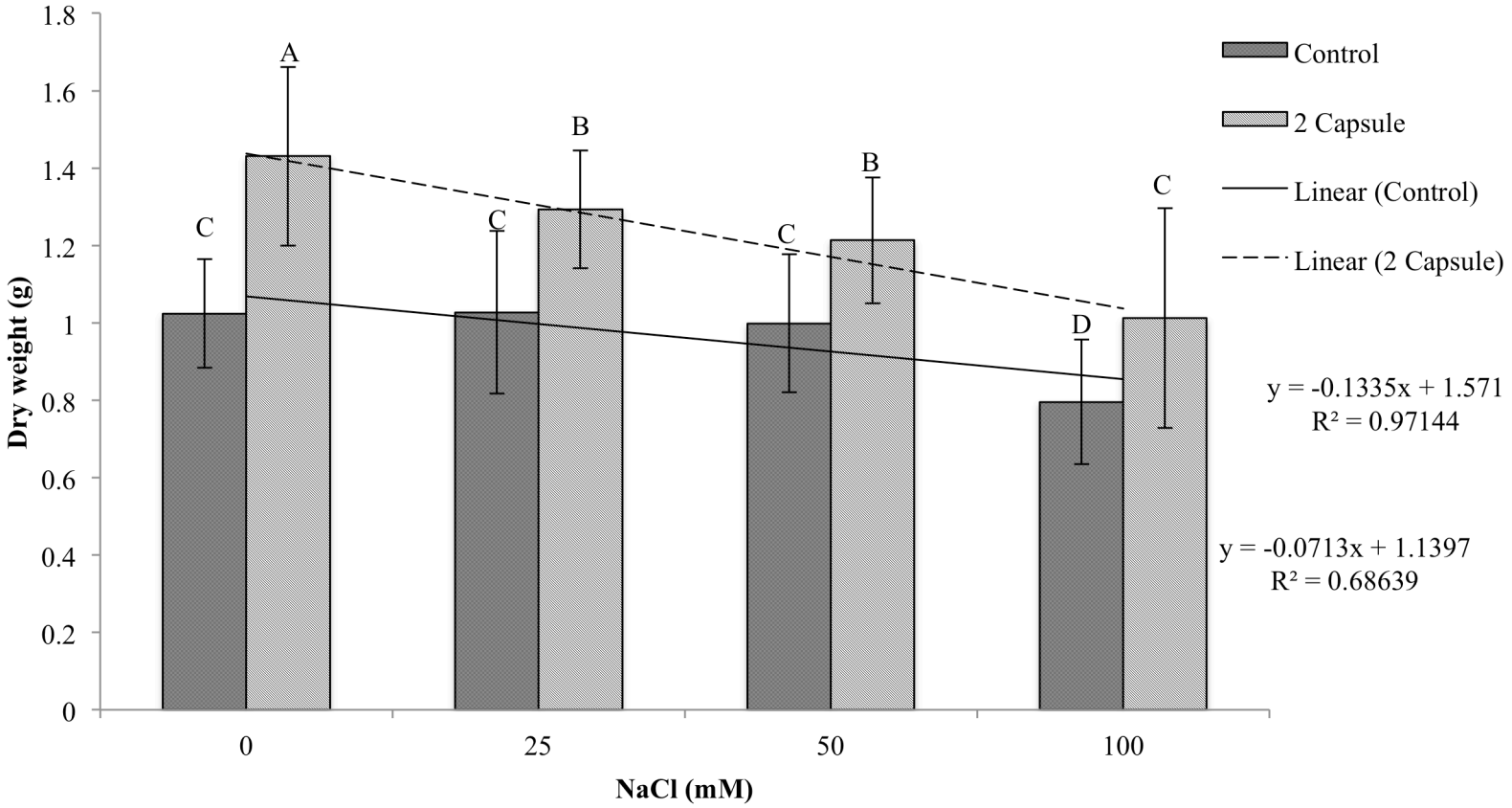

Figure 4.3: Mean dry weight percent comparison of cucumber ('Vlaspik') 28 days after emergence between control and two gelatin capsule treatment under four levels of salt stress: 0 mM, 25 mM, 50 mM, and 100 mM. Data analyzed by ANOVA ($\alpha = 0.05$) and Tukey's HSD test was used to compare mean separation. Letters not associated with same letter are significantly different. Bars represent standard deviation. Linear regression was used to determine correlation.

Effect of gelatin capsule treatment on total nitrogen content and sodium in cucumber plant tissue under salt stress

Similar trends were observed with fresh weight and dry weight as nitrogen content. The nitrogen content had a significant decrease ($p<0.0001$) in plants treated with gelatin with increased salt stress. In control plants, the only significant decrease ($p<0.0001$) in nitrogen content was a 32% decrease at 100 mM NaCl compared to 0 mM NaCl (Figure 4.4). There was a significant decrease ($p<0.0001$) in total nitrogen content in plants treated with gelatin with increased salt stress, with a 16% decrease at 25 mM NaCl compared to plants not subjected to salt stress, a 25% decrease at 50 mM NaCl, and a 42% decrease at 100 mM NaCl. No significant difference in total nitrogen content was measured between 25 mM and 50 mM of plants treated with gelatin capsules. Comparing the control with the gelatin treated plants, there was also a significant difference ($p<0.0001$) in nitrogen content. There was a 75%, 44%, 36% and 50% increase in nitrogen content in gelatin capsule treated plants compared to control plants at 0, 25, 50 and 100 mM NaCl, respectively (Figure 4.4). There was no significant difference between nitrogen content between control plants not subjected to salt stress, and gelatin treated plants at 100 mM NaCl (Figure 4.4). The linear regression analysis revealed that the gelatin treated plants had a significantly ($p<0.0001$) higher y-intercept (39.8) than control plants (23.6) which indicates that the treated plants had a higher nitrogen content at 0 mM. The gelatin treated plants also had a significantly ($p<0.0001$) lower slope (-4.72) compared to the control (-2.05), which reveals that there was a greater decrease in nitrogen content in treated plants than the control as salinity increased.

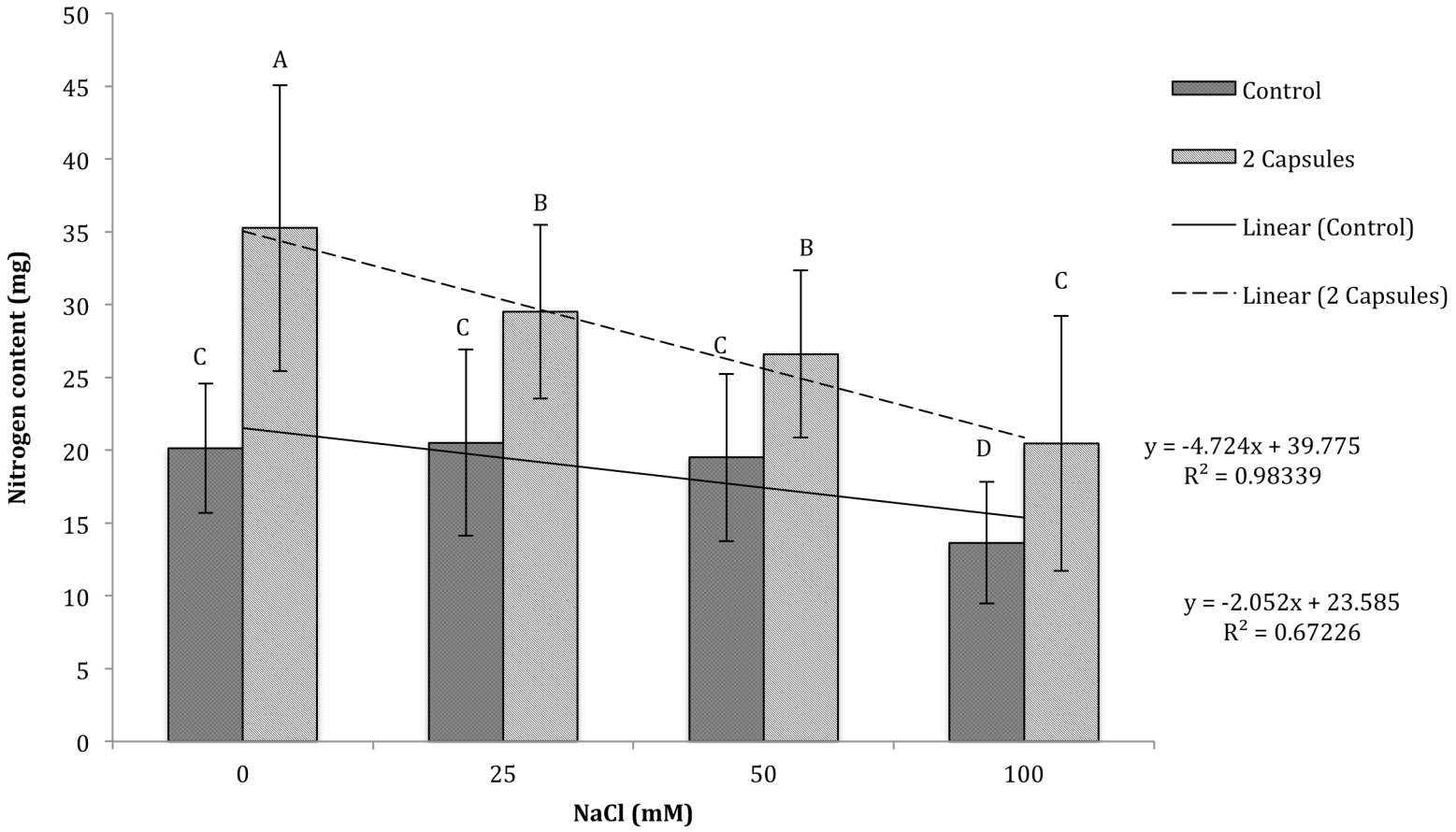

Figure 4.4: Total nitrogen content comparison of cucumber ('Vlaspik') 28 days after emergence between control and two gelatin capsules treatment under four levels of salt stress: 0 mM, 25 mM, 50 mM, and 100 mM. Data analyzed by ANOVA ($\alpha = 0.05$) and Tukey's HSD test was used to compare mean separation. Letters not associated with same letter are significantly different. Bars represent standard deviation. Linear regression was used to determine correlation.

Sodium analysis results exhibited an opposite trend compared to total nitrogen content. There was a significant increase in Na^+ concentration in plant tissue with increased salt stress in both the control and treated plants (Figure 4.5). In control plants there was a 11.5 fold increase in Na^+ concentration from plants that were not subjected to salt stress compared to 25 mM NaCl, a 12 fold increase at 50 mM NaCl and the greatest increase of 13 fold was measured at 100 mM NaCl plants. The increase in sodium content was not as great in plants treated with gelatin capsules compared to the control. A 10.5 fold increase in Na^+ concentration from plants not subjected to salt stress compared to 25 mM NaCl, a 11.7 fold increase at 50 mM NaCl, and 12.5 fold increase at 100 mM NaCl (Figure 4.5). No significant differences in Na^+ concentration accumulation in plant tissue were observed between the treatments in plants not subjected to salt stress at 25 mM NaCl or 50 mM NaCl. A significant difference was observed between the treatments at 100 mM NaCl with a 23% decrease in Na^+ concentration in plants treated with gelatin capsules compared to control (Figure 4.5). Regression analysis revealed that there was no significant difference in the y-intercept value of the Na^+ concentration between control and treated plants; however, there was significant difference ($p<0.0178$) in the slope of the regression with gelatin treated taking up less sodium than the control with increased NaCl concentration (Figure 4.5).

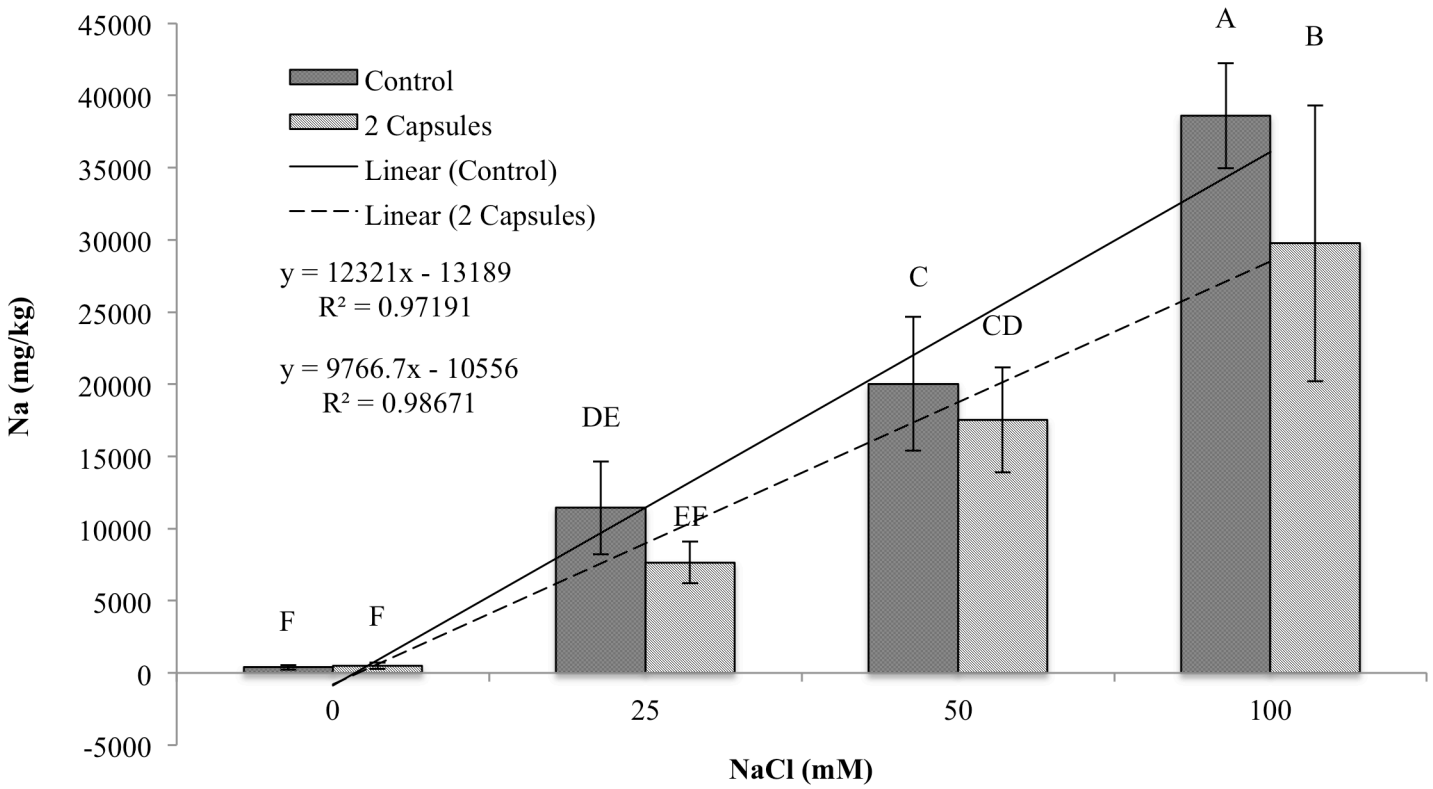

Figure 4.5: Na^+ concentration (mg/kg) comparison of cucumber ('Vlaspik') 28 days after emergence between control and two gelatin capsule treatment under four levels of salt stress: 0 mM, 25 mM, 50 mM, and 100 mM. Data analyzed by ANOVA (α = 0.05) and Tukey's test was used to compare mean separation. Letters not associated with same letter are significantly different. Bars represent standard deviation.

Effect of gelatin capsule treatment on proline content in cucumber plant tissue under salt stress

There was a significant change in proline content with increased salt stress in both control plants and plants treated with gelatin capsules. In the control plants, a significant increase in proline content was measured between plants that were not subjected to salt stress with all other salinity levels tested (Figure 4.6). In plants treated with gelatin capsules, no significant differences in proline was measured between plants not subjected to salt stress and 25 mM NaCl, but there was a 47% and 42% increase at 50 mM and 100 mM, respectively. No significant differences in proline were measured between the control plants and plants treated with gelatin capsules at a particular stress level. The exception was at 25 mM NaCl with 30% less proline in the gelatin treatment compared to the control (Figure 4.6).

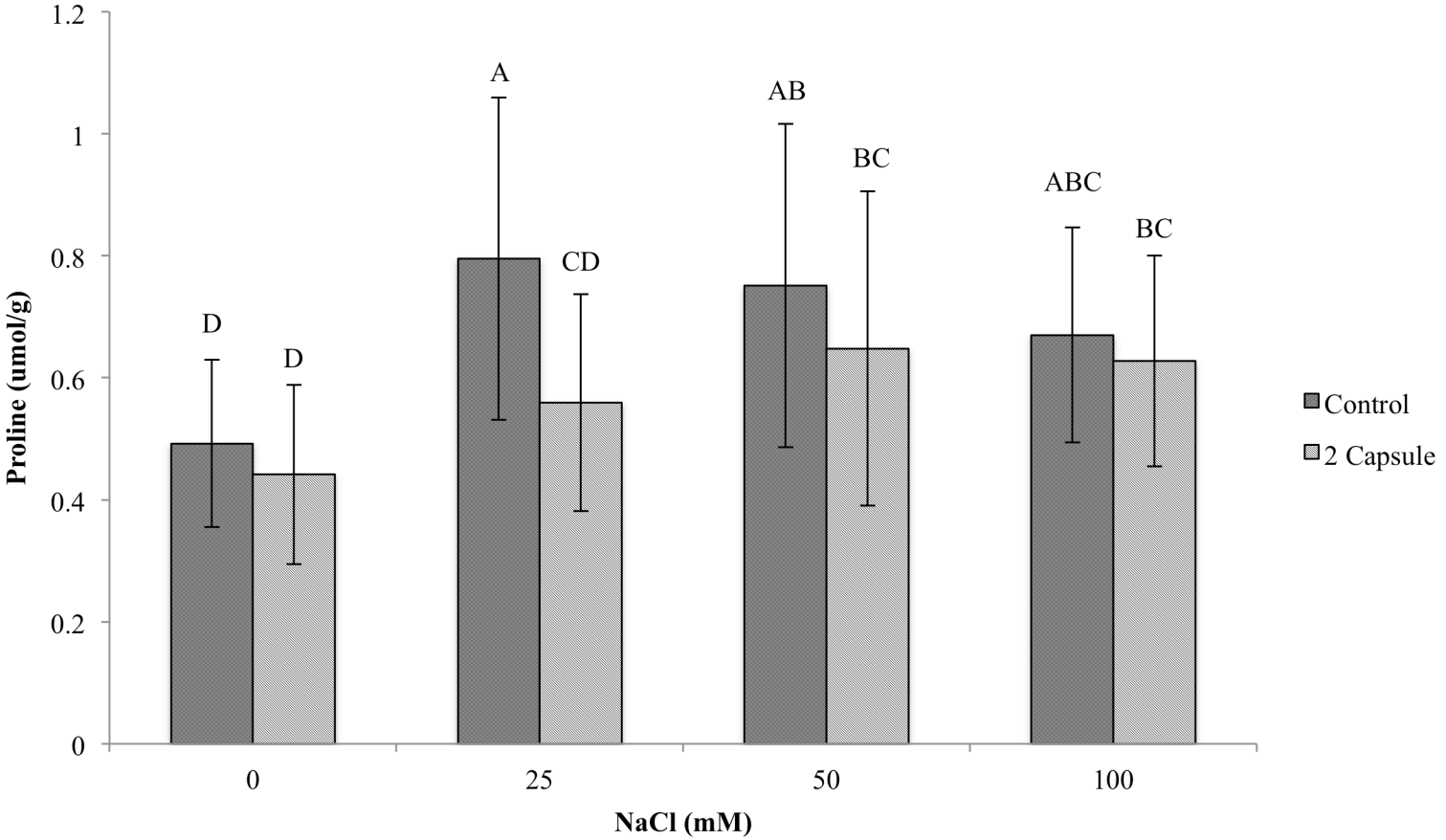

Figure 4.6: Proline concentration (umol/g) comparison of cucumber ('Vlaspik') 28 days after emergence between control and two gelatin capsule treatment under four levels of salt stress: 0 mM, 25 mM, 50 mM, and 100 mM. Data analyzed by ANOVA ($\alpha = 0.05$) and Tukey's test was used to compare mean separation. Letters not associated with same letter are significantly different. Bars represent standard deviation.

DISCUSSION

Salt stress negatively affected cucumber plant growth. Total leaf area, fresh weight and dry weight all decreased significantly with increased salt stress in both control plants and plants treated with gelatin capsules (Figures 4.1, 4.2 and 4.3). Consistent with these results, there was a reduction in total leaf area and leaf expansion was reduced by salinity in barley (Munns et al. 1982) and sugar-beet (Papp, Ball, and Terry 1983).

Salt stress affects plant growth and metabolism, which was partially attributed to a decrease in osmotic potential by the salt resulting in decreased availability of water to plants causing secondary osmotic stress (Tiwari et al. 2010; Zhu 2001). Salt stress also injures plants by ion cytotoxicity (Na^+, Cl^-, SO_4^{2-}) which results in nutritional deficiencies and metabolic imbalance (Zhu 2002). Salt stress leads to a reduction in photosynthesis and respiration by stomatal closure to reduce transpirational water loss by post-translational modulation of ion channels in guard cells, which is mediated by ABA (Janicka-Russak and Ktobus 2007; Grabov and Blatt 1998).

As reported in the previous studies (Wilson 2015, chap. 2, 3), gelatin capsule treated plants exhibited larger plants with increased total leaf area, fresh weight and dry weight, compared to the control plants. These results are supported by the increase in nitrogen content in gelatin capsules plants compared to the control (Figure 4.4), which suggest that plants treated with protein hydrolysate accumulate higher amount of nitrogen in the tissue. The increased salt stress; however, caused a decline in nitrogen content, which may be related to the reduction in nitrate reductase (NR) activity in leaves. NR activity in leaves is largely dependent on nitrate

flux from roots and is severely affected by osmotic shock induced by NaCl (Silveira et al. 2001; Ferrario, Valadier, and Foyer 1998). The nitrate assimilation process is more sensitive to water stress than photosynthetic assimilation, and NR activity could be lowered by enzyme degradation and the reduction in gene expression and NR protein synthesis (Ferrario, Valadier, and Foyer 1998).

Sodium ion concentration in leaf tissue increased with increased salt stress (Figure 4.5) and is consistent with results from Zhu et al. (2008). This suggests that the discriminative nature of the Na^+ transport is active under high salt stress. At low K^+ concentration, Na^+ competes with K^+ to pass through high-affinity K^+ transporters (HKT) (Rus et al. 2001; Platten et al. 2006). However, at high Na^+ concentration, higher uptake of Na^+ is achieved by both non-selective cation channels (NSCC) and HKT (Wakeel 2013; Munns and Tester 2008; Tester and Davenport 2003). Although NSCC are permeable to both Na^+ and K^+, it has a high affinity for Na^+ (Tester and Davenport 2003), hence the plants accumulates higher concentration of Na^+ under severe salt stress. There were no significant differences in Na^+ accumulation between control plants and capsule treated plants under mild salt stress (25 mM NaCl) and medium salt stress (50 mM NaCl) (Figure 4.5). Only under high salt stress (100 mM NaCl) the gelatin treated plants accumulated less Na^+ in the leaf tissue compared to the control plants. Further, the rate of sodium uptake was less from gelatin treatment compared the control as salinity increased. This result suggests a protective nature of gelatin capsule treatment in reducing sodium accumulation in alleviating salt stress.

An increase in proline accumulation in cucumber leaf tissue with increased salt stress, was demonstrated in tomato (Claussen 2005), *Pancratium maritium* (Khedr et al. 2003), and cucumber (Zhu, Bie, and Li 2008), and its protective function has been emphasized in numerous reviews (Kishor, Polavarapu, and Sreenivasulu 2014; Szabados and Sacvoure 2009; Liang et al. 2013; Kishor et al. 2005). Proline is one of the most common osmoprotectant in plants, and its increased accumulation under various abiotic stress such as drought stress, salinity stress and heat stress (Nounjan, Nghis, and Theerakulpisut 2012; Liang et al. 2013). Zhu et al. (2008) reported a steady increase in proline under increased salt stress in salt tolerant cultivar of cucumber, and only a slight increase in a salt sensitive cultivar. In this study, there was a significant increase in proline content between the control plants that were not subjected to salt stress and all plants subjected to salt stress (25 mM, 50 mM, and 100 mM) (Figure 4.6). Increased proline accumulation under salt stress was attributed to both the stimulation of proline biosynthesis, but also to the repression of proline catabolism (Kishor et al. 2005). In Arabidopsis, P5CS1, the enzyme responsible for first step in proline biosynthesis is induced by salt stress and was shown to accumulate a higher amount of transcript under salinity conditions (Yoshiba et al. 1995). Proline catabolism, on the other hand, is activated during re-hydration and stress relief, and is controlled by proline dehydrogenase (PDH). PDH activity is repressed during stress, and thus prevents proline degradation for the duration of the abiotic stress (Verbruggen, Villarrole, and Van Montagu 1993; Kiyosue et al. 1996). The results from this study revealed that proline accumulation increased from 0 mM to 25 mM NaCl, and instead of a steady increase as described by Zhu et al. (2008), it remained at the same level for 50 mM and 100 mM NaCl. This

suggests that proline biosynthesis was induced by the salt stress and proline catabolism repressed by the salt stress, proline accumulated in the leaf tissue.

Exogenous proline was reported to protect plants under stress (Huang et al. 2009); however, this study result indicates that plants treated with gelatin capsules had significantly less proline than the control plants only under mild stress (25 mM NaCl) (Figure 4.6). This is hypothesized to be due the plants treated with gelatin capsules experienced less stress from the salt treatment, and thus accumulated less proline in the tissue. Proline has multiple functions in plant stress tolerance, and proline was shown to act as a protein chaperone to protect structural integrity and enhance enzyme activities (Szabados and Sacvoure 2009), and act as singlet oxygen quencher in ROS scavenging (Kishor et al. 2005; Szabados and Sacvoure 2009). Arabidopsis plants with mutation on P5CS1 gene, unable to accumulate proline under stress, had enhanced oxidative damage due to accumulation of ROS (Székely et al. 2008). Proline also acts as a reserve source of carbon, nitrogen and energy during recovery from stress (Zhang, Lu, and Verma 1997). Proline biosynthesis from glutamate regenerates $NADP^+$ that is needed to support the oxidative steps of the pentose phosphate pathway (PPP), and high concentration of $NADP^+$ are necessary for PPP for regeneration of NADPH and to supply ribose-5-phosphate for the synthesis of purines. Thus proline accumulated under stress conditions serves as a sink for excess reductants providing the NAD^+ and $NADP^+$ required for maintenance of respiratory and photosynthetic process (Kishor et al. 2005). The amount of proline that plants accumulate was correlated to the stress imposed, which means plants that are under mild stress, will accumulate significantly lower amount of proline compared to plants under high stress (Zhu, Bie, and Li

2008). Thus we can assume that the plants treated with gelatin experienced less stress and hence accumulated less proline, compared to the control plants.

The total leaf area, fresh weight and dry weight results suggests that the gelatin capsule treatment was able to compensate for the damages caused by the salt stress. Plant growth parameters at highest salt stress (100 mM NaCl) with the application gelatin capsule were not significantly different from that of control plants with no salt stress (Figure 4.1, 4.2, 4.3), which indicates that plant growth was comparable under the two contrasting stress conditions. This effect may be attributed to the effect of protein hydrolysates on overall plant growth and development. The linear regression analysis revealed that the gelatin treated plants had greater nitrogen content at 0 mM compared to the control (Figure 4.4). Protein hydrolysates were shown to stimulate carbon and nitrogen metabolism and increase nitrogen assimilation in plants (Schiavon, Ertani, and Nardi 2008; Ertani et al. 2009). NAD-dependent glutamate dehydrogenase, nitrate reductase and malate dehydrogenase in maize were reported following application of animal epithelial hydrolysate (Maini 2006). Alfalfa protein hydrolysate applied to hydroponically-grown maize increased the activity malate dehydrogenase, isocitrate dehydrogenase, and citrate synthase, which are enzymes found in TCA cycle; as well as nitrogen metabolism enzymes, nitrate reductase, nitrite reductase, glutamine GS, GOGAT, and aspartate aminotransferase (Schiavon, Ertani, and Nardi 2008). Ertani et al. (2009) reported that both alfalfa protein hydrolysate and animal connective tissue hydrolysate stimulated plant growth, and also increased the nitrate conversion into organic nitrogen by inducing nitrate reductase and GS activities. The treatments enhanced especially the GS2 isoform, which is responsible for assimilation of ammonia produced by nitrate reduction, confirms that protein hydrolysate

enhances plant growth by up regulating nitrate assimilation (Ertani et al. 2009; Calvo, Nelson, and Kloepper 2014). The linear regression analysis of nitrogen content also revealed that, although the gelatin treated plants had greater nitrogen content at 0 mM, it also had a greater decrease in nitrogen content with increasing NaCl concentration (Figure 4.4). This suggests that the plants treated with gelatin are susceptible to salt stress; however, are better able to withstand the stress due to the overall general growth enhancement regardless of stress .

