MITIGATION OF DROUGHT STRESS IN TOMATO WITH *ASCOPHYLLUM NODOSUM (L.) LE JOL.* EXTRACTS

by

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To

My beloved parents

My lovely wife Nileeka

My sister Indunee

I couldn't have done this without your love & care

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Abstract

Growth and development of a plant can be affected due to various biotic and abiotic stresses such as water, salinity, frost and pathogens. Application of seaweeds and their extracts to mitigate plant stresses in agriculture is one of the promising strategies. However, there is less evidence associated with seaweed extract application on drought stress management. In the current study, investigate the effect of brown alga *Ascophyllum nodosum* (L.) Le Jolis. extract (ANE) to mitigate drought stress in tomato plants using physiological, biochemical and molecular biological aspects. The results indicated a rapid recovery (85% compared to 30-40% in controls) of ANE treated plants. It also evident high stomatal conductance , high plant water potential and less wilting in ANE treated plants over controls. Moreover, ANE treatments helped in lowering antioxidant enzyme activities, low lipid peroxidation, low proline accumulation and the lower expression of stress responsive genes under the stress. In conclusion, it is evident that ANE treated plants perform well and recover well under drought stress compare to control plants.

List of Abbreviations and Symbols Used

ABA	Abscisic acid
ACT	Actin
ANOVA	Analysis of variance
ANE	*Ascophyllum nodosum* extract
APX	Ascorbate peroxidase
β	Beta
BSA	Bovine serum albumin
CO_2	Carbon dioxide
CAT	Catalase
°C	Celsius
CAO	Chlorophyll *a* oxygenase
cDNA	Complimentary deoxyribonucleic acid
CAM	Crassulacean-acid-metabolism
m^3/m^3	Cubic meter per cubic meter
P5CS	*Delta-pyrroline-5-carboxylate synthetase*
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
FBPase	Fructose-1,6-bisphosphatase
GR	Glutathione reductase
g	Grams or gravitational acceleration
g/L	Grams per liter
GPOD	Guaiacol Peroxidase
h	Hours
H_2O_2	Hydrogen peroxide
$OH^{\cdot}$	Hydroxyl radicals
LEA	*Late embryogenesis abundant* genes
LP	Lipid peroxidation
L	Liters
LANS	Long-Ashton nutrient solution
ltpg2	*Lpiid transfer protein 2*
LeMCA1	*lycopersicon esculentum metacaspase 1*
MDA	Malondialdehyde
MPa	Mega Pascals
µL	Micro liters
µmol	Micro moles
mg/mL	Milli grams per milli liter
mL	Milli liters

mM	Milli molar
min	Minutes
MAP	Mitogen-activated protein
M	Molar
MS	Murashige and Skoog media
NADP-ME	NADP-malic enzyme
nm	Nano meters
P	P value
mM^{-1} cm^{-1}	Per milli molar per centimeter
%	Percentage
PEPCase	Phosphoenolpyruvate carboxylase
PSII	Photosystem II
PEG	Polyethylene glycol
PCR	Polymerase chain reaction
PUFA	Polyunsaturated fatty acids
PVP	Polyvinylpyrrolidone
PPDK	Pyruvate orthophosphate dikinase
ROS	Reactive oxygen species
RWC	Relative water content
RNA	Ribonucleic acid
Rubisco	Ribulose-1,5-bisphosphate carboxylase/oxygenase
s	Seconds
SlAREB-1	*solanum lycopersicum ABA-response element binding protein 1*
SEM	standard errors of the mean
SOD	Superoxide dismutase
O_2^-	Superoxide radicals
UAE	Ultrasound acoustic emissions
VPD	Vapor pressure difference
v/v	Volume by volume
w/v	Weight by volume

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CHAPTER 1

INTRODUCTION

Agriculture is indispensable for food security. The human population is expected to reach nine billion by the year 2050 (www.un.org, www.fao.org) and, therefore, food production has to increase by 70% to feed the population (www.fao.org). However, this increase in food production has to be achieved with the existing, or most likely, reduced land area. A number of factors, such as variety, management practices, pests and diseases as well as environmental conditions, affect crop yield.

Unfavorable conditions can be considered as a stress. Stress can be categorized as biotic or abiotic stress: i) pests, including insects and pathogens such as bacteria, virus and fungal infections, are considered as biotic stresses; ii) abiotic stresses include drought, salinity, flood, chill, oxidative stress and chemical toxicity; these stresses can potentially reduce crop yields by more than 50% (Bray *et al*., 2000; Wang *et al*., 2003). Plants undergo physiological, biochemical and genetic changes when subjected to stress that are detrimental to productivity (Plant *et al*., 1991; Bray, 2002; Rizhsky *et al*., 2002; Bray, 2004).

Water is the most important input for plants' growth and development. Rainfall and irrigation are the two main sources of water in agriculture. Rainfed crops contribute to 65% of world food production and the remaining 35% of food is produced from irrigation agriculture. Only 17% of total cultivated areas are irrigated (Smith, 2000). Thus, most of the land under cultivation depends on natural precipitation. In recent years, there has been a major shift in global rainfall pattern leading to unprecedented drought in many crop production areas of the world.

Water scarcity creates stress inside plants. Depending on the severity of drought stress, plants' responses vary from reduced growth, temporary wilting to death. Drought stress effects vary from species to species depending on their morphology and physiological adaptations. For example, cactus thrives in arid conditions and their morphological adaptations, like the presence of thick cuticle and thorns minimize water loss. In contrast, the majority of crop plants lack physiological adaptations to reduced water availability (Guillen *et al.,* 2013; Nawaz *et al.,* 2013).

There are currently several approaches that potentially reduce the impact of drought stress on crop production, such as cultivation of drought tolerant varieties, rain water harvesting, adopting agronomic practices like mulching, efficient irrigation systems like drip irrigation, sprinkler irrigation. However, none of these approaches are fully successful and needs to come up with a novel solution to avoid reduced water supplies.

Use of organic inputs to mitigate plant stresses is being intensively explored. Application of seaweed extracts is one of the promising approaches among the organic inputs that have been found so far. Seaweeds have been used for centuries as a fertilizer, stress tolerant enhancer or because of their plant growth regulatory properties (Temple and Bomke, 1989; Crouch and Van Staden, 1992; Rayirath *et al.*, 2009; Fan *et al.*, 2011; Wally *et al.,* 2013).

Ascophyllum nodosum (L.) Le Jol. is one of the widely used seaweeds in agriculture. It is a brown seaweed and is limited to the North Atlantic Ocean (Ugarte and Sharp, 2001). *A. nodosum* is frequently applied in agriculture, especially in the form of extracts. In previous studies, *A. nodosum* extracts (ANE) were shown to enhance freezing and salinity tolerance in plants (Rayirath *et al.*, 2009, Jithesh *et al.,* 2012).

The current study is focused on the potential use of ANE to mitigate drought stress. The main objective of the study was to understand physiological, biochemical and molecular mechanisms of ANE mediated drought stress tolerance in tomatoes (*Solanum lycopersicum* L.).

CHAPTER 2

LITERATURE REVIEW

Plant growth and development is affected by a number of biotic and abiotic factors. Water stress is one of the major abiotic factors that negatively affect crop production. Drought stress is the main form of water stress; however, water stress can result from other stresses such as salinity and freezing (Rayirath *et al.,* 2009; Pan *et al.,* 2012).

Rainfall is the main source of water for plant growth and unpredictable weather patterns and improper water use in agriculture are the main causes for drought (Smith, 2000). Drought impacts all stages of plant growth. During germination, drought stress delays the germination process and at extreme water deficit, germination ceases (Blum, 1996). At the vegetative stage, it reduces plant vigor and growth (Boutraa and Sanders, 2001). In the early reproductive stage, drought affects fertilization, leading to reduction in seed set and at a later stage, it affects seed filling and hence, reduced yield (Garrity and O'Toole, 1995; Sheoran and Saini, 1995; Boutraa and Sanders, 2001). Drought stress also reduces the quality and economic value of the crop.

Besides affecting plant growth and productivity, drought stress also causes secondary stresses like oxidative stress, which in turn leads to denaturation of functional and structural proteins (Wang *et al*., 2003). Some plants like corn tend to produce toxic chemicals, such as nitrates under water deficiency, which are lethal to livestock (Livingston *et al*., 1995).

To mitigate drought stress, it is important to know the changes that happen inside the plants during drought stress. The changes vary from morphology to the expression of

genes which includes stomatal response, metabolic adjustments, changes in photosynthesis (Figure 2.1). It is better to consider all aspects separately to get a clear picture on how to mitigate drought stress.

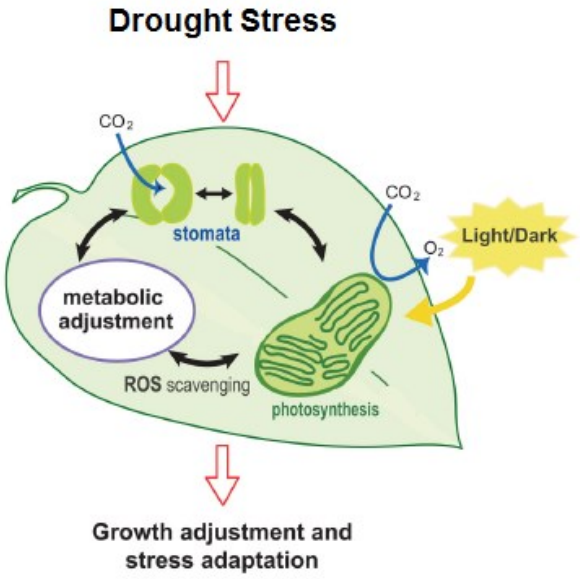

Figure 2.1. Schematic diagram representing major changes inside a plant under drought stress (Adapted from Osakabe *et al*., 2014).

2.1 Plant water relations and mechanisms of drought tolerance

Water movement through a plant is a passive process, where it is driven by water potential differences between the soil, plant and atmosphere and the hydraulic conductivities between each component (Lobet *et al*., 2014). Water moves from high water potential to a lower potential. Plants absorb water from soil through roots and absorbed water moves to xylem vessels through radial water movement. After entering to xylem vessels, water moves from roots to leaves through the xylem and release to atmosphere as water vapor through stomata (Figure 2.2).

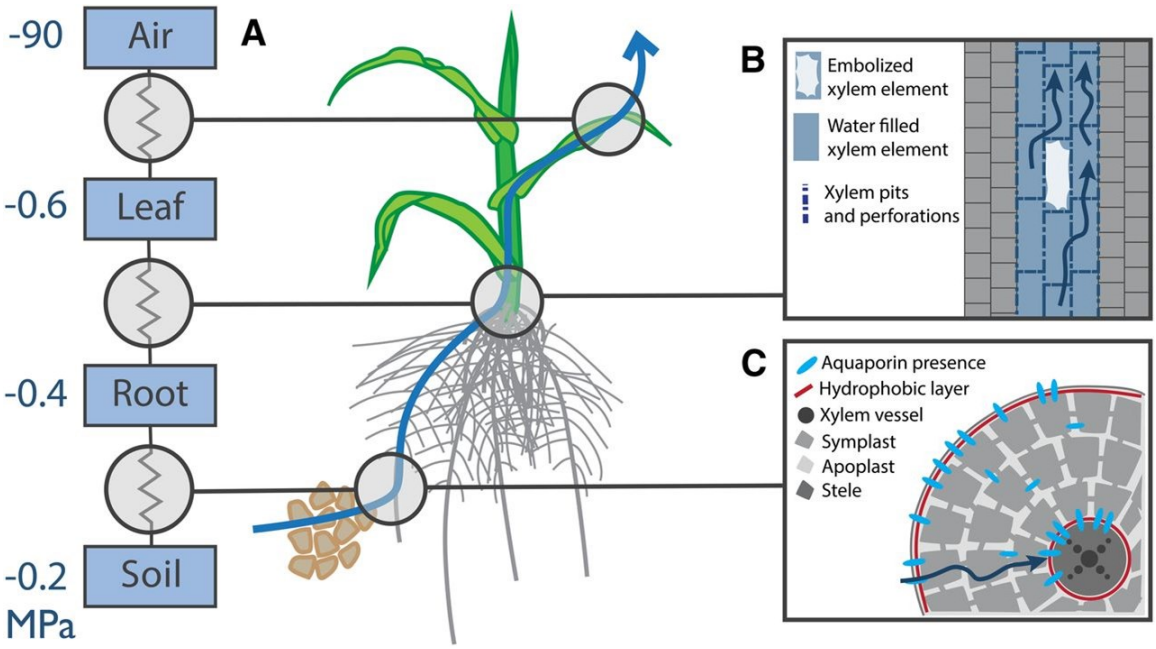

Figure 2.2. A schematic diagram showing water movement through a plant (Adapted from Lobet *et al*., 2014). A. Water movement inside the plant. B. Axial water movement in xylem vessels. C. Radial water movement in roots.

Plants prefer adequate water supply for functioning. However, under drought conditions, create high water pressure difference between soil and plant roots initially and it continues towards canopy with the progression of the stress. This is not favorable to its normal functioning. Plants have different mechanisms to overcome drought stress and can involve either tolerance or avoidance. Tolerance describes those physiological and biochemical adaptations that allow plants to survive under drought stress. Avoidance is concerned with maintaining a favorable water status in the plant by adopting different physiological and biochemical processes (Malinowski and Belesky, 2000).

Normally, plants are grouped into three categories depending on water use; hydrophytes, mesophytes and xerophytes. Hydrophytes are the plants which grow under adequate supply of water and xerophytes grow under water scarcity. Mesophytes are

adapted to neither high water supplied conditions not the arid conditions. Most of the plants belong to mesophytes. (Seddon, 1974; Runhaar *et al*,. 1997; Scremin-Dias *et al*., 2011). Xerophytes have different mechanisms to avoid drought stress, such as longer root systems, follow crassulacean-acid-metabolism (CAM) pathways and leaf architecture to minimize transpiration. Mesophytes also have adaptive mechanisms to avoid drought stress. Some mesophytes grow in the night to avoid stress. Furthermore, mesophytes adapt mechanisms, such as promote root growth and inhibit shoot growth, reduce respiration, translocation of assimilates and keep very low CO_2 assimilation to almost zero, under drought stress (Plant *et al*., 1991; Khan *et al*., 1993).

Different parts of a plant respond differently to water deficit. Leaves have different strategies when they are under drought stress. Leaf rolling, leaf shedding or low stomatal conductance are the main responses of the leaf to drought stress (Morgan, 1984, Hu *et al*, 2006). Stomatal closure helps to minimize transpiration. Root growth increase with drought stress. Accumulation and translocation of assimilates, maintaining cell wall elasticity and osmotic adjustment are some of the other drought stress tolerance mechanisms exhibited by plants (Malinowski and Belesky, 2000).

2.2 Plant Anatomy and Physiological Functions

Drought stress affects plant morphology, anatomy and physiology. These changes can be either visible or measurable and spread from canopy to root, depending on the intensity of the stress. Almost all the changes are unfavorable to plants and damage can be different depending on the intensity, duration of drought stress and the plant species.

2.2.1 Canopy, roots and vascular bundle

Stomatal closure is the initial response from a plant to drought stress (Osakabe *et al*., 2014). Stomatal closure stimulated by the turger pressure change in guard cells due to low water supply. This is induce by the secretion of abscisic acid where it can activate different signalling molecules to trigger stress tolerance through activation of stress responsive genes in the system (Figure 2.3)

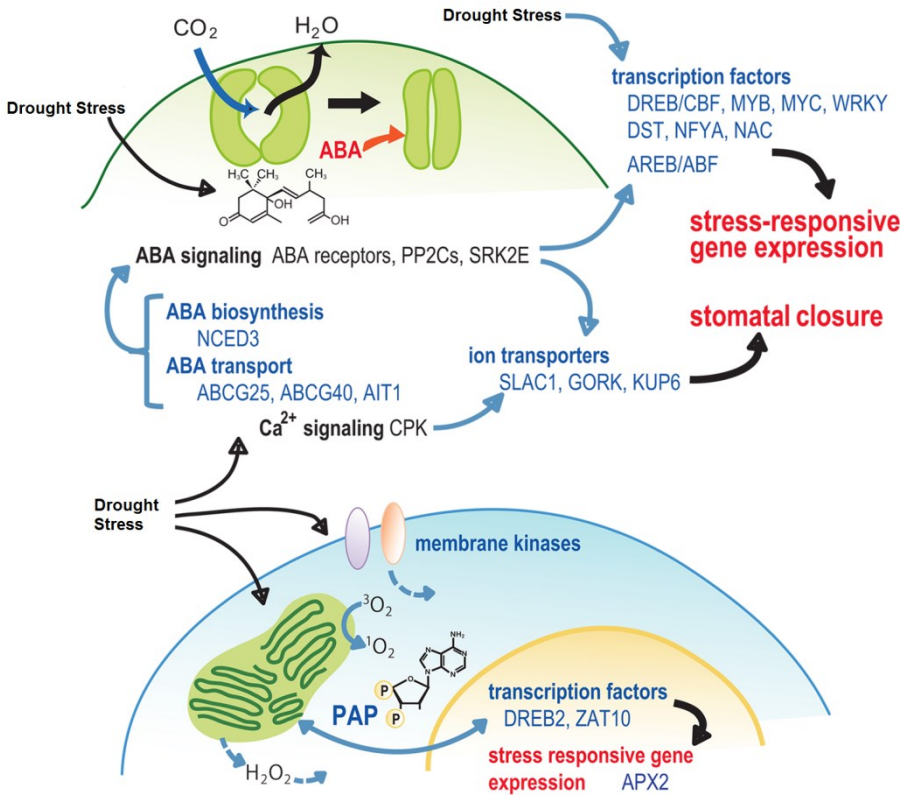

Figure 2.3. A schematic diagram representing stomatal closure and factors associate with stomatal closure during drought stress (Adapted from Osakabe *et al*., 2014).