Another hypothesis is that gelatin treatment increased the synthesis of osmoprotectants. The identity of the osmoprotectant is unknown; however, proline is ruled out as the accumulating osmolyte as our results indicate reduced proline accumulation in plants treated with gelatin capsule treatment compared to the control. The proline present in gelatin may have acted as a protectant to salt stress by increasing antioxidant activity in the plant. Huang et al. (2009) reported that exogenous application of proline on cucumber plants subjected to salt stress significantly alleviated the growth inhibition of plants induced by salt-stress by improving water status and increasing peroxidase enzyme activity in the leaf. Although the plants in this study did not accumulate more proline with the addition of gelatin capsules as expected, gelatin increased plant growth that may be attributed to decreased damage caused by salt stress.

Overall, plants treated with gelatin were better able to withstand increased salt stress and produced larger biomass compared to the control plants. The gelatin treatment may have acted as salt stress protectant to the plants, inducing synthesis of osmoprotectants. The gelatin treated plants also had lower Na^+ accumulation. This is evident from the significant difference ($p<0.0178$) in the slope of the regression analysis of Na^+ concentration between control and

treated plants. The control plants accumulated Na^+ at a higher rate with increased NaCl concentration compared to the treated plants, which suggests that gelatin treatment restricted Na^+ transport to the shoot and excluded Na^+ accumulation in the tissue. Seed encapsulation and application of gelatin may be used in cucumber transplant production or by direct seeding in a saline soil environment for enhanced growth promotion and early seedling establishment.

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CHAPTER 5: Transcriptome analysis of beneficial effects of gelatin seed treatment on cucumber plant growth

ABSTRACT

Growth enhancement of gelatin capsule treatment on cucumber plants was characterized in previous studies (Chapter 2 and Chapter 3) and under salinity stress (Chapter 4); however, the underlying molecular mechanisms of the plant growth promotion by gelatin are still unknown. The effect of gelatin capsule treatment on cucumber growth and development was studied at the transcriptome level using whole genome gene expression profiling (RNA-sequencing). Using pairwise comparison and weighted gene co-expression network analysis, modules of co-expressed genes and 16 hub genes were identified for plants treated with gelatin. The upregulation of MYB, NAC, and WRKY transcription factors have been associated with plants treated with gelatin capsules. Amino acid transport genes and nitrogen transport gens were identified as having strong correlation with increased leaf area and nitrogen content in plants treated with gelatin capsules that may explain the increased biomass and high nitrogen content in treated plants. The analysis also identified genes involved in xenobiotic biodegradation that may explain the increased salinity tolerance of gelatin treated plants. This work together with the previous studies involving gelatin treatments provides insights on mechanisms on growth enhancement and increased salinity stress tolerance.

INTRODUCTION

Seed gelatin encapsulation is a technology developed by Alliance Seed Capsule, a consortium between Coating Supply and Sakata, in which raw and/or processed seeds are encapsulated in gelatin capsule (Illustration 5.1 and 5.2). The encapsulation provides several advantages such as improved handling and sowing, faster plant growth, precision planting, precise seed quantities per sowing unit, and also provides the capability of combining seed enhancement technologies and other chemicals or biological additives such as pesticides, fertilizers, and Rhizobium (Takahashi and Trias 2012).

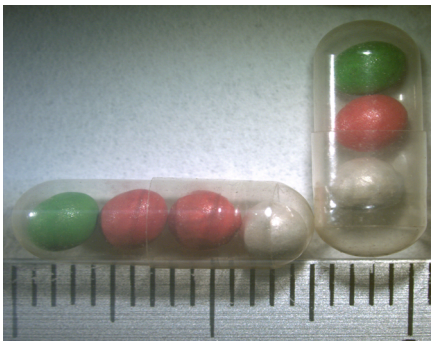


Illustration 5.1: Seed Encapsulation of pelleted seeds

Illustration 5.2: Seed Encapsulation of multiple seeds

From a regulatory standpoint, application of gelatin as a seed treatment falls under the category of plant biostimulants as it uses animal based protein hydrolysate, a hydrolyzed form of protein, as the main component. Plant biostimulants are a broad class of substances and microorganisms that enhance plant growth. The European biostimulants industry council (EBIC) has defined plant biostimulants as substance(s) and/or microorganisms whose function when applied to plants or the rhizosphere is to stimulate natural processes to enhance/benefit nutrient

uptake, nutrient efficiency, tolerance to abiotic stress, and crop quality. Biostimulants have no direct action against pests, and therefore do not fall within the regulatory framework of pesticides (du Jardim, 2012; EBIC, 2014).

Protein hydrolysate and other protein-based product application were reported to enhance plant growth and yield in field tomato (Parrado et al. 2008), greenhouse tomato (Koukounararas, Tsouvaltzis, and Siomos 2013), papaya (Morales-Payan and Stall 2003), maize seedlings (Ertani, Schiavon, et al. 2013; Ertani, Pizzeghello, et al. 2013), and hydroponic lettuce (Colla et al. 2012). A positive effect of protein hydrolysate on abiotic stress also was reported, and cucumber plants subjected to suboptimal pH level and temperature performed better with the application of humic acid containing substance LACTOFOL™, with notable increase in leaf area, shoot and root mass (Boehme, Schevschenko, and Pinker 2008; Boehme, Schevtschenko, and Pinker 2005). An alfalfa plant-derived biostimulant increased maize plant biomass under salinity condition, and enhanced Na^+ accumulation and reduced K^+ accumulation in roots and leaves (Ertani, Schiavon, et al. 2013). Perennial ryegrass treated with Macro-Sorb Foliar (FOLIAR™), an animal membrane hydrolysate subjected to high air temperature stress exhibited increased photochemical efficiency and membrane thermostability compared to plants without the treatment (Kauffman, Kneivel, and Watschke 2007).

Protein hydrolysate was shown to stimulate carbon and nitrogen metabolism and increase nitrogen assimilation in plants (Schiavon, Ertani, and Nardi 2008; Ertani et al. 2009). NAD-dependent glutamate dehydrogenase, nitrate reductase and malate dehydrogenase in maize had higher activities following application of animal epithelial hydrolysate (Maini 2006). Alfalfa

protein hydrolysate applied to hydroponically-grown maize increased the activity of malate dehydrogenase, isocitrate dehydrogenase, and citrate synthase, which are enzymes found in the TCA cycle; as well as nitrogen metabolism enzymes: nitrate reductase, nitrite reductase, glutamine, glutamine synthetase (GS), glutamine synthase, and aspartate aminotransferase (Schiavon, Ertani, and Nardi 2008). Ertani et al. (2009) reported that both alfalfa protein hydrolysate and animal connective tissue hydrolysate stimulated plant growth, and also increased nitrate conversion into organic nitrogen by inducing nitrate reductase and GS activities. The treatments especially enhanced the GS2 isoform, which is responsible for assimilation of ammonia produced by nitrate reduction; thus confirming that protein hydrolysate enhances plant growth by up regulating nitrate assimilation (Ertani et al. 2009; Calvo, Nelson, and Kloepper 2014).

Seed encapsulation using gelatin capsules is a novel approach as a seed treatment to enhance plant growth (Takahashi and Trias 2012). The effect of gelatin applied at time of sowing on cucumber plant growth was previously been characterized (Wilson 2015, chap. 2,3,4). The gelatin capsule treatment increased leaf surface area, fresh and dry weight as compared to the non-treated control, and increased salinity tolerance in plants treated with gelatin capsules. However, the underlying molecular mechanisms of the plant growth promotion by protein hydrolysate are unknown. The objective of this study was to characterize the effect of gelatin capsules on cucumber growth and development at the transcriptome level using whole genome gene expression profiling (RNA-sequencing).

MATERIALS & METHODS

Plant materials and evaluation of growth and nitrogen content

Cucumber seeds 'Vlaspik' (Seminis, Oxnard, CA) were planted in a controlled greenhouse maintained at 24°C/21°C temperature with 14/10 hours photoperiod at New York State Agricultural Experiment Station in Geneva, NY in the winter of 2013. Two size #3 gelatin capsules (Capsule line, Pompano Beach, FL) were placed adjacent to each seed in a 4-inch pot (Illustration 5.3); these are referred to as treated plants. Capsules were placed adjacent to the seeds to prevent decrease in germination rate, and for controlling the amount of gelatin used in the treatment. Total weight of two gelatin capsules were 93.7 mg with a nitrogen content of 14.2 mg and protein content of 80.0 mg. Non-treated, control plants did not receive gelatin capsule treatment. Thirty-two plants (16 control and 16 treated plants) were placed in the greenhouse following a completely randomized design. First (L1) and second (L2) true leaves from 8 plants in each treatment group were sampled at each of two different time points (one and two weeks after emergence: time point 1 (T1) and time point 2 (T2)). Total leaf area was measured using CI-202 Leaf Area Meter (CID Bio-Science, Camas, WA) and fresh weight was determined. Samples were flash frozen in liquid nitrogen and stored at -80°C. Frozen samples were weighed to determine frozen weight. A subsample (~100 mg) of the frozen leaf tissue was placed in an oven to dry at 100°C over night and sent to the Cornell University Stable Isotope Laboratory (Ithaca, NY) for elemental analysis of N by combustion method. All sampling data were then statistically analyzed by ANOVA ($\alpha = 0.05$) and Tukey's HSD test was then used to determine significance.

Illustration 5.3: Two gelatin capsules (four halves) placed adjacent to the seeds in 4-inch pot.

RNA isolation and Strand-specific RNA-seq library construction and sequencing

Three biological replicates of each treatment of every leaf of at every time point were selected for RNA-seq, a total of 24 samples (two treatments, two time point, two leaf, and three biological replicates). Total RNA was isolated from ~5 g of ground leaf tissue using the Spectrum™ Plant Total RNA Kit (Sigma Aldrich, St Louis, MO) following the manufacture's protocol. The isolated RNA quantity and quality were evaluated by electrophoresis (2% Agarose gel) followed by ethidium bromide staining and quantification by Nanodrop 1000 (Thermo Scientific, Waltham, MA).

A 5µg subsample of the isolated total RNA from each sample were treated with 1 µl DNase I (Invitrogen, Carlsbad, CA) incubated at RT for 15 minutes followed by 70°C for 10 minutes. The DNase treated total RNA samples were used to isolate mRNA in order to construct strand-specific RNA-seq library using NEBNext Poly(A) mRNA Magnetic Isolation Module and NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA). Briefly, the mRNA was isolated with 14 µl of NEBNext Magnetic Oligo $d(T)_{25}$ and eluted

with 16 µl of Elution Buffer. Five-microliter of the isolated mRNA was fragmented in NEBNext First Strand Synthesis Buffer by heating at 94°C for 15 minutes, and then used for the first-stranded cDNA synthesis by reverse transcription using random primers. The synthesized first strand cDNA was then used as a template to synthesize double stranded cDNA. The resulting double stranded cDNA was end repaired, dA-tailed and then ligated with NEBNext adaptor. Small size (approximately 300bp) fragments were selected using Agencourt AMPure XP beads (Bechman Coulter, Pasadena, CA) in 0.6 volumes of ligation reaction. Another round of size selection was performed with 0.25 volumes of the cDNA library solution, and then digested with 1 µl of NEBNext USER enzyme at 37°C for 15 minutes. The cDNA library was enriched by PCR with NEBNext index primers following conditions: 98°C for 30 seconds; 15 cycles of 98°C for 10 seconds, 65°C for 30 seconds, 72°C for 30 seconds; 72°C for 5 minutes; and held at 4°C. The PCR-enriched cDNA libraries were purified by 1.4 volumes of Agencourt AMPure XP beads and eluted in 22 µl Elution Buffer. The purified cDNA library was analyzed along with Quick-Load® 1 kb Extended DNA Ladder (New England Biolabs, Ipswich, MA) by electrophoresis (2% Agarose gel) followed by ethidium bromide staining to visualize the optimal size range of the cDNA library (250 bp - 400 bp). The cDNA library of optimal sizes was quantified by Qubit 2.0 Fluorometer using the dsDNA HS Assay Kit (Invitrogen, Carlsband, CA). Three sets of 8 multiplexed libraries (30 ng per library) were configured and sent to Cornell University Biotechnology Resource Center (Ithaca, NY) for single-end sequencing using three lanes (one lane per set of the multiplexed libraries) on Illumina HiSeq 2000 (Illumina, San Diego, CA).

Reads processing and data analysis

The Illumina pipeline generated a total of 387 million raw reads for the 24 samples. On average there were 16 million (±1361774.7) raw reads per library, with the longest read of 101 bp (Table5.1). Data was analyzed with CLC Genomics Workbench (CLC) version 6.5 (CLCBio, Cambridge, MA). The cucumber reference genome (Chinese long, version 2.0) (Li et al. 2011) and annotation file was downloaded from the International Cucurbit Genomic Database (www.icugi.org). MapMan Gene ontology of the reference transcriptome of 23,248 genes were obtained via the web-based search tool Mercator (http://mapman.gabipd.org), which searched several databases, including Arabidopsis TAIR 10 (The Arabidopsis Information Resources), SwissProt/Uniref 90 (UniProt Reference Clusters), CDD (Conserved Doman Database, NCBI), KOG (Eukaryotic orthologous groups of proteins, NCBI), InterPro (EMBL-EBI).

Reads mapping against the reference genome (Chinese long, version 2.0) were conducted using the following parameters. The limit for read unspecific match to the reference genome was set to 10, minimum length fraction was 0.9 and minimum similarity fraction was 0.98. Normalized read counts RPKM (reads per kilobase of transcript per million) for each gene was calculated, and genes with RPKM lower than 1.0 was considered not expressed and excluded from analysis. In order to identify differentially expressed genes (DEGs), pairwise comparisons of RPKMs were performed between control and treated plants at each leaf tissue in each time points (control T1-L1 vs. treated T1-L1, control T1-L2 vs. treated T1-L2, control T2-L1 vs. treated T2-L1, control T2-L2 vs. treated T2-L2). The results were weighted by the t-type test statistics (p-value) and FDR (false discovery rate) corrected p-values using Baggerley's test (Baggerly et al. 2003). The cutoff for defining a DEG is $P_{FDR}<0.05$.

Gene network construction and visualization

Unsigned co-expression networks were constructed using WGCNA (v1.41) package in R (Langfelder and Horvath 2008) using square root normalized RPKM data from the 24 samples. Total of 620 differentially expressed genes (DEG) were used. The adjacency matrix was created by calculating the Pearson's correlation between all genes and raised to a default power of 6. The Topological Overlap Measure (TOM), which is a measure of overlap in shared neighbors, was calculated using the adjacency matrix. The dissimilarity TOM was used as input for the dendrogram, and modules were detected using the DynamicTreeCut algorithm. Modules are defined as clusters of highly interconnected genes, and genes within the same cluster have high correlation coefficients among one another (Hollender et al. 2014; Langfelder and Horvath 2008). All modules were assigned a unique color (blue, yellow, brown, turquoise, and gray). The module eigengenes were used to represent each module, which were calculated by the first principal component, capturing the maximal amount of variation of the module. The eigengenes were then used to estimate the correlations between module eigengenes and the traits of interest: total leaf area (TLA), fresh weight (FW), frozen weight (DW), and total nitrogen percent (PercentN). The gene network of the blue module was visualized using Cytoscape (v.3.0.0), the most relevant module in explaining the trait variations, such as the effect of the treatments.

Expression anaysis using qRT-PCR

Expression profile of 5 genes (Csa1G066570, Csa1G590300, Csa5G589260, Csa5G140480, Csa5G319910) were confirmed in the 12 samples taken at time point 2 using qRT-PCR. A

housekeeping gene (Csa6M484600) encoding ACTIN was used as reference. Primers were designed using BatchPrimer 3v1.0 software (You et al. 2008) (Table 5.1). Genes were selected from the 620 DEG identified pairwise comparisons of RPKMs were performed between control and treated plants at each leaf tissue in each time points. The RPKM and qRT-PCR expression values were square root transformed.

Table 5.1: qRT-PCR Primer sequence (forward, and reverse) by gene (feature ID). Primers design with BatchPrimer 3v1.0

Feature ID	Forward (5'-3')	Reverse (5'-3')
ACT	CCGTTCTGTCCCTCTACGCTAGTG	GGAACTGCTCTTTGCAGTCTCGAG
Csa1G066570	TGAGCAATGCCCTGTCCAAC	ATCACCTTCCTGGCCCACAA
Csa1G590300	ATTGCTGGCGTTGTCACTGG	TCCCAAGCATGGCTTCACAA
Csa5G140480	CTGCATTTCCCCGGATTCTG	GCGGGGTCGAACTTGTCATC
Csa5G319910	AGCCCATGTGCCTTCGTTGT	GATCGCCTGTTCCCCGATTT
Csa5G589260	GGTGTTGGCCGGTTTTTGAA	CTTTCAGTGCGGCAGCTCCT

The mRNA were purified from 5 µg of total RNA for each of the 12 samples and reverse transcribed in a final volume of 20 µl using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), according to the manufacturer's protocol. The qRT-PCR were performed using 50 ng of cDNA in a final volume of 20 µl containing 10 µl of LightCycler® 480 SYBR Green MasterMix (Roche Applied Science, Germany) and 2 µl of 10

mmol of specific primers (Table 5.2). Triplicates of each sample were run in the LightCycler® 480 instrument (Roche Applied Science, Germany). The total ammount of cDNA were normalized by the co-amplification of the ACTIN gene and by calibrating the relative expression using the relative quantification method described by Pfaffl (2001). (Fraga, Meulia, and Fenster 2008; Pfaffl 2001).

RESULTS

Evaluation of growth and nitrogen content

The growth measurements of the plants treated with gelatin capsules were greater than the control in both time points (T1 and T2) (Figure 5.1, 5.2, 5.3, 5.4). One week after emergence (T1), there were significant differences between the first leaf (L1) of control and plants treated by gelatin capsules, with a 36% increase in total leaf area ($p<0.0314$) (Figure 5.1). There were no significant difference in leaf area between the control and the treated plants on the second leaf (L2) (Figure 5.1). Two weeks after emergence (T2), both L1 and L2 were significantly different between the control and treated plants ($p<0.0230$ and $p<0.0292$), respectively (Figure 5.2). There was a 28% and a 40% increase in leaf area from the control to the treated for L1 and L2, respectively (Figure 5.2).

There were no significant differences in fresh weight one week after emergence (T1) between the control and treated plants. However, a significant difference ($p<0.0076$) was

measured on the second week after emergence (T2) with a 52% increase in the treated plant compared to the control (Figure 5.3).

Frozen weight also increased with the gelatin capsule treatments, with a 16% increase one week after emergence and a 28% increase two weeks after emergence. There were no significant differences in frozen weight between control and treated plants during T1, but significant differences were measured in T2 ($p<0.0076$) (Figure 5.4).

The nitrogen content increase was less significant with only a 4% increase in nitrogen in the treated plants compared to the control one week after emergence, and a 12% increase two weeks after emergence. However, there was not a significant increase at T1, but a significant difference in nitrogen content was measured in T2 ($p<0.0076$) (Figure 5.5).

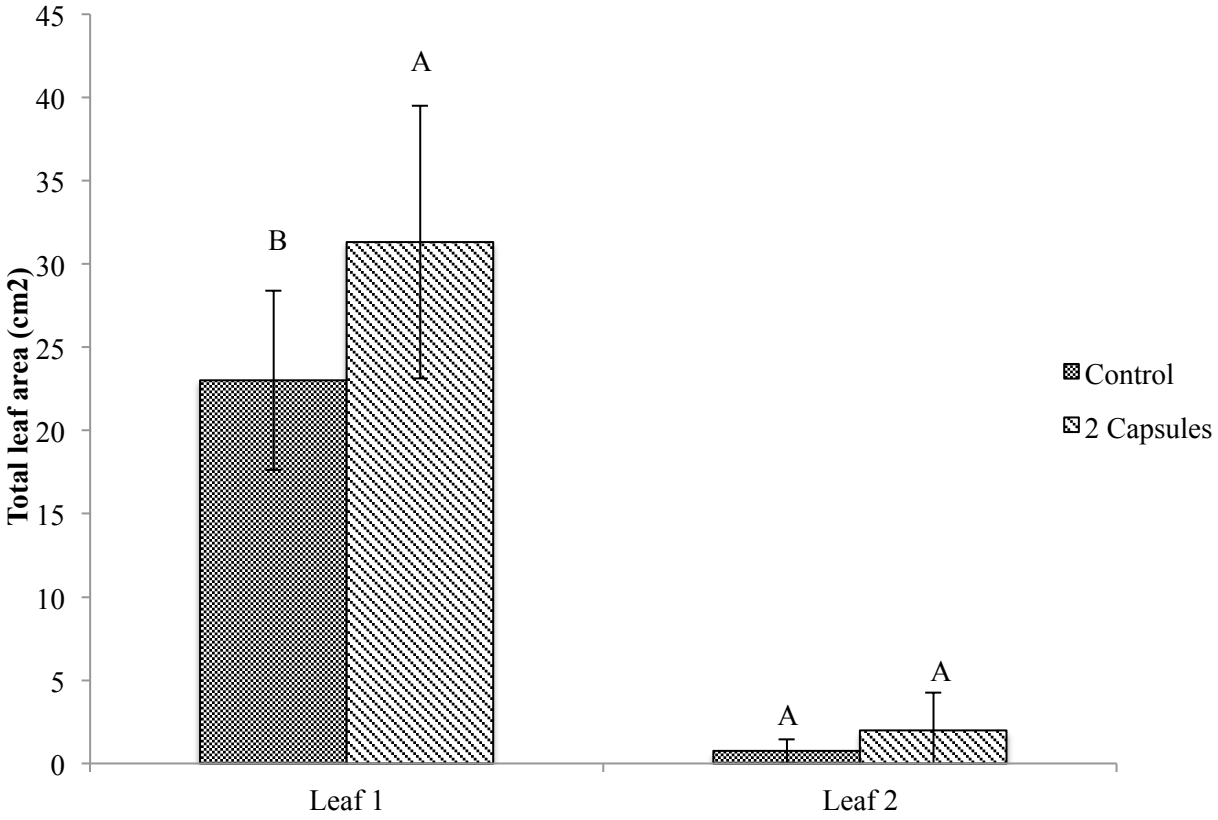


Figure 5.1: Total leaf area comparison of cucumber ('Vlaspik') one week after emergence (T1) between control and gelatin capsule treatment (2 Capsules). Samples were harvested one week after emergence; first leaf (Leaf 1) and second leaf (Leaf 2) were harvested separately. Data analyzed by ANOVA ($\alpha = 0.05$) and Tukey's HSD test was then used to compare mean separation. Letters not associated with same letter were significantly different. Bars represent standard deviation.

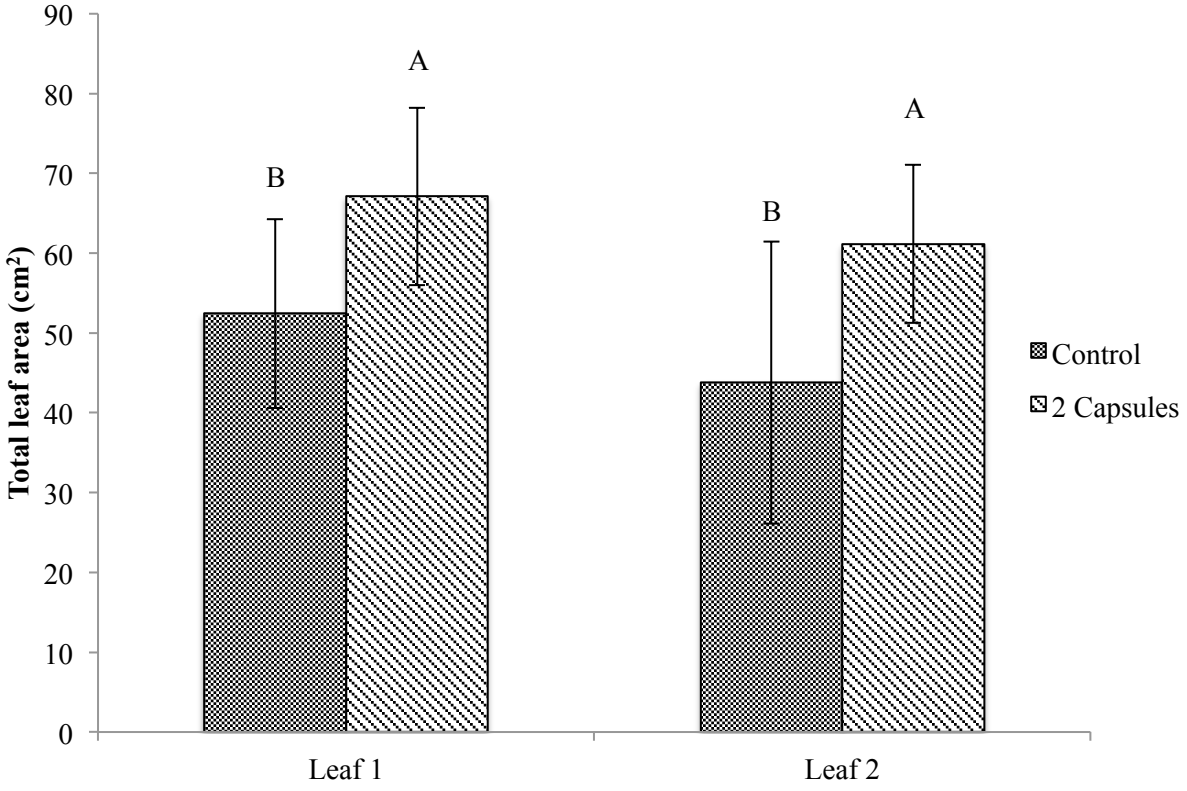


Figure 5.2: Total leaf area comparison of cucumber ('Vlaspik') two weeks after emergence (T2) between control and gelatin capsule treatment (2 Capsules). Samples were harvested one week after emergence; first leaf (Leaf 1) and second leaf (Leaf 2) were harvested separately. Data analyzed by ANOVA ($\alpha = 0.05$) and Tukey's HSD test was then used to compare mean separation. Letters not associated with same letter were significantly different. Bars represent standard deviation.