Smit and Singles (2006) studied how canopy development was affected by drought stress in sugarcane. Poor canopy development reduces light interception, and their photosynthesis. Furthermore, they showed that the drought stress increased leaf

senescence and led to yield reduction. Bosabalidis and Kofidis (2002) proved that drought stress results in a decrease in size of both mesophyll and epidermal cells in olive, however, the cell density increased.

When the plant is under drought stress, the root pushes deeper in search of water. It was found that the root length increases with drought stress (Turkan *et al*., 2005; Bahrami *et al*., 2012). Sharp and LeNoble (2002) observed an increase in the rate of root tip elongation with the increase in drought stress in maize. However, the root volume and the dry weight reduced significantly under the drought stress (Geetha *et al*., 2012; Hadi *et al*., 2012).

Drought stress also affects shoot length. Under water deficient, shoot length in sesame (*Sesamum indicum* L.) was reduced (Bahrami *et al*., 2012), but in some cases, it showed reduction at the initial stage and then an increase in shoot length. Further, some plants increased shoot length initially and then reduced (Turkan *et al*., 2005).

Plant vascular bundles have a major role in the transport of water and nutrients in tomatoes. It was found that the rate of flow of xylem fluids was reduced and hydraulic resistance at the pedicel and the peduncle increased with drought stress (Van Ieperen *et al*., 2003). Salleo *et al.* (2000) tested the effects of xylem cavitation on stomatal conductance in Laurel (*Laurus nobilis* L.). Cavitation was measured using ultrasound acoustic emissions (UAE) and when water potential was reduced, UAE level increased. Increased UAE level indicates, that the high loss of hydraulic conductance due to reduced rate of xylem fluid flow.

2.2.2 Growth and development

There is a significant reduction in plant growth under drought stress. Initially, turgor pressure is reduced and this results in reduction of cell elongation (Farooq *et al*., 2009). Also, drought stress causes damage in mitosis which results in limited cell division. Both reduced cell elongation and limited cell division negatively impacts plant growth (Farooq *et al*., 2009), as shown in Figure 2.4.

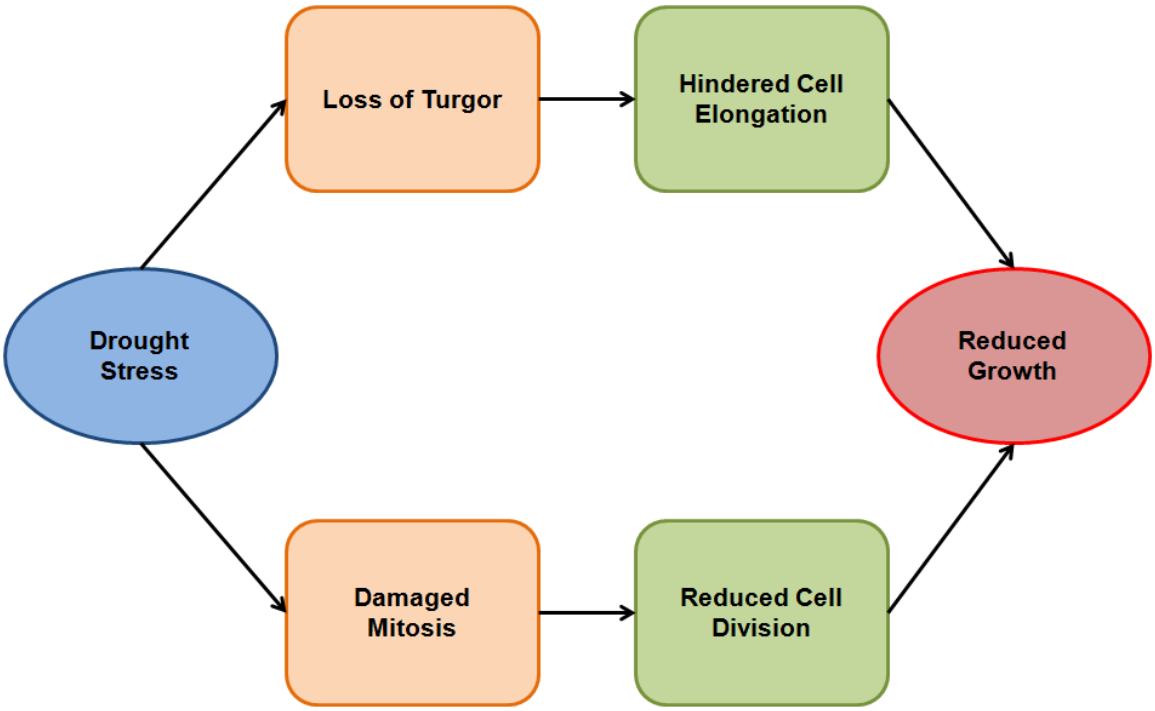

Figure 2.4. A schematic diagram showing the process how drought stress reduces plant growth (Adapted from Farooq *et al*., 2009).

2.2.3 Physiological functions

Plant water potential influences physiological functions of plants, including photosynthesis, transpiration, respiration, photorespiration, stomatal conductance (Chaves *et al*., 2002; Blanke and Cooke, 2004; Flexas *et al*., 2004).

With optimal water supply, plants functions, such as photosynthesis, respiration, transpiration and stomatal conductance, happen well. Moreover, continuous water supply allows high transpiration and low leaf to air water vapor pressure difference (VPD). Low VPD increases photosynthesis and under drought stress, reduces photosynthesis, due to high VPD (Brunce, 1988). In tomatoes, high transpiration rates reduce photosynthetic capacity and induce drought stress (Blanke and Cooke, 2004). Large water potential gradients between the xylem and the site of evaporation (leaves) result in reduced photosynthesis (Sharkey, 1984, Blanke and Cooke, 2004). It was observed that the net photosynthetic rate and transpiration rate declined with an increase in drought stress (Teraza *et al*., 1999; Rao *et al.,* 2000 and Flexas *et al*., 2004). In tomatoes, both drought stress and potassium deficiency leads to limited growth due to significant reduction in photosynthesis (Behboudian and Anderson 1990).

Under drought stress, stomata close and this affects CO_2 flux. Stomatal closure is one of the first responses to drought stress (Hommel *et al*., 2014; Xie *et al.,* 2014). Stomata close when plant water potential reduces or if the leaf turgor reduces. The response limits CO_2 exchange in leaves (Chaves *et al*., 2002). Low CO_2 flux causes an increase in ROS. On the other hand, plant tissue water potential is reduced by drought. Low tissue water potential reduce the activities of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), phosphoenolpyruvate carboxylase (PEPCase), NADP-

malic enzyme (NADP-ME), fructose-1,6-bisphosphatase (FBPase) and pyruvate orthophosphate dikinase (PPDK) enzymes. Both ROS production and reduced activity of enzymes lower the carboxylation. Further, drought causes a down-regulation of non-cyclic electron transport, which negatively affects ATP synthesis. As a result of low carboxylation and low ATP levels, photosynthesis drops under drought conditions (Figure 2.5) (Farooq *et al*., 2009).

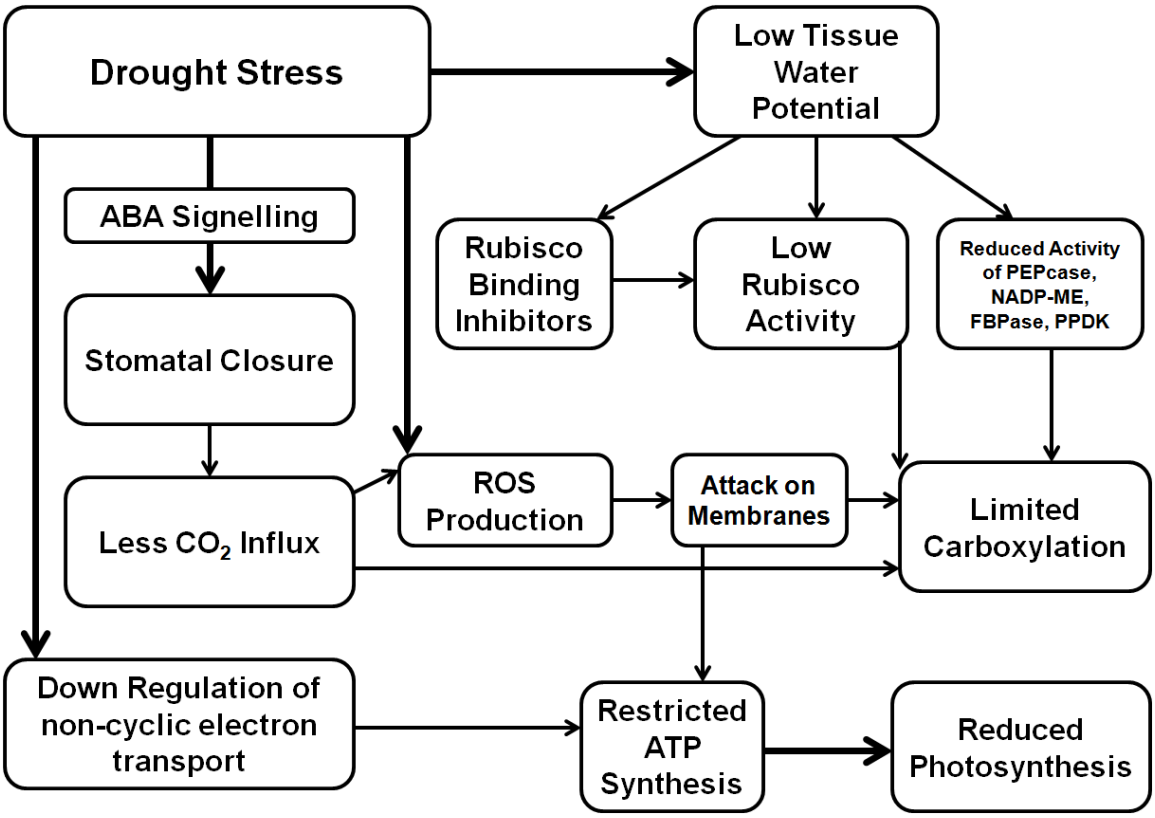

Figure 2.5. A schematic diagram showing the photosynthesis reduction under drought stress (Adapted from Farooq *et al*., 2009).

Rizhsky *et al.* (2002) observed that respiration was reduced with drought stress. Bell *et al*. (1971) noted that the mitochondrial oxygen uptake declined with an increase in drought stress in maize. Furthermore, Burton *et al.* (1998) observed limited root respiration with drought in sugar maple. Ribas-Carbo *et al*. (2005) found that respiration rate was diminished with a rise in drought stress in soybean leaves. However, photorespiration was greater in drought stressed soybean than in non-stressed plants (Wingler *et al*., 1999; Haupt-Herting *et al*., 2001).

As drought stress progresses, it reduces the leaf water potential and stomatal conductance (Medrano *et al*., 2002; Bota *et al.,* 2004; Flexas *et al*., 2004; Miyashita *et al*., 2005; Smit and Singles, 2006). Blanke and Cooke (2004) found that the leaf water potential reduced under severe drought stress, but Miyashita *et al*. (2005) discovered that the leaf water potential stayed constant for a period after the onset of water and then reduced rapidly. Furthermore, the recovery after re-watering declined gradually when the drought stress progressed. Reduction of leaf water potential with stress also affected leaf relative water content. Leaf relative water content was reduced with drought (Teraza *et al*., 1999; Bota *et al.,* 2004; Turkan *et al*., 2005; Valentovič *et al*., 2006).

It was found that the germination rate and seedling growth was reduced with drought stress (Bahrami *et al*., 2012). Moreover, Hadi *et al*. (2012) showed seed length, seed width, seed length to width ratio, seed diameter, seed weight, kernel weight, shell weight, kernel to shell ratio, hypocotyl length, dry weight of hypocotyl and cotyledon leaves were significantly lowered under drought stress.

2.3 Biochemical changes under drought stress

Drought stress affects biochemical processes in a plant. Plant hormonal activation, antioxidant enzyme activation, reduction of sugar, protein, amino acid levels or degradation of genetic materials are some of the biochemical pathways affected during drought stress.

2.3.1 Abscisic acid (ABA) Signaling under drought Stress

When a plant is subjected to drought, there is an increase in ABA biosynthesis, leading to elevated ABA levels in the tissues (Plant *et al*., 1991). The increase in the ABA concentration in leaves results in stomatal closure and minimal water loss from the plant. However, the stomatal closure reduces photosynthesis (Bray, 1988; Plant *et al*., 1991; Zegzouti *et al*., 1997). High ABA concentration in root tips was observed in plants subjected to drought stress (-1.6 MPa) (Sharp and LeNoble, 2002). Bray (1988) studied the role of ABA in drought, using an ABA deficient tomato. In optimal growth conditions, ABA concentration in this mutant was 50% of the wild type plant. When both the wild type and mutant were exposed to drought stress, there was a significant increase in the synthesis of ABA in the wild type but reduced in the mutant. It was recorded that the ABA concentration of the mutant under drought stress was 6% of its ABA concentration grown under optimal conditions.

Bray (2002[a]) proposed a model to explain the mechanism of ABA accumulation in *Arabidopsis thaliana* leaves and ABA regulates gene expression in the leaves. First, ABA should accumulate under drought stress in *Arabidopsis* leaves (Figure 2.6 - a). *ATHK1* gene, which is involved in phosphorylation of histidine and aspartate, works as a part of the sensing molecules. ATHK1 mRNA up regulates with drought stress in

Arabidopsis. ATHK1 consists of two domains: histidine kinase domain and a receiver domain, to regulate the responses to drought stress. ATHK1 interacts with a phosphorelay intermediate ATHP1 for transfer the signal. ATHP1 is well known for binding with histidine kinases in plants. Mitogen-activated protein (MAP) kinase sense the received signal from ATHP1 and MAP kinase cascade is present in drought response. MAP kinases are bound to an unknown substance in the nucleus. This results in activation of *AtNCED* gene (unknown gene) and production of AtNCED in the nucleus. Meanwhile, in the chloroplast, zeaxanthin converts into violaxanthin using zeaxanthin epoxidase enzyme (AtZEP). Violaxanthin is converted into 9-*cis*-epoxycarotenoids. Generally, 9-*cis*-epoxycarotenoid dioxygenase enzyme (NCED) breaks down carotenoids. AtNCED cleave 9-*cis*-epoxycarotenoids to xanthoxin. Xanthoxin is the 1st precursor of ABA. It is converted into abcisic aldehyde. Finally, abscisic aldehyde oxidase (AAO) cleaves abcisic aldehyde and produces ABA.

Accumulated ABA involves in signal transduction pathway in *Arabidopsis* leaves to regulate genes (Figure 2.6 - b). Produced ABA binds with the receptors in the cell membrane and cytoplasm. ABA-receptor complex molecules move through a signal cascade and express ABA responsive genes, such as Abscisic acid response element (ABRE) genes, in *Arabidopsis.* Serine/ threonine protein phosphatases (ABI1 and ABI2) and ERA1 are important molecules involved in this signaling cascade. Abscisic acid response element (ABRE) genes, which are located in the nucleus, contain a G-box site with a –ACGT sequence. Two G-box sites bind together through –ACGT sequence with the aid of bZIP transcription factors and for a dimer. Formed dimers can be either *cis*- or *trnas*- acting elements, however, only two *cis*- acting elements able to facilitate ABA

response. There are proteins involved in the dimer formation, which contains DNA binding domain and a leucine zipper. These proteins either form homodimers or heterodimers, which indicate positive or negative regulation of genes. Moreover, under severe drought stress, dehydration-responsive gene *RD22* is activated and is responsible for production of MYB and MYC elements. These elements are also involved in ABA response. All these G-box, MYB and MYC elements are involved in the signal transduction pathway under ABA signaling, but these mechanisms require more investigation.