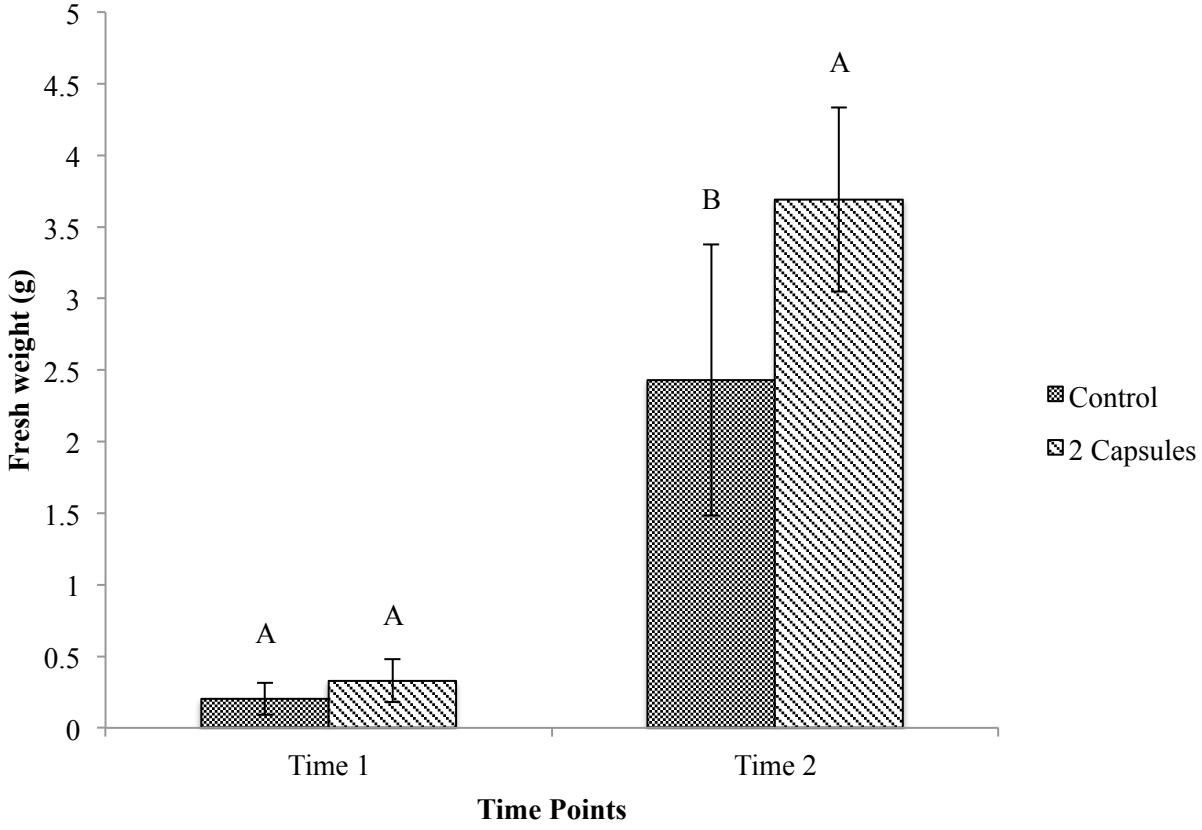


Figure 5.3: Fresh weight comparison of cucumber ('Vlaspik') between control and gelatin capsule treatment (2 Capsules). Samples were harvested one week after emergence (Time 1) and two weeks after emergence (Time 2). Data analyzed by ANOVA ($\alpha = 0.05$) and Tukey's HSD test was then used to compare mean separation. Letters not associated with same letter were significantly different. Bars represent standard deviation.

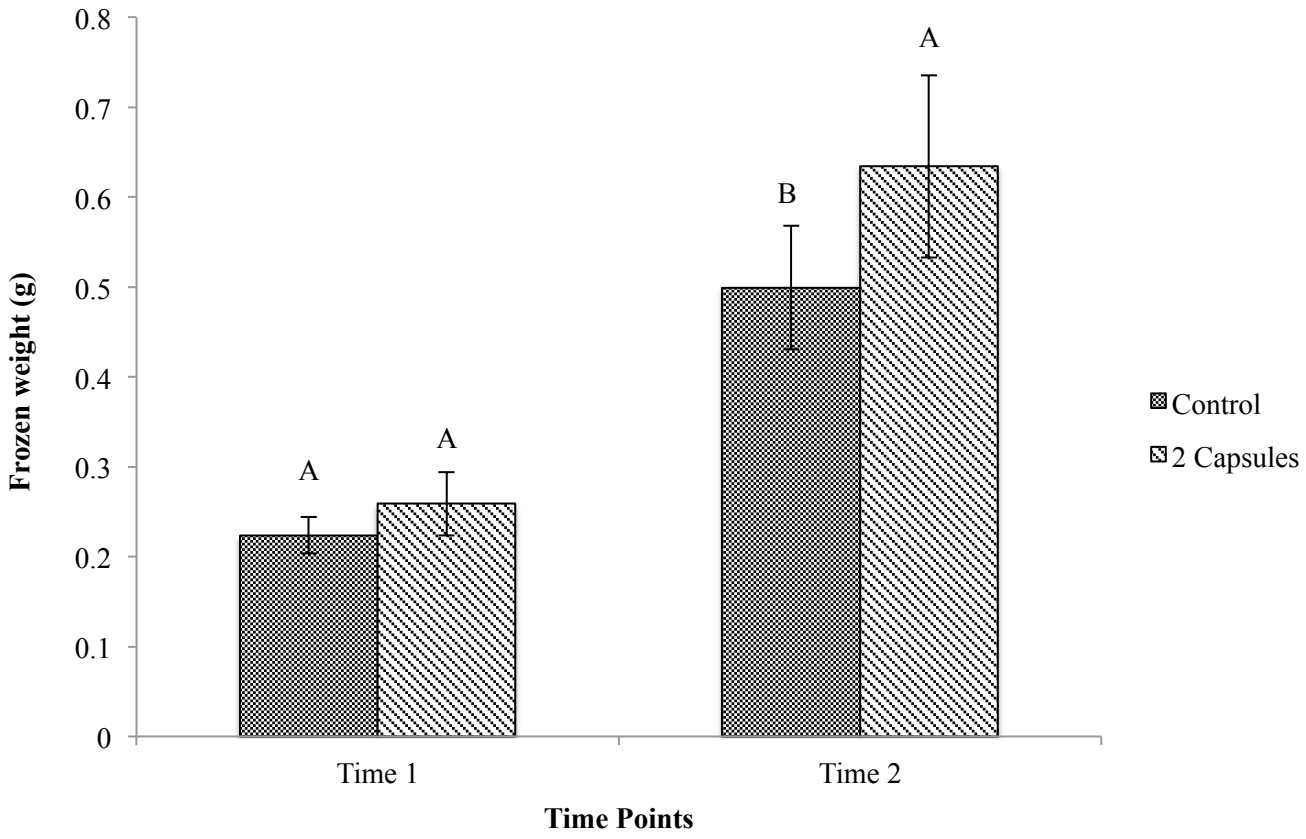


Figure 5.4: Frozen weight comparison of cucumber ('Vlaspik') between control and gelatin capsule treatment (2 Capsules). Samples were harvested one week after emergence (Time 1) and two weeks after emergence (Time 2). Data analyzed by ANOVA ($\alpha = 0.05$) and Tukey's HSD test was then used to compare mean separation. Letters not associated with same letter were significantly different. Bars represent standard deviation.

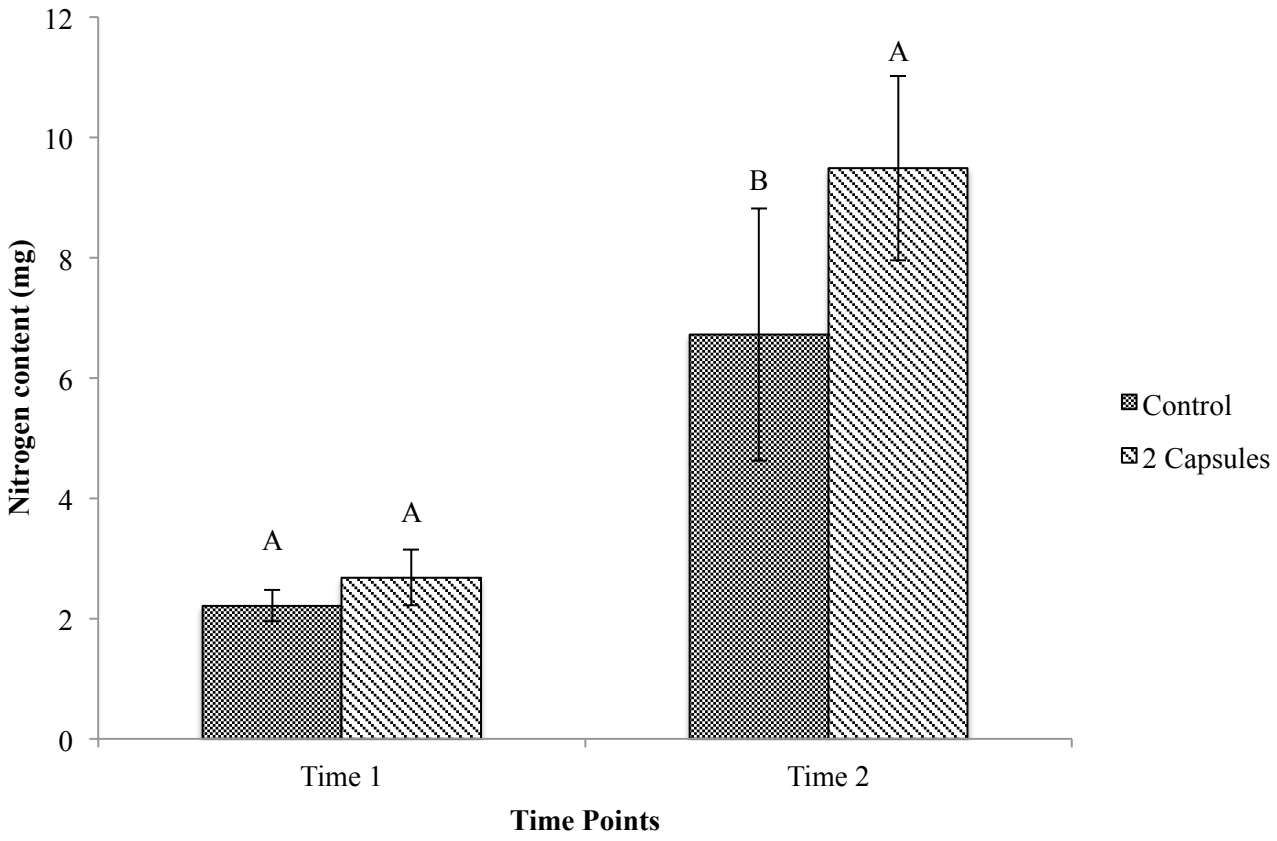


Figure 5.5: Nitrogen content comparison of cucumber ('Vlaspik') between control and gelatin capsule treatment (2 Capsules). Samples were harvested one week after emergence (Time 1) and two weeks after emergence (Time 2). Data analyzed by ANOVA ($\alpha = 0.05$) and Tukey's HSD test was then used to compare mean separation. Letters not associated with same letter were significantly different. Bars represent standard deviation.

Transcriptome analysis

RNA sequencing of the 24 samples (two treatments, two time point, two leaf, and three biological replicates) yielded a total of 377 million reads, translating to a mean of 16 million reads per samples. Mapping of the 377 million reads against the reference genome indicated that 69% (260.1 million) were mapped of which 98.8% (257.0 million) were mapped to exons and 1.2% (3.1 million) to introns. Excitingly, 97.7% (254 million) of the 260.1 million mapped reads were mapped uniquely to the reference genome (Table 5.2).

The first leaf of the control plants (L1) at T1 had a total of 14,864 expressed genes (RPKM>1.0), whereas L1 of the treated plants at T1 had the fewest number of expressed genes in the whole group with only 13,885 expressed genes. Of these genes 13,803 overlapped between the control and treated plants (Figure 5.6). The samples from L2 in T1 had the highest number of expressed gene, with 15,764 expressed genes in the control and 15,874 expressed genes in the treated plants, of which 15,508 expressed genes overlapped (Figure 5.6). The control L1 from T2 had 14,713 expressed genes, and the treated had 14,843 expressed genes, of which 14,535 expressed genes overlapped (Figure 5.7). The control L2 from T2 had 14,756 expressed genes, and the treated had 15,287 expressed genes, of which 14,537 expressed genes overlapped (Figure 5.7). There were 12,650 total genes that over lapped with all four groups (T1-L1, T1-L2, T2-L1, T2-L2) (Figure 5.8).

Table 5.2: RNA-seq results of 24 samples: two treatments (control and treated with 2 capsules), two time points (T1 and T2), two leaves (L1, and L2) and three replications of each. Mean, average and total (±standard deviation) read number, mean, average and total (±standard deviation) read length (bp), mean and average uniquely mapped total gene, mean and average percent to total fragments uniquely mapped to the reference, mean and average percent fragments mapped to exons, and mean and average percent fragment mapped to introns (Illumina HiSeq 2000).

Sample	Treatment	Time point/Leaf	Rep	Number of reads	Uniquely mapped total gene	Percent fragment uniquely mapped to reference (%)	Percent fragment uniquely mapped to exons (%)	Percent fragment uniquely mapped to introns (%)
1	Control	T1-L1	1	12019363	9350227	77.79	98.71	1.29
2	Control	T1-L1	2	8624277	6592181	76.44	98.61	1.39
3	Control	T1-L1	3	14970567	10499989	70.14	99.36	0.64
4	Control	T1-L2	1	10128190	7724322	77.03	98.73	1.27
5	Control	T1-L2	2	9216330	6472955	70.23	98.33	1.67
6	Control	T1-L2	3	14144786	9498041	67.15	98.86	1.14
7	Treated	T1-L1	1	17361035	13764974	79.29	99.1	0.9
8	Treated	T1-L1	2	8794498	4518865	51.38	98.99	1.01
9	Treated	T1-L1	3	16744466	12843001	76.7	99.17	0.83
10	Treated	T1-L2	1	21897381	16628402	75.94	98.64	1.36
11	Treated	T1-L2	2	8393223	5533323	65.93	98.58	1.42
12	Treated	T1-L2	3	16743616	12757850	76.2	98.09	1.91
13	Control	T2-L1	1	13141492	10676776	81.24	98.62	1.38
14	Control	T2-L1	2	13736384	10695841	77.87	98.61	1.39
15	Control	T2-L1	3	23334193	17068298	73.15	98.98	1.02
16	Control	T2-L2	1	12638497	10042325	79.46	99.19	0.81
17	Control	T2-L2	2	16590125	2877719	17.35	99.06	0.94
18	Control	T2-L2	3	32818854	23045970	70.22	99.17	0.83
19	Treated	T2-L1	1	8454166	5946132	70.33	98.5	1.5
20	Treated	T2-L1	2	14493239	11039888	76.17	98.61	1.39
21	Treated	T2-L1	3	25311395	19265850	76.12	98.53	1.47
22	Treated	T2-L2	1	8279148	6515229	78.69	99.11	0.89
23	Treated	T2-L2	2	25182470	4169826	16.56	98.99	1.01
24	Treated	T2-L2	3	24438920	17400788	71.2	98	2
			Average	15727359	10622032	68.86	98.77	1.23
			Total	377,456,615 ± 6671306.5	254928772± 5195188			

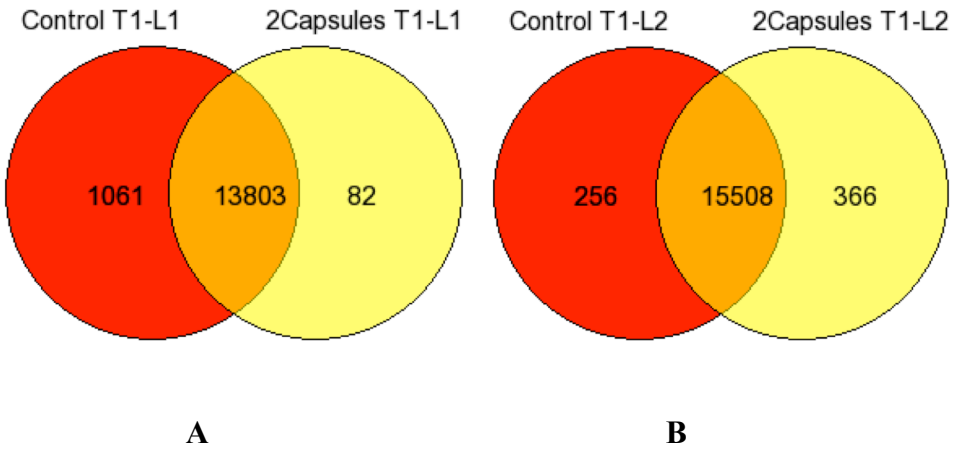


Figure 5.6: Venn diagram of gene expressed (RPKM≥1) in control and gelatin capsule treatments (2 Capsules) one week after emergence (T1), A) first leaf (L1) B) second leaf

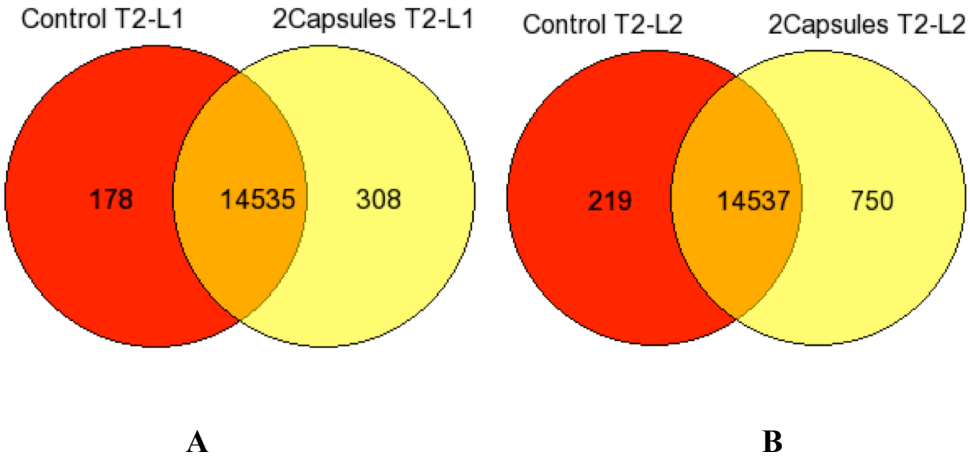


Figure 5.7: Venn diagram of gene expressed (RPKM≥1) in control and gelatin capsule treatments (2 Capsules) two weeks after emergence (T1), A) first leaf (L1) B) second leaf (L2). Expressed genes expressed in both groups are shown in the overlapped area.

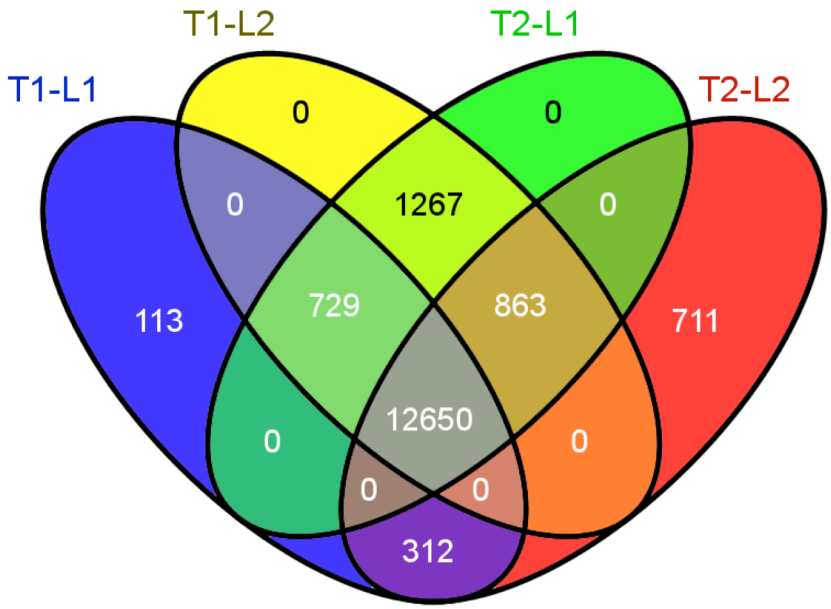


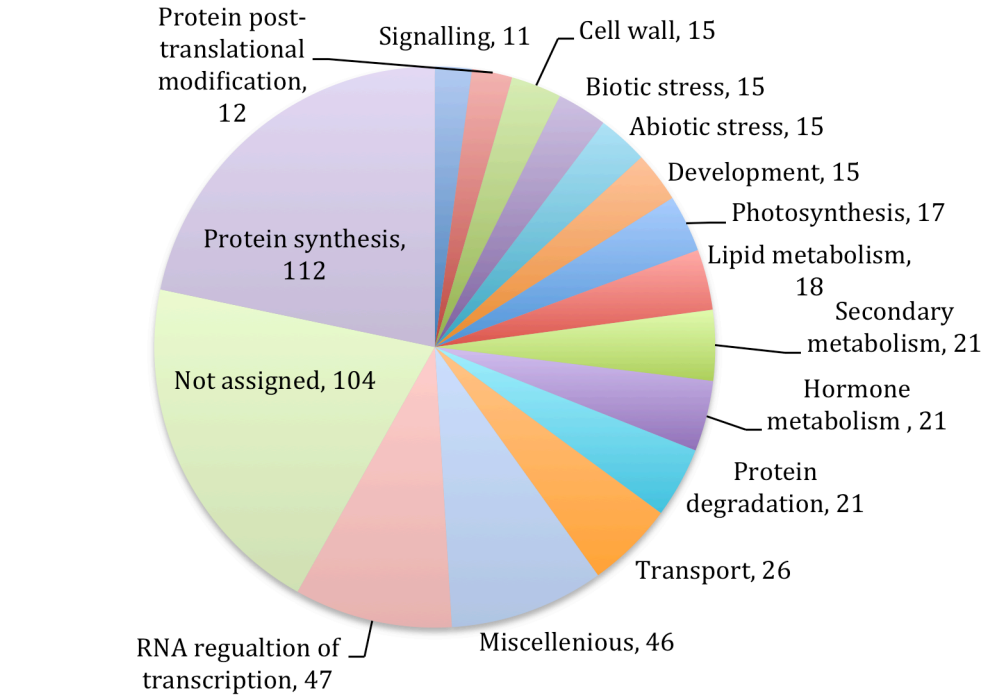
Figure 5.8: Venn diagram of gene expressed (RPKM≥1) in four groups: one and two week(s) after emergence (T1/T2), first and second leaf (L1/L2). Expressed genes expressed in both groups are shown in the overlapped area.

Genes expressed differentially in plant tissue one week after emergence (T1)

In this study a cutoff of $P_{FDR}<0.05$ in Baggerley's test was used to judge the significant differences in gene expression. A total of 22 genes (Appendix table 5.1) were differentially expressed between the treated and the control in the first leaf (L1) one week after emergence (T1). Out of 22 differentially expressed genes (DEG), nine genes (Csa4G622870, Csa2G258100, Csa1G049960, Csa5G623650, Csa4G124910, Csa7G398090, Csa5G171700, Csa3G872080, Csa5G207960) were downregulated in treated samples (lower expression in treated compared to control), and 13 genes (Csa6G109650, Csa2G079660, Csa6G425840, Csa3G120410, Csa7G039260, Csa2G247040, Csa7G447100, Csa7G047970, Csa6G118330, Csa2G355030, Csa6G194150, Csa4G000030, Csa3G822300) were upregulated (higher expression in treated compared to control). The DEGs were involved in development (Csa7G398090 and Csa4G124910), photosynthesis (Csa2G079660), hormone metabolism (Csa2G258100), cell wall degradation (Csa1G049960), signaling (Csa6G194150), and biotic stress (Csa6G425840) (Appendix table 5.1). The second leaf (L2) had 13 DEGs (Appendix table 5.1), two of which were downregulated (Csa5G161900 and Csa3G027190) and ten genes (Csa1G569290, Csa1G569270, Csa6G150530, CsaUNG029290, Csa7G009730, Csa3G047780, Csa2G024440, Csa3G128920, Csa1G172630, Csa1G540870, Csa5G576740) were upregulated (Appendix table 5.1) in the treated plants as compared with controls. The MapMan GO classification revealed that DEGs belonged to protein synthesis (Csa1G540870), cell wall related (Csa7G009730, Csa6G150530), abiotic stress (Csa1G569270, Csa1G569290), hormone metabolism (Csa2G024440) and photosynthesis (Csa5G576740) (Appendix table 5.1).

Genes expressed differentially two weeks after emergence (T2)

Samples from second leaf (L2) at T2 had 588 DEG between treatment and controls of which 146 were downregulated in treated, and 442 were upregulated (Appendix table 5.1, Figure 5.9) despite of the first leaf (L1) revealed no DEG at the set threshold value. The MapMan GO classification revealed that largest function categories were protein synthesis, transcription factor, transport, hormone and secondary metabolism, as well as lipid metabolism. The list of DEG at T2 (588 genes) was much more extensive compared to that of T1 (22 genes). Despite the limited number of DEG at T1, there were six overlapping DEG between T1 (L1) and T2 (L2), including Csa2G247040, Csa2G355030, Csa4G000030, Csa6G109650, Csa6G425840, and Csa7G398090 (Tables 5.3).



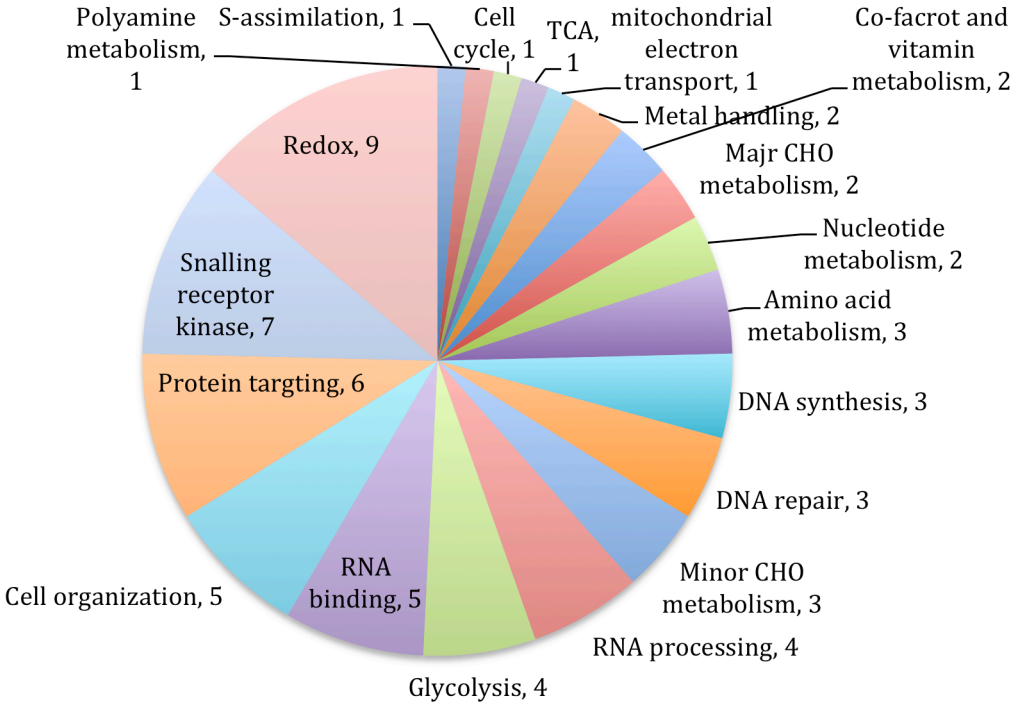


Figure 5.9: Number of DEG grouped by gene ontology (GO)

Identification of gene modules and hub genes highly associated with enhanced growth by gelatin seed treatment

Weighted gene co-expression network analysis (WGCNA) of the 24 RNA-seq samples using the 620 DEGs (Tables 5.3) showed four clusters along the lines between two time points and two leaf positions, i.e. T1-L1, T1-L2, T2-L1 and T2-L2 (Figure 5.10). There was no sub-clustering by the treatment for T1-L1, T1-L2, and T2-L1. However for T2-L2, the sub clustering is clearly separated according to the treatment, and all control samples are clustered under one branch, which are separated from the treated samples, reflecting the fact that 588 of the 620 genes were DEGs between control and treated in T2-L2 (Figure 5.10).

The WGCNA analyses further identified that the 620 DEGs were clustered into five modules, which are labeled by colors: blue, yellow, brown, turquoise and gray (Figure 5.11). Each branch in the dendogram constitutes a module and each leaf in the branch represents one gene (Figure 5.11). There were 208 genes in the blue module, 90 genes in the brown module, 249 genes in the turquoise module, 66 genes in the yellow module, and 4 genes in the grey module (Appendix table 5.1).

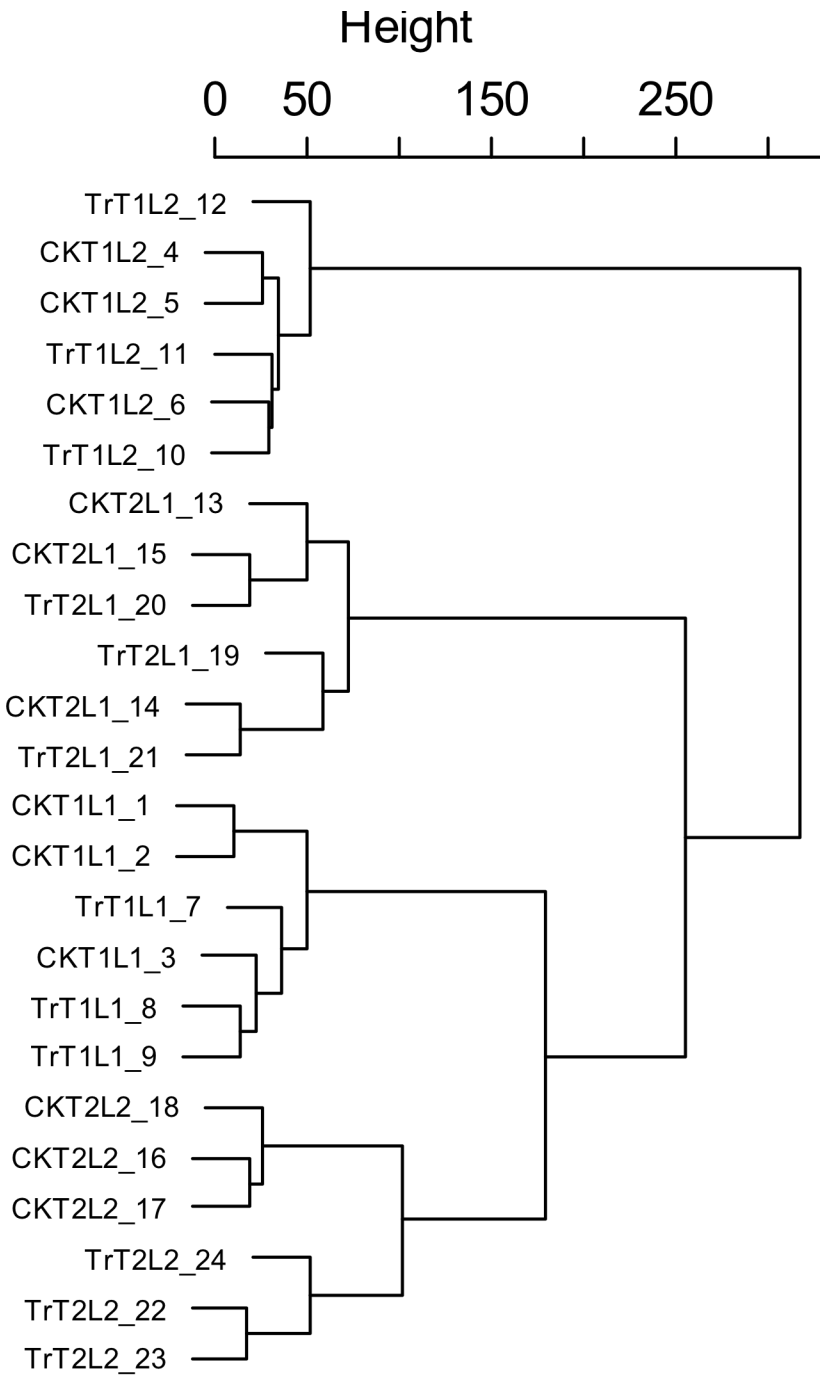


Figure 5.10: Cluster dendrogram illustrating the relationship of leaf tissue from harvested at different time points (T1/T2), leaf position (L1/L2) of control plants (CK) and plants treated with gelatin capsule (Tr).

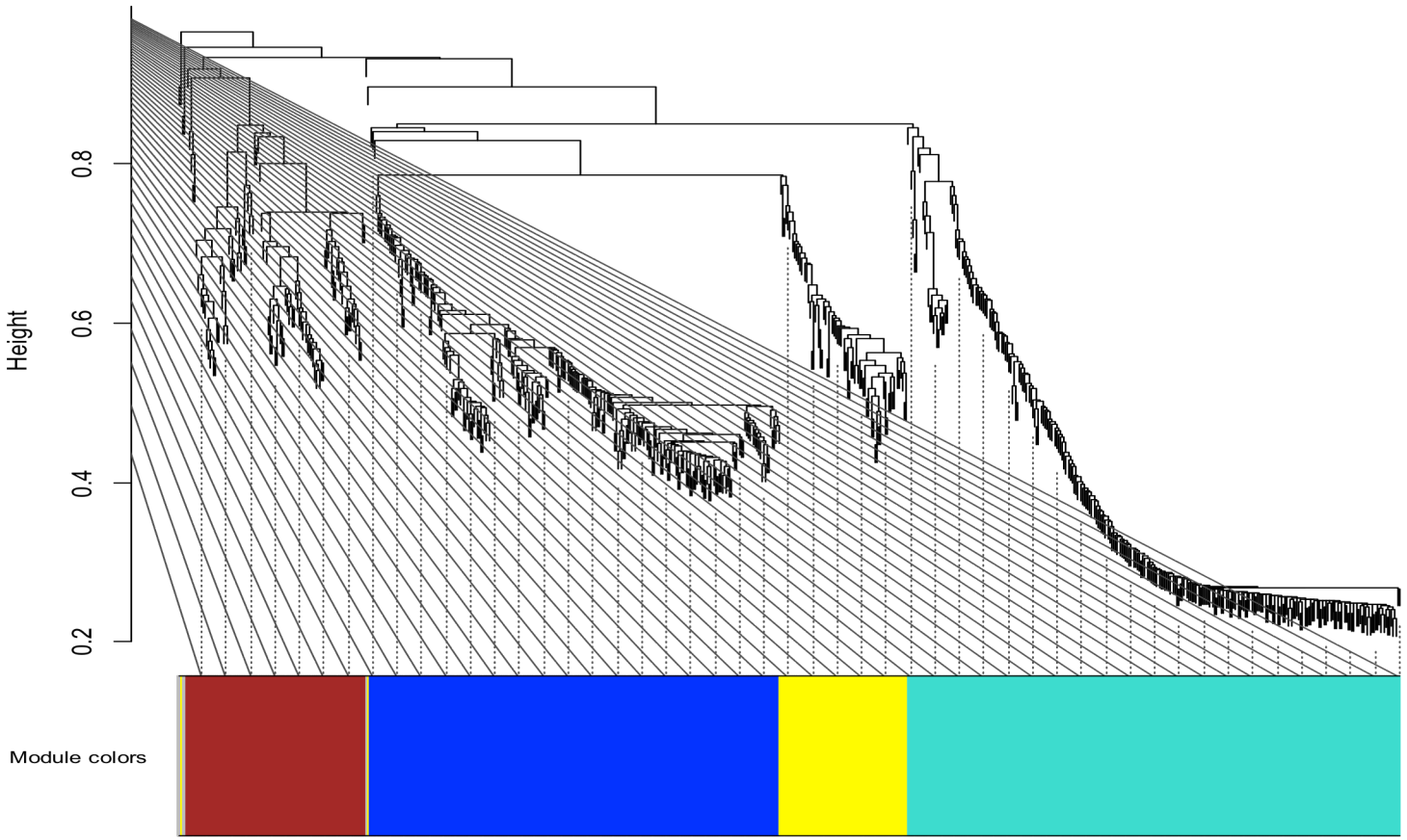


Figure 5.11: Hierarchical cluster tress showing co-expression modules identified by WGCNA. Each leaf in the tree is one gene; the major branches constitute 5 modules labeled by different colors. Each branch in the dendogram constitutes a module and each leaf in the branch represents one gene.

The module-trait (TLA, FW, DW, PercentN) relationship (Figure 5.12) revealed that the blue module had a strong positive correlation with the leaf area (TLA; R^2= 0.86; $p<1x10^{-7}$) and nitrogen percent (PercentN; R^2= 0.85; $p<2x10^{-7}$) and a moderate positive correlation with fresh weight (FW; R^2= 0.64; $p<7x10^{-4}$) and frozen weight (DW; R^2= 0.64; $p<7x10^{-4}$). The yellow module had a moderate positive correlation to all the traits. The turquoise module on the other hand had a strong negative correlation to TLA (R^2= -0.83; $p<6x10^{-7}$) and PercentN (R^2= -0.77; $p<1x10^{-5}$) and the brown module showed a moderate negative correlation to FW (R^2= -0.61; $p<0.002$) and DW (R^2= -0.61; $p<0.002$) (Figure 5.12).

To study the relationships among modules and how the modules were related to the four growth traits, an eigengene network was inferred by calculating their adjacencies (Figure 5.13). The network indicated that there were two major branches: one comprising of the blue and yellow module eigengenes, and the other comprising of the brown and turquoise module eigengenes. The four growth traits were collectively determined to be closer to the blue and yellow module eigengenes than to those of the brown and turquoise modules eigengenes. The FW and DW were extremely close to each other, and were nearly equally related to the eigengenes of blue module and those of yellow module. However, the TLA and PercentN were highly related to each other and were markedly close to the blue module eigengenes. These results strongly suggests that the blue module eigengenes were most closely related to the four growth traits, therefore the blue module was determined to be of the greatest importance, and was investigated in more detail (Figure 5.13).

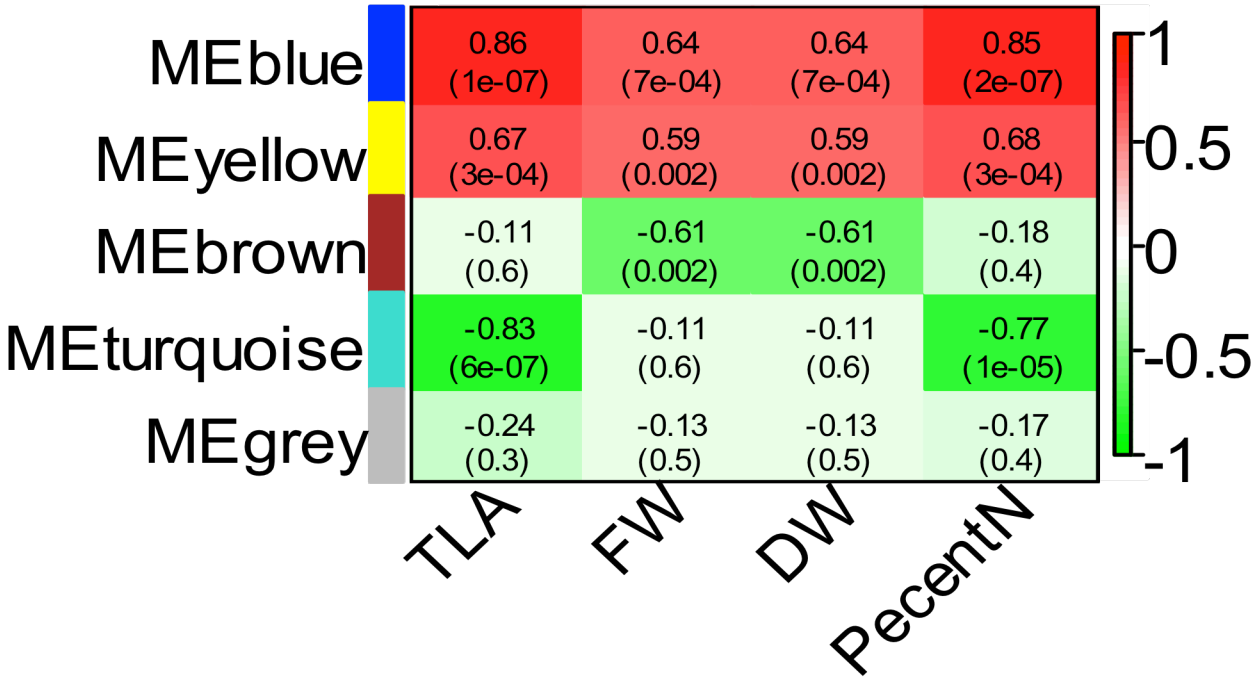


Figure 5.12: Module-trait association. Each row corresponds to a module and each column corresponds to a specific trait: total leaf area (TLA), fresh weight (FW), frozen weight (DW), and percent nitrogen (PercentN). The color of the cell indicates the correlation coefficient between the module and trait, dark red color indicates high degree of positive correlation and dark green indicates high degree of negative correlation between each module and trait. The numbers in the cell indicate the R^2 value (top) and p-value (below).

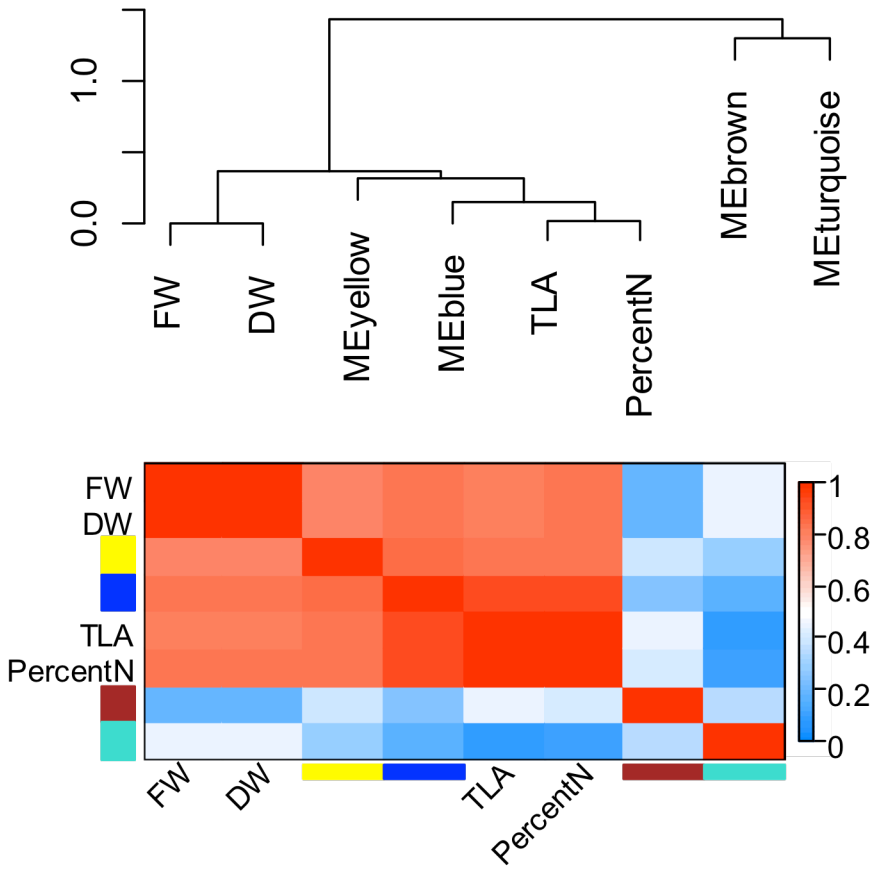


Figure 5.13: Module eigengene network and its relationship with growth traits. The dendrogram on the top illustrates the relationship among the module eigengenes and traits total leaf area (TLA), fresh weight (FW), frozen weight (DW) and percent nitrogen (PercentN). Each row and column on the heat map corresponds to a module or a trait. The color of the cell indicates the adjacencies among the module eigengenes and traits; dark red color indicates close ardencies and dark blue no or little adjacencies

The intramodular analysis of gene significance (GS), the absolute value of the correlation between the gene and the trait, and module membership (MM), the correlation of the module eigengene and the gene expression profile of the 208 genes in the blue module identified a number of genes of high significance for the growth traits as well as high module membership in the module (Figure 5.14). The data suggested that GS and MM were highly correlated with all four growth traits, especially for Percent N and TLA (Figure 5.13).

For better visualization of the intramodular connectivity (network degree), the gene networks in the blue module were exported into Cytoscape 3.1 (Figure 5.15). Network analysis using Network Analyzer (Assenov et al. 2008), a Cytoscape plugin, identified 16 hub genes of the highest network degree (60-92) in the blue module; Csa2G357860, Csa5G615830, Csa2G358860, Csa4G618490, Csa6G538630, Csa1G212830, Csa2G351820, Csa5G011650, Csa2G163170, Csa7G395800, Csa2G345990, Csa6G127320, Csa1G007850, Csa2G359890, Csa3G848170, Csa4G056640 (Figure 5.15, Table 5.4). Of particular interest, Csa1G007850 encodes a putative MYB-related transcription factor, Csa6G127320 encodes a NAC transcription factor, Csa5G615830 an amino acid transporter, and Csa2G163170 a plasma membrane localized ammonium transporter (Figure 5.15, Table 5.4).

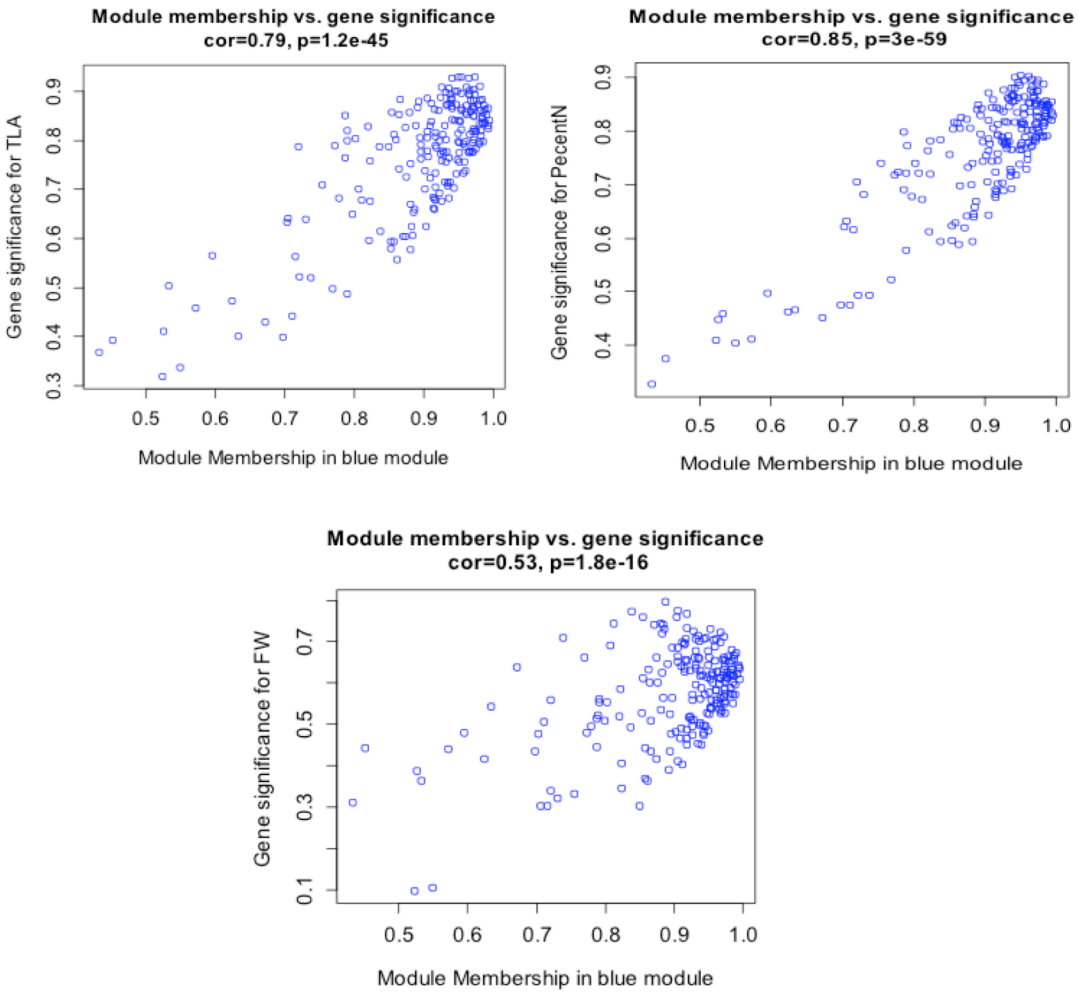


Figure 5.14. Intramodular analysis of gene significance (GS) and module membership (MM) of the genes in the blue module. Data for trait DW was not included, which is nearly identical to that of FW.

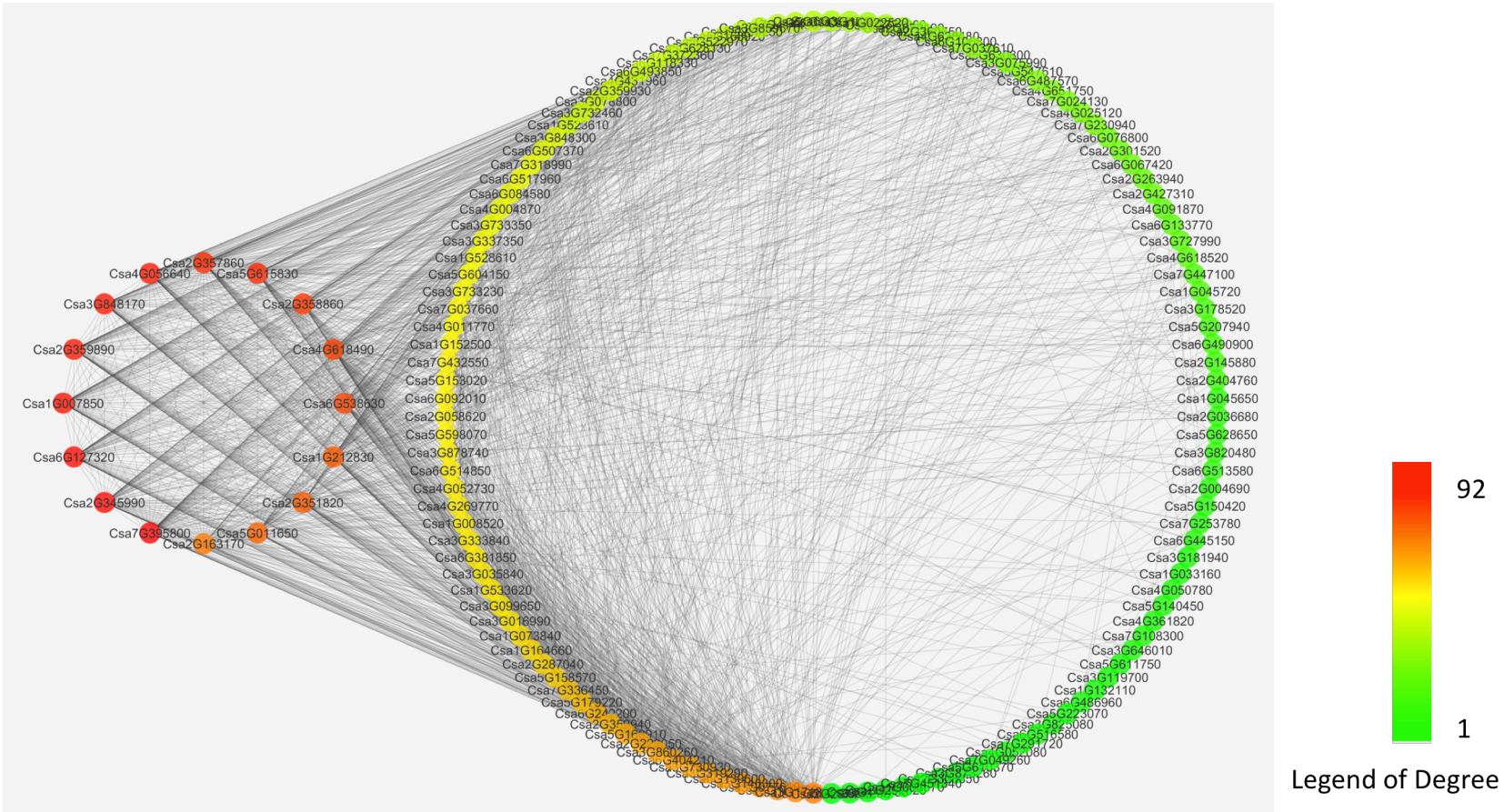


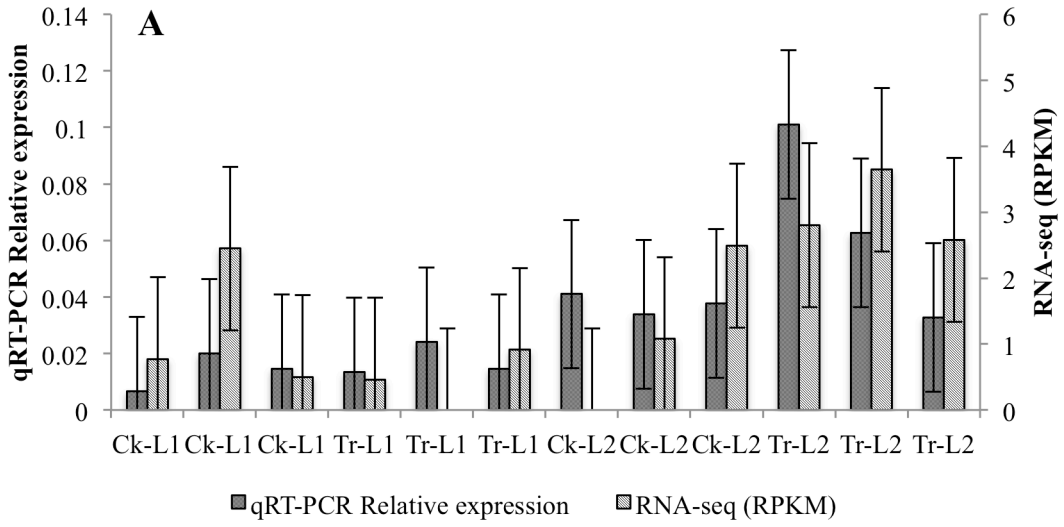
Figure 5.15. Visualization of a co-expression network of 150 of 208 genes in the blue module. The network TOM similarity was calculated with a power of 30 and edges are of weight higher than 0.25. The 16 hub genes of the highest network degree (60-92) are shown on the left panel of the figure

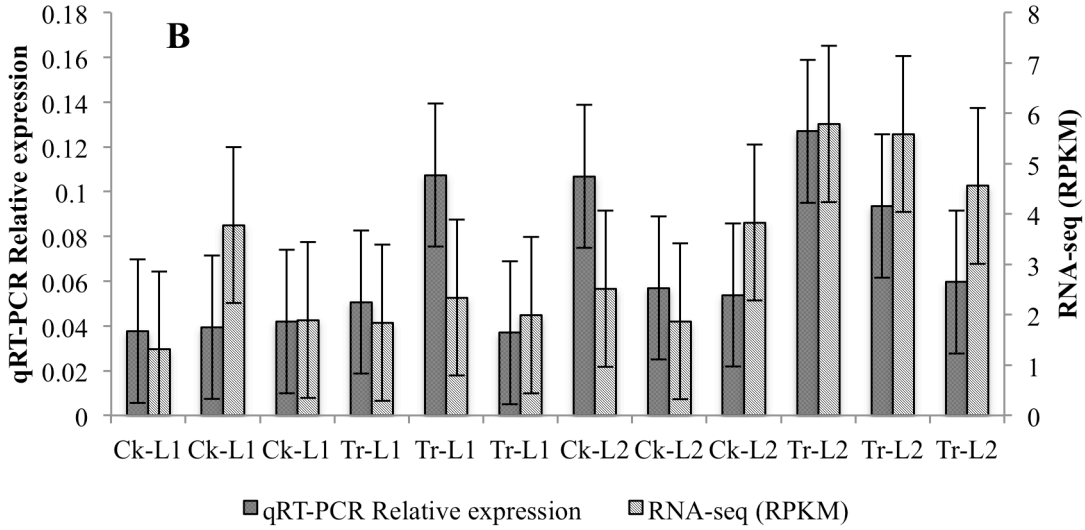
Table 5.4: List of 16 hub genes identified by the co-expression network of genes in the blue module. Degree represents the number of edges between genes, which represent co-expression correlations.