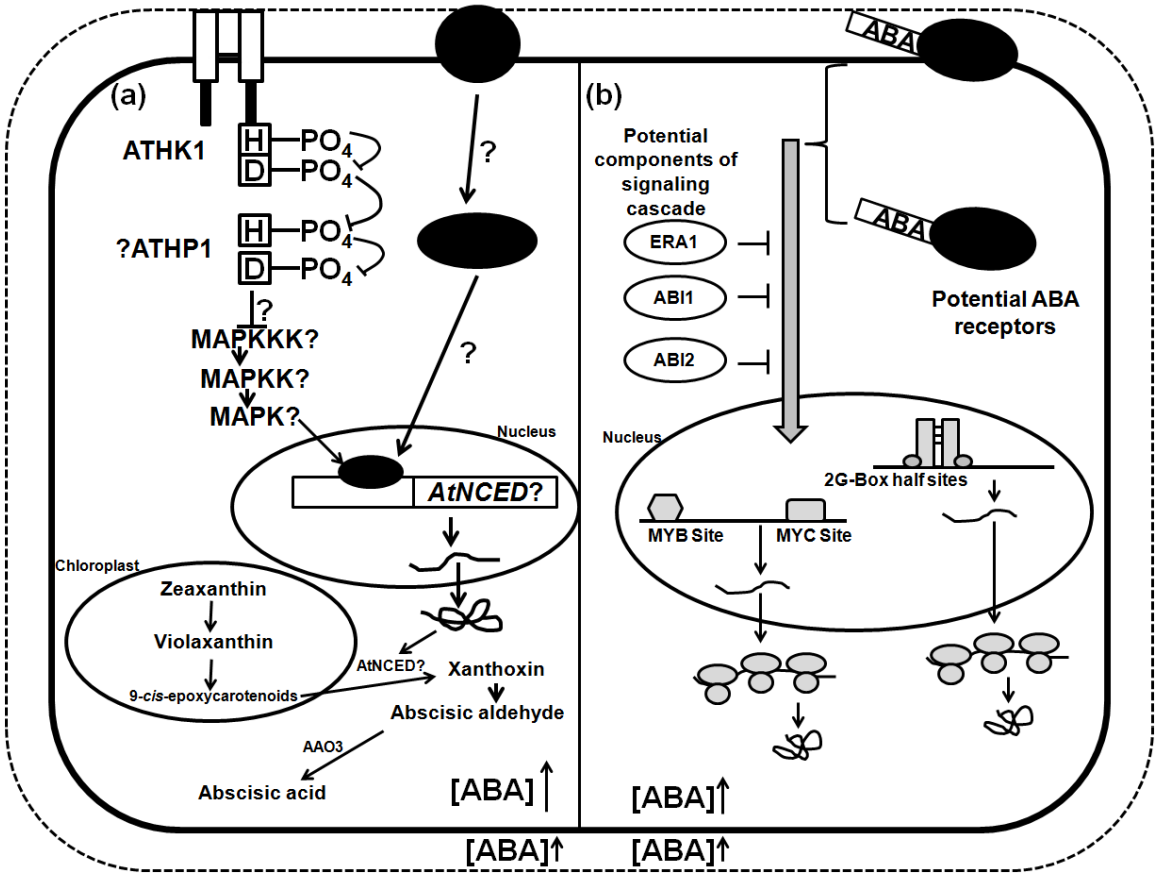

Figure. 2.6. A schematic diagram showing the proposed mechanisms of model *Arabidopsis thaliana* cell subject to drought stress (Adapted from Bray, 2002[a]). (a) Proposed mechanism for accumulation of ABA under drought. H – Histidine, D-Aspartate, AtNCED? – Unknown gene, (b) Proposed signal transduction pathway that ABA recognize and lead to ABA regulate gene expression, ? – unidentified function or substance.

2.3.2 Other phytohormones and drought Stress

Ethylene, cytokinins, gibberellic acid, jasmonic acid, salicylic acid and brassinolids are involved in stress responses in plants. Their roles in drought stress listed in Table 2.1.

Table 2.1 Plant growth regulators and their functions under drought stress

PGR	Functions under drought stress	Reference
Auxins	Enhance expression of *LEA (late embryogenesis abundant)* genes (*LEA* aids in drought tolerance)	Peleg and Blumwald (2011)
Ethylene	Inhibit root growth (root growth increase when ethylene inhibitor present)	Sharp and LeNoble (2002)
Cytokinins	Decrease stomatal regulation of gas exchange Increase shoot/root ratio Delay leaf senescence by drought stress Reverse leaf or fruit abscission caused drought stress Prevent photosynthesis	Pospisilova *et al.* (2000), Ha *et al.* (2012) Ha *et al.* (2012)
Gibberellic acid	Maintain membrane permeability Enhance chlorophyll content Enhance relative water content Enhance macro-nutrient contents in leaves	Kaya *et al.* (2006)
Jasmonic acid	Induce antioxidant enzymes activity Induce betain biosynthesis Regulate ascorbate and glutathione metabolism	Sedghi *et al*. (2012) Gao *et al.* (2004) Shan and Liang (2010)

Table 2.1 Plant growth regulators and their functions under drought stress (Continued)

PGR	Functions under drought stress	Reference
Salicylic acid	Induce antioxidant enzymes activity	Sedghi *et al*. (2012), Hayat *et al.* (2008)
Brassinolids	Enhance SOD, CAT, ascorbate peroxidase and glutathione reductase enzyme levels	Bajguz and Hayat (2009),
	Enhance ascorbic acid, carotenoids and glutathione levels	Sedghi *et al*. (2012)
	Enhance net photosynthetic rate	
	Enhance ABA and proline levels	
	Induce stress related genes	Peleg and Blumwald (2011)

2.3.3 Antioxidant enzymes

Under optimal water supply, plants function normally and there is a balance between reactive oxygen species (ROS) and antioxidant enzymes. This equilibrium shifts with the onset of drought stress and ROSs increase (Gill and Tuteja, 2010). When a plant undergoes stress, abscisic acid (ABA) biosynthesis is activated (Jiang and Zhang, 2002). Elevated ABA induces the production of reactive oxygen species (ROS); these toxic compounds includes substances such as hydrogen peroxide (H_2O_2), superoxide radicals (O_2^-) and hydroxyl radicals (OH^-) that damage plant cells (Jiang and Huang, 2001; Jiang and Zhang, 2002; Turkan *et al.,* 2005). Antioxidant enzymes are activated inside the plant to suppress ROSs and break them into nontoxic compounds.

There are a number of antioxidant enzymes, each with a different role in relation to plant defense against stress (Table 2.2). Superoxide ions ($O_2^{\cdot-}$) catalyze to hydrogen peroxide (H_2O_2) using the superoxide dismutase (SOD) enzymes. There are different forms of SOD which have similar function, such as FeSOD, Cu/ZnSOD and MnSOD (Choi *et al*., 2004; Abedi and Pakniyat, 2010). H_2O_2 is then broken down by catalase (CAT) and peroxidases (Glutathione peroxidase, Ascorbate peroxidase, Guaiacol peroxidase). CAT breaks H_2O_2 into water and oxygen. Peroxidases reduce H_2O_2 into non-harmful substances such as water and polyunsaturated fatty acids, depending on peroxidase type. Enzymes such as SOD, CAT, peroxidases and glutathione peroxidases scavenge ROS, while enzymes like glutathione S-transferese and ascorbate peroxidase help in the detoxification of lipid peroxidation (LP) products (Blokhina *et al.,* 2003).

Table 2.2 Antioxidant enzymes and their reactions (Blokhina *et al.*, 2003).

Enzyme	Reaction Catalyzed
Superoxide dismutase	$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \leftrightarrow 2H_2O_2 + O_2$
Catalase	$2H_2O_2 \leftrightarrow O_2 + 2H_2O$
Glutathione peroxidase	$2GSH + PUFA\text{-}OOH \leftrightarrow GSSG + PUFA + 2H_2O$
Glutathione s-transferase	$RX + GSH \leftrightarrow HX + R\text{-}S\text{-}GSH$
Ascorbate peroxidase	$AA + H_2O_2 \leftrightarrow DHA + H_2O$
Guaiacol peroxidase	$Donor + H_2O_2 \leftrightarrow Oxidized\ donor + H_2O$
Glutathione reductase	$NADPH + GSSG \leftrightarrow NADP^+ + 2GSH$

** R- an aliphatic, aromatic or heterocyclic group, X – Sulfate, nitrite or halide group, AA - Ascorbic acid

Superoxide dismutase (SOD), Catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR) activites increase under drought stress (Jiang and Huang, 2001; Jiang and Zhang, 2002; Choi *et al*., 2004). In some cases, SOD activity increases with the stress and remains same, even as stress progresses (Turkan *et al.,* 2005). In general, CAT activity increases with the stress but occasionally it is observed to be unchanged or reduced with drought stress (Dwivedi *et al*., 1979; Turkan *et al.,* 2005).

Dhindsa (1991) found that those enzymes responsible for glutathione metabolism, such as glutathione reductase, glutathione peroxidase and glutathione S-transferase, increase levels during drought stress and oxidized glutathione (GSSG) level increases in the plant when glutathione reductase level diminishes. Oxidized glutathione (GSSG) has been reported to negatively affect protein synthesis but increases lipid peroxidation and solute leakage. Furthermore, all glutathione enzyme activities increase with slow drying

but it is kept constant with rapid drying. When re-watered, all enzyme activities were reduced with slow drying but in rapid drying, enzyme activities were increased initially and then decreased.

2.3.4 Lipid peroxidation

Lipids are important component in plants' structure and function. Lipids are involved in cell membranes, organelle biosynthesis in plant cells and mediating plant growth and development (Welti *et al.,* 2002; Wang, 2004; Benning 2009). Plants under drought stress leads to secondary stress such as oxidative stress. Oxidative stress can affect on cellular lipids and oxidative degradation of lipids is known as the lipid peroxidation (Niki *et al.,* 2005). Free radicals gain electrons from cell membrane lipids and cause damage to the cell (Niki, 2009). Membrane lipid peroxidation increases under drought stress. The accumulation of malondialdehyde (MDA) is used as an indicator of lipid peroxidation. Sudden increase of MDA reduces the activity of the antioxidant enzymes (Jiang and Huang, 2001; Turkan *et al.,* 2005). Valentovič *et al*. (2006) measured lipid peroxidation in both drought tolerant and susceptible varieties of maize. It was found that the lipid peroxidation increased in leaves, roots and mesocotyls (seedlings) in both varieties.

Polyunsaturated fatty acids (PUFA) are the most common form of lipids found in plants. They are highly susceptible to free radicals (Dotan *et al*., 2004). Sattler *et al.* (2006) studied the mechanism of lipid peroxidation. First, free radicals attack the double bonds of PUFA acyl chains and create lipid radicals. This forms lipid peroxyl radicals reacting with oxygen molecules which attack adjacent PUFAs in the absence of lipid soluble antioxidants, especially tocopherols. These extend as a chain reaction.

Tocopherols donate hydrogen atoms to lipid peroxyl radicals to avoid further extension of the reaction. This creates either lipid peroxides or phytoprostane G1 (PPG1). Lipid peroxides convert into hydroxyl fatty acids, either enzymatically or spontaneously. Formed PPG1 decay and form either malondialdehydes (MDA) or different forms of alkanes or alkines, such as PPF1, PPD1, PPE1 (Figure 2.7).

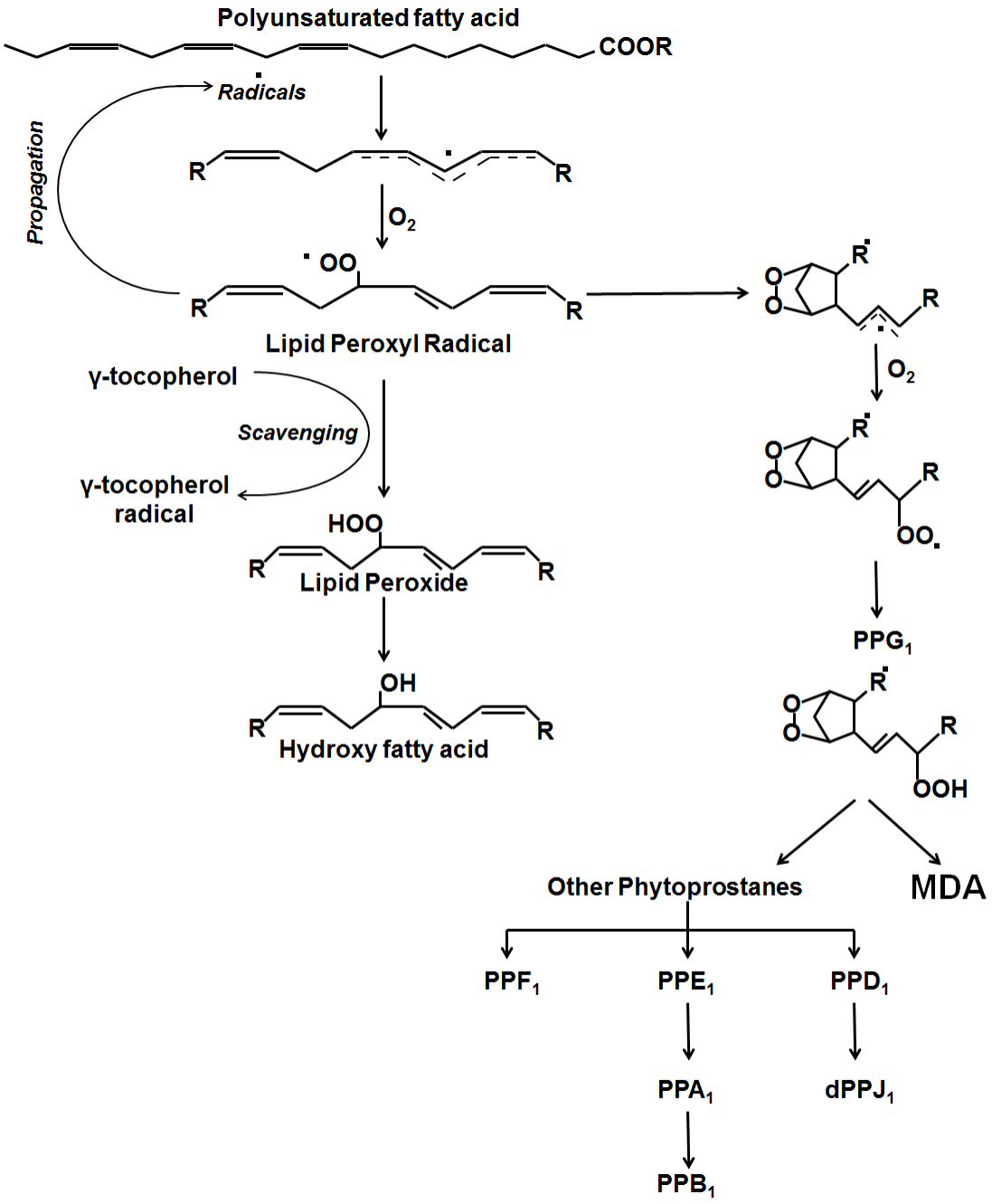

Figure 2.7. Schematic representation of steps involved in lipid peroxidation. (Adapted from Sattler *et al.,* 2006.) MDA – malondialdehydes, PPG_1 - phytoprostane G1, PPF_1 - phytoprostane F1, PPE_1 - phytoprostane E1, PPA_1 - phytoprostane A1, PPB_1 - phytoprostane B1, PPD_1 - phytoprostane D1, $dPPJ_1$ - phytoprostane J1.

2.3.5 Electrolyte leakage

Drought stress can cause negative impacts on physiochemical properties in cell membrane, such as tissue injury, electrolyte leakage, loss of functionality of the membrane and damage to membrane bound proteins (Fan and Sokorai, 2005). Electrolyte leakage is used as an indirect measure of membrane stability under stress conditions. Lipid peroxidation due to ROS damage is the main cause if electrolyte leakage in stressed plants (Houmli *et al.,* 2010). However, electrolyte leakage is widely used as a measurement in pant tolerance to various stresses (Arvin and Donnelly, 2008).

Electrolyte leakage increases with the increase of the stress (Gulen and Eris, 2004). Bajji *et al*. (2001) observed in Durum wheat, that electrolyte leakage reduces with time and re-watering increases it for a certain level and remains constant. Valentovič *et al*. (2006) found that electrolyte leakage in roots and leaves in both tolerant and susceptible maize varieties increases with the stress.