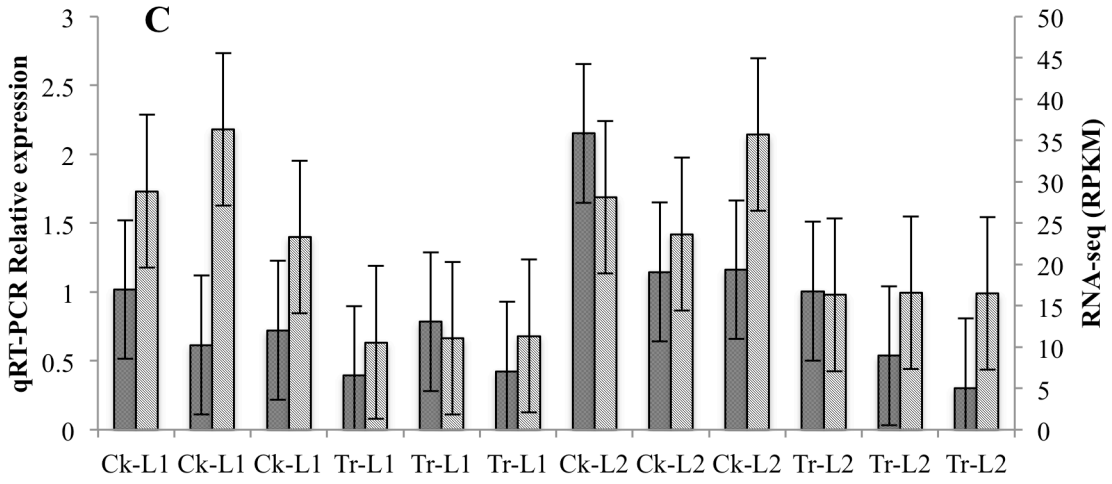
Gene	Gene Ontology	Annotation	Degree
Csa7G395800	Miscellaneous	Glutathione S-transferase PARB	92
Csa2G345990	Not assigned	Unknown protein	90
Csa6G127320	Development	NAC domain containing protein 36 (NAC036)	89
Csa1G007850	Transcription factor	Myb-like DNA binding domain	87
Csa2G359890	Not assigned	Unknown protein	86
Csa4G056640	Hormone metabolism	Nine-cis-epoxycarotenoid dioxygenase 4 (NCED4)	85
Csa3G848170	Protein degradation	Aminopeptidase M1 (APM1)	85
Csa2G357860	Not assigned	Unknown protein	81
Csa5G615830	Transport	Transmembrane amino acid transporter family protein	81
Csa2G358860	Not assigned	Unknown protein	77
Csa4G618490	Transport	Zinc transporter 5 precursor (ZIP5)	75
Csa6G538630	Co-factor and vitamine metabolism	Molybdenum cofactor sulfurase family protein	74
Csa2G351820	Protein targeting	Vacuolar-processing enzyme precursor	68
Csa1G212830	Secondary metabolism	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	68
Csa5G011650	Miscellaneous	NAD(P) –binding Rossmann-fold superfamily protein	66
Csa2G163170	Transport	Ammonium transporter 1;1 (AMT1;1)	60

Expression anaysis using qRT-PCR

Five genes (Csa1G066570, Csa1G590300, Csa5G589260, Csa5G140480, Csa5G319910) were selected from the 620 DEG identified by pairwise comparisons of RPKMs between control and treated plants at each leaf tissue in each time points. The genes were tested for the qRT-PCR validation of expression levels measured by RNA-seq (RPKM). The correlation value (R^2 value) of the qRT-PCR relative expression value to the RPKM of RNA-seq was relatively low (R^2 of 0.48 to 0.79); however, all the genes had significance in p-values ranging $p > 0.001$ to 0.05 validating the expression ratio measured by RNA-seq (Figure 5.16).


A
qRT-PCR Relative expression
RNA-seq (RPKM)
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
6
5
4
3
2
1
0
Ck-L1 Ck-L1 Ck-L1 Tr-L1 Tr-L1 Tr-L1 Ck-L2 Ck-L2 Ck-L2 Tr-L2 Tr-L2 Tr-L2
qRT-PCR Relative expression
RNA-seq (RPKM)


B
qRT-PCR Relative expression
RNA-seq (RPKM)
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
8
7
6
5
4
3
2
1
0
Ck-L1 Ck-L1 Ck-L1 Tr-L1 Tr-L1 Tr-L1 Ck-L2 Ck-L2 Ck-L2 Tr-L2 Tr-L2 Tr-L2
qRT-PCR Relative expression
RNA-seq (RPKM)


C
qRT-PCR Relative expression
RNA-seq (RPKM)
3
2.5
2
1.5
1
0.5
0
50
45
40
35
30
25
20
15
10
5
0
Ck-L1 Ck-L1 Ck-L1 Tr-L1 Tr-L1 Tr-L1 Ck-L2 Ck-L2 Ck-L2 Tr-L2 Tr-L2 Tr-L2

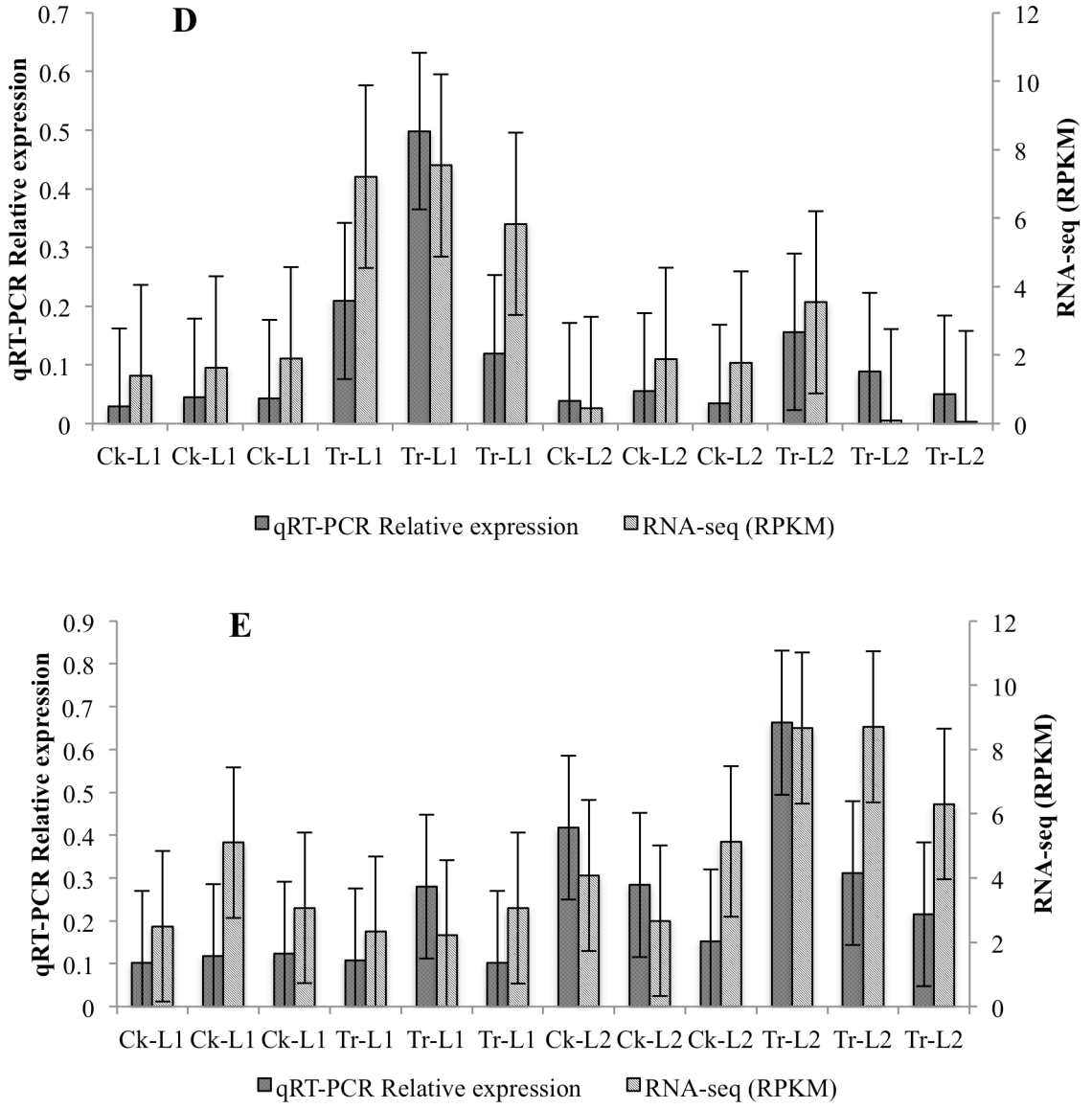


Figure 5.16: Comparison between gene expression from RNA-seq data and qRT-PCR data. Data has been transformed by square root. Bars represent standard deviation. A) Csa1G066570 (R^2= 0.597, $p<0.020$), B) Csa1G590300 (R^2=0.519, $p<0.042$), C) Csa5G589260 (R^2=0.483, $p<0.05$), D) Csa5H140480 (R^2=0.798, $p<0.001$), and E) Csa5G319910 (R^2=0.591, $p<0.021$)

DISCUSSION

Amino acid and nitrogen transport

The increased expression of nitrogen source transporters such as amino acid transport proteins: amino acid transporter (Csa5G615830; Appendix table 5.1), amino acid permease 3 (AAP3; Csa6G381850; Appendix table 5.1), amino acid permease 6 (AAP6; Csa3G894480; Appendix table 5.1), as well as ammonium transporter 1;1 (AMT1;1; Csa2G163170; Appendix table 5.1), ammonium transporter 2 (AMT2; Csa3G730930; Appendix table 5.1) in plants treated with gelatin capsules provided an insight on the mechanism of amino acid transport from the gelatin capsule to the plant as well as the nitrogen translocation within the plant.

Gelatin capsules are composed of hydrolyzed collagen, a polypeptide found primarily in the flesh and connective tissues of animals (Balian and Bowes 1977; Schrieber and Gareis 2007). Collagen can be broken down through hydrolysis to form hydrolyzed collagen, and further hydrolysis will break down the peptide to single amino acids (Regenstein and Boran 2010; Balian and Bowes 1977). Plants are capable of acquiring nitrogen as nitrate (NO_3) and ammonium (NH_4^+), but also as organic forms such as amino acids and proteins from the soil (Nasholm, Kielland, and Ganeteg 2009). Assimilation pathways of amino acids have been demonstrated with ^{15}N-labelled amino acids (Schmidt and Stewart 1999; Thornton et al. 2007), as well as through metabolic profile analysis using GC-MS (Wang et al. 2007; Wang et al. 2014). Transport studies with plant tissues have demonstrated the presence of multiple transport systems for amino acids in plants (Ortiz-Lopez, Chang, and Bush 2000; Liu and Bush 2006; Guo 2004; Fischer 1998). The amino acids enters the xylem in roots (Schobert, Kockenberger, and

Komor 1988) and are exported from the xylem to the surrounding tissue (Okumoto et al. 2002). The amino acids are then transported from xylem to the phloem, leading to cycling within the plant (Okumoto et al. 2002).

One of the amino acid transporters upregulated with the gelatin capsule treatment, AAP3 is preferentially expressed in the phloem and has been associated with long distance transport of basic amino acids such as arginine, histidine, and lysine (Okumoto et al. 2004; Marella et al. 2013; Fischer et al. 2002). The long distance transport by AAP3 has been attributed to amino acid loading from the apoplast into the phloem (Okumoto et al. 2002; Okumoto et al. 2004; Fischer 1998). AAP6, on the other hand is expressed mainly in xylem parenchyma cells in sink tissues such as sink leaf, cauline leaf, and roots (Rentsch et al. 1996). Unlike AAP3, which is responsible for high affinity transport of basic amino acids, AAP6 is a high affinity transporter of acidic and neutral amino acids such as aspartic acid, proline, alanine and valine (Fischer et al. 2002; Okumoto et al. 2002; Rentsch et al. 1996). The relatively low concentration of amino acids in the xylem compared to that in the phloem sap means that a high affinity transporter would be necessary in the xylem parenchyma cells to mediate the amino acid transfer from the xylem to the phloem (Okumoto et al. 2002). The high affinity nature of AAP6, as well as its expression in the xylem parenchyma cells, suggests that AAP6 plays a role in the xylem to phloem transfer of amino acids. Hunt et al. (2010) demonstrated that AAP6 mutant (aap6) had a reduced amino acid content in the sieve element sap.

The increased expression of amino acid transporters AAP3 and AAP6 in the plants treated with gelatin capsules suggests that amino acid transport in the plants were positively

affected by the treatment, possibly by the amino acids from the treatment being taken up by the plants. Wang et al. (2014) demonstrated that in pak choi, amino acid supply was considered an N-limiting condition compared to N source from NO_3^-, which further enforces the effect of availability of amino acids in plant growth promotion.

AAPs are selectively expressed in areas where expression of H^+ATPases are localized, as AAPs is driven by a proton gradient and requires a high proton concentration to fuel the active transport involved in uptake of amino acids from the apoplast into the phloem (Okumoto et al. 2002; Niittylä et al. 2007; Vitart et al. 2001). Expression of both AAP3 and AAP6 are localized in the plasma membrane (Okumoto et al. 2002), which is where the expression of H^+ATPase 4 (AHA4; Csa1G008520; Appendix table 5.1), another gene highly expressed in plants treated with gelatin capsules, is found to be localized (Vitart et al. 2001). Expression of AAP1, AAP2, and AAP8 correlates with the expression pattern of two H^+ATPase genes AHA10 and AHA3 (Dewitt et al. 1996; Harper, Manney, and Sussman 1994). This suggests coupling of H^+ATPases with AAPs, which have been characterized as proton symporters energized by H^+ATPase (Boorer et al. 1996; Boorer and Fischer 1997). It is possible that AAP3 and AAP6 transport activity is coupled with AHA4, generating proton motive force required for high affinity transport of the amino acids between xylem to phloem; however, further examination is necessary to link these genes together in a concrete manner.

AMT2 (Csa3G730930; Appendix table 5.1) and AMT1;1 (Csa2G163170; Appendix table 5.1) were another gene pair involved in nitrogen metabolism that showed high degree of differential expression in plants treated with gelatin capsule, and were also shown to have high

correlation in WGCNA analysis. Ammonium is an important intermediate in nitrogen metabolism, and the transport of and distribution of NH_4^+ across cellular membranes depends on ammonium transporters (AMT). In plants, AMT-mediated ammonium transport is critical for providing sufficient nitrogen for optimal growth (Loqué and von Wirén 2004; Neuhäuser, Dynowski, and Ludewig 2009). GFP studies with AMT2, demonstrated its expression in the vascular tissue in roots, pith of stem, petiole and leaf hydathodes, and it mediated electro-neutral ammonium transport in the form of NH_3 (Neuhäuser, Dynowski, and Ludewig 2009). This is especially advantageous for the plant as there is no need to actively export co-transported protons by energy consuming H^+ pumps, allowing AMT2 to function as a high affinity NH_4^+ transporter in the plasma membrane (Neuhäuser, Dynowski, and Ludewig 2009; Sohlenkamp et al. 2002; Khademi et al. 2004). Root AMT2 expression has been reported to be regulated by N-supply by Sohlenkamp et al. (2002), in which the AMT2 transcript increased in roots after N-deprivation; however, transcript level in shoots remained unchanged. The increase in AMT2 transcript in plants treated with gelatin capsules suggests an increase in N-transport, exporting NH_4^+ from the vascular tissue and importing NH_3 into the cytoplasm. The increase in total N observed in plants treated with gelatin capsules is hypothesized to be due to the increase in N-assimilation. However, none of the genes associated with N-assimilation (GS/GOGAT) were differentially expressed in the plants treated with gelatin capsules. As the GS/GOGAT system is dependent upon the N-concentration in the tissue, it may be that the concentration of NH_3 in the tissue was not high enough to up regulate gene involved in N-assimilation.

Biotic and abiotic stress

The plants treated with gelatin capsules had an upregulation of genes involved in jasmonic acid metabolism. Jasmonic acid (JA) is an important plant hormone (McConn et al. 1997; Creelman and Mullet 1997) that functions as a regulatory molecule to mediate multiple plant developmental processes (McConn et al. 1997; Cheng et al. 2009; Creelman and Mullet 1997; Rao et al. 2000). It acts as a defense signal to mediate plant resistance against biotic and abiotc stress such as insect attack, UV damage, pathogen infection, and wounding (McConn et al. 1997; Vijayan et al. 1998; Rao et al. 2000; Reymond et al. 2000; Farmer 2001; Schilmiler and Howe 2005; Wasternack 2007; Kim, Park, and Kim 2009). Jasmonate-zim-domain protein 3 (JAZ; Csa5G28650, Appendix table 5.1) function as negative regulator to repress diverse JA responses, probably by directly inhibiting various transcriptional regulators (Chini et al. 2007; Thines et al. 2007; Yan et al. 2009; Song et al. 2011). Such transcriptional regulator belonged to WRKY family: WRKY51 (Csa6G486960; Appendix table 5.1; Csa2G297180; Appendix table 5.1), WRKY53 (Csa3G119700; Appendix table 5.1), WRKY54 (Csa5G223070; Appendix table 5.1), WRKY70 (Csa3G727990; Appendix table 5.1), which were all upregulated in the treated plant during the second week after emergence in the second leaf (T2-L2). The WRKY transcription factors are one of the largest families of transcriptional regulators in plants and are a key part of signaling webs that modulate many plant responses to pathogen infection and abiotic stresses such as drought and salinity (Rushton et al. 2010; Eulgem et al. 2000). Increased levels of WRKY mRNA, protein and DNA-binding activity has been reported to be induced by infection with viruses (Wang 1998), bacteria (Tao et al. 2009; Shimono et al. 2007), oomycetes (Eulgem et al. 1999), fungal elicitors (Rushton 1996; Fukuda 1997), and by phytohormones such as

salicylic acid (SA) (Yang 1999) and jasmonic acid (JA) (Shim and Choi 2013). WRKY genes expression has been shown to be upregulated in response to wounding, and induced WRKY mRNA accumulation is often extremely rapid and transient (Hara 2000). This expression behavior indicates a role for WRKY proteins in regulating secondary-response genes, whose products carry out the protective and defensive reactions (Eulgem et al. 2000). Specifically WRKY53 and WRKY70 have been shown to be involved in the SA-signaling pathway, which precedes systemic acquired resistance (SAR) (Hu, Dong, and Yu 2012; Jones and Dangl 2006). Increase in endogenous SA and accumulation of SA induces a subset of pathogenesis-related (PR) genes, which triggers SAR (Heath 2000; Jones and Dangl 2006; Ward et al. 1991). SA-induced SAR also depends on NPR1, a key protein involved in SA-signaling (Ward et al. 1991; Cao et al. 1994). WRKY70 and WRKY53 expression was reduced in npr1 mutants after functional analog of SA induction, which means the NPR1-dependent pathway is responsible for SA-induced expression of the WRKY genes (Hu, Dong, and Yu 2012; Wang, Amornsiripanitch, and Dong 2006).

JA and ethylene are also involved in plant defense responses, and SA-mediated and JA-mediated defense pathways are often described as mutually antagonistic (Kunkel and Brooks 2002). Blocking SA signaling enhances JA-responsive gene expression while, disrupting the JA signaling regulator enhances SA accumulation and PR expression (Spoel et al. 2003; Kloek et al. 2001). Hu et al. (2012) showed with a wrky46wrky70wrky53 triple mutant, JA-induced PDF1.2 (JA-inducible defensin) expression was upregulated, which suggests that WRKY46, WRKY70 and WRKY53 work together to suppress the expression of JA-responsive PDF1.2 expression. Thus WRKY70 plays an important role in balancing the signaling branches promoting SA-

dependent and suppressing JA-dependent responses (Eulgem and Somssich 2007). Like WRKY70, WRKY51 also plays a role in the cross talk between SA and JA signaling. Gao et al. (2011) demonstrated that repression of JA-mediated signaling under reduction in oleic acid, which simultaneously up-regulates SA-mediated response, and inhibits JA-induced defense, is mediated via WRKY51 proteins. WRKY51 negatively regulated the repression of JA-inducible PDF1.2 expression under low oleic acid conditions in the presence of SA (Gao et al. 2011).

WRKY proteins are characterized by the presence of one or two highly conserved domains carrying the WRKYGQK sequence and zinc-binding motif at the N-terminal end (Eulgem et al. 2000; Rushton et al. 2010). WRKY proteins bind specific DNA sequences (W-box elements) in the promoters of the target genes, and promoters of defense-related genes including PR (Eulgem and Somssich 2007; Machens et al. 2014). Evidence from others also suggests that WRKY genes are not only able to induce gene expression, but WRKY proteins can also serve as transcriptional repressors (Rushton et al. 2010). This raises the possibility that WRKY 51, WRKY70 and WRKY53 directly interacts with expression of SA- and/or JA-mediated defense genes, which can be induced by abiotic stress such as salt stress (Fujita et al. 2009). This may explain the salt stress tolerance observed in plants treated with gelatin capsules in the previous chapter (Wilson 2015, chap. 4). The plants treated with the gelatin capsules appeared to exhibit priming of defense genes, which may have aided in their ability to withstand a high concentration of salt stress.

Salt and drought stresses usually induce the production of abscisic acid (ABA), which plays a major role in various aspects of stress tolerance (Lim et al. 2012; Zhu 2002). ABA, like JA and

SA, serves as a biotic signaling molecule, which act as integrator of abiotic stress such as salinity and drought stress. When ABA levels increase during abiotic stress, ABA molecules binds to its receptors and inhibit protein phosphatase 2C (PP2C) activity (Lim et al. 2012). This inhibition of PP2C activity causes stomatal closure (Lim et al. 2012; Lee et al. 2009). Group A PP2C is a negative regulator of ABA (Schweighofer, Hirt, and Meskiene 2004; Gosti et al. 1999; Merlot et al. 2001; Wasilewska et al. 2008). The identification of PP2C (Csa7G049260; Appendix table 5.1) and Highly ABA-induced PP2C gene 2 (HAI2; Csa1G524760; Appendix table 5.1) as genes highly associated with plants treated with gelatin capsules, suggests that increased plant biomass and nitrogen in the treated plants are due to the negative regulation of ABA.

According to an expression pattern study conducted by Chen et al. (2006), MYB73 (Csa2G427310; Appendix table 5.1) another gene with high correlation with the increased biomass in plants treated with gelatin capsules was constitutively expressed in all organs during JA and SA treatments. Jia et al. (2011) demonstrated that MYB73 expression, which encodes for R2R3 MYB transcription factor increased by SA, JA and ethylene treatments, suggesting MYB73 gene participated in the JA and SA signaling pathways (Jia et al. 2011). Additionally, the MYB expression was also upregulated in salt stress, but not with other stresses. MYB73 mutants had a high accumulation of salt overly sensitive (SOS) transcripts, which indicates that MYB73 is a negative regulator of SOS induction in response to salt stress (Kim et al. 2013). MYB proteins are a superfamily of transcription factors that, like WRKY, play a regulatory role in developmental processes and defense response in plants. Specifically R2R3-MYB genes, such as Csa1G007850 (Appendix table 5.1), a member of the MYB family transcription factor, have been reported to be involved in physiological process such as regulation of secondary

metabolism (Borevitz et al. 2000; Jin and Martin 1999; Nesi et al. 2001), control of cell morphogenesis and cell cycle (Oppenheimer et al. 1991; Noda et al. 1994; Glover, Perez-Rodriguez, and Martin 1998; Payne et al. 1999; Ito et al. 2001; Araki et al. 2004), biotic and abiotic stress responses (Urao et al. 1993; Sugimoto, Takeda, and Hirochika 2000; Vailleau et al. 2002; Abe et al. 2003; Denekamp and Smeekens 2003; Nagaoka and Takano 2003), light and hormone signaling pathways (Chen et al. 2006; Gubler et al. 1995; Wang et al. 1997; Ballesteros et al. 2001).

NAC transcription factors

NAC (NAM, ATAF1,2, CUC2) proteins are one of the largest families of plant-specific transcription factors, and the family is present in a wide range of plants, with more than 100 predicted members in *Arabidopsis thaliana* (Kato et al. 2010; Olsen et al. 2005). Due to its large number, only a small portion of the NAC proteins have been characterized, and yet the family has been implicated in various plant processes including developmental programs, defense, and abiotic responses (Olsen et al. 2005). NAC proteins commonly have highly conserved sequences named NAC domains in their N-terminal regions (Aida et al. 1997). NAC domains consist of five subdomains (A-E) (Kikuchi et al. 2000), and subdomains D and E are required for DNA-binding, while the C-terminal regions can function as a transcriptional activation domain (Duval et al. 2002; Kato et al. 2010). NAC proteins have been classified into 18 subgroups by similarity in the amino acid sequence in the NAC domains (Ooka et al. 2003). The NAC036 gene (Csa6G127320, Appendix table 5.1) is one of the hub genes identified in the WGCNA analysis, is a member of the ONAC022 subgroup. RNA gel blot analysis have revealed that NAC036 gene is strongly expressed in leaves, and plants overexpressing NAC036 gene has a semi dwarf

phenotype (Kato et al. 2010). Kato et al. (2010) through microscopic observation determined that dwarfing in these plants were due to reduction in cell size; however, the mechanism by which overexpression of NAC036 gene causes reduction of cell size is unclear. Microarray data has confirmed that expression of NAC036 gene was induced by abiotic stress such as osmotic stress and salt stress, which lead the authors to speculate that the overexpression of NAC036 leads to excessive stress responses, which subsequently compromises cell growth. In this study, NAC036 expression was downregulated in the treated plants in T2-L2, which agrees with the leaf area data, in which there was a increase in leaf area in the treated plants in T2-L2. It is possible that the NAC036 gene are involved in other abiotic stress response pathways, such as ABA, SA and JA; however, this relationship is unclear without further investigation.

Detoxifying proteins

Plants are continually exposed to potentially toxic chemicals (xenobiotic). These chemicals cannot be used for nutrition or source of energy, thus plants have found a way to remove such compounds from its system by either storing in a secure place (i.e. vacuole) or destroying them by biodegradation (Coleman, Blake-Kalff, and Davies 1997). The process of chemical modification of xenobiotic compounds in plants is classified by three phases: phase I (activation reaction, which involves hydrolysis or oxidation), phase II (conjugation reaction), and phase III (compartmentalization and processing). In phase I, the hydrolysis reactions are catalyzed by esterases, but most of the reactions are oxidation, which are catalyzed by cytochrome P450 system (Coleman, Blake-Kalff, and Davies 1997). Two cytochrome P450 genes CYP72A8 (Csa3G825080; Appendix table 5.1) and CYP77B1 (Csa6G514850; Appendix table 5.1) have been found to be highly correlated with plants treated with gelatin capsule treatments. In phase II,

the phase I activated metabolites are deactivated by covalent linkage to an endogenous molecules such as glutathione, to form a water-soluble conjugate (Wilce and Parker 1994). Glutathione S-transferase (GST), for instance is a soluble enzyme that is often thought of as detoxification enzyme with ability to metabolize a wide variety xenobiotics via GHS conjugation (Wilce and Parker 1994; Dixon and Edwards 2010; Dixon, Lapthorn, and Edwards 2002). GSTs are grouped into four classes: phi, zeta, tau and theta. Zeta and theta GST are found in both animals and plants, but tau and phi classes are only found in plants (Dixon, Lapthorn, and Edwards 2002). Phi and tau GSTs are induced after exposure to biotic and abiotic stresses. Three GSTs were found to be highly associated with plant treated with gelatin capsules: GST tau 9 (GSTU9; Csa1G033160; Appendix table 5.1), GST tau 25 (GSTU25; Csa4G064650; Appendix table 5.1), and GST phi 8 (GSTF8; Csa7G395800; Appendix table 5.1), which were all upregulated in the second leaf of the treated plants, second week after emergence (T2-L2). GSTF8 in particular was one of the hub genes, and it is used as a marker for early stress and defense responses. GSTF8 expression can be induced by a range of biotic and abiotic stresses including SA, auxin, herbicides, and microbial infections (Thatcher et al. 2007). The endogenous function of GSTF8 is still poorly understood. It is cytosolic and highly expressed in roots, where it may act to detoxify products of oxidative stress resulting from pathogen attach or foreign chemicals in the soil (Thatcher et al. 2007).

The inactive, water-soluble conjugates formed in phase II are then exported from the cytosol by membrane-located transport proteins such as plasma membrane intrinsic protein 1C (PIP1C; Csa5G53020; Appendix table 5.1) which initiate phase III, the compartmentalization and processing of the detoxification system (Coleman, Blake-Kalff, and Davies 1997). The transport

of glutathione conjugates from the cytosol to the vacuole requires passing through the tonoplast. The tonoplast intrinsic proteins and ABC transporters are thought to be involved in this process. There were numerous genes associated with detoxifying function in the list of genes that are highly associated with gelatin capsule treatments, such as ABC protein 9 (NAP9; Csa1G181360; Appendix table 5.1), tonoplast intrinsic protein 2;3 (TIP2;3; Csa7G447100; Appendix table 5.1), tonoplast intrinsic protein 2 (TIP2; Csa6G486670; Appendix table 5.1). The NAP9 and TIP2 genes were upregulated in the second leaf of the treated plant during the second week after emergence (T2-L2), whereas TIP2;3 was upregulated in the first leaf during the first week after emergence in the treated plants. ABC transporter superfamily mediates excretion of potentially toxic compounds, conferring heavy-metal tolerance (Martinoia et al. 2002). The UDP-glycotransferase (UGT; Csa4G618520; Appendix table 5.1), a gene involved in biodegradation of xenobiotics, is one of the genes highly associated with gelatin capsule treatment, which further confers with the hypothesis that gelatin capsule treatment increases the expression of genes associated with detoxification, which allows for increased plant growth and accumulation of biomass. The UGT gene was upregulated in the second leaf during the second week after emergence in the treated plants. It is possible that GSTF8 and other GSTs are upregulated in plants treated with gelatin capsule treatments, and is involved in detoxifying products of oxidative stress in the root, which allows for efficient uptake of amino acids and nitrogen from the soil.