2.3.6 Total proteins, sugars and nucleic acids

Total protein, soluble protein, soluble sugars, total carbohydrates and starch reduced with the progress of stress. It was found that reducing sugars increased initially and was lowered with the progression of the stress but non-reducing sugars declined from the beginning (Dwivedi *et al*., 1979). In contrast, Valentovič *et al*., 2006 showed that soluble sugar content rises in maize leaves with drought stress. α-amino nitrogen level increase with the time (Dwivedi *et al*., 1979). Proline content rose with the stress in leaves and roots (Turkan *et al.,* 2005; Valentovič *et al*., 2006). Further, polyamine, and glycine betaine contents increase with drought stress (Krasensky and Jonak, 2012).

Drought stress damages nucleic acids in the cells. Schutter *et al*. (2007) studied how drought stress affects the cell cycle in *Arabidopsis*. Moreover, Bray and West (2005), stated that abiotic stresses are main cause of genotoxic stress which cause damage to DNA.

2.3.7 Chlorophyll and other photosynthetic pigments

Under drought stress, the leaf water content is reduced and leaf chlorophyll content declined due to degradation of chlorophyll pigments (Dwivedi *et al*., 1979; Jiang and Huang, 2001). This loss is higher in mesophyll cells compared to bundle sheath cells (Alberte *et al*., 1977). Chlorophyll loss leads to low or inactive photosynthetic activity and changes the fluorescence characteristics of the upper leaves. This is mainly due to its effect on photosystem II (PSII), because of its influence on photochemistry of PSII (Angelopoulos *et al*., 1996). Chlorophyll *a* functions as a reaction center pigment and chlorophyll *b* functions as an antenna pigment. Other than chlorophyll *b*, pigments belong to carotenoids and xanthophylls act as antenna pigments. β-carotene, zeaxanthin, lycopene and lutein are some of the pigments in these groups. Both the reaction center pigment (chlorophyll *a*) and the antenna pigments together create a photosystem. Antenna pigments help to transfer captured light energy to chlorophyll *a*. There is a balance between chlorophyll *a* and chlorophyll *b* molecules which keeps the chlorophyll *a:b* ratio constant. Chlorophyll *a* oxygenase (CAO) enzyme helps to convert chlorophyll a to b (Tanaka *et al*., 1998; Tanaka and Tanaka, 2011).

Ledford and Niyogi (2005) observed plants' tendency to be subjected to photo-oxidative stress under drought stress. High light causes stress to plant chloroplasts. Activation of reactive oxygen species (ROS) inside the chloroplast causes secondary

oxidative stress and damages the chloroplasts. However, constant plant water content allows for increasing leaf chlorophyll content in stressed plants, over time (Alberte *et al*., 1977).

2.4 Gene Expression and Drought Stress

There are different genes responsible for plant stresses. Under drought stress, stress responsive genes activate inside the plant cell. Activation of the stress responsive genes is reflected as visual symptoms in a stressed plant. Some of them express specifically under water stress or drought. Expression can be site specific in the plants, such as within the leaves or roots.

2.4.1 Expression of drought responsive genes

Researchers observed that drought stress induced the transcription of a number of specific genes. Some of them were specific to drought stress while others were general stress response genes (Plant *et al.,* 1991; Khan *et al.,* 1993; Lu *et al*, 2010). In tomatoes, genes *le16*, *le4, le20* and *le25* were expressed in response to drought stress and these genes were regulated by abscisic acid (ABA) (Plant *et al.,* 1991; Khan *et al.,* 1993). Khan *et al.* (1993) noted that the genes *le16, le20* were highly expressed in leaves, while *le4* was expressed in root tissues and *le25* is expressed both in the root and leaf tissues. The transcription of genes may also depend on the severity of drought stress. For example, the expression of *le20* was induced even under mild stresses, while the transcription of *le25* was initiated only under extreme stress (Thompson and Corlett, 1995). Furthermore, high expression of ethylene-responsive gene (ER5) was found under drought in tomato (Zegzouti *et al.,* 1997). Iuchi *et al*., (2000) used 5 clones of CPRD (cowpea responsive to

dehydration), *CPRD8, CPRD14, CPRD22, CPRD12, CPRD4* and high expression was recorded with drought. It was found that there are 277 genes associated with drought in *Arabidopsis*. Among them, 128 genes were expressed in response to drought, whereas 119 genes were expressed in drought and salinity, 8 genes were expressed in both cold and drought and 22 genes for drought, cold and salinity (Seki *et al.,* 2002).

Mitochondrial and chloroplast associated genes also involved in drought stress response. In *Arabidopsis ERD5* (early responsive to dehydration stress) gene was seen to be prominent in mitochondria and its expression under drought stress induced proline synthesis (Kiyosue *et al*., 1996). CDSP 32 (chloroplastic drought-induced stress protein associate *CDSP32* gene) in chloroplasts showed high expression when potato plants were under drought stress (Rey *et al.,* 1998).

A number of stress response genes code functional proteins, such as water channel proteins, chaperones, LEA proteins, proteinases, detoxicating enzymes, while others are regulatory proteins like protein kinases (MYB, MYC, bZlP), transcription factors (MAPK, CDPK), phospholipase C, 14-3-3 protein. These facilitate stress tolerance or stress response (Shinozaki and Yamaguchi-Shinozaki, 1997).

2.4.1.1 Selected Genes in the Study

There were five genes selected for this study to understand how the expression level chances under drought stress with ANE application. The selected genes are, Δ-*pyrroline-5-carboxylate synthetase* (*P5CS*), *late embryogenesis abundant* (*LEA*), *lipid transfer protein gene 2* (*ltpg2*), *ABA-response element binding protein 1* (*SlAREB-1*) and *metacaspase-1* (*MCA1)*.

Δ-pyrroline-5-carboxylate synthetase (P5CS)

In plants, proline is accumulated as a result of biotic and abiotic stresses (Kishor *et al*., 2005; Verbruggen and Hermans, 2008). Glutamate or ornithine is used to produce proline in plants and glutamate pathway is the most prominent for proline synthesis in higher plants (Strizhov *et al*., 1997; Parvaiz and Satyawati, 2008) (Figure 2.8). Initially, L-glutamate is reduced to L – glutamate γ – semialdehyde by Δ-pyrroline-5-carboxylate synthetase (P5CS) enzyme and L – glutamate γ – semialdehyde spontaneously converts into Pyrroline 5 – carboxylate (P5C). Produced P5C is converted into L-proline by Δ-pyrroline-5-carboxylate reductase (P5CR). This proline synthesis is occur in cytosol and plastids in the cell.

The *P5CS* gene expresses under stress and produce P5CS enzyme to aid in production of proline (Delauney and Verma, 1993; Strizhov *et al*., 1997; Zheng *et al*., 2014). In general, it was found two *P5CS* genes: *P5CS1* and *P5CS2* in *Arabidopsis* and several other species (Strizhov *et al*., 1997; Iskandar *et al*., 2014; Planchet *et al*., 2014). It was evident that *P5CS1* more abundant in leaves, stems and flowers, but *P5CS2* found in root-derived callus and cell suspension cultures (Strizhov *et al*., 1997).

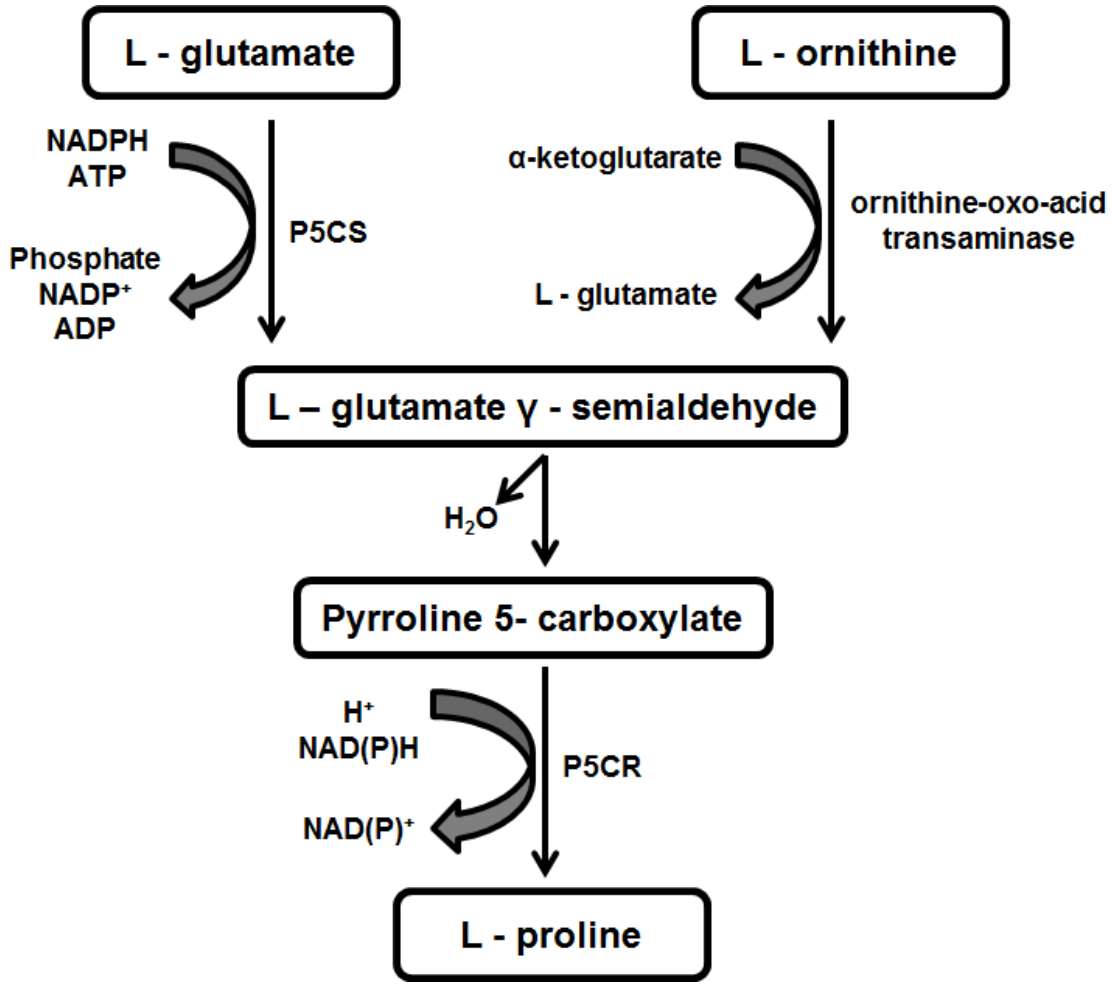

Figure 2.8. Schematic diagram representing proline synthesis in higher plants (Adapted from Parvaiz and Satyawati, 2008).

Late embryogenesis abundant (LEA)

Late embryogenesis abundant (LEA) proteins involve in protecting plants from damage caused by different stresses, in particular from drought stress (Hand *et al*., 2011). LEA proteins are low molecular weight proteins (~10-30 kDa) and mainly localized in cytoplasm and nucleus (Hong-Bo *et al.,* 2005). These proteins were categorized in to seven different groups (group 1 – 7) and further subdivided based on the LEA protein super families they belong (Battaglia *et al.,* 2008). Main functions of LEA proteins

involves provide resistance to drought or salinity or protect seeds from dehydration (Wise, 2004; Wise and Tunnacliffe, 2004; Hong-Bo *et al.,* 2005). However, LEA proteins involve in different functions (Table 2.3). Each LEA protein consists of different motifs and there are not many similarities between these LEA groups (Figure 2.9)

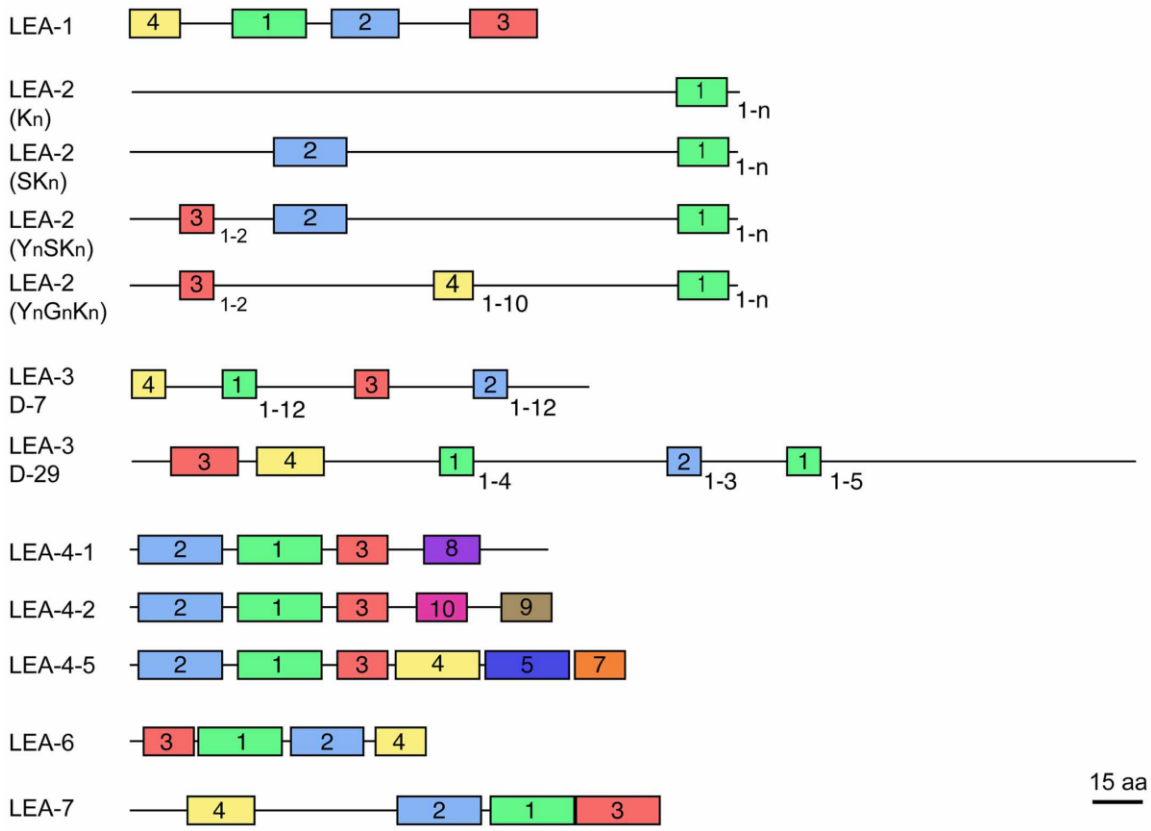

Figure 2.9. Schematic representation of different motifs in LEA proteins groups (Adapted from Battaglia and Covarrubias, 2013). Different colors indicate distribution of different motifs in each LEA group; similar color size does not indicate similar sequence. Numbers in right bottom – maximum number of repetitions.

Table 2.3. Functions of different LEA proteins (Adapted from Wise, 2004; Wise and Tunnacliffe, 2004; Battaglia *et al*., 2008)

LEA Group	LEA Superfamily	Functions	Remarks
1 (D-19)	4, 6	DNA binding nuclear protein, Nucleic acid unwinding or nucleic acid repair, Molecular chaperone	Accumulate during embryo development
2 (D-11)	1,10, 3	DNA unwinding or repair, DNA-binding nuclear protein; regulation of transcription, Cytoskeleton, DNA unwinding or repair, Ca^{2+} binding, Molecular chaperone	Known as "dehydrins" Accumulate in seed desiccation Present in all vegetative tissues
3 (D-7/ D-29)	2, 5	Cytoskeleton, Ca^{2+} binding, Molecular chaperone, Chromatin-associated nuclear protein, Filament, Kinase or phosphatase	Accumulate in mature seeds
4 (D-113)	-	Control dehydration	Accumulate in dry embryos, leaves Respond to ABA
5	-	Aid in late stages of seed development	Contain higher hydrophobic residues

Table 2.3. Functions of different LEA proteins (Adapted from Wise, 2004; Wise and Tunnacliffe, 2004; Battaglia et al., 2008) (Continued)

LEA Group	LEA Superfamily	Functions	Remarks
6 (PVLEA18)	7	Molecular chaperone, Chromatin-associated nuclear protein; transcription factor	Accumulate in dry seeds and pollen grains
7 (ASR1)	-	Induce in desiccation	Accumulate in seeds during late embryogenesis

The *LEA* gene expression starts at late maturation, reach to maximum level by initiation period of drying and decrease after germination (Brands *et al.,* 2002; Wang *et al*., 2003). Hong-Bo *et al,* 2005 demonstrate *LEA* gene expression it involve four steps: signal recognition, signal transduction, signal amplification and integration, LEA gene expression responses and its product formation. There are different factors affect on LEA gene expression and ABA is the main factor among them (Wise, 2004, Kaur *et al*., 2014). ABA can involve in three different pathways in *LEA* gene expression as ABA dependent type, ABA-induced type or ABA-irresponsive type (Shinozaki and Yamaguchi-Shinozaki, 1997; Bray, 2002[a]).

Induced *LEA* genes lead to production of LEA proteins. These proteins function as unfolded proteins, but desiccation can induce folding in them (Wise and Tunnacliffe, 2004). Desiccation induces folding of unfolded group 3a LEA protein into an α-helical

structure and it can form a coiled-coil like structure. Formed coiled-coil like structures unions to form filaments, which is essential component in cytoskeletal (Figure 2.10)

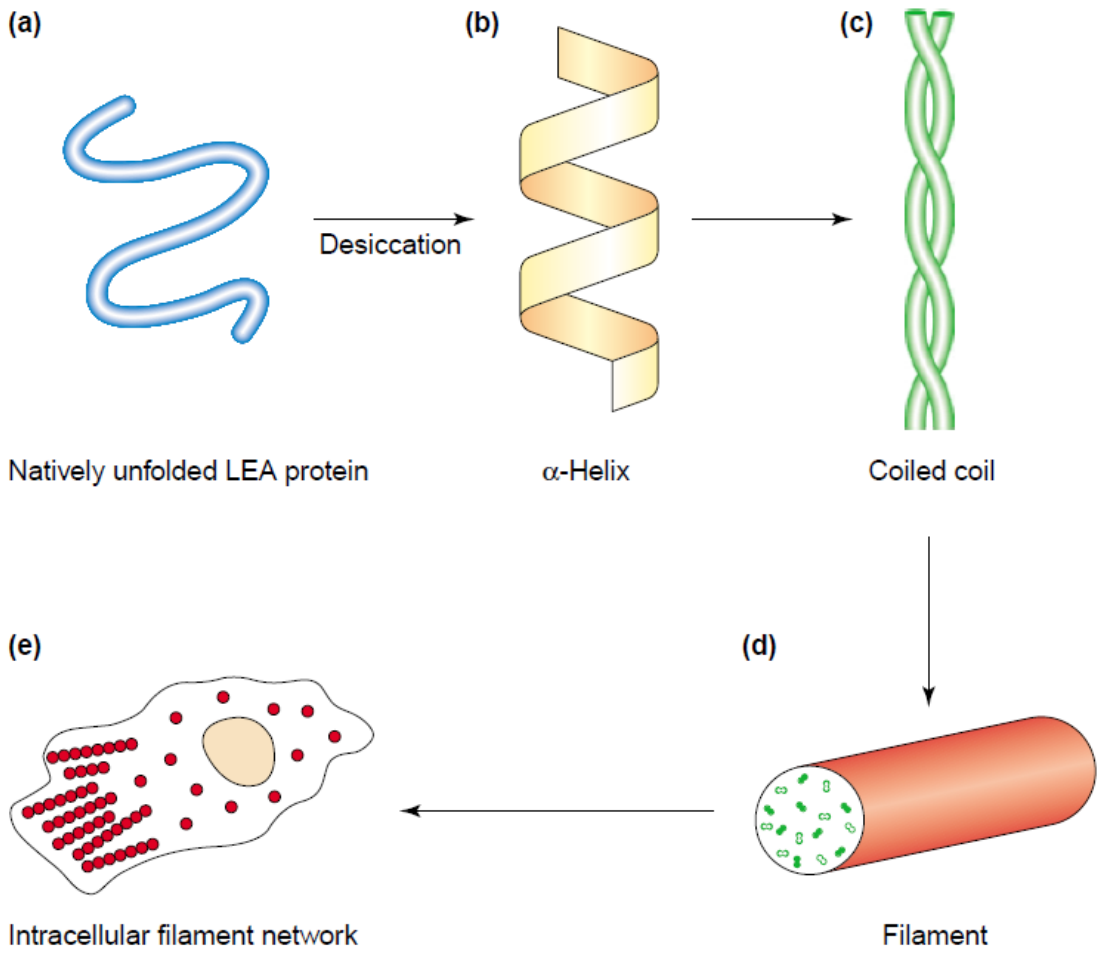

Figure 2.10. Schemetic diagram of formation of intracellular filaments from group 3a LEA proteins with desiccation (Adapted from Wise and Tunnacliffe, 2004).

Lipid transfer protein gene 2 (ltpg2)

Non-specific lipid transfer proteins (nsLTPs) are small molecular weight proteins (Edstam *et al*., 2011). LTPs involve in various processes in plants including phospholipid transfer, cutin formation, embryogenesis, sexual reproduction, defense against pathogens, and the adaptation of plants to various environmental conditions (Kader, 1997; Kim *et* al.,

2012; Ambrose *et* al., 2013; Lee and Suh, 2013; Yeats and Rose, 2013; Li *et al*., 2014). In general, they are divided into two groups as type 1 and type 2. Type 1 proteins consist around 90 amino acids and type 2 around 70 amino acids (Edstam *et al.,* 2014). The LTP found in different membranes in the cell such as mitochondria, but in general, they are extracellular proteins (Kader, 1997).

There are several LTP genes present in different plant species (Table 2.4).

Table 2.4. Genes encoding lipid transfer proteins in plants (Adapted from Kader, 1997).

Plant	Gene or cDNA	Accession Number	Protein*
Arabidopsis thaliana	LTP1 (cDNA)		110
	LTP1 (gene)	S73825	111
Tomato *Solanum lycopersicum*	TSW12 (cDNA)	X56040	114 (23)
Tobacco *Nicotiana tabacum*	LTP1 (cDNA)	D13952	
	LTP1 (gene)	X62395	118 (23)
Rice *Oryza sativa*	LTP (gene)	Z23271	117
Wheat *Triticum durum*	pTd4.90 (gene)	X63669	113 (23)
Maize *Zea mays*	9c2 (cDNA)	J04176	120 (27)
	6B6 (cDNA)	M57249	99
Carrot *Daucus carota*	EP2 (cDNA)	M64746	120 (26)

* number of amino acids and signal peptide (in parenthesis) indicated.

The *LTP* express in roots, seedlings, cotyledons, leaves, stems or shoot meristems (Kader, 1997). Further, LTP expression can be cell specific, where it is expressed in epidermis of coleoptiles, leaf veins, somatic and zygotic embryos, anthers, and floral apical meristem (Kader, 1997). Under stress, *LTP* expression increase. It was found that, under drought *ltpg2* expression level increased compared to unstressed plants (Lu *et al*., 2010).

ABA-response element binding protein 1 (SlAREB-1)

In plants ABA accumulate under drought and it play a key role in drought stress tolerance (Nakashima *et al*., 2014). ABA regulate most of the genes responsible for drought tolerance and ABA-response element (ABRE) act as the major *cis*-element in ABA related gene expression (Yoshida *et al*., 2010). There are several ABREs: ABRE1, ABRE2 in nature, which acts as transcriptional factors (bZip-type) against drought stress in plants (Yoshida *et al*., 2010). Both *ABRE1* and *ABRE2* highly expressed in vegetative tissues (Uno *et al.,* 2000). With drought, ABA accumulates and high ABA concentrations up-regulate the expression of *ABRE1* gene. It produces ABRE1 and it undergoes conformational changes before transcriptional activation of target genes for stress response in plants (Figure 2.11) (Fujita *et al*., 2005).

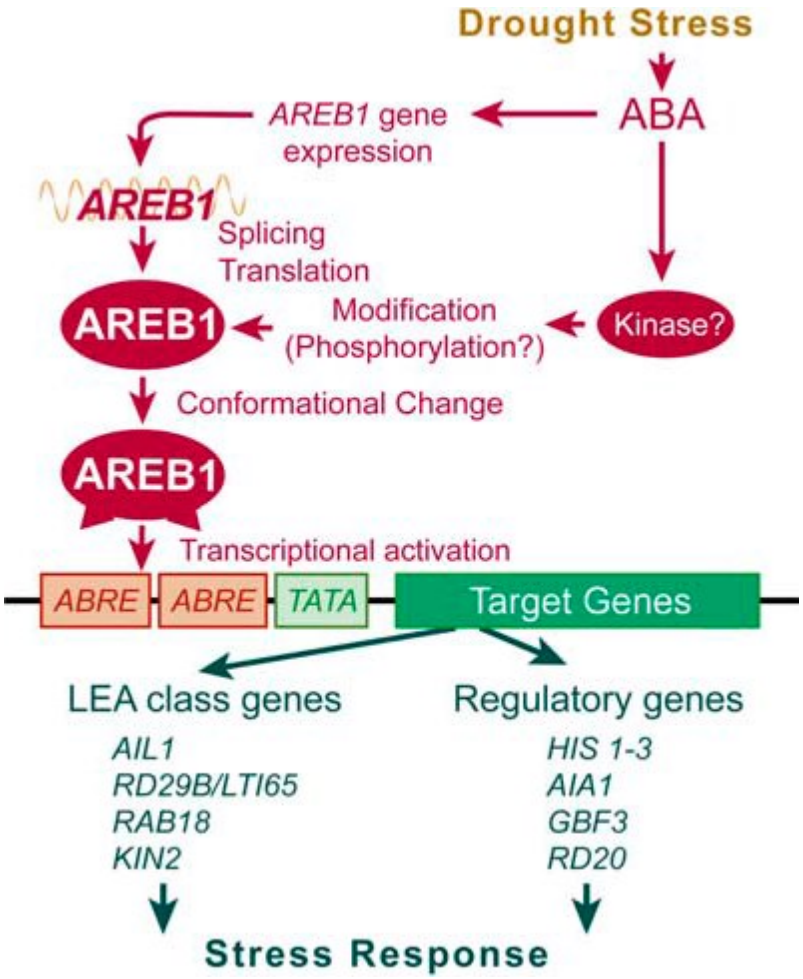

Figure 2.11. Schematic diagram of model of regulation of ABA signalling by AREB1 (Adapted from Fujita *et al*., 2005).

Metacaspase 1 (LeMCA1)

Caspases are major components of both plant and animal apoptosis (Bonneau *et al*., 2008). Caspases are cysteine proteases and use the sulfur atom in cysteine to cleave polypeptide chains (Goodsell, 2000). Later, two caspase-related proteases were found: paracaspases and metacaspases, where paracaspases found in animals, however, metacaspases prominent in yeast, fungi, and plants (Uren *et* al., 2000; Bonneau *et al*.,

2008). There are two types of metacaspases as type I and type II, where fungi consists type I only (Tsiatsiani *et al*., 2011).

It was found that the metacaspase genes highly up-regulate under stress (Tsiatsiani *et al*., 2011; Watanabe and Lam, 2011). Hoeberichts *et al*. 2003 proved that *LeMCA1* mRNA level increase with the *B. cinerea* attack in tomato leaves. Under pathogen attack, nucleotide-binding domain and leucine-rich repeat (NB-LRR)-type immune receptors activate inside the plant cell (Coll *et al*., 2014). It can increase the ROS and salicylic acid level inside the cell. The NB-LRR activation can lead to resistance inside the cell or activation of metacaspase 1. Activated metacaspase 1 can lead the cell to hypersensitive response (HR) cell death. Elevated ROS and salicylic acid levels in the cell can increase the levels or ROS and salicylic acid in neighboring cells as an immune response (Figure 2.12) (Coll *et al*., 2014).

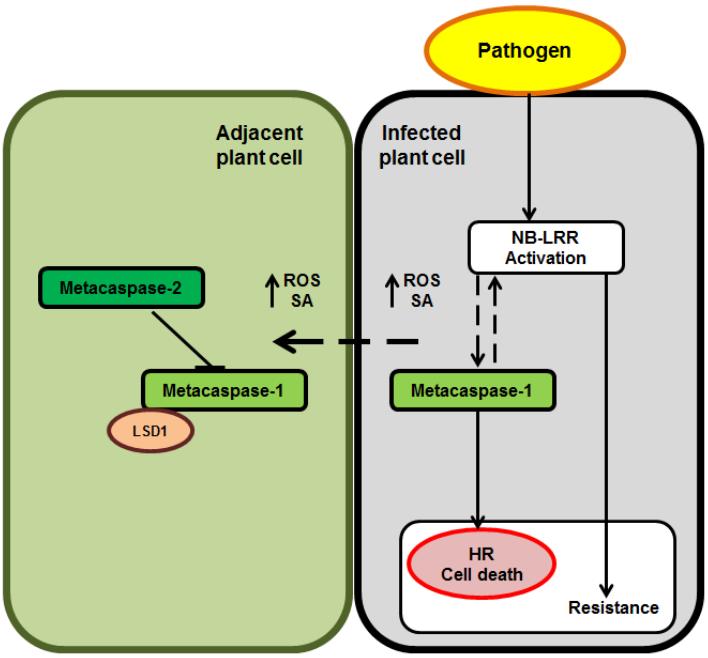

Figure 2.12. Metacaspase activation in *Arabidopsis* under pathogen attack (Adapted from Coll *et al*., 2014). ROS – Reactive oxygen species, SA – Salicylic acid, HR - hypersensitive response, LSD1 - lysine-specific demethylase 1.

2.4.2 Signal Transduction

When a plant undergoes drought stress, a general stress signal transduction pathway is induced (Xiong *et al.,* 2002). Drought creates secondary stress in the form of oxidative or osmotic stress in plants. These stresses disturb plants' homeostasis and cause damage to cell membranes and structural and functional proteins. Receptors such as osmosensors, including Histidine kinases, as well as ion channel proteins and G protein coupled receptors, activate signaling cascade. These produce messenger molecules like ABA, Ca^{2+} and H_2O_2. These messenger molecules activate phosphorylation cascade via a calcium dependent protein kinase (CDPK), salt overly sensitive kinase 3 (SOS3) and mitogen-activated protein kinase (MAPK). Phosphorylation cascades activate genes through transcription factors such as MYC/MYB, CBF/DREB, ABF and HSF (Shinozaki and Yamaguchi-Shinozaki, 1997; Vinocur and Altman, 2005). Activation of genes re-establishes homeostasis inside the cell. Products of these stress-regulator genes generate regulatory molecules, such as ABA, salicylic acid and ethylene, which regulate stresses. As a result, plants gain stress tolerance or stress resistance (Figure 2.13) (Shinozaki and Yamaguchi-Shinozaki, 1997; Xiong *et al.,* 2002).