CONCLUSION

Gelatin capsule treatment of cucumber seeds has induced many biochemical pathways, and up regulation of genes involved in numerous pathways. MYB and WRKY family transcription factors that regulate diverse pathways including abiotic stress tolerance and other responses were upregulated in plants with gelatin treatment. The result is an increase in biotic and abiotic stress tolerance in plants treated with gelatin as described in the previous chapter (Wilson 2015, chap. 4).

Numerous authors have reported that exogenous application of amino acids such as glutamate, proline, and glycine betaine provide abiotic stress protection (Vranova et al. 2011; Rhodes, Verslues, and Sharp 1999; Liang et al. 2013) Application of amino acids have been shown to enhance abiotic stress tolerance (Chen and Murata 2011; Reis, Lima, and Medeiros de Souza 2012; Ahmad, Lim, and Kwon 2013; Calvo, Nelson, and Kloepper 2014; Ashraf and Foolad 2007).

Most notably cucumber plants treated with gelatin capsules had increased the amino acid uptake and transport by up regulation of AAP3 and AAP6. Together with the increase in ammonium transport by AMT1;1 and AMT2, it provides possible explanation for the increase in total N content in plants treated with gelatin capsules.

The detoxification system of xenobiotic by GST might also play a role in the increased plant biomass by the application of gelatin capsule treatment, as they remove oxidative stress in the plant and thus allows for more efficient usage of nutrient by the plant and thus maximizing plant growth. Upregulation of transcription factors NAC036 is thought to be somehow involved in

plant biomass increase seen in treated plants, as these transcription factors are key factor in numerous regulatory networks, including cell development.

The RNA-sequence data has highlighted some possible mechanisms of how gelatin capsules affect plant growth; however, more in depth investigation based on the molecular results are necessary to shed light on the specific growth promotion mechanism of the treatment. For example, using AAP mutants would be an interesting venue to determine the function of AAPs on growth promotion by the gelatin capsules.

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CHAPTER 5 APPENDIX

Appendix table 5.1: List of DEG in the first leaf (L1) and second leaf (L2) in first time point (T1), and second leaf (L2) in second time point (L2) between control and gelatin capsule treatment that show high correlation to the growth parameters (TLA, FW, PercentN). FDR p-value ha been corrected by Baggerley's test. Mapman classification is used for gene ontology (GO). Annotation contains annotation from BLAST and the e-value, which is parameter that describes the number of hits one can "expect" to see by chance when searching a database. The module color refers to the module in which the gene is classified to.

Differentially expressed	Module Color	Gene	Fold change	Baggerley's test: FDR p-value correction	MapMan GO Classification	Annotations	e-value
T1-L1	blue	Csa4G431960	3.195	0	Protein targeting	Sigma factor binding protein 1 (SIB1)	2.00E-14
T1-L1	blue	Csa2G072490	1.618	0.049	Protein degradation	Insulinase (Peptidase family M16) family protein	0
T1-L1	blue	Csa3G782680	1.16	1.20E-04	Cell vesicle transport	Syntaxin related protein 1 (SYR1)	7.00E-123
T1-L1	brown	Csa4G124910	1.404	0.000006196	Miscellaneous	Polyamine oxidase 2 (PAO2)	3.00E-228
T1-L1	brown	Csa3G120410	3.575	0.00005376	Transcription factor	WRKY DNA-binding protein 53 (WRKY53)	1.00E-102
T1-L1	brown	Csa7G448050	NA	Not assigned	unknown		
T1-L1	brown	Csa2G247040	1.987	9.97E-04	RNA binding	Glycine rich protein 7 (GRP7)	1.00E-58
T1-L1	brown	Csa4G000030	1.544	1.91E-03	Not assigned	DUF1005	1.00E-118
T1-L1	brown	Csa6G109650	-1.75	1.71E-05	Miscellaneous	Bi-functional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein	6.00E-41
T1-L1	brown	Csa6G425840	3.098	6.03E-08	Biotic stress	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family	2.00E-65
T1-L1	turquoise	Csa7G047970	2.18	0.0009962	Transport	MATE efflux family protein	4.00E-205
T1-L1	turquoise	Csa5G207960	2.293	3.454E-10	Transcription factor	Salt-inducible zinc finger 1 (SZF1)	1.00E-116
T1-L1	turquoise	Csa6G194150	5.147	1.779E-09	Cell wall	Expansin A4 (EXPA4)	2.00E-137
T1-L1	turquoise	Csa2G355030	1.363	4.70E-03	Transcription factor	MYB domain protein 77 (MYB77)	4.00E-115
T1-L1	turquoise	Csa7G398090	1.705	0.041	Development	Senescence-associated gene 21 (SAG21)	2.00E-20
T1-L1	yellow	Csa6G118330	1.98	4.437E-10	Amino acid metabolism	Embryo defective 1017 (emb1075)	7.00E-235
T1-L1	yellow	Csa5G623650	2.467	7.05E-03	Not assigned	unknown	
T1-L2	blue	Csa1G533620	-2.242	5.36E-03	Not assigned	unknown	
T1-L2	blue	Csa6G517180	1.68	0.000001159	Miscellaneous	alpha/beta-hydrolases superfamily protein	1.00E-128
T1-L2	blue	Csa5G523190	5.13	0.00002257	Redox	Glutaredoxin	9.00E-41
T1-L2	blue	Csa3G016990	1.432	6.40E-03	Development	unknown	1.00E-23
T1-L2	turquoise	Csa5G161900	-2.457	1.80E-06	Miscellaneous	Plant invertase/pectin methylesterase inhibitor superfamily protein	3.00E-24

Appendix table 5.1 (Continued)

T1-L2	turquoise	Csa1G569290	1.313	3.46E-10	Abiotic stress	A member of heat shock protein 90 (HSP90) gene family (*Arabidopsis thaliana*)	0
T1-L2	turquoise	Csa1G569270	2.042	7.34E-05	Protein synthesis	Ribosomal protein L18e/L15 superfamily protein	0
T2-L2	blue	Csa4G638480	-71.384	1.63E-03	Biotic stress	ADR1-like 1 (ADR1-L1)	8.00E-257
T2-L2	blue	Csa1G231530	-5.347	1.18E-04	Hormone metabolism	Indole-3acetic acid inducible 12 (IAA12)	
T2-L2	blue	Csa5G139420	-3.252	4.53E-05	Protein degradation	Prolyl oligo-peptidase family protein	3.00E-69
T2-L2	blue	Csa7G336450	-3.105	5.63E-05	Not assigned	unknown	
T2-L2	blue	Csa6G517960	-2.436	7.19E-06	Development	Squamosa promoter binding protein-like 8 (SPL8)	4.00E-162
T2-L2	blue	Csa3G337350	-2.409	5.31E-05	Biotic stress	MLP-like protein 34 (MLP34)	2.00E-26
T2-L2	blue	Csa1G524760	-2.299	7.66E-05	Hormone metabolism	highly ABA-induced PP2C gene 2 (HAI2)	
T2-L2	blue	Csa3G062590	-2.236	2.65E-10	Transcription factor	NAC domain transcription factor family	1.00E-50
T2-L2	blue	Csa5G522970	-2.079	4.50E-04	Miscellaneous	Beta-1,4-N-acetylglucosaminyltransferase family protein	4.00E-144
T2-L2	blue	Csa2G263940	-2.007	7.86E-09	Cell wall	Proline-rich protein 4 (PRP4)	6.00E-65
T2-L2	blue	Csa2G357860	-1.953	1.01E-06	Not assigned	unknown	
T2-L2	blue	Csa6G076800	-1.921	8.36E-03	Cell wall	Fasciclin-like arabinoglaaractan protein 8 (FLA8)	2.00E-54
T2-L2	blue	Csa6G448110	-1.869	1.83E-05	Transport	Gamma tonoplast intrinsic protein (GAMMA-TIP)	2.00E-67
T2-L2	blue	Csa3G073930	-1.822	7.68E-04	Protein degradation	Small ubiquitin-like modifier 1 (SUMO1)	1.00E-32
T2-L2	blue	Csa3G690300	-1.686	4.28E-05	Not assigned	unknown	
T2-L2	blue	Csa7G108300	-1.647	9.38E-06	Cell wall	Fasciclin-like arabinogalaractan protein 1 (FLA1)	9.00E-70
T2-L2	blue	Csa5G598070	-1.634	1.24E-07	Not assigned	unknown	
T2-L2	blue	Csa1G420360	-1.607	6.30E-03	Biotic stress	Cystein-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 protein	
T2-L2	blue	Csa6G507370	-1.596	8.96E-03	Development	Senescence 1 (SEN1)	5.00E-138
T2-L2	blue	Csa1G073840	-1.59	2.16E-03	Glycolysis	Phosphoglycerate/bisphosphoglycerate mutase family protein	5.00E-91
T2-L2	blue	Csa5G615830	-1.576	1.20E-05	Transport	Transmembrane amino acid transporter family protein	1.00E-102
T2-L2	blue	Csa6G487570	-1.558	9.20E-04	Transport	General control non-repressible 5 (GCN5)	7.00E-122
T2-L2	blue	Csa2G145880	-1.522	4.76E-03	Photosynthesis	Serine hydroxymethyltransferase 4 (SHM4)	3.00E-137
T2-L2	blue	Csa3G075990	-1.49	8.18E-03	Miscellaneous	GDSL-like Lipase/Acylhydrolase superfamily protein	7.00E-17
T2-L2	blue	Csa2G361590	-1.274	3.12E-03	Not assigned	unknown	
T2-L2	blue	Csa2G036680	-1.209	1.49E-03	Polyamine metabolism	S-adenosylmethionine decarboxylase proenzyme	4.00E-165
T2-L2	blue	Csa3G134910	1.136	6.18E-03	Not assigned	unknown	

Appendix table 5.1 (Continued)

T2-L2	blue	Csa1G574970	1.827	1.18E-05	Major CHO metabolism	Hexokinase 2 (HXK2)	7.00E-51
T2-L2	blue	Csa4G618520	1.837	0.0001765	Biodegradation of xenobiotic	UDP glycosyltransferase (UGT)	1.00E-132
T2-L2	blue	Csa2G003610	1.841	0.0003098	Lipid metabolism	Abnormal inflorescence meristem (AIM1)	3.00E-294
T2-L2	blue	Csa3G730930	1.847	0.00004243	Transport	Ammonium transporter 2 (AMT2)	8E-220
T2-L2	blue	Csa6G493850	1.86	1.50E-05	Not assigned	unknown	
T2-L2	blue	Csa3G159420	1.871	1.61E-03	Not assigned	unknown	
T2-L2	blue	Csa1G528610	1.874	0.00435	Lipid metabolism	Thylakoid membrane anion transporter 1 (ANTR1)	7.00E-236
T2-L2	blue	Csa1G181360	1.875	0.001947	Transport	non-intrinsic ABC protein 9 (NAP9)	3.00E-156
T2-L2	blue	Csa3G154410	2.05	1.63E-10	Not assigned	unknown	
T2-L2	blue	Csa3G183950	2.092	0.0004285	Abiotic stress	Heat shock protein 83	0.00E+00
T2-L2	blue	Csa2G296090	2.143	2.44E-04	Not assigned	unknown	
T2-L2	blue	Csa7G291720	2.169	0.006566	Lipid metabolism	Choline kinase	4.00E-133
T2-L2	blue	Csa2G427310	2.238	0.007581	Transcription factor	MYB domain protein 73 (MYB73)	1.00E-88
T2-L2	blue	Csa3G881750	2.24	0.009561	Lipid metabolism	PR5-like receptor kinase (PR5K)	4.00E-154
T2-L2	blue	Csa5G589260	2.243	0	Amino acid metabolism	O-acetylserine(thiol) lyase (OAS-TL)	4.00E-162
T2-L2	blue	Csa3G733350	2.317	0.00004435	Cell organization	RHO guanyl-nucleotide exchange factor 11 (ROPGEF11)	9.00E-41
T2-L2	blue	Csa2G004690	2.522	0.000004593	Transcription factor	Heat shock transcription factor C1 (HSFC1)	5.00E-67
T2-L2	blue	Csa7G387690	2.631	0.000001654	Transport	Sulfate transporter 3;1 (SULTR 3;1)	3.00E-307
T2-L2	blue	Csa1G008520	2.771	0.00804	Transport	Plasma membrane proton ATPase 4 (HA4)	0.00E+00
T2-L2	blue	Csa6G514850	3.048	0.00009803	Miscellaneous	Cytochrome P450 family 77 subfamily B polypeptide 1 (CYP77B1)	1.00E-241
T2-L2	blue	Csa5G604150	3.14	0.01	Redox	Thioredoxin 2 (TRX2)	
T2-L2	blue	Csa5G223070	3.216	0.003151	Transcription factor	WRKY DNA-binding protein 54 (WRKY54)	3.00E-96
T2-L2	blue	Csa2G359890	3.289	3.70E-03	Not assigned	unknown	
T2-L2	blue	Csa1G596520	3.4	0.0002165	Protein degradation	Cystein proteinases superfamily protein	2.00E-168
T2-L2	blue	Csa7G395800	3.415	0.001823	Miscellaneous	Glutathione S-transferase phi 8 (GSTF8)	3.00E-82
T2-L2	blue	Csa3G119700	3.575	0.00005376	Transcription factor	WRKY DNA-binding protein 53 (WRKY53)	1.00E-102
T2-L2	blue	Csa6G381850	3.831	0.001156	Transport	Amino acid permease 3 (AAP3)	2.00E-234
T2-L2	blue	Csa1G033160	4.495	0.006049	Miscellaneous	Glutathione S-transferase tau 9 (GSTU9)	3.00E-68
T2-L2	blue	Csa6G486960	4.496	0.000002502	Transcription factor	WRKY DNA-binding protein 51 (WRKY51)	1.00E-36
T2-L2	blue	Csa2G058620	5.345	0.003945	Abiotic stress	DNAJ heat shock N-terminal domain-containing protein	2.00E-137

Appendix table 5.1 (Continued)

T2-L2	blue	Csa3G064240	5.658	3.97E-13	Not assigned	unknown	
T2-L2	blue	Csa3G727990	6.451	4.501E-07	Transcription factor	WRKY DNA-binding protein 70 (WRKY70)	4.00E-166
T2-L2	blue	Csa6G093640	7.096	0.003228	Transcription factor	PCF (TCP)-domain family protein 20 (TCP20)	6.00E-72
T2-L2	blue	Csa3G825080	7.272	0.003456	Miscellaneous	Cytochrome P450 family 72 subfamily A polypeptide 8 (CYP72A8)	5.00E-38
T2-L2	blue	Csa1G002860	8.184	0.002006	Signaling	Suppressor of BIR1 (SOBIR1)	9.00E-255
T2-L2	blue	Csa2G297180	12.788	0.0003204	Transcription factor	WRKY DNA-binding protein 51 (WRKY51)	3.00E-51
T2-L2	blue	Csa7G037610	14.168	7.21E-03	Not assigned	unknown	
T2-L2	blue	Csa7G414490	17.197	0.001714	Miscellaneous	NAD(P)-binding Rossmann-fold superfamily protein	1.00E-84
T2-L2	blue	Csa1G007850	1.783	0.026	Transcription factor	MYB-like DNA-binding domain protein	7.00E-61
T2-L2	blue	Csa1G022520	3.614	0.025	Not assigned	unknown	2.00E-30
T2-L2	blue	Csa1G043040	1.212	0.027	Transcription factor	Homeobox protein 22 (HB22)	3.00E-22
T2-L2	blue	Csa1G045650	-3.2	1.04E-07	Protein degradation	AAA-ATPase 1 (AATP1)	1.00E-149
T2-L2	blue	Csa1G132110	1.644	0.015	Not assigned	unknown	1.00E-43
T2-L2	blue	Csa1G164660	-1.478	0.04	Miscellaneous	Glycosyl hydrolase superfamily protein	6.00E-149
T2-L2	blue	Csa1G165230	1.466	0.016	Transcription factor	NAC domain containing protein 90 (NAC090)	8.00E-62
T2-L2	blue	Csa1G212830	1.554	0.025	Secondary metabolism	2-oxoglutarase (2OG) and Fe(II)-dependent oxygenase superfamily protein	6.00E-92
T2-L2	blue	Csa1G523610	1.418	0.031	Transcription factor	GRAS family transcription factor	1.00E-144
T2-L2	blue	Csa1G560830	-4.342	1.61E-05	RNA processing	RNA helicase 2	2.00E-282
T2-L2	blue	Csa1G575160	1.173	0.027	Transcription factor	SNF7 family protein	7.00E-104
T2-L2	blue	Csa1G611290	1.417	0.014	Miscellaneous	Beta glucosidase 15 (BGLU15)	9.00E-159
T2-L2	blue	Csa2G163170	1.75	0.014	Transport	Ammonium transporter 1;1 (AMT1;1)	2.00E-241
T2-L2	blue	Csa2G287040	1.909	0.032	Cell organization	Ankyrin repeat family protein	5.00E-14
T2-L2	blue	Csa2G345990	1.632	0.019	Not assigned	Thioredoxin superfamily protein	5.00E-67
T2-L2	blue	Csa2G351820	1.889	0.031	Protein targeting	Vacuolar-processing enzyme precursor (VPE)	2.00E-219
T2-L2	blue	Csa2G358860	1.58	0.049	Not assigned	unknown	
T2-L2	blue	Csa2G359940	1.788	0	Not assigned	unknown	
T2-L2	blue	Csa2G360750	1.962	6.24E-03	Not assigned	Transducing/WD40 repeat-like superfamily protein	1.00E-141
T2-L2	blue	Csa2G423560	1.685	0.012	Transcription factor	B-box type zinc finger family protein	5.00E-21
T2-L2	blue	Csa3G035840	-3.171	3.41E-03	Miscellaneous	receptor serine/threonine kinase	4.00E-19
T2-L2	blue	Csa3G061000	5.308	0.016	Protein postranslational modification	Calcium-binding EF-hand family protein	1.00E-85

Appendix table 5.1 (Continued)

T2-L2	blue	Csa3G099650	1.434	3.40E-03	Abiotic stress	Wound-responsive family protein	1.00E-17
T2-L2	blue	Csa3G145780	10.118	0.012	Signaling	Early light-inducible protein (ELIP1)	7.00E-55
T2-L2	blue	Csa3G146300	1.261	3.15E-05	Metal handling	Acireductone dioxygenase 2 (ARD2)	1.00E-92
T2-L2	blue	Csa3G171820	1.309	0.023	Hormone metabolism	SAUR-like auxin-responsive protein family	1.00E-45
T2-L2	blue	Csa3G178520	1.315	3.66E-03	Protein degradation	Subtilase 1.3 (SBT1.3)	0
T2-L2	blue	Csa3G181940	1.399	0.012	Abiotic stress	Heat stress transcription factor (Hsf) family	3.00E-55
T2-L2	blue	Csa3G182150	-1.65	9.72E-06	Signaling	PAR1 protein	3.00E-55
T2-L2	blue	Csa3G239250	-2.005	1.63E-04	Not assigned	Unknown	
T2-L2	blue	Csa3G316280	2.061	7.95E-07	Transcription factor	Calcium-dependent lipid-binding (CaLB domain) family protein	4.00E-64
T2-L2	blue	Csa3G319290	1.31	0.04	Biotic stress	Polyketide cyclase/dehydrase and lipid transport superfamily protein	3.00E-20
T2-L2	blue	Csa3G333840	3.405	6.16E-05	Biotic stress	MLP-like protein 34 (MLP34)	3.00E-13
T2-L2	blue	Csa3G588450	1.584	0.047	Protein synthesis	Glucose hypersensitive 1 (GHS1)	3.00E-45
T2-L2	blue	Csa3G646010	1.758	0.013	Hormone metabolism	Dormancy/auxin associated family protein	3.00E-15
T2-L2	blue	Csa3G646660	2.135	0.048	Not assigned	Unknown	
T2-L2	blue	Csa3G732460	1.343	0.046	Lipid metabolism	Sulfoquinovosyldiacylglycerol 2 (SQD2)	7.00E-209
T2-L2	blue	Csa3G733230	-2.994	0.046	Abiotic stress	Heat shock protein 20 (HSP20)	2.00E-13
T2-L2	blue	Csa3G776960	3.355	0.014	Not assigned	Thioredoxin family protein	4.00E-81
T2-L2	blue	Csa3G820480	1.617	0.026	Protein targeting	Sigma factor binding protein 1 (SIB1)	1.00E-10
T2-L2	blue	Csa3G848170	1.744	0.024	Protein degradation	Amino peptidase M1 (APM1)	3.00E-284
T2-L2	blue	Csa3G848300	-2.377	0.021	Miscellaneous	nitrile lyases, berberine bridge enzymes, reticuline oxidases, troponine reductases	9.00E-238
T2-L2	blue	Csa3G859670	1.268	0.024	Not assigned	unknown	4.00E-10
T2-L2	blue	Csa3G860260	4.271	0.028	Abiotic stress	Wound-responsive family protein	1.00E-127
T2-L2	blue	Csa3G872070	-1.57	0.046	Cell wall	Glycosyl hydrolase 9B5 (GH9B5)	2.00E-228
T2-L2	blue	Csa3G873260	2.16	0.026	Not assigned	unknown	1.00E-06
T2-L2	blue	Csa3G878740	3.003	0.014	Development	DNAJ/HSP40 cystein-rich domain superfamily protein	6.00E-32
T2-L2	blue	Csa4G011770	3.01	0.015	Transcription factor	NAC domain containing protein 83 (NAC083)	4.00E-62
T2-L2	blue	Csa4G025120	-1.865	0.027	Not assigned	unknown	4.00E-44
T2-L2	blue	Csa4G052080	3.146	0.035	Not assigned	unknown	
T2-L2	blue	Csa4G056640	1.53	0.011	Hormone metabolism	Nine-cis-epoxycarotenoid dioxygenase 4 (NCED4)	3.00E-226
T2-L2	blue	Csa4G063480	1.772	0.028	Biotic stress	NPR1-like protein 3	2.00E-37

Appendix table 5.1 (Continued)

T2-L2	blue	Csa4G091870	3.269	1.43E-04	Secondary metabolism	Downy mildew resistant 6 (DMR6)	5.00E-140
T2-L2	blue	Csa4G269770	2.17	0.047	Protein postranslational modification	Protein kinase 2B (APK2B)	2.00E-125
T2-L2	blue	Csa4G361820	3.071	0.03	Development	NAC domain containing protein 32 (NAC32)	2.00E-88
T2-L2	blue	Csa4G630010	-1.901	0.039	Hormone metabolism	Ethylene response factor 8 (ERF8)	4.00E-32
T2-L2	blue	Csa4G646340	1.828	0.013	Abiotic stress	Heat shock protein 17.6A (HSP17.6II)	1.00E-49
T2-L2	blue	Csa4G651750	-3.219	0.02	Transcription factor	Aspartyl protease family protein	4.00E-136
T2-L2	blue	Csa5G011650	1.376	0.021	Miscellaneous	NAD(P)-binding Rossmann-fold superfamily protein	3.00E-65
T2-L2	blue	Csa5G097460	-1.366	1.15E-06	Biotic stress	P-loop containing nucleotide triphosphate hydrolases superfamily protein	1.00E-65
T2-L2	blue	Csa5G140450	7.962	0.02	DNA repair	Heavy metal transport/detoxification superfamily protein	3.00E-24
T2-L2	blue	Csa5G150420	2.011	0.048	Transcription factor	Apetala2/Ethylene-responsive element binding protein family	1.00E-48
T2-L2	blue	Csa5G152250	1.603	2.95E-05	DNA repair	Heavy metal transport/detoxification superfamily protein	2.00E-27
T2-L2	blue	Csa5G152260	-1.607	0.016	DNA repair	Heavy metal transport/detoxification superfamily protein	3.00E-27
T2-L2	blue	Csa5G153020	1.177	0.016	Transport	Plasma membrane intrinsic protein 1C (PIP1C)	4.00E-149
T2-L2	blue	Csa5G158570	1.562	3.77E-09	Miscellaneous	Copper amine oxidase family protein	2.00E-113
T2-L2	blue	Csa5G167120	1.63	0.019	Hormone metabolism	Ethylene response factor 104 (ERF104)	4.00E-45
T2-L2	blue	Csa5G168910	-1.606	0.021	Not assigned	unknown	
T2-L2	blue	Csa5G168920	2.675	0.026	Signaling	Leucine-rich repeat receptor -like protein kinase family protein	2.00E-76
T2-L2	blue	Csa5G179220	1.348	0.015	Lipid metabolism	Alpha/beta-hydrolases superfamily protein	1.00E-131
T2-L2	blue	Csa5G207940	2.293	3.45E-10	Transcription factor	Salt-inducible zinc finger 1 (SZF1)	2.00E-44
T2-L2	blue	Csa5G547610	-1.559	0.04	Nucleotide metabolism	Nudix hydrolase homolog 17 (NUDT17)	2.00E-67
T2-L2	blue	Csa5G610370	4.368	0.034	Lipid metabolism	PR5-like receptor kinase (PR5K)	1.00E-98
T2-L2	blue	Csa5G611750	1.952	0.016	Metal handling	Heavy metal transport/detoxification superfamily protein	3.00E-49
T2-L2	blue	Csa5G628650	7.218	0.02	Hormone metabolism	Jasmonate-zim-domain protein 3 (JAZ3)	4.00E-18
T2-L2	blue	Csa5G630800	-1.502	0.035	Miscellaneous	Oxidoreductase, zinc-binding dehydrogenase family protein	2.00E-111
T2-L2	blue	Csa6G084580	3.26	1.04E-10	Biotic stress	Disease resistance-responsive (dirigent-like protein) family protein	7.00E-47

Appendix table 5.1 (Continued)

T2-L2	blue	Csa6G106800	2.214	3.81E-05	Signaling	Calcium-binding EF-hand family protein	3.00E-41
T2-L2	blue	Csa6G127320	1.484	0.019	Development	NAC domain containing protein 36 (NAC036)	3.00E-73
T2-L2	blue	Csa6G133770	2.243	1.52E-07	Hormone metabolism	Cytokinin response factor 4 (CRF4)	2.00E-34
T2-L2	blue	Csa6G404210	10.38	0.029	Not assigned	SPFH/Band 7/ PHB domain-containing membrane-associated protein family	5.00E-143
T2-L2	blue	Csa6G445150	-1.623	4.96E-04	Not assigned	Aquaporin 2	2.00E-118
T2-L2	blue	Csa6G486670	1.638	0.024	Transcription factor	Tonoplast intrinsic protein 2 (TIP2)	2.00E-101
T2-L2	blue	Csa6G490900	3.584	0.021	Transcription factor	Remorin family protein	8.00E-35
T2-L2	blue	Csa6G495000	-3.252	0.027	Miscellaneous	Root hair specific 19 (RHS19)	5.00E-138
T2-L2	blue	Csa6G505280	1.841	9.08E-06	DNA synthesis	Histone H1-3 (HIS1-3)	4.00E-34
T2-L2	blue	Csa6G513580	1.269	2.01E-10	Protein targeting	Peroxisomal membrane 22kDa family protein	7.00E-64
T2-L2	blue	Csa6G514860	1.318	0.033	Transport	Glucose-6-phosphate/phosphate translocator 2 (GPT2)	4.00E-162
T2-L2	blue	Csa6G516580	1.417	0.044	Signaling	Cysteine-rich receptor-like protein kinase 29 (CRK29)	1.00E-55
T2-L2	blue	Csa6G538630	1.859	0.015	Co-factor and vitamin metabolism	Molybdenum cofactor sulfurase family protein	9.00E-120
T2-L2	blue	Csa7G037660	1.854	0.036	Protein synthesis	Nucleic acid-binding, OB-fold-like protein	1.00E-76
T2-L2	blue	Csa7G049260	2.259	0.022	Protein postranslational modification	Protein phosphatase 2C family protein	1.00E-109
T2-L2	blue	Csa7G230940	1.146	0.023	Redox	Glutaredoxin family protein	2.00E-30
T2-L2	blue	Csa7G253780	1.374	0.045	Transport	Polyol/monosaccharide transporter 1 (PMT1)	2.00E-190
T2-L2	blue	Csa7G318990	1.676	0.023	Biotic stress	Pathogenesis-related family protein	4.00E-62
T2-L2	blue	Csa7G352410	-1.471	0.026	Not assigned	unknown	6.00E-71
T2-L2	blue	Csa7G372360	-4.168	2.77E-06	Miscellaneous	Defective in induced resistance 1 (DIR1)	6.00E-21
T2-L2	blue	Csa7G429630	1.754	0.017	Not assigned	unknown	5.00E-35
T2-L2	blue	Csa7G432550	1.637	0.013	Not assigned	Tetratricopeptide repeat (TPR)-like superfamily protein	3.00E-50
T2-L2	brown	Csa4G297410	-4.596	1.61E-05	Abiotic stress	Pollen Ole e 1 allergen and extensin family protein	4.00E-62
T2-L2	brown	Csa3G179180	-4.461	1.86E-10	Not assigned	unknown	
T2-L2	brown	Csa7G390010	-3.971	1.12E-05	Not assigned	unknown	
T2-L2	brown	Csa1G062340	-2.872	2.35E-04	Miscellaneous	GDSL-like Lipase/Acylhydrolase superfamily protein	3.00E-171
T2-L2	brown	Csa6G139210	-2.397	2.49E-03	Miscellaneous	Indole-3-acetate beta-D-glucosyltransferase (IAGLU)	1.00E-27