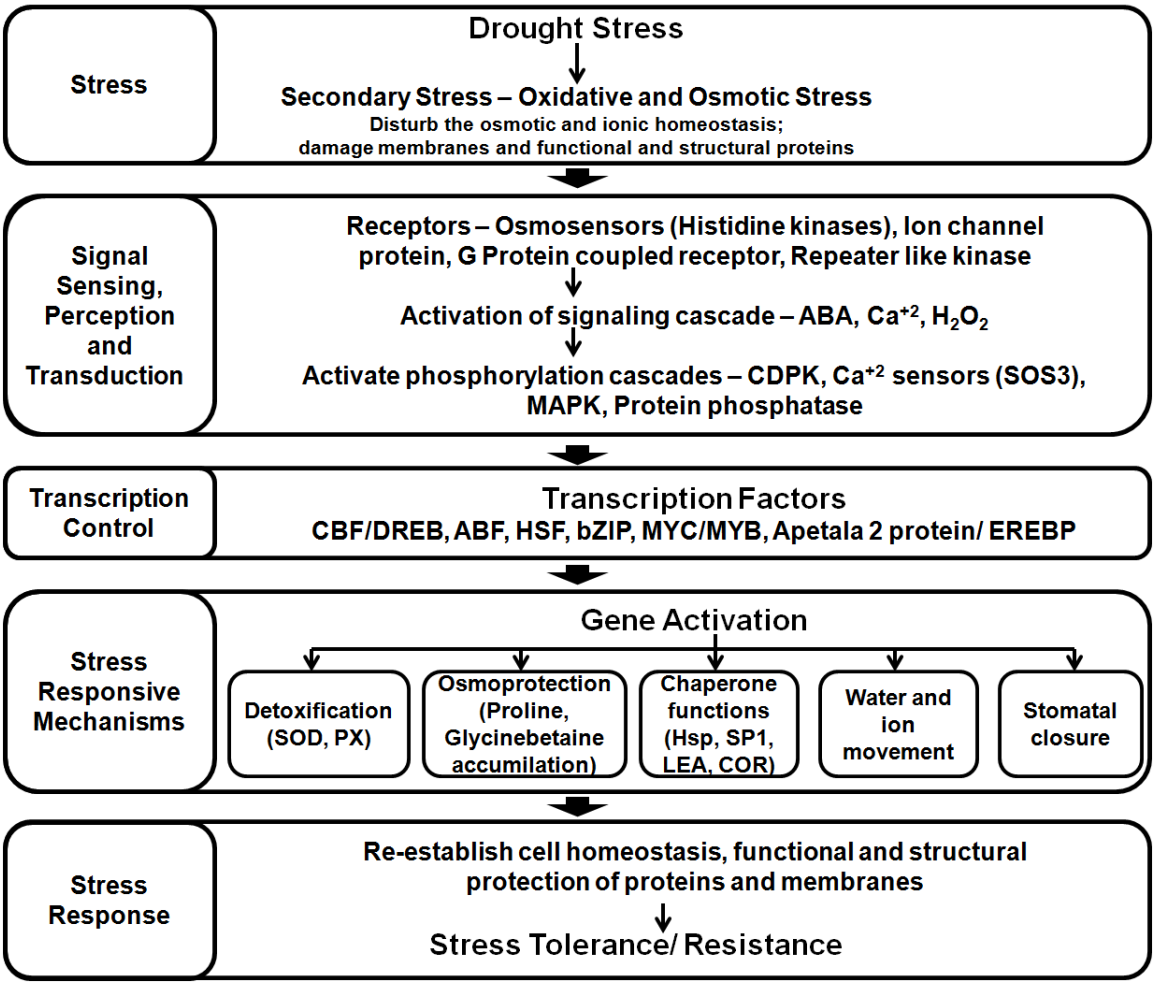

Figure 2.13. A schematic diagram showing general signal transduction pathway in plants under drought stress (Adapted from Xiong *et al.,* 2002; Vinocur and Altman, 2005; Farooq *et al*., 2009). Abbreviations: ABA - Abscisic acid, CDPK - Calcium dependent protein kinase, SOS3 - salt overly sensitive kinase 3, MAPK - mitogen-activated protein kinase, CBF/DREB - C-repeat-binding factor/dehydration-responsive binding protein, ABF - ABRE binding factor, HSF – Heat shock factor, bZIP - basic leucine zipper transcription factor, EREBP - ethylene responsive element binding protein, SOD - superoxide dismutase, PX – peroxidase, Hsp - heat shock protein, SP1 - stable protein 1, LEA - late embryogenesis abundant, COR - cold-responsive protein.

2.4.3 Drought and stress resistant transgenic crops

Transgenic crops are not a novel concept and there are different transgenic tomato varieties found in present day agriculture (Hsieh *et al*., 2002; Lee *et al*., 2003; Cortina and Culia´n˜ez-Macia, 2005; Mishra *et al*, 2012). Tomato is the first commercially available transgenic crop in the history and cultivar "Flavr-Savr"^{TM} was released in 1994 (Kramer and Redenbaugh, 1994; Bai and Lindhout, 2007). However, there are different drought tolerant tomato cultivars such as, Zarina (a cherry tomato cultivar) (Sánchez-Rodríguez *et al*., 2010), *L. pennellii* (LA0716), *L. chilense* (LA1958, LA1959, LA1972), *S. sitiens* (LA1974, LA2876, LA2877, LA2878, LA2885), *S. pimpinellifolium* (LA1579) (Razdan and Mattoo, 2007) available.

Even though, there are various drought tolerance and resistant tomato varieties available, different research carried out to produce a range of transgenic tomato varieties with the incorporation of different drought tolerance genes from other plants species. *Arabidopsis* C repeat/dehydration-responsive element binding factor 1 incorporated with *nos* terminator from cauliflower mosaic virus 35s promoter was used to create drought tolerant transgenic CBF1 tomato variety (Hsieh *et al.,* 2002). Further, artificially constructed ABRC3-*CBF1* gene (ABR3 from *HAV22* gene in barley and *CBF1* from *Arabidopsis*) transferred into tomato plants reflected high survival rate against drought stress, high chlorophyll fluorescence, low ion leakage and high yield compared to untransformed wild type (cultivar CL5915–93D4-1-013) (Lee *et al*., 2003). Transgenic tomato, expressing *yeast trehalose-6-phosphate synthase (TPS1) gene*, under drought stress, exhibited the same growth pattern as unstressed plants (Cortina and Culia´n˜ez-Macia, 2005). Overexpression of the stress response gene, *LeERF1* improved plant

tolerance by inducing the expression of similar genes *P5CS, LEA, ltpg2* and *tdi-6,* which led to stress tolerance in tomatoes (Lu *et al*, 2010).

In addition to transgenic tomato varieties, transgenic tobacco, rice and wheat varieties are also common in the present agriculture (Mundy and Chua, 1988; Abebe *et al.,* 2003; Badawi *et al*., 2004). Two transgenic tobacco lines, 35S:DREB1A and *rd29A*:DREB1A, constructed from the *DREB1A* gene in *Arabidopsis,* were used against drought stress. They performed better than wild type plants (Kasuga *et al*., 2004). Alvim *et al.* (2001) used 35S-BiPS sense, and 35S-BiPAS antisense transgenic tobacco plants with a pBI121-transformed control. High photosynthesis, high transpiration rate, high relative water content and high stomatal conductance were observed in stressed 35S-BiPS sense plants compared to the control and non-sense ones.

2.5 Tomato (*Solanum lycopersicum* L.)

The tomato has been used as a model plant in various physiological studies (Arie *et al*., 2007). Tomato is highly susceptible to drought stress (Rao *et al*, 2000; Sam *et al*, 2000). There are cultivars used in drought stress related studies and cultivar Scotia is one of the main candidates among them (MacDonald *et al*., 2009; MacDonald *et al*., 2010). Scotia is an early maturing variety (around 60 days) (Pilley and Nowak, 1997). There are different mutant tomato varieties developed for drought stress related studies such as $ACO1_{AS}$, INCA-9, *sitiens* (Sobeih *et* al., 2004; Aroca *et al*., 2008; Gonzalez *et al.,* 2008).

Around 400 genes have been identified related to drought stress in tomatoes (Gong *et al*., 2010). Genes, such as *le4, le16, le20* and *le25*, are expressed under drought stress, which was found to be more prominent with high level of ABA (Khan *et al.,* 1993). The *le20* gene is especially responsible for the production of histone H1 (Corlett *et*

al., 1998). Furthermore, *LeERF1* gene expression in tomato can be found in drought stress and it is responsible for tolerance. Overexpression of the *LeERF1* gene activates the stress related genes *P5CS, LEA, ltpg2* and *tdi-65* (Lu *et al.,* 2010).

2.6 *Ascophyllum nodosum* (L.) Le Jol.

Ascophyllum nodosum, a macro brown algae, belongs to the family Fucaceae and it is the only species in the genus *Ascophyllum*. It is a dominant perennial seaweed which is found in the intertidal zone, along the North Atlantic Basin and parts of the north-western coast of Europe. It is commonly known as rockweed, Norwegian kelp, knotted kelp, knotted wrack or egg wrack (Taylor 1957, Ugarte *et al*., 2006). Shoots of *A. nodosum* arise from a holdfast and later, grow as a dichotomous and lateral branching system. It forms flat strap shaped fronds which have a single long bladder and these fronds hang downwards, draping sheltered intertidal rocks. *A. nodosum* generates most fronds from its base. When larger fronds get damaged, the plant generally regenerates new fronds from the base (Ugarte and Sharp, 2001).

Ascophyllum nodosum is the most common seaweed in commercial extracts used in agriculture. There are many beneficial effects of *Ascophyllum* products (Rayirath *et al.,* 2009). Previous studies on commercially available *A. nodosum* extracts enhanced plant shoot growth and branching (Temple and Bomke, 1989), increased root growth (Metting *et al*., 1990), increased nutrient uptake (Yan, 1993), and improved resistance to diseases (Featonby-Smith and Van-Staden, 1983; Jayaraman *et al*., 2011) and abiotic stresses like drought, salinity and frost (Nabati *et al*., 1994, Rayirath *et al.,* 2009).

Ascophyllum nodosum is rich in bioactive compounds, including betaines [like γ-aminobutyric acid betaine, δ-aminovaleric acid betaine, laminine (N^6, N^6, N^6-

trimethyllysine) and glycinebetaine] (Blunden *et al*., 1985), as are the commercial products of *A. nodosum* (Blunden *et al*., 1986). Fucoidan is one of the beneficial polysaccharides from *A. nodosum* (Foley *et al.,* 2011). Radwan *et al.* (1997) found nine different humic acid substances in *A. nodosum* extracts. Moreover, *A. nodosum* extracts showed plant growth regulator-like properties and also induced the plant growth regulators (Wally *et al*., 2013). Commercial *A.nodosum* extract rich in organic matter such as, alginic acid, mannitol and amino acids, and inorganic compounds (Table 2.5).

Table 2.5 Organic and inorganic composition of *Ascophyllum nodosum* extract (Technical information, Acadian Seaplants LTD, Dartmouth, Canada)

Component	Amount (% from dry powder)
Organic matter	**45% - 55%**
Alginic acid	12-16 %
Fucose Polymers	13-17%
Mannitol	4%-6%
Amino acids	4%- 6%
Other organic compounds	**10% - 12%**
Ash	**45% -55%**
Nitrogen	0.8-1.5 %
Phosphorous	0.5-1.0 %
Potassium	14-18 %
Calcium	0.3-0.6 %
Iron	75-250 ppm
Magnesium	0.2-0.5 %
Manganese	8-12 ppm
Sodium	3.0-5.0 %
Sulfer	1.0-2.0 %
Zinc	10-25 ppm

CHAPTER 3

OBJECTIVES

Water is one of the major inputs in agriculture and drought stress reduces the productivity in a considerable amount in all over the world. Among different approaches to mitigate drought stress, application of seaweed extracts is one of the novel and effective approaches. *Ascophyllum nodosum* (L.) Le Jol. extract (ANE) is a highly effective seaweed extract product which is used to mitigate abiotic stresses such as salinity and cold. Further, it is an effective and environmentally friendly method to overcome plant stresses. Since ANE had positive responses against plant stresses, it is a good candidate to use it in this study.

It was hypothesized that ANE enhances plants' tolerance to drought stress. The overall objective of the study was to investigate the effect of ANE on the plant performance under drought stress. There were 3 specific objectives set in this study to understand the ANE mediated drought stress tolerance.

The first objective was to study the effects of ANE on phenotypic changes such as wilting and physiological changes such as stomatal conductance, plant water potential, relative water content, dry matter content and root growth of tomato during the vegetative stage, under drought stress conditions.

The second objective was to investigate the biochemical changes in antioxidant enzymes, lipid peroxidation, proline content and leaf photosynthetic pigments content (chlorophyll *a*, chlorophyll *b* and carotenoids) in tomatoes during the vegetative stage, under drought stress, and in response to ANE application.

The third objective was to investigate the effect of ANE on the regulation of stress responsive and specifically, drought responsive genes, in tomatoes, during the vegetative stage. The examination of gene expression is a major component of the study since the expression of the stress responsive genes determines the state of the plant under stress. Transcript levels of particular genes were analyzed to understand the expression patterns of the genes.

CHAPTER 4

MATERIALS AND METHODS

4.1 Seeds and Chemicals

Tomato (*Solanum lycopersicum*) seeds (cultivar Scotia) were purchased from Halifax Seed Company (Halifax, NS, Canada). All the chemicals and reagents were of analytical grade and purchased from Sigma Aldrich (Oakville, ON, Canada), unless otherwise stated.

4.2 Sea Weed Extract

Alkaline *Ascophyllum nodosum* extract (ANE); Acadian® was a kind gift from Acadian Seaplants LTD. (Dartmouth, NS, Canada) (batch no. 12119 J).

4.3 Plant Material and Growth Conditions

Tomato seeds were seeded in the seeding trays and kept until they germinated. Seeding trays were covered with dark trays to avoid light and daily watering was done. Germinated seedlings were kept in the seeding trays until they reach the two leaf stage. Two leaf stage tomato plants were transplanted into 4 inch plastic pots containing 100±2 g of PRO-MIX BX MYCORRHIZAE™ (Premier tech Horticulture, Rivière-du-Loup, QC, Canada). Plants were grown under controlled environmental conditions 16/8 h light/dark cycle using fluorescent tubes of approximately 350 - 400 µmol photons m^{-2} s^{-1} in a growth room at 26±2°C. On the day of transplanting and after 10-12 days of transplanting, plants were fertilized with 100 mL of 20-20-20 fertilizer (1g/L) per pot. When plants reached 10 inch (±2) in height, they were used for treatments.

4.4 Plant Treatments and Re-watering

4.4.1 Treatments

Six different treatments, as listed in Table 4.1, were applied. Fertilizer (20-20-20) was used at a rate of 1g/L. LANS-modified solution was equivalent to inorganic fraction of ANE and its concentration was equivalent to 3.5 mL/L ANE (recommended concentration for plants). Desired concentrations of modified LANS and ANE were mixed with 20-20-20 fertilizer and used for the treatments.

Table 4.1: Treatments Used in the Study

Treatment No.	Type	Rate
1	20-20-20	1 g/L
2	20-20-20+ LANS -Modified	1 g/L + 100 mL/L (0.1 x)
3	20-20-20+ ANE	1 g/L + 0.875 mL/L
4	20-20-20+ ANE	1 g/L + 1.75 mL/L
5	20-20-20+ ANE	1 g/L + 3.5 mL/L
6	20-20-20+ ANE	1 g/L + 7.0 mL/L

4.4.2 Application of Six Treatments and Re-watering

Six treatments were applied twice during the first 48 hours (36 plants/ treatment). Plants were treated with six treatments during each of the two applications, as mentioned in Table 4.1. From the second application until the end of Day 5, plants were not watered.

At the end of Day 5, plants were saturated and brought back to field capacity, with the objective of examining the ability of the plants to recover from drought stress after the different treatments (Figure 4.1).

Figure 4.1. Schematic diagram of experiment time line**. Plants treated twice in 48 h interval (Between Day -2 and Day 0). Day 1 - 1st sample/ measurement. Day 5 - Re-watering at the end of Day 5. Day 6 – final sampling day for biochemical and gene expression studies. Day 7 – final sampling day for physiological parameters except stomatal conductance. Day 8- final day for visual ratings and stomatal conductance measurements. Sampling Days 3, 5.25b(6 h after re-watering) and 5.5 (12 h after re-watering) not shown.
** *In-vitro* rooting experiment not followed the above time line.

4.5 Determination of Volumetric Water Content

Volumetric water content was recorded throughout the experiment. EM50 data collection system and EC-5 soil moisture sensors (Decagon Devices, Inc, USA) were used to record the soil moisture data.

4.6 Experiment 1: Physiological Examinations

Visual observations, stomatal conductance and recovery percentage were determined using the same set of plants.

4.6.1 Statistical Design and Sampling

"Row + Column" design, with 3 replicates and 2 sub samples, was applied in the experiments. Blocking was done according to the three shelves and three sampling days to minimize the variability. Two sampling methods were used. For non-destructive sampling experiments (visual observations and stomatal conductance), readings were taken daily. For destructive sampling experiments (plant water potential, leaf relative water content and plant dry matter content), reading were taken at Days 1, 3 and 5 before re-watering and Days 5.5 (12 h), 6 and 7 after re-watering. Data analysis was carried out using one way analysis of variance (ANOVA), with the repeated measures at the 5% level of significance and GenStat® (16^{th} edition) software (VSN International Ltd, Hempstead, UK) was used.

4.6.2 Visual Observations

Wilting intensity was graded using a scale made according to Engelbrecht *et al.* (2007), with minor modifications, (wilting was graded in to five groups instead of six groups) (Figure 4.2). Plants were observed daily from the first day of treatments until the end of the experiment. During the experiment, wilting of the plants and recovery after re-watering were observed. Visual observations were taken daily from Day 1 to Day 5 before re-watering. Observations were then made at 3h, 6h, 12h, 24 h (Day 6), 48 h (Day 7) and 72 h (Day 8) after re-watering.

Figure 4.2. Visual Scale for wilting intensity. 1. Healthy plant. 0% wilted. 2. 25% wilted. 3. 50% wilted. 4. 75% wilted. 5. Totally (100%) wilted.

4.6.3 Stomatal Conductance

A portable steady state diffusion porometer (Model SC-1, Decagon Devices, Inc, USA) was used to measure stomatal conductance in tomato plants. Data was collected from each plant from each block daily from Day 1 until Day 5 before re-watering and 6 h (Day 5.25), 12 h (Day 5.5), 24 h (Day 6), 48 h (Day 7) and 72 h (Day 8) after re-watering.

4.6.4 Recovery Percentage

If one or more leaves indicated un-wilted after re-watering, the plant was considered as a recovered plant. It was found during preliminary experiments that at least one leaf was able to recover after re-watering, that plant was able to survive in future. Numbers of recovered plants after re-watering were checked at 6 h, 12 h, 18 h, 24 h, 48 h and 72 h and was expressed as a percentage. Data was analyzed using one way analysis of variance (ANOVA), with the repeated measures at the 5% level of significance, performed using SAS software (version 9.4, SAS Institute Inc., NC, USA). Tukeys multiple mean comparison method was used to analyze the difference between the means.

4.6.5 Determination of Plant Water Potential

A pressure chamber instrument (Model 615, PMS instrument company, USA) was used to measure plant water potential. Readings were taken at Days 1, 3 and 5 before re-watering and 12 h (Day 5.5), 24 h (Day 6) and 48 h (Day 7) after re-watering.

4.6.6 Leaf Relative Water Content

Tomato leaves were used to measure relative water content (RWC). First, all the leaves from each experimental unit were removed using a pair of scissors and fresh weight was recorded. They were then floated on deionised water for 5 h under low irradiance and the leaves were blotted firmly until all the surface was water removed. Then turgid weight was recorded from the blotted leaf samples. All the leaf samples were oven dried at 70°C for 72 h and dry weights were determined. Sampling was done on Days 1, 3 and 5 before re-watering and 12 h (Day 5.5), 24 h (Day 6) and 48 h (Day 7) after re-watering. By using these data, RWC was calculated

$$\text{RWC (\%)} = \left[\frac{Fresh\ Weight - Dry\ Weight}{Turgid\ Weight - Dry\ Weight}\right] \text{X } 100$$

4.6.7 Plant Dry Matter Content

All the leaves and shoots were removed from plants. First, fresh weights were recorded and all the samples were oven dried at 70°C for 72 h. After 72 h, dry weights were recorded in all the samples. Total dry matter content was calculated on Days 1, 3 and 5 before re-watering and 12h (Day 5.5), 24h (Day 6) and 48 h (Day 7) after re-watering.

$$\text{Dry matter (\%)} = \left[\frac{Dry\ Weight}{Fresh\ Weight}\right] \text{X } 100$$

4.6.8 Root Growth

The experiment was performed in-vitro. Sterilized tomato seeds were grown on Petri plates containing ½ MS media, when plants were 2 weeks old, they were assigned to 6 different treatments, as mentioned in Table 4.2.

Table 4.2 Treatments used in root growth assay

Treatment no.	Type	Amount
1	MS	30 mL
2	MS + PEG	30 mL + 45 mL
3	MS + ANE (0.175 mL/L)	30 mL
4	MS + ANE + PEG	30 mL + 45 mL
5	MS + LANS-M (Equivalent to ANE)	30 mL
6	MS + LANS-M + PEG	30 mL + 45 mL

Plants were transferred into 100 mm X 100 mm square plates containing 30 mL of MS medium, MS medium supplemented with different combinations of Polyethylene glycol (PEG), ANE, ANE LANS. Media was prepared without adding sucrose. PEG 8000 was used to induce drought stress in the plates. ANE 0.175 mL/L, LANS-modified (equivalent to ANE 0.175 mL/L) and control plates with and without PEG were used in the experiment.

By using 2:3 ratio (media: PEG) in 440 g/L concentration of PEG, -0.7 MPa drought stress was supplied to the stressed plates, as mentioned by Verslues *et al.,* in

2006 and all the controls were not supplemented with PEG. MS plates were filled with 45 mL of PEG and kept overnight. The remaining PEG was drained before the plants were introduced into the media.

Root length and area were observed daily from Day 1 to Day 7, using the WinRHIZO® software (Epson Expression 10000 XL scanner). Each experimental unit consists of 2 sub samples and each treatment had 6 replicates in a completely randomized design. Data was analyzed using one way analysis of variance (ANOVA), with the repeated measures at the 5% level of significance, performed using SAS software (version 9.4, SAS Institute Inc., NC, USA). Tukeys multiple mean comparison method was used to analyze the difference between the means.

4.7 Experiment 2: Biochemical Analysis

All the biochemical analyses were based on spectrophotometric detection methods and they were carried out in 96 well plates. All the samples were run in triplicate and a BioTek Power XS2 microplate reader (VT, USA) with Gen5™ software was used to measure the absorbance in respective wave lengths.

4.7. 1 Tissue Collection and Storage

Tomato leaves were cut with scissors and wrapped in aluminum foil to avoid contamination. Tomato leaves were flash frozen in liquid nitrogen. Frozen leaves were stored at -80 ºC until use.

4.7.2 Statistical Design and Sampling

"Row + Column" design with 3 replicates and 2 sub samples was applied in the experiments. Samples were taken at Days 1, 3 and 5 before re-watering and 6h (Day 5.25), 12 h (Day 5.5) and 24 h (Day 6) after re-watering. Data was analyzed, as mentioned above.

4.7.3 Antioxidant Enzyme Assays

4.7.3.1 Crude Protein Extraction

Frozen leaf samples were lyophilized and soluble proteins were extracted by homogenizing 30 mg of sample in 1 ml of 0.1 M potassium phosphate buffer (pH 7.5) containing 3 mM EDTA and 0.5% polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 12,000 X g for 20 min at 4°C and the supernatant was used for the antioxidant enzyme assays (He *et al*., 2009; Sarkar *et al*., 2009).

4.7.3.2 Determination of Total Protein

The supernatant was collected as the crude enzyme extract. Protein concentration was measured using the Coomassie Plus – The Better Bradford™ Assay Kit (Pierce, Rockford, IL, USA). Two hundred micro liters of the Bradford reagent was added to 35µL of H_2O and 5µl of crude enzyme extract the absorbance was read at 595 nm. The amount of protein per sample was calculated using bovine serum albumin (BSA) standard (0.1–2 mg/mL) (Figure 4.3). A concentration gradient of BSA (0.1 mg/mL, 0.2 mg/mL, 0.3 mg/mL, 0.4 mg/mL, 0.5 mg/mL, 0.6 mg/mL, 0.7 mg/mL, 0.8 mg/mL, 0.9 mg/mL, 1 mg/mL. 1.5 mg/mL and 2 mg/mL) was prepared by dissolving BSA in water. After

reacting with Bradford™ reagent, absorbance was measured. Absorbance values were plotted against BSA concentration to obtain the standard curve

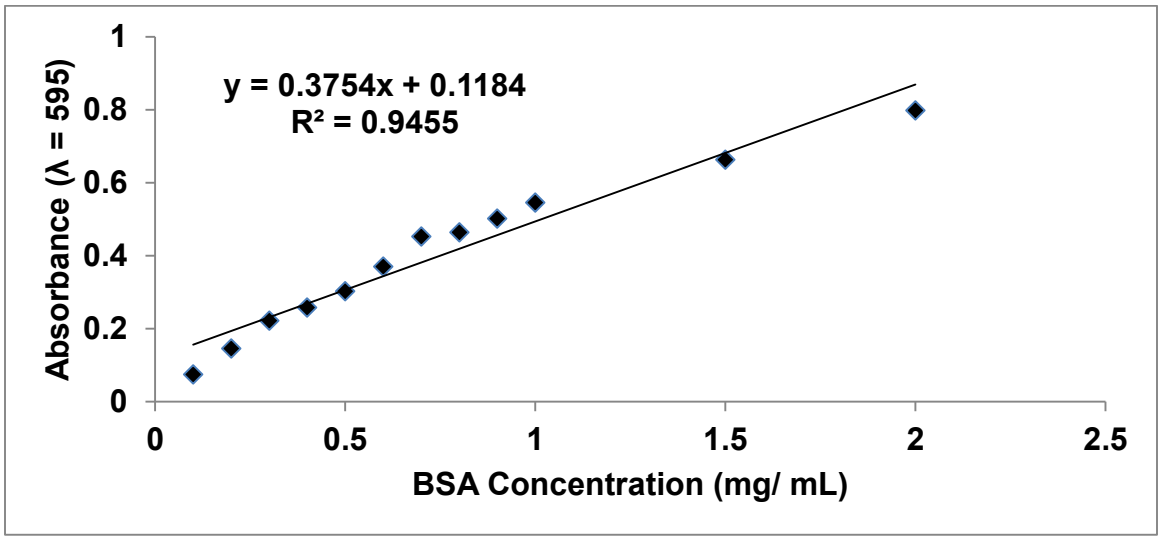

Figure 4.3. BSA Standard Curve

4.7.3.3 Estimation of Superoxide Dismutase (SOD) Activity

Total SOD activity was determined using a SOD determination kit (Sigma-Aldrich) and SOD amounts were determined using a standard curve, developed by using SOD from bovine erythrocytes (S7571) from Sigma-Aldrich. Absorbance was measured at 450 nm.

4.7.3.4 Estimation of Catalase (CAT) Activity

The activity of CAT was calculated as a decrease in absorbance at 240 nm for 3 min, following the decomposition of H_2O_2 (Chance and Maehly, 1955). The absorbance of the 205µL reaction mixture, containing 200 µL 0.059 M hydrogen peroxide, in 0.05 M

potassium phosphate buffer at pH 7.0, and 5 µL of crude enzyme extract, was measured at 240 nm every 20 s for a period of 3 min (extinction coefficient 39.4 mM^{-1} cm^{-1}) (Yordanova *et al*., 2004).

4.7.3.5 Estimation of Guaiacol Peroxidase (GPOD) Activity

GPOD activity was determined as a decrease in absorbance at 470 nm for 3 min, using the procedure described by Elegy *et al* (1983) with slight modifications. The absorbance of the 205µL reaction mixture, containing 5 µL of crude enzyme extract and 200µL 0.05 M guaicol solution, in 25 mM sodium acetate buffer at pH 5.0 and 8.8 mM hydrogen peroxide, was measured at 470 nm every 20 s for a period of 3 min (extinction coefficient 26.6 mM^{-1} cm^{-1}) (He *et al*., 2009).

4.7.4 Determination of Lipid Peroxidation

Lipid peroxidation was measured in terms of malondialdehyde (MDA) content following a method described by Dhindsa *et al*. (1981). Briefly, 200 µL of supernatant of leaf extracts was mixed with 800 µL of 20% (w/v) trichloroacteic acid containing 0.5% (w/v) thiobarbituric acid. The mixture was heated at 100 $^{\circ}C$ for 30 minutes, quickly cooled, and then centrifuged at 10,000 g for 10 minutes. The absorbance of the supernatant was measured at 532 nm and 600 nm (extinction coefficient of 155 mM^{-1} cm^{-1}) (Health and Packer, 1968).

4.7.5 Determination of Proline

Proline content was determined using the cold extraction method described by Carillo *et al*. (2008). First, 30 mg of freeze dried sample was taken and transferred into a

2 mL screw cap micro centrifuge tube (VWR, Mississauga, ON, Canada). The sample was extracted using 1 mL of 70% ethanol. Each sample was centrifuged at 12,000 g for 15 minutes at 4 °C and stored in ice until use. Both standards and extracted samples were analyzed in same manner and 500 µL of sample was mixed with 1 mL of reaction mixture, which contains 1% ninhydrin (w/v) in 60% glacial acetic acid (v/v) and 20% ethanol (v/v). All the samples were sealed and heated at 95 °C for 20 minutes in a water bath. After 20 minutes, samples were transferred into ice, cooled and spun at 2,500 g for 1 minute. Absorbance of the product was measured at 520 nm and proline amount was measured using the L-proline standard curve (0.01 mM – 2mM).

4.7.5.1 Preparation of Standard Curve for L-Proline

A concentration gradient of L-proline (0.01 mM, 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, 0.6 mM, 0.7 mM, 0.8 mM, 0.9 mM, 1 mM and 2 mM) was prepared by dissolving L- proline in 70% ethanol. Each standard (500 µL) was reacted with the 1mL of reaction mixture, as described above and absorbance values were recorded. Absorbance values were plotted against L-proline concentration to obtain the standard curve (Figure 4.4).

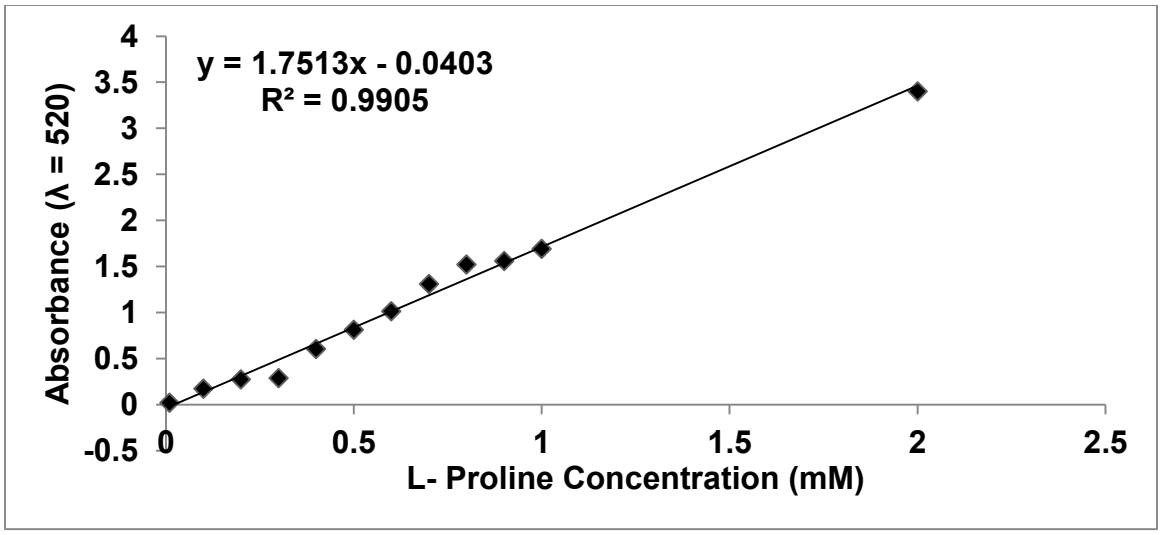

Figure 4.4. Standard curve for L-proline

4.7.6 Determination of Total Chlorophyll and Total Carotenoids

Total photosynthetic pigments (Chlorophyll *a*, chlorophyll *b*, total chlorophyll and total carotenoids) were determined using 100% dimethyl sulfoxide (DMSO) (Wellburn, 1994).

Briefly, DMSO was pre-heated to 65 °C. Leaves were removed from respective treatments using scissors. Removed leaves were transferred into 15 mL Falcon™ centrifuge tubes (VWR, Mississauga, ON, Canada) and 6 mL of pre-heated DMSO was added to sample. Tubes were kept in 65 °C water bath for 30 minutes. Absorbance of extracts was measured at 665 nm, 649 nm and 480 nm. The following equations were used to calculate the pigment amount in each sample.

$$Chlorophyll\ a = (12.47\ X\ A665) - (3.62\ X\ A649)$$

$$Chlorophyll\ b = (25.06\ X\ A649) - (6.50\ X\ A665)$$

$$Total\ Chlorophyll = Chlorophyll\ a + Chlorophyll\ b$$

$$Total\ Carotenoids = \frac{1000A480 - 1.29\ Chlorophyll\ a - 53.78\ Chlorophyll\ b}{220}$$

$$Chlorophyll\ a{:}\ b = \frac{Chloropgyll\ a}{Chlorophyll\ b}$$

$$Carotenoids : Total\ Chlorophyll\ = \frac{Total\ Carotenoids}{Total\ Chlorophyll}$$

4.8 Experiment 3: Quantitative Gene Expression Analysis

4.8.1 Tissue Collection and Storage

Tomato leaves were collected and stored at -80°C until use, as described in section 4.7.1.

4.8.2 Isolation of RNA

Total RNA was extracted using a RNeasy plant mini kit (Qiagen, Mississauga, ON, Canada). RNA concentration was quantified using a NanoDrop 2000

spectrophotometer (Thermo Scientific, USA) and RNA quality was confirmed by visualized RNA bands after resolving on 1.5% agarose gel.

4.8.3 RNA Purification and cDNA Synthesis

RQ1 DNAse (Promega Inc., USA) was used to treat RNA according to the manufacturer's instructions. DNAse treated RNA was reverse transcribed using a high capacity cDNA reverse transcript kit (Applied Biosystems, ON, Canada), according to the manufacturer's instructions.