Appendix table 5.1 (Continued)

T2-L2	brown	Csa5G642130	-1.918	1.35E-03	Miscellaneous	Peroxidase superfamily protein	1.00E-98
T2-L2	brown	Csa3G035890	-1.737	1.64E-04	Transcription factor	WRKY DNA-binding protein 40 (WRKY40)	2.00E-32
T2-L2	brown	Csa6G148170	-1.707	9.44E-13	Photosynthesis	Chlorophyll A/B binding protein 1 (CAB1)	6.00E-119
T2-L2	brown	Csa3G239280	-1.465	7.68E-03	Miscellaneous	Peroxidase superfamily protein	8.00E-14
T2-L2	brown	Csa4G126430	-1.413	1.80E-03	development	flowering-time gene CONSTANS (CO) encoding zinc-finger proteins	3.00E-86
T2-L2	brown	Csa1G066480	-1.393	7.30E-03	Not assigned	unknown	
T2-L2	brown	Csa2G369210	-1.274	3.12E-03	Not assigned	unknown	
T2-L2	brown	Csa7G451340	1	0	Miscellaneous	Nitrile lyases, berberine bridge enzymes, reticuline oxidases, troponine reductases	8.00E-213
T2-L2	brown	Csa6G109750	1.185	0.046	Miscellaneous	Bif-unctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein	6.00E-41
T2-L2	brown	Csa6G318680	1.312	0.0007313	Protein degradation	Small ubiquitin-like modifier 1 (SUMO1)	1.00E-45
T2-L2	brown	Csa6G446450	1.369	4.52E-03	biotic stress	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family	2.00E-65
T2-L2	brown	Csa3G150800	1.389	1.57E-03	not assigned	unknown	
T2-L2	brown	Csa6G150550	1.445	0.000008129	Redox	Copper/zinc superoxide dismutase 2 (CSD2)	1.00E-89
T2-L2	brown	Csa7G024130	1.47	2.38E-03	Not assigned	unknown	
T2-L2	brown	Csa2G336130	1.485	0.011	RNA binding	glycine-rich RNA binding protein (GLYCINE RICH PROTEIN 7)	1.00E-58
T2-L2	brown	Csa5G171740	1.562	3.769E-09	Miscellaneous	Copper amine oxidase family protein	4.00E-114
T2-L2	brown	Csa1G212840	1.58	0	Protein degradation	Cystatin/monellin superfamily protein	5.00E-07
T2-L2	brown	Csa7G452200	1.581	0.024	transport	Delta tonoplast intrinsic protein, functions as a water channel and ammonium (NH3) transporter	4.00E-111
T2-L2	brown	Csa6G521090	1.68	0.000001159	Miscellaneous	alpha/beta-hydrolases superfamily protein	1.00E-128
T2-L2	brown	Csa3G710200	1.705	3.66E-03	Not assigned	unknown	
T2-L2	brown	Csa7G025720	1.772	0.0001424	Protein postranslational modification	Protein kinase superfamily protein	4.00E-163
T2-L2	brown	Csa5G631510	1.776	2.25E-03	Not assigned	unknown	
T2-L2	brown	Csa2G009470	1.841	0.0003098	Lipid metabolism	Abnormal inflorescence meristem (AIM1)	3.00E-294
T2-L2	brown	Csa2G351840	2.309	0.00001605	Miscellaneous	Glutathione S-transferase zeta 1 (GSTZ1)	9.00E-78
T2-L2	brown	Csa4G001980	2.335	7.43E-04	not assigned	unknown	
T2-L2	brown	Csa3G736660	2.599	2.35E-04	Not assigned	unknown	
T2-L2	brown	Csa3G778440	2.637	0.001429	Miscellaneous	Glutathione S-transferase L3 (GSTL3)	1.00E-99

Appendix table 5.1 (Continued)

T2-L2	brown	Csa1G228960	3.136	0.00003412	Transport	Pyrophosphate-energized vacuolar membrane proton pump	0.00E+00
T2-L2	brown	Csa5G319910	3.216	0.003151	Transcription factor	WRKY DNA-binding protein 54 (WRKY54)	3.00E-96
T2-L2	brown	Csa6G148290	3.755	1.73E-05	Not assigned	unknown	
T2-L2	brown	Csa2G361590	5.96	1.72E-06	Not assigned	unknown	
T2-L2	brown	Csa7G447100	8		Transport	Tonoplast intrinsic protein 2;3 (TIP 2;3)	5.00E-107
T2-L2	brown	Csa6G356490	9.406	7.34E-04	Not assigned	unknown	
T2-L2	brown	Csa5G547610	9.656	0.0000344	Lipid metabolism	AMP-dependent synthetase and ligase family protein	6.00E-236
T2-L2	brown	Csa7G037660	14.168	7.21E-03	Not assigned	unknown	
T2-L2	brown	Csa1G109330	-3.47	0.013	Protein degradation	Serine carboxypeptidase-like 48 (SCPL48)	3.00E-109
T2-L2	brown	Csa1G123470	1.58	0	Protein degradation	Cystatin/monellin superfamily protein	
T2-L2	brown	Csa2G003610	1.841	3.10E-04	Lipid metabolism	Abnormal inflorescence meristem (AIM1)	3.00E-294
T2-L2	brown	Csa2G120410	1.347	3.65E-09	Protein synthesis	Zinc-binding ribosomal protein family protein	7.00E-47
T2-L2	brown	Csa2G382650	-1.737	1.64E-04	Transcription factor	WRKY DNA-binding protein 40 (WRKY40)	8.00E-147
T2-L2	brown	Csa3G119700	3.575	5.38E-05	Transcription factor	WRKY DNA-binding protein 53 (WRKY53)	1.00E-102
T2-L2	brown	Csa3G600020	-2.306	4.96E-04	Secondary metabolism	Chalcone synthase 2 (CHS2)	5.00E-212
T2-L2	brown	Csa3G734160	2.599	2.35E-04	Not assigned	Unknown	2.00E-17
T2-L2	brown	Csa3G814360	2.296	0.013	Not assigned	Unknown	8.00E-26
T2-L2	brown	Csa3G903520	-1.154	0.029	Photosynthesis	Glycine decarboxylase complex H (GDCH)	2.00E-78
T2-L2	brown	Csa3G911280	1.219	3.02E-03	Development	DUF581	2.00E-44
T2-L2	brown	Csa4G011770	3.01	0.015	Transcription factor	NAC domain containing protein 83 (NAC083)	3.00E-81
T2-L2	brown	Csa4G062920	1.404	6.20E-06	Miscellaneous	Polyamine oxisase 2 (PAO2)	3.00E-228
T2-L2	brown	Csa4G361820	3.071	0.03	Development	NAC domain containing protein 32 (NAC32)	5.00E-120
T2-L2	brown	Csa4G630010	-1.901	0.039	Hormone metabolism	Ethylene response factor 8 (ERF8)	2.00E-34
T2-L2	brown	Csa4G651750	-3.219	0.02	Transcription factor	Aspartyl protease family protein	2.00E-142
T2-L2	brown	Csa5G140450	7.962	0.02	DNA repair	Heavy metal transport/detoxification superfamily protein	1.00E-61
T2-L2	brown	Csa5G158570	1.562	3.77E-09	Miscellaneous	Copper amine oxidase family protein	4.00E-114
T2-L2	brown	Csa5G223070	3.216	3.15E-03	Transcription factor	WRKY DNA-binding protein 54 (WRKY54)	3.00E-96
T2-L2	brown	Csa5G605150	1.346	0.021	RNA processing	Ribonuclease T2	6.00E-105
T2-L2	brown	Csa5G608610	1.776	2.25E-03	Not assigned	Unknown	4.00E-31

Appendix table 5.1 (Continued)

T2-L2	brown	Csa6G014540	1.836	0.019	Cell wall	Expansin A8 (EXPA8)	6.00E-12
T2-L2	brown	Csa6G057170	-1.227	0.027	Photosynthesis	Light harvesting complex photosystem II subunit 6 (LHCB6)	3.00E-13
T2-L2	brown	Csa6G139750	-1.707	9.44E-13	Photosynthesis	Chlorophyll A/B binding protein 1 (CAB1)	1.00E-14
T2-L2	brown	Csa6G152330	1.508	2.61E-03	Protein synthesis	Ribosomal protein L1p/L10e family	1.00E-11
T2-L2	brown	Csa6G157680	1.312	7.31E-04	Protein degradation	Small ubiquitin-like modifier 2 (SUMO2)	1.00E-45
T2-L2	brown	Csa6G404210	10.38	0.029	Not assigned	SPFH/Band 7/ PHB domain-containing membrane-associated protein family	2.00E-15
T2-L2	brown	Csa6G404270	1.408	0.049	Abiotic stress	Germin-like protein 7 (GLP7)	7.00E-71
T2-L2	brown	Csa6G405340	-3.365	0.028	Cell wall	Glycosyl hydrolase superfamily protein	1.00E-20
T2-L2	brown	Csa6G517180	1.68	1.16E-06	Miscellaneous	Alpha/beta-hydrolases superfamily protein	1.00E-12
T2-L2	brown	Csa6G538750	1.47	2.38E-03	Not assigned	DUF23	2.00E-20
T2-L2	brown	Csa7G037610	14.168	7.21E-03	Not assigned	Unknown	2.00E-45
T2-L2	brown	Csa7G066240	3.449	0.036	Not assigned	Unknown	
T2-L2	brown	Csa7G071460	1.556	1.42E-04	Protein synthesis	Ribosomal protein S14p/S29e family protein	1.00E-28
T2-L2	brown	Csa7G073430	-3.971	1.12E-05	Not assigned	DUF579	3.00E-78
T2-L2	brown	Csa7G428940	1.343	0.033	Protein synthesis	Ribosomal protein L6 family protein	2.00E-11
T2-L2	brown	Csa7G430130	-1.498	0.047	Lipid metabolism	Bi-functional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein	8.00E-48
T2-L2	grey	Csa6G407650	1.837	0.0001765	Biodegradation of xenobiotic	UDP glycosyltransferase (UGT)	1.00E-13
T2-L2	grey	Csa1G050250	1.106	6.41E-03	Glycolysis	Glyceraldehyde-3-phosphate dehydrogenase	2.00E-18
T2-L2	grey	Csa3G680120	1.221	0.031	Minor CHO metabolism	Galactinol synthase 1 (GolS1)	1.00E-15
T2-L2	grey	Csa4G618520	1.837	1.77E-04	Hormone metabolism	UDP-Glycosyltransferase superfamily protein	1.00E-13
T2-L2	turquoise	Csa3G122470	-6.667	5.69E-04	Not assigned	unknown	
T2-L2	turquoise	Csa4G167980	-4.114	1.13E-06	Not assigned	unknown	
T2-L2	turquoise	Csa5G152260	-4.109	7.06E-04	Cell wall	Pectin lyase-like superfamily protein	1.00E-79
T2-L2	turquoise	Csa5G139600	-3.252	4.53E-05	Protein degradation	Prolyl oligopeptidase family protein	3.00E-69
T2-L2	turquoise	Csa4G000930	-3.167	4.50E-04	Not assigned	unknown	
T2-L2	turquoise	Csa7G387690	-3.105	5.63E-05	Not assigned	unknown	
T2-L2	turquoise	Csa3G849920	-2.921	6.11E-16	Transcription factor	MYB domain protein 30 (MYB30)	7.00E-20
T2-L2	turquoise	Csa6G190340	-2.765	8.71E-05	Miscellaneous	UDP-Glycosyltransferase superfamily protein	3.00E-84
T2-L2	turquoise	Csa6G518250	-2.436	7.19E-06	Development	Squamosa promoter binding protein-like 8 (SPL8)	4.00E-16
T2-L2	turquoise	Csa7G450510	-2.276	9.15E-03	Secondary metabolism	Cinnamoyl Coenzyme A reductase 1 (CCR1)	5.00E-15

Appendix table 5.1 (Continued)

T2-L2	turquoise	Csa5G505200	-2.248	1.07E-03	Protein degradation	Anaphase-promoting complex/cyclosome 2 (APC2)	8.00E-148
T2-L2	turquoise	Csa4G416430	-2.238	1.75E-03	Lipid metabolism	Nonspecific lipid-transfer protein precursor (LTP)	1.00E-109
T2-L2	turquoise	Csa7G049260	-1.969	6.68E-05	Hormone metabolism	Ethylene responsive element binding factor 4 (ERF4)	7.00E-113
T2-L2	turquoise	Csa3G202730	-1.907	1.38E-12	Secondary metabolism	Beta-hydroxylase 1 (BETA_OHASE1)	3.00E-89
T2-L2	turquoise	Csa1G420360	-1.884	5.86E-03	Not assigned	unknown	
T2-L2	turquoise	Csa7G043640	-1.87	9.28E-04	Protein degradation	Xylem cysteine peptidase 1 (XCP1)	7.00E-150
T2-L2	turquoise	Csa4G639870	-1.85	6.66E-03	Not assigned	unknown	
T2-L2	turquoise	Csa6G309950	-1.797	8.01E-05	Light signaling	Phototropin 1 (PHO1)	3.00E-45
T2-L2	turquoise	Csa1G050420	-1.733	4.47E-06	Not assigned	unknown	
T2-L2	turquoise	Csa3G252490	-1.725	6.57E-03	Not assigned	unknown	
T2-L2	turquoise	Csa6G109650	-1.652	7.76E-03	Secondary metabolism	Flavanone 3-hydroxylase (F3H)	7.00E-156
T2-L2	turquoise	Csa7G230940	-1.647	9.38E-06	Cell wall	Fasciclin-like arabinoglaaractan protein 1 (FLA1)	9.00E-70
T2-L2	turquoise	Csa2G000230	-1.618	1.21E-03	Cell wall	Fasciclin-like arabinogalaaractan protein 17 precursor (FLA17)	2.00E-119
T2-L2	turquoise	Csa1G462020	-1.607	6.30E-03	Biotic stress	Cystein-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 protein	
T2-L2	turquoise	Csa6G517960	-1.596	8.96E-03	Development	Senescence 1 (SEN1)	5.00E-138
T2-L2	turquoise	Csa2G173070	-1.522	4.76E-03	Photosynthesis	Serine hydroxymethyltransferase 4 (SHM4)	3.00E-137
T2-L2	turquoise	Csa5G207940	-1.5	5.58E-08	Photosynthesis	RuBisCO activase (RA)	
T2-L2	turquoise	Csa3G182240	-1.494	5.11E-03	Secondary metabolism	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	7.00E-93
T2-L2	turquoise	Csa5G162630	-1.494	0.021	miscellaneous	extensin-like protein (ELP) (Arabidopsis thaliana)	3.00E-52
T2-L2	turquoise	Csa3G563300	-1.485	7.90E-03	Lipid metabolism	Lysophospholipase 2 (LysoPL2)	1.00E-126
T2-L2	turquoise	Csa3G035840	-1.464	0.021	miscellaneous	Bi-functional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein (Arabidopsis thaliana)	5.00E-10
T2-L2	turquoise	Csa5G174640	-1.419	9.38E-06	Abiotic stress	Bcl2-associated anthanogene (ABG) protein	3.00E-77
T2-L2	turquoise	Csa7G336450	-1.375	6.08E-03	Photosynthesis	Non-photochemical quenching 4 (NPQ4)	4.00E-107
T2-L2	turquoise	Csa6G013960	-1.334	5.05E-03	Photosynthesis	RuBisCO activase (RA)	1.00E-69
T2-L2	turquoise	Csa3G539670	-1.282	7.34E-04	Photosynthesis	Photosystem I subunit H2 (PSAH2)	0
T2-L2	turquoise	Csa7G398090	-1.261	7.43E-04	development	Late embryogenesis abundant 3 (LEA3) family protein	2.00E-20
T2-L2	turquoise	Csa5G171730	-1.229	1.50E-04	not assigned	unknown	
T2-L2	turquoise	Csa3G826640	-1.228	1.14E-04	Lipid metabolism	Fatty acid desaturase 3	2.00E-108
T2-L2	turquoise	Csa3G874340	-1.221	3.64E-04	protein folding	DnaJ/Hsp40 cysteine-rich domain superfamily protein	6.00E-73

Appendix table 5.1 (Continued)

T2-L2	turquoise	Csa5G212590	-1.194	0.012	lipid metabolism	Omega-3 fatty acid desaturase, chloroplast precursor	1.00E-274
T2-L2	turquoise	Csa3G134920	1.136	6.18E-03	Not assigned	unknown	
T2-L2	turquoise	Csa6G381850	1.251	1.62E-03	Not assigned	unknown	
T2-L2	turquoise	Csa1G569380	1.278	2.06E-04	Abiotic stress	A member of heat shock protein 90 (HSP90) gene family (Arabidopsis thaliana)	3.00E-270
T2-L2	turquoise	Csa1G043180	1.285	0.00657	Hormone metabolism	Jasmonate-zim-domain protein 3 (JAZ3)	0.00E+00
T2-L2	turquoise	Csa6G289760	1.294	0.000364	Photosynthesis	ATP synthase protein 1-related	6.00E-131
T2-L2	turquoise	Csa5G179220	1.295	0.009199	RNA Processing	Fibrillarin 2 (FIB2)	1.00E-151
T2-L2	turquoise	Csa1G046840	1.3	6.55E-05	Not assigned	unknown	
T2-L2	turquoise	Csa6G151730	1.311	4.19E-04	Hormone metabolism	Multiprotein bridging factor 1B (MBF1B)	2.00E-42
T2-L2	turquoise	Csa3G483790	1.33	0.006473	RNA binding	RNA-binding (RRM/RBD/RNP motifs) family protein	7.00E-46
T2-L2	turquoise	Csa2G247040	1.332	1.62E-03	Not assigned	unknown	
T2-L2	turquoise	Csa1G039240	1.338	0.004886	RNA binding	Glycine-rich RNA-binding protein 3 (GR-RBP3)	3.00E-62
T2-L2	turquoise	Csa3G826690	1.409	6.998E-11	Transport	Silicon transpoter LSI1	3.00E-145
T2-L2	turquoise	Csa3G101810	1.426	0.003945	Protein synthesis	Translation initiation factor SUI family protein	9.00E-54
T2-L2	turquoise	Csa3G110620	1.426	0.002703	Transcription factor	NAC domain containing protein 3 (NAC3)	7.00E-102
T2-L2	turquoise	Csa4G000800	1.451	0.001625	RNA binding	Hyaluronan/mRNA binding family	2.00E-128
T2-L2	turquoise	Csa2G258680	1.466	0.01	Hormone metabolism	SAUR-like auxin-responsive protein family	0.00E+00
T2-L2	turquoise	Csa6G151110	1.503	9.23E-03	cell wall	cellulose synthase-like A02 (CSLA02) (Arabidopsis thaliana)	5.00E-251
T2-L2	turquoise	Csa3G153170	1.515	0.00005673	Redox	Thioredoxin superfamily protein	2.00E-39
T2-L2	turquoise	Csa6G421800	1.523	0.0007678	Protein degradation	Ubiquitin-like protein 5 (UBL5)	2.00E-35
T2-L2	turquoise	Csa4G651760	1.53	0.01	Transcription factor	MYB domain protein R1 (MYBR1)	
T2-L2	turquoise	Csa3G118080	1.532	9.679E-12	Development	Squamosa promoter binding protein-like 10 (SPL10)	1.00E-95
T2-L2	turquoise	Csa6G046270	1.562	0.0006147	Transcription factor	Constants-like zinc finger family	2.00E-186
T2-L2	turquoise	Csa1G041530	1.567	2.71E-03	Not assigned	unknown	
T2-L2	turquoise	Csa6G088070	1.582	1.49E-06	Not assigned	unknown	
T2-L2	turquoise	Csa3G808360	1.638	0.0002177	TCA	Putative alpha-carbonic anhydrase (CAH1)	4.00E-81
T2-L2	turquoise	Csa5G140480	1.639	0.00005501	Development	Nodulin MtN3 family	5.00E-85
T2-L2	turquoise	Csa3G127210	1.687	1.29E-03	Not assigned	unknown	
T2-L2	turquoise	Csa7G049230	1.702	0.036	not assigned	unknown	
T2-L2	turquoise	Csa2G368880	1.706	0.003583	Redox	Thioredoxin H-type 1 (TRX1)	1.00E-32
T2-L2	turquoise	Csa4G017110	1.754	4.35E-04	Not assigned	unknown	
T2-L2	turquoise	Csa1G569250	1.778	9.18E-03	Not assigned	unknown	

Appendix table 5.1 (Continued)

T2-L2	turquoise	Csa2G362490	1.788	0	Not assigned	unknown	
T2-L2	turquoise	Csa1G021950	1.803	0	RNA binding	Glycine-rich RNA-binding protein 3 (GR-RBP3)	6.00E-74
T2-L2	turquoise	Csa2G006870	1.822	0.000168	Cell organization	Profillin 3	3.00E-68
T2-L2	turquoise	Csa2G359940	1.869	0.027	RNA regulation of transcription	Member of the R2R3 factor MYB gene family; myb domain protein r1 (MYBR1)	4.00E-115
T2-L2	turquoise	Csa2G012120	1.884	0	Miscellaneous	Uclacyanin 1 (UCC1)	3.00E-38
T2-L2	turquoise	Csa2G028480	1.908	9.15E-03	hormone metabolism	Lipoxygenase 1 (EC 1.13.11.12) - (Solanum tuberosum)	2.00E-25
T2-L2	turquoise	Csa6G242200	1.979	2.91E-03	signaling	Rapid Alkalinization Factor (RALF) 23	4.00E-32
T2-L2	turquoise	Csa2G258610	1.987	0.0009968	RNA binding	Glycine rich protein 7 (GRP7)	1.00E-58
T2-L2	turquoise	Csa3G739040	2.068	0.00483	Biodegradation of xenobiotic	Gloxal oxidase-related protein	1.00E-258
T2-L2	turquoise	Csa2G431070	2.23	0.000006487	Abiotic stress	SHEPHERD (SHD)	0.00E+00
T2-L2	turquoise	Csa3G129540	2.231	0.021	protein synthesis	chloroplast ribosomal protein S7 (Arabidopsis thaliana)	3.00E-81
T2-L2	turquoise	Csa5G604980	2.243	0	Amino acid metabolism	O-acetylserine(thiol) lyase (OAS-TL)	4.00E-162
T2-L2	turquoise	Csa1G024920	2.253	0.007294	Protein postranslational modification	RACK1B	7.00E-174
T2-L2	turquoise	Csa1G386590	2.258	3.86E-04	not assigned	unknown	
T2-L2	turquoise	Csa1G587400	2.496	0.00002831	Protein degradation	Metallopeptidase M24 family protein	3.00E-172
T2-L2	turquoise	Csa7G395800	2.631	0.000001654	Transport	Sulfate transporter 3;1 (SULTR 3;1)	3.00E-307
T2-L2	turquoise	Csa1G600980	2.792	0.00737	Protein postranslational modification	AGC (cAMP-dependent, cGMP-dependent and protein kinase C) kinase family protein	2.00E-235
T2-L2	turquoise	Csa5G589260	2.953	0.000001814	Miscellaneous	Plant invertase/pectin methylesterase inhibitor superfamily protein	5.00E-62
T2-L2	turquoise	Csa5G139420	3.156	6.39E-05	Not assigned	unknown	
T2-L2	turquoise	Csa5G615230	3.177	2.18E-03	Not assigned	unknown	
T2-L2	turquoise	Csa4G620640	3.195	0	Protein targeting	Sigma factor binding protein 1 (SIB1)	2.00E-14
T2-L2	turquoise	Csa4G420110	3.334	0	Protein degradation	Metacaspase 9 (MC9)	1.00E-120
T2-L2	turquoise	Csa7G397040	3.415	0.001823	Miscellaneous	Glutathione S-transferase phi 8 (GSTF8)	3.00E-82
T2-L2	turquoise	Csa6G445040	3.81	6.53E-03	Not assigned	unknown	
T2-L2	turquoise	Csa6G382970	3.831	0.001156	Transport	Amino acid permease 3 (AAP3)	2.00E-234
T2-L2	turquoise	Csa1G039120	4.104	0.00004243	Development	Nascent polypeptide-associated complex NAC	4.00E-68
T2-L2	turquoise	Csa2G072490	4.767	5.874E-08	Protein amino acid activation	Glycyl-tRNA synthetase	0.00E+00

Appendix table 5.1 (Continued)

T2-L2	turquoise	Csa3G026690	4.954	0.006704	Lipid metabolism	Palmitoy (protein) hydrolase	5.00E-77
T2-L2	turquoise	Csa5G567800	5.13	0.00002257	Redox	Glutaredoxin	9.00E-41
T2-L2	turquoise	Csa2G070890	5.345	0.003945	Abiotic stress	DNAJ heat shock N-terminal domain-containing protein	2.00E-137
T2-L2	turquoise	Csa3G002850	5.719	0.00004243	Biotic stress	Thaumatin-like protein 1	2.00E-166
T2-L2	turquoise	Csa7G429630	9.428	8.95E-05	Not assigned	unknown	
T2-L2	turquoise	Csa6G008680	9.581	0.004781	Protein postranslational modification	CBL-interacting protein kinase 1 (CIPK1)	3.00E-169
T2-L2	turquoise	Csa5G470570	14.58	0.00536	Transcription factor	Response regulator 9 (ARR9)	7.00E-57
T2-L2	turquoise	Csa4G639890	15.851	0.00001712	Lipid metabolism	Triacylglycerol lipase like 1 (TLL1)	2.00E-131
T2-L2	turquoise	Csa7G009740	125.334	0.009597	Transcription factor	Cryptochrome-interacting basic-helix-loop-helix (CIB1)	1.00E-115
T2-L2	turquoise	Csa1G000010	1.357	0.029	Protein synthesis	60S acidic ribosomal protein family	5.00E-36
T2-L2	turquoise	Csa1G009690	1.458	3.16E-08	Protein synthesis	Ribosomal protein L34	1.00E-59
T2-L2	turquoise	Csa1G013160	1.322	0.01	Not assigned	Unknown	3.00E-42
T2-L2	turquoise	Csa1G013770	1.803	0	RNA binding	Glycine rich RNA-binding protein 5 (GR-RBP5)	6.00E-74
T2-L2	turquoise	Csa1G024170	1.522	3.55E-06	Protein synthesis	Ribosomal protein S7e family protein	2.00E-92
T2-L2	turquoise	Csa1G024830	2.253	7.29E-03	Protein postranslational modification	Receptor for activated C kinase 1B (RACK1B)	7.00E-174
T2-L2	turquoise	Csa1G031860	4.104	4.24E-05	Transcription factor	Basic transcription factor 3	4.00E-68
T2-L2	turquoise	Csa1G039190	1.338	4.89E-03	RNA binding	Glycine rich RNA-binding protein 5 (GR-RBP5)	3.00E-62
T2-L2	turquoise	Csa1G039250	1.567	2.71E-03	Not assigned	DUF1118	7.00E-70
T2-L2	turquoise	Csa1G042360	1.281	2.35E-03	Protein synthesis	Ribosomal protein S7e family protein	2.00E-91
T2-L2	turquoise	Csa1G042920	1.285	6.57E-03	Hormone metabolism	Jasmonate-zim-domain protein 3 (JAZ3)	2.00E-37
T2-L2	turquoise	Csa1G045540	1.3	6.55E-05	Not assigned	DUF1118	5.00E-67
T2-L2	turquoise	Csa1G050240	-1.256	0.029	Glycolysis	Glyceraldehyde-3-phosphate dehydrogenase (GAP-DH)	4.00E-189
T2-L2	turquoise	Csa1G050270	-1.733	4.47E-06	Not assigned	Unknown	1.00E-149
T2-L2	turquoise	Csa1G051810	1.38	0.012	Signaling	Calnexin 1 (CNX1)	4.00E-256
T2-L2	turquoise	Csa1G059750	2.634	0.024	Not assigned	Unknown	
T2-L2	turquoise	Csa1G063560	1.22	0.043	Abiotic stress	Heat shock protein 20 (HSP20)	1.00E-34
T2-L2	turquoise	Csa1G163140	-1.401	8.89E-03	Protein synthesis	Ribosomal protein L1p/L10e family	4.00E-111
T2-L2	turquoise	Csa1G168900	1.214	0.028	Protein synthesis	Ribosomal protein 5A	3.00E-100
T2-L2	turquoise	Csa1G479110	1.778	9.18E-03	Not assigned	Unknown	
T2-L2	turquoise	Csa1G570180	3.012	5.39E-05	Transport	Heavy metal transport/detoxification superfamily protein	1.00E-47
T2-L2	turquoise	Csa1G573590	1.361	3.54E-05	Protein synthesis	Ribosomal protein S25 family protein	7.00E-51

Appendix table 5.1 (Continued)

T2-L2	turquoise	Csa1G574950	1.58	0.014	Biotic stress	Receptor like protein 52	4.00E-80
T2-L2	turquoise	Csa1G575160	1.173	0.027	Transcription factor	SNF7 family protein	1.00E-105
T2-L2	turquoise	Csa1G586750	2.496	2.83E-05	Protein degradation	Metallopeptidase M24 family protein	3.00E-172
T2-L2	turquoise	Csa1G589720	2.792	7.37E-03	Protein postranslational modification	AGC (cAMP-dependent, cGMP-dependent and protein kinase C) kinase family protein	2.00E-235
T2-L2	turquoise	Csa1G604040	2.041	0.037	Redox	L-ascorbate oxidase precursor (ASO)	0
T2-L2	turquoise	Csa1G611290	1.417	0.014	Miscellaneous	Beta glucosidase 15 (BGLU15)	2.00E-187
T2-L2	turquoise	Csa1G629740	-1.618	1.21E-03	Cell wall	fasciclin-like arabinogalactan-protein precursor 17 (Fla17)	3.00E-199
T2-L2	turquoise	Csa2G000570	1.314	6.05E-03	Protein synthesis	Ribosomal L28e protein family	1.00E-65
T2-L2	turquoise	Csa2G000600	1.391	6.47E-04	Protein synthesis	Ribosomal protein L31e family protein	4.00E-59
T2-L2	turquoise	Csa2G000770	1.822	1.68E-04	Cell organization	Profilin 3 (PRF3)	3.00E-68
T2-L2	turquoise	Csa2G012710	1.521	3.62E-04	Transcription factor	NOP56-like pre RNA processing ribonucleoprotein	1.00E-290
T2-L2	turquoise	Csa2G013290	3.167	0.042	Lipid metabolism	Acyl carrier protein 4 (ACP4)	1.00E-37
T2-L2	turquoise	Csa2G033940	1.812	3.15E-05	Signaling	Calreticulin 1a (CRT1a)	4.00E-216
T2-L2	turquoise	Csa2G036680	-1.209	1.49E-03	Polyamine metabolism	S-adenosylmethionine decarboxylase proenzyme	7.00E-136
T2-L2	turquoise	Csa2G145880	-1.522	4.76E-03	Photosynthesis	Serine hydroxymethyltransferase 4 (SHM4)	2.00E-263
T2-L2	turquoise	Csa2G193310	1.498	6.85E-06	Protein synthesis	Ribosomal protein S15	1.00E-75
T2-L2	turquoise	Csa2G222050	1.332	1.62E-03	Not assigned	RmlC-like cupins superfamily protein	2.00E-88
T2-L2	turquoise	Csa2G285350	1.262	0.046	Protein synthesis	Translation protein SH3-like family protein	5.00E-71
T2-L2	turquoise	Csa2G302060	1.523	4.38E-07	Protein synthesis	Ribosomal protein L10 protein	9.00E-82
T2-L2	turquoise	Csa2G433350	-2.293	0.043	Secondary metabolism	4-coumarate-CoA ligase 2 (4CL2)	1.00E-187
T2-L2	turquoise	Csa3G002400	7.592	0.017	Lipid metabolism	AMP-dependent synthetase and ligase family protein	4.00E-137
T2-L2	turquoise	Csa3G002750	5.719	4.24E-05	Biotic stress	Thaumatin-like protein 1	2.00E-166
T2-L2	turquoise	Csa3G019340	4.954	6.70E-03	Lipid metabolism	Alpha/beta-hydrolases superfamily protein	5.00E-77
T2-L2	turquoise	Csa3G044520	-1.737	0.015	C1-metabolism	Ribonuclease E inhibitor RraA/Dimethylmenaquinone methyltransferase	2.00E-72
T2-L2	turquoise	Csa3G077610	1.426	3.95E-03	Protein synthesis	Translation initiation factor SUI family protein	9.00E-54
T2-L2	turquoise	Csa3G117960	1.532	9.68E-12	Development	Squamosa promoter binding protein-like 10 (SPL10)	1.00E-95
T2-L2	turquoise	Csa3G118140	1.413	9.80E-05	Protein synthesis	Ribosomal protein S6	7.00E-134
T2-L2	turquoise	Csa3G122460	-6.667	5.69E-04	Not assigned	Unknown	5.00E-18
T2-L2	turquoise	Csa3G134910	1.136	6.18E-03	Not assigned	Unknown	3.00E-46
T2-L2	turquoise	Csa3G134930	1.756	1.64E-04	Protein synthesis	Ribosomal protein L14	4.00E-63
T2-L2	turquoise	Csa3G135010	1.319	0.028	Protein synthesis	Ribosomal protein L30/L7 family protein	6.00E-125

Appendix table 5.1 (Continued)

T2-L2	turquoise	Csa3G149910	-2.523	0.033	Not assigned	DUF679	6.00E-20
T2-L2	turquoise	Csa3G152090	1.515	5.67E-05	Redox	Thioredoxin superfamily protein	2.00E-39
T2-L2	turquoise	Csa3G183920	-1.907	1.38E-12	Secondary metabolism	Beta-hydroxylase 1 (BETA-OHASE1)	4.00E-161
T2-L2	turquoise	Csa3G214020	-1.725	6.57E-03	Not assigned	Maternal effect embryo arrest 9 (MEE9)	1.00E-56
T2-L2	turquoise	Csa3G403980	1.33	6.47E-03	RNA binding	RNA-binding (RRM/RBD/RNP motifs) family protein	7.00E-46
T2-L2	turquoise	Csa3G483830	-1.282	7.34E-04	Photosynthesis	Photosystem I subunit H-1	6.00E-67
T2-L2	turquoise	Csa3G639040	1.71	0.016	Protein synthesis	Stress response suppressor 1 (STRS1)	1.00E-213
T2-L2	turquoise	Csa3G653380	1.422	5.67E-08	Protein synthesis	Zinc-binding ribosomal protein family protein	2.00E-57
T2-L2	turquoise	Csa3G653460	1.642	1.62E-03	Protein synthesis	Nucleolin-like 2 (NUC-L2)	1.00E-162
T2-L2	turquoise	Csa3G698560	1.52	5.30E-04	Protein synthesis	Ribosomal protein L6 family protein	3.00E-111
T2-L2	turquoise	Csa3G710180	2.47	0.02	Not assigned	Unknown	1.00E-37
T2-L2	turquoise	Csa3G733320	2.068	4.83E-03	Biodegradation of xenobiotic	Glyoxal oxidase-related protein	1.00E-258
T2-L2	turquoise	Csa3G748210	1.46	9.83E-09	Protein synthesis	Ribosomal protein L24e family protein	3.00E-82
T2-L2	turquoise	Csa3G776970	1.638	2.18E-04	TCA	Alpha carbonic anhydrase 1 (CAH1)	4.00E-81
T2-L2	turquoise	Csa3G855430	1.847	0.019	Miscellaneous	O-Glycosyl hydrolases family 17 protein	1.00E-162
T2-L2	turquoise	Csa3G895680	3.397	0.026	Transcription factor	Apetala2/Ethylene-responsive element binding protein family	2.00E-56
T2-L2	turquoise	Csa3G914560	1.451	1.63E-03	RNA binding	Hyaluronan/mRNA binding family	2.00E-128
T2-L2	turquoise	Csa4G004870	1.754	4.35E-04	Not assigned	Unknown	8.00E-34
T2-L2	turquoise	Csa4G025120	-1.865	0.027	Not assigned	Unknown	3.00E-50
T2-L2	turquoise	Csa4G052080	3.146	0.035	Not assigned	Unknown	1.00E-08
T2-L2	turquoise	Csa4G091870	3.269	1.43E-04	Secondary metabolism	2-oxoglutarase (2OG) and Fe(II)-dependent oxygenase superfamily protein	1.00E-144
T2-L2	turquoise	Csa4G152270	-4.114	1.13E-06	Not assigned	Unknown	
T2-L2	turquoise	Csa4G269770	2.17	0.047	Protein postranslational modification	Protein kinase 2B (APK2B)	1.00E-153
T2-L2	turquoise	Csa4G290160	1.607	0.011	Protein postranslational modification	Receptor-like protein kinase 1 (RLK1)	4.00E-27
T2-L2	turquoise	Csa4G297420	2.307	0.037	Not assigned	Cysteine/Histidine-rich C1 domain family protein	4.00E-47
T2-L2	turquoise	Csa4G307390	-2.045	0.022	Cell organization	Tubulin beta chain 3	5.00E-256
T2-L2	turquoise	Csa4G333620	-2.238	1.75E-03	Lipid metabolism	Lipid-transfer protein 6 (LTP6)	2.00E-29
T2-L2	turquoise	Csa4G431960	3.195	0	Protein targeting	Sigma factor binding protein 1 (SIB1)	2.00E-14

Appendix table 5.1 (Continued)

T2-L2	turquoise	Csa4G641690	1.538	0.01	Transcription factor	MYB domain protein 77 (MYB77)	1.00E-72
T2-L2	turquoise	Csa4G651970	1.399	3.03E-08	Protein synthesis	Eukaryotic translation initiation factor 5A-2 (eIF-5A-2)	2.00E-85
T2-L2	turquoise	Csa5G097970	3.156	6.39E-05	Not assigned	Unknown	5.00E-12
T2-L2	turquoise	Csa5G153020	1.177	0.016	Transport	Plasma membrane intrinsic protein 1;4 (PIP1;4)	2.00E-147
T2-L2	turquoise	Csa5G160150	-2.457	1.80E-06	Miscellaneous	Plant invertase/pectin methylesterase inhibitor superfamily protein	1.00E-102
T2-L2	turquoise	Csa5G168920	2.675	0.026	Signaling	Leucine-rich repeat receptor -like protein kinase family protein	3.00E-93
T2-L2	turquoise	Csa5G171160	1.599	1.44E-04	Protein synthesis	Ribosomal protein S10p/S20e family protein	2.00E-59
T2-L2	turquoise	Csa5G174560	-1.419	9.38E-06	Abiotic stress	BCL-2-associated athaogene 3 (BAG3)	3.00E-93
T2-L2	turquoise	Csa5G180850	1.312	0.05	Protein synthesis	Ribosomal protein L23AA	6.00E-73
T2-L2	turquoise	Csa5G182730	-1.5	5.58E-08	Photosynthesis	RuBisCO activase (RA)	1.00E-204
T2-L2	turquoise	Csa5G320430	-2.045	3.44E-07	Secondary metabolism	Phytoene synthase (PSY)	5.00E-232
T2-L2	turquoise	Csa5G409620	1.474	2.76E-05	Protein synthesis	Ribosomal protein L7Ae/L30e/S12e/Gadd45 family protein	9.00E-54
T2-L2	turquoise	Csa5G434550	14.58	5.36E-03	Transcription factor	Response regulator 3	7.00E-57
T2-L2	turquoise	Csa5G523190	5.13	2.26E-05	Redox	Glutaredoxin family protein	9.00E-41
T2-L2	turquoise	Csa5G606660	1.869	0.037	Miscellaneous	Carbohydrate-binding X8 domain superfamily protein	9.00E-53
T2-L2	turquoise	Csa5G606810	2.66	0.024	Not assigned	Unknown	3.00E-06
T2-L2	turquoise	Csa5G610410	3.177	2.18E-03	Not assigned	Unknown	1.00E-08
T2-L2	turquoise	Csa5G638420	1.444	6.70E-03	Protein synthesis	Ribosomal protein S19e family protein	3.00E-73
T2-L2	turquoise	Csa5G645120	1.554	2.58E-04	Protein synthesis	Ribosomal protein L16p/L10e family protein	2.00E-74
T2-L2	turquoise	Csa5G649850	1.378	6.08E-03	Protein synthesis	Ribosomal protein S4	4.00E-106
T2-L2	turquoise	Csa5G652280	1.316	0.029	Transcription factor	MYB domain protein 6 (MYB6)	4.00E-84
T2-L2	turquoise	Csa6G001220	9.581	4.78E-03	Protein postranslational modification	CBL-interacting protein kinase 1 (CIPK1)	3.00E-169
T2-L2	turquoise	Csa6G014790	1.439	2.35E-04	Protein synthesis	R-protein L3B (RPL3B)	5.00E-214
T2-L2	turquoise	Csa6G039540	1.562	6.15E-04	Transcription factor	Constant-like zinc finger family	2.00E-186
T2-L2	turquoise	Csa6G055370	1.611	1.54E-06	Protein synthesis	Ribosomal protein S18C	2.00E-82
T2-L2	turquoise	Csa6G077430	1.669	2.33E-03	Protein synthesis	Ribosomal protein S13	1.00E-78
T2-L2	turquoise	Csa6G087710	1.582	1.49E-06	Not assigned	Ribosome associated membrane RAMP4	1.00E-26
T2-L2	turquoise	Csa6G091290	1.404	7.56E-03	Protein synthesis	Ribosomal protein S27	7.00E-45
T2-L2	turquoise	Csa6G108510	-1.652	7.76E-03	Secondary metabolism	Flavanone 3-hydroxylase (F3H)	4.00E-118
T2-L2	turquoise	Csa6G133770	2.243	1.52E-07	Hormone metabolism	Cytokinin response factor 4 (CRF4)	9.00E-38
T2-L2	turquoise	Csa6G151720	1.43	2.74E-09	Protein synthesis	60S acidic ribosomal protein family	

Appendix table 5.1 (Continued)

T2-L2	turquoise	Csa6G181560	-2.765	8.71E-05	Miscellaneous	UDP-Glycosyltransferase superfamily protein	6.00E-145
T2-L2	turquoise	Csa6G301020	-1.797	8.01E-05	Signaling	Phototropin 2 (PHOT2)	0
T2-L2	turquoise	Csa6G365120	1.251	1.62E-03	Not assigned	Unknown	5.00E-45
T2-L2	turquoise	Csa6G405880	1.523	7.68E-04	Protein degradation	Ubiquitin-like protein 5 (UBL5)	2.00E-35
T2-L2	turquoise	Csa6G507370	-1.596	8.96E-03	Development	Senescence 1 (SEN1)	2.00E-80
T2-L2	turquoise	Csa6G519680	1.421	2.95E-06	Protein synthesis	Ribosomal protein L16B	8.00E-96
T2-L2	turquoise	Csa6G538630	1.859	0.015	Co-factor and vitamin metabolism	Molybdenum cofactor sulfurase family protein	2.00E-128
T2-L2	turquoise	Csa7G000520	125.334	9.60E-03	Transcription factor	Cryptochrome-interacting basic-helix-loop-helix (CIB1)	1.00E-115
T2-L2	turquoise	Csa7G027790	-1.87	9.28E-04	Protein degradation	Xylem cysteine peptidase 1 (XCP1)	1.00E-162
T2-L2	turquoise	Csa7G108300	-1.647	9.38E-06	Cell wall	Fasciclin-like arabinogalactan-protein 1 (FLA1)	2.00E-153
T2-L2	turquoise	Csa7G267900	-1.375	6.08E-03	Photosynthesis	Photosystem II 22kDa protein chloroplast precursor (CP22)	3.00E-110
T2-L2	turquoise	Csa7G397560	1.605	7.19E-06	Protein synthesis	Ribosomal L22e protein family	2.00E-58
T2-L2	turquoise	Csa7G414420	9.428	8.95E-05	Not assigned	Unknown	
T2-L2	turquoise	Csa7G432010	-2.276	9.15E-03	Secondary metabolism	Cinnamoyl CoA reductase	4.00E-168
T2-L2	turquoise	Csa7G451340	2.619	1.72E-06	Miscellaneous	Nitrile lyases, berberine bridge enzymes, reticuline oxidases, troponine reductases	0
T2-L2	yellow	Csa5G011650	-71.384	1.63E-03	Biotic stress	ADR1-like 1 (ADR1-L1)	8.00E-257
T2-L2	yellow	Csa5G603370	-4.312	2.12E-03	Cell wall	RXF12	1.00E-144
T2-L2	yellow	Csa3G859730	-3.412	9.15E-03	Miscellaneous	Cytochrome P450, family 82, subfamily C, polypeptide 4 (CYP82C4)	7.00E-83
T2-L2	yellow	Csa1G033120	-3.352	4.23E-03	Transcription factor	Aspartyl protease family protein	6.00E-130
T2-L2	yellow	Csa6G312550	-2.678	1.24E-03	Secondary metabolism	Phenylalanine ammonia-lyase 1 (PAL2)	9.00E-30
T2-L2	yellow	Csa3G300600	-1.888	2.47E-04	Miscellaneous	Plant invertase/pectin methylesterase inhibitor superfamily protein	4.00E-52
T2-L2	yellow	Csa5G623670	-1.447	0.018	not assigned	unknown	
T2-L2	yellow	Csa2G028500	1.232	7.93E-03	Hormone metabolism	Lipoxygenase 1 (LOX1)	0.00E+00
T2-L2	yellow	Csa6G324880	1.335	0.0001866	Protein targeting	Importing alpha isoform 2	2.00E-281
T2-L2	yellow	Csa7G398150	1.351	0.003881	Cell wall	Expansin B3 (EXPB3)	2.00E-117
T2-L2	yellow	Csa4G292970	1.368	4.84E-03	Not assigned	unknown	
T2-L2	yellow	Csa6G445150	1.382	7.92E-04	Not assigned	unknown	
T2-L2	yellow	Csa7G047420	1.409	0.031	cell wall	fasciclin-like arabinogalactan-protein 6 (Fla6)	7.00E-49
T2-L2	yellow	Csa6G007450	1.436	0.003383	Cell wall	MYB domain protein 6 (MYB6)	4.00E-80
T2-L2	yellow	Csa3G643770	1.534	0.001616	Transport	Lysine histidine transporter 2 (LHT2)	5.00E-213

Appendix table 5.1 (Continued)

T2-L2	yellow	Csa5G609110	1.732	0.004961	Hormone metabolism	IAA-Alanine resistant 3 (IAR3)	2.00E-173
T2-L2	yellow	Csa1G590300	1.827	1.18E-05	Major CHO metabolism	Hexokinase 2 (HXK2)	7.00E-51
T2-L2	yellow	Csa6G121970	1.841	0.02	hormone metabolism	EXORDIUM like 5 (EXL5)	4.00E-159
T2-L2	yellow	Csa6G495000	1.86	1.50E-05	Not assigned	unknown	
T2-L2	yellow	Csa3G002330	2.238	0.007581	Transcription factor	MYB domain protein 73 (MYB73)	1.00E-88
T2-L2	yellow	Csa3G481240	2.483	0.000002215	Cell wall	Maternal effect embryo arrest 31 (MEE31)	4.00E-159
T2-L2	yellow	Csa1G003490	2.619	0.000001719	Miscellaneous	nitrile lyases, berberine bridge enzymes, reticuline oxidases, troponine reductases	0.00E+00
T2-L2	yellow	Csa4G000030	2.908	0.00547	Hormone metabolism	S-adenosyl-L-methionine: jasmonic acid carboxyl methyltransferase	0
T2-L2	yellow	Csa3G850600	2.992	2.07E-03	Not assigned	unknown	
T2-L2	yellow	Csa1G066560	3.395	0.0001601	Signaling	Cystine-rich RLK 29 (CRK29)	8.00E-79
T2-L2	yellow	Csa2G028490	3.4	0.0002165	Protein degradation	Cystein proteinases superfamily protein	2.00E-168
T2-L2	yellow	Csa3G665100	6.455	2.944E-09	Secondary metabolism	Transparent testa 4 (TT7)	9.00E-146
T2-L2	yellow	Csa1G073810	6.575	0.002475	Secondary metabolism	Terpene synthase 14 (TPS14)	2.00E-144
T2-L2	yellow	Csa1G596520	6.671	1.497E-07	Glycolysis	Phenylalanine ammonia-lyase 2 (PAL2)	0.00E+00
T2-L2	yellow	Csa3G852600	8.469	0	Miscellaneous	Cytochrome P450 family 82 subfamily C polypeptide 4 (CYP82C4)	8.00E-145
T2-L2	yellow	Csa2G033340	9.201	6.89E-03	Not assigned	unknown	
T2-L2	yellow	Csa6G127320	9.393	0.000009377	Secondary metabolism	MYB domain protein 10 (MYB10)	7.00E-51
T2-L2	yellow	Csa1G066570	11.211	4.726E-08	Secondary metabolism	Terpene synthase 14 (TPS14)	2.00E-133
T2-L2	yellow	Csa3G589590	12.087	1.93E-05	Not assigned	unknown	
T2-L2	yellow	Csa6G057160	15.701	0.00008718	Hormone metabolism	UDP-glucosyltransferase 75B1 (UGT75B1)	3.00E-128
T2-L2	yellow	Csa1G068570	26.653	0.001096	Secondary metabolism	Terpene synthase 14 (TPS14)	8.00E-12
T2-L2	yellow	Csa1G006320	2.533	2.29E-05	Biotic stress	Enhanced disease susceptibility 1 (EDS1)	2.00E-159
T2-L2	yellow	Csa1G022490	-3.352	4.23E-03	Transcription factor	Aspartyl protease family protein	3.00E-135

Appendix table 5.1 (Continued)

T2-L2	yellow	Csa1G064830	3.424	0.029	Signaling	Cystein-rich receptor like kinase 10 (CRK10)	
T2-L2	yellow	Csa1G065930	3.395	1.60E-04	Signaling	Cystein-rich receptor like kinase 29 (CRK29)	
T2-L2	yellow	Csa1G085390	1.609	5.43E-03	Transcription factor	C2H2-like zinc finger protein	
T2-L2	yellow	Csa1G574970	1.827	1.18E-05	Major CHO metabolism	Hexokinase 2 (HXK2)	
T2-L2	yellow	Csa2G351740	1.399	0.013	Transcription factor	MYB-like transcription factor family protein	8.00E-51
T2-L2	yellow	Csa2G369060	1.582	6.50E-03	Protein synthesis	Ribosomal protein S6	5.00E-133
T2-L2	yellow	Csa2G369220	3.723	0.04	Not assigned	ENTH/ANTH/VHS superfamily protein	1.00E-72
T2-L2	yellow	Csa2G427310	2.238	7.58E-03	Transcription factor	MYB domain protein 73 (MYB73)	1.00E-88
T2-L2	yellow	Csa3G016990	1.432	6.40E-03	Development	DUF581	7.00E-26
T2-L2	yellow	Csa3G166350	-3.315	0.017	Not assigned	Unknown	2.00E-116
T2-L2	yellow	Csa3G176260	-1.888	2.47E-04	Miscellaneous	Plant invertase/pectin methylesterase inhibitor superfamily protein	5.00E-63
T2-L2	yellow	Csa3G852590	8.469	0	Miscellaneous	Cytochrome P450 family 82 subfamily C polypeptide 4 (CYP82C4)	8.00E-145
T2-L2	yellow	Csa4G056640	1.53	0.011	Hormone metabolism	Nine-cis-epoxycarotenoid dioxygenase 4 (NCED4)	1.00E-249
T2-L2	yellow	Csa4G280660	1.368	4.84E-03	Not assigned	Unknown	3.00E-31
T2-L2	yellow	Csa4G638480	-71.384	1.63E-03	Biotic stress	Apoptosis defense response1-like 1 (ADR1-L1)	3.00E-292
T2-L2	yellow	Csa5G097460	-1.366	1.15E-06	Biotic stress	P-loop containing nucleotide triphosphate hydrolases superfamily protein	6.00E-82
T2-L2	yellow	Csa5G146260	3.213	0.02	Protein degradation	RING/U-box superfamily protein	8.00E-40
T2-L2	yellow	Csa5G240140	-4.312	2.12E-03	Cell wall	Glycosyl hydrolase superfamily protein	0
T2-L2	yellow	Csa5G651640	1.436	3.38E-03	Transcription factor	MYB domain protein 6 (MYB6)	4.00E-80
T2-L2	yellow	Csa6G147460	-2.678	1.24E-03	Secondary metabolism	Phenylalanine lyase 4 (PAL4)	0
T2-L2	yellow	Csa6G365170	1.397	2.67E-03	Protein synthesis	Ribosomal protein L22p/L17e family protein	2.00E-93
T2-L2	yellow	Csa6G398200	1.382	7.92E-04	Not assigned	Unknown	9.00E-102
T2-L2	yellow	Csa6G445170	1.436	0.012	Protein synthesis	Ribosomal protein L11 family protein	5.00E-85
T2-L2	yellow	Csa6G493850	1.86	1.50E-05	Not assigned	Unknown	9.00E-136

CONCLUSION

Seed encapsulation using gelatin capsules is a novel approach to seed treatment but the effect of gelatin on crop growth was widely undetermined. This project aimed to characterize the effect of gelatin capsule on selected crop seeds, determine the effect of different types of gelatin commercially available, determine the effect of gelatin capsules on crops under abiotic stress (salt stress) and to characterize the effect of gelatin capsules on cucumber growth and development at the transcriptome level using whole genome expression profiling.

Enhanced plant growth was measured in the aboveground portions of plants with the application of gelatin capsules applied at time of sowing adjacent to seeds. Crops tested (cucumber, tomato, broccoli, corn, arugula, pepper) showed increased plant growth as measured by leaf area, fresh and dry weight. The magnitude of plant growth enhancement was crop specific, and cucumber was used as the model crop for all further studies. Plants treated with gelatin capsules exhibited increased nitrogen content, and increased salinity tolerance compared to the non-treated control. Different types of hydrolyzed collagen, including granulated gelatin, gelatin hydrolysate, and amino acid mixtures containing amino acids present in gelatin were compared and revealed that granulated gelatin treatment had the greatest plant growth compared with other treatments. Plants were treated with two gelatin capsules and equivalent amount of nitrogen in the form of urea revealed that increased plant growth from the application of gelatin capsule was not solely due to the nitrogen.

The results from the studies indicate that gelatin capsules and other gelatin products may be used in greenhouse or transplant production. The gelatin treatment would specifically benefit crops in which the leaves were consumed as all six crops examined had increased leaf area. The effect of gelatin capsules on crop yield was not studied and field investigations are warranted. The effect of the gelatin capsules on the nutritional content of the crop would also be of interest for future study, since only nitrogen was measured in this study. A more in depth study should be examined on fate of nitrogen from the applied gelatin treatment through plant growth and development. In particular, measuring the leachate from the pot to determine if leaching of

nitrogen from applied urea occurred resulting in less growth than an equivalent amount of nitrogen from gelatin capsule treated plants. A biochemical approach measuring the activity of nitrogen assimilation enzymes would help determine if gelatin capsules affects nitrogen assimilation enzymes activity as reported for other protein hydrolysates.

The RNA-sequencing results provided some insights on the mechanisms of the growth promotion from the gelatin capsule treatments. Genes were upregulated from the gelatin treatment involved in nitrogen transport including ammonium transporters and amino acid transporters, and MYB and WRKY family transcription factors that regulate diverse pathways including abiotic stress tolerance and other responses were upregulated in plants with gelatin treatment. Genes involved in detoxification such as Glutathione S-transferase exhibited a high positive correlation with increased leaf area and nitrogen content in plants treated with gelatin.

A more detailed study is required to determine if the genes identified by the RNA-sequencing, such as amino acid transporters, ammonium transporter, are actually responsible for increased nitrogen content and leaf area of plants treated with gelatin. Amino acid transporter mutants, as well as ammonium transporter mutants would be powerful tools to study the involvement of the amino acid and ammonium transporters in the gelatin capsule treated plants.