4.8.4 Real Time qPCR Analysis

A StepOne™ Real-Time qPCR System (Applied Biosystems, CA) was used to perform real time qPCR. Ten microliters of total reaction mixture, containing 50 ng of cDNA, 20 ng of gene specific primers, 5 µL of 2X SYBR green reagent (GoTaq® qPCR Master Mix, Promega, Madison, WI, USA) and 2.5 µL DEPC water, was prepared. Transcript levels of each gene were normalized to the expression of *ACT* (Actin) gene (U60480) and β-*Tubulin* gene (DQ205342). Fold changes of treated plants were relative to fertilizer control plants.

P5CS (Δ-pyrroline-5-carboxylate synthetase) (AY897574), *LEA* (late embryogenesis-like protein) (Z46654), *ltpg2* (lipid transfer protein) (U81996), (Lu *et al.,* 2010), *SlAREB-1* (ABA-response element binding protein 1) (NM001247667), (Bastias, *et al*., 2011), *LeMCA1* (metacaspase 1) (AY114141) (Wen *et al.,* 2013; Hoeberichts, *et al.,* 2003) were picked for quantitative PCR and gene specific primers were used for all the genes (table 4.3).

Real Time qPCR conditions for gene specific primers were as follows; heat activation at 95 ºC for 10 min, denaturation at 95 ºC for 15 s, annealing and final extension at 60 ºC for 1 min followed by 40 cycles. Relative transcript levels were analyzed using the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001).

Table 4.3: Primers Used in the Study

Gene	Gene Specific Primer
ACT	F 5' TGGGATGATATGGAGAAGATATGG 3'
	R 5' GGCTTCAGTTAGGAGGACAGGA 3'
β-*Tubulin*	F 5' TGGATCTGGCATGGGAACAC 3'
	R 5' TGCACCGACAGTGTAGCATT 3'
P5CS	F 5' ATCAAAGTGCTGCATGGGGA 3'
	R 5' GCCTGAAGACGTCTGGAACA 3'
LEA	F 5' CAGCTCAGGCAACCCATGAT 3'
	R 5' TGCTCCACTAGCCTTCTCCT 3'
ltpg2	F 5' CCCAGAAGACCGGAAGACAG 3'
	R 5' GAGCAATCAGTGGAAGGGCT 3'
SlAREB1	F 5' GTTGGTGTTCCAGGTGGTCA 3'
	R 5' TTTGGTGGCACTCCAACACT 3'
LeMCA1	F 5' GAGGTCTTCCAGACAGTGGC 3'
	R 5' ACTGGACCATCTGATTCGGC 3'

4.8.5 Statistical Analysis

One way analysis of variance (ANOVA), with the repeated measures at the 5% level of significance, was performed using SAS software (version 9.4, SAS Institute Inc., NC, USA). Tukeys multiple mean comparison method was used to analyze the difference between the means.

4.9 Experiment 4: Greenhouse Experiments

4.9.1 Plant Materials and Treatments

All the recovered plants, after re-watering from the recovery experiment, and all the same age unstressed plants were used. Plants were transferred into 10 inch plastic pots. Recovered plants were treated with 500 mL of respective treatments and unstressed plants with 500 mL of 20-20-20 (1g/L) fertilizer bi-weekly until the fruits matured. Daily watering was carried out from transferring until the final day of harvest.

4.9.2 Experimental Design

Experimental units were arranged according to Completely Randomized Design. One way analysis of variance (ANOVA), at the 5% level of significance, was performed using SAS software (version 9.4, SAS Institute Inc., NC, USA). Duncan's multiple mean comparison method was used to analyze the difference between the means.

4.9.3 Determination of Fruit Number and Yield

The total number of set fruits was recorded weekly for 16 weeks, from the day of transplanting, and the total number of fruits harvested was recorded for 7 weeks. Fruits

were harvested based on the color change from orange-red to deep red. Individual fruit weights from each harvested plant from each treatment were recorded for 7 weeks. By using the following equations, parameters were calculated for each treatment.

$$Mean\ number\ of\ fruits\ set = \frac{Total\ number\ of\ fruits\ set}{Number\ of\ platns}$$

$$Mean\ number\ of\ fruits\ harvested = \frac{Total\ number\ of\ fruits\ harvested}{Number\ of\ platns}$$

$$Mean\ fruit\ weight = \frac{Total\ harvested\ fruit\ weight}{Number\ of\ harvested\ fruits}$$

$$Total\ yield = \frac{Total\ harvested\ fruit\ weight}{Number\ of\ plants}$$

CHAPTER 5

RESULTS

In this study, a series of experiments were conducted to evaluate the effects of ANE on mitigating drought stress in tomato. To examine the potential use of ANE, experiments were carried out under three different aspects: phenotypic and physiological, biochemical and expression of stress responsive genes. Moreover, ANE was applied to plants which were recovering from stress to evaluate the yields, compared to control treatments.

5.1 Volumetric Water Content

Volumetric water content was determined from Day 1 to Day 8, to examine how ANE treatments affected moisture retention. Volumetric water content was significantly different between treatments ($P=0.0097$), and days ($P < 0.0001$) but did not show any significant difference in interaction between treatments and days ($P=1.0000$).

On Day 1, the volumetric water content of all the groups ranged from 0.35 m^3/m^3 to 0.4 m^3/m^3 (Figure 5.1). Soil moisture decreased gradually and by the end of Day 5, it reached around -0.07 m^3/m^3. Moisture levels of highest concentrations of ANE (3.5 mL/L and 7.0 mL/L) groups were slightly higher (at day 3, 0.0197 m^3/m^3 and 0.0538 m^3/m^3 respectively), compared to the rest of the groups, up to Day 3. After re-watering, all the groups increased moisture levels, but they did not reach to the original levels (~ 3 m^3/m^3).

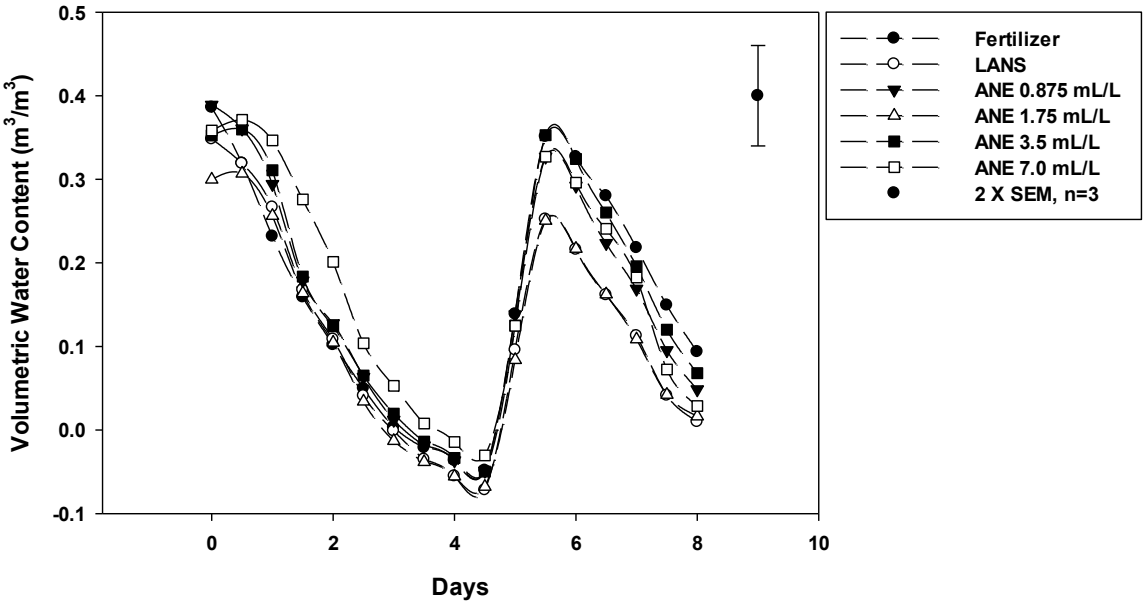

Figure 5.1. Soil moisture level variation in pots treated with fertilizer (20-20-20), LANS and 4 levels of ANE (0.875 mL/L, 1.75 mL/L, 3.5 mL/L and 7.0 mL/L). Moisture readings were recorded at every 10 min from Day 1 to Day 8. n=3.Vertical bar represents two standard errors of the mean (SEM).

5.2 Experiment 1: Physiological Examinations

With adequate supply of water, plants function normally. However, under stress, their physiological functions were reduced compared to non-stressed plants, and this was clearly evident morphologically in stressed plants. These set of experiments were conducted with the objective to examine how tomato plants function under drought stress with the application of ANE, compared to LANS and fertilizer treatments.

5.2.1 Visual Observations

Wilting was observed from Day 1 to Day 5 before re-watering and 6 h (Day 5.25), 12 h (Day 5.5), 24 h (Day 6), 48 h (Day 7) and 72 h (Day 8) after re-watering. It was rated with the objective of determining the effect of different treatments on plants under drought stress. An interaction was found between the treatments and the days (Figure 5.2) and a positive quadratic relationship was evident with the wilting rating ($P<0.001$).

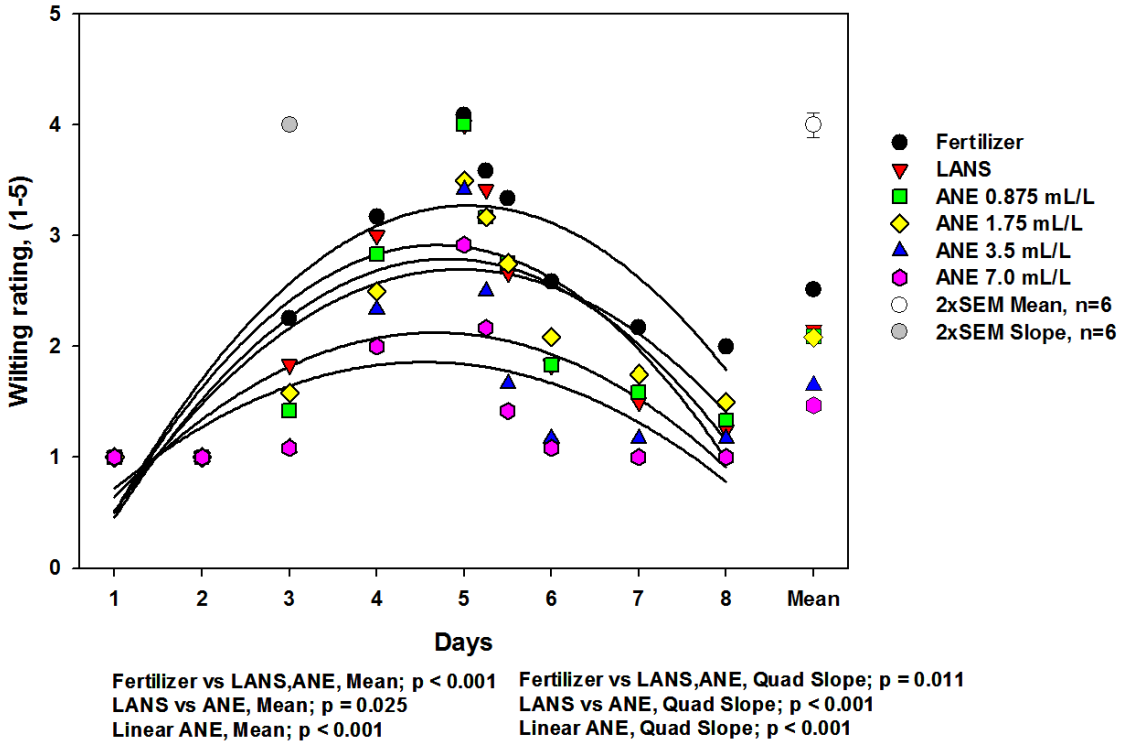

Figure 5. 2. Wilting level change with fertilizer (20-20-20), LANS and 4 levels of ANE (0.875 mL/L, 1.75 mL/L, 3.5 mL/L and 7.0 mL/L) treatments. Number of replicates (n) = 6, vertical bar represents two standard errors of the mean (SEM). Significant treatment effects for wilting at fertilizer vs rest ($P < 0.001$), with quadratic responses ($P = 0.011$) and LANS vs ANE ($P =0.025$), with quadratic response ($P < 0.001$).

For the first two days (Days 1 and 2), none of the treatments exhibited wilting symptoms and all the plants appeared the same. On Day 3, fertilizer, LANS and the two

lowest concentration of ANE treated plants indicated 25% of wilting, but 3.5 mL/L and 7.0 mL/L ANE treated plants still appeared healthy. By Day 4, all the plants showed signs of wilt, but 3.5 mL/L and 7.0 mL/L ANE treated plants appeared less wilted, compared to the rest of the treatments. By the end of the Day 5, 7.0 mL/L ANE treated plants appeared less wilted compared to others (50% vs 60-75%). Six hours after re-watering, all ANE treated plants showed less wilting compared to fertilizer and LANS treated plants. Among them, 3.5 mL/L and 7.0 mL/L ANE treated plants reported less wilting intensity (2) compared to 0.875 mL/L and 1.75 mL/L ANE treated plants. Twelve hours after re-watering, 3.5 mL/L and 7.0 mL/L ANE treated plants appeared normal but rest of the groups appeared wilted. Though they showed wilting symptoms, the intensity was lesser than when they were at 6 h after re-watering. By Day 6 (24h after re-watering), fertilizer, LANS, 0.875 mL/L ANE and 1.75 mL/L ANE treated plants displayed lesser wilting (2). By Day 7, all the ANE treated plants recovered but fertilizer and LANS treated ones remained wilted (2). Fertilizer treated plants did not recover by the end of Day 8 and remained at 25% wilted level (Figure 5.3).

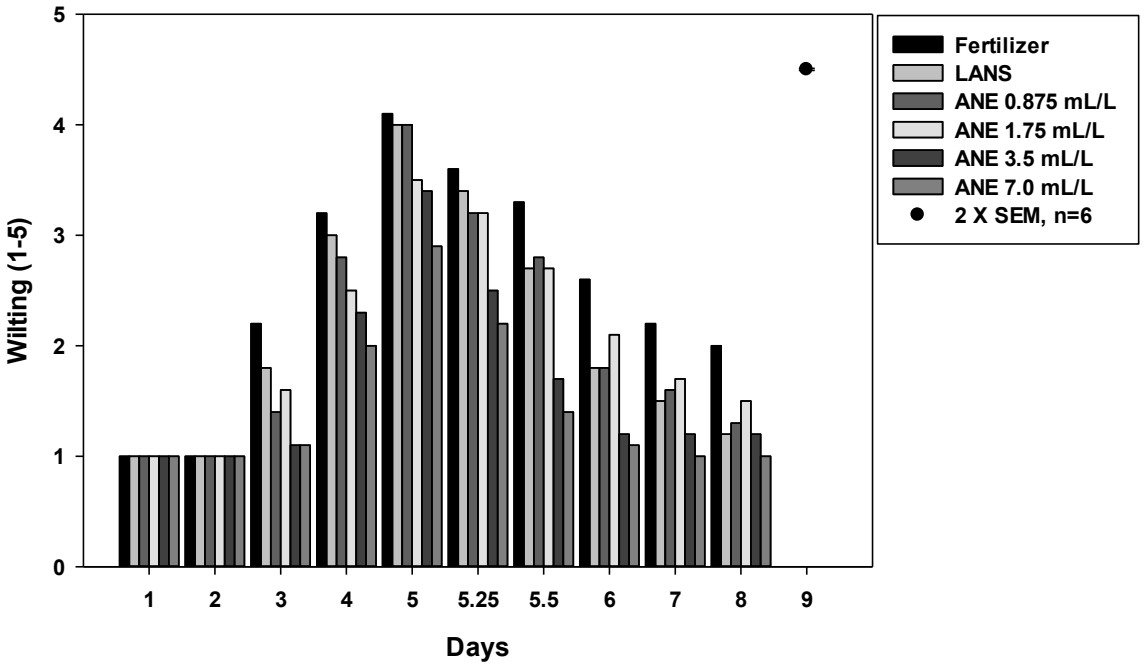

Figure 5.3. *Ascophyllum nodosum* extract reduce plant wilting. 1 represent very outstanding healthy plant and 5 represent very poor quality plant (100% wilted). Six treatments; fertilizer (20-20-20), LANS and 4 levels of ANE (0.875 mL/L, 1.75 mL/L, 3.5 mL/L and 7.0 mL/L) applied. Number of replicates (n) = 6 and experiment was repeated 2 times. Vertical bar represents two standard errors of the mean (SEM).

5.2.2 Stomatal Conductance

Stomatal conductance is a measure of plant air movement through stomata and under drought stress it is reduced by a considerable amount. Stomatal conductance was measured with an objective of understanding how ANE treatments affected plant air movement under drought stress. Stomatal conductance showed significant difference between treatments ($P = 0.024$) and a negative linear relationship was observed between the treatments and days (Figure 5.4).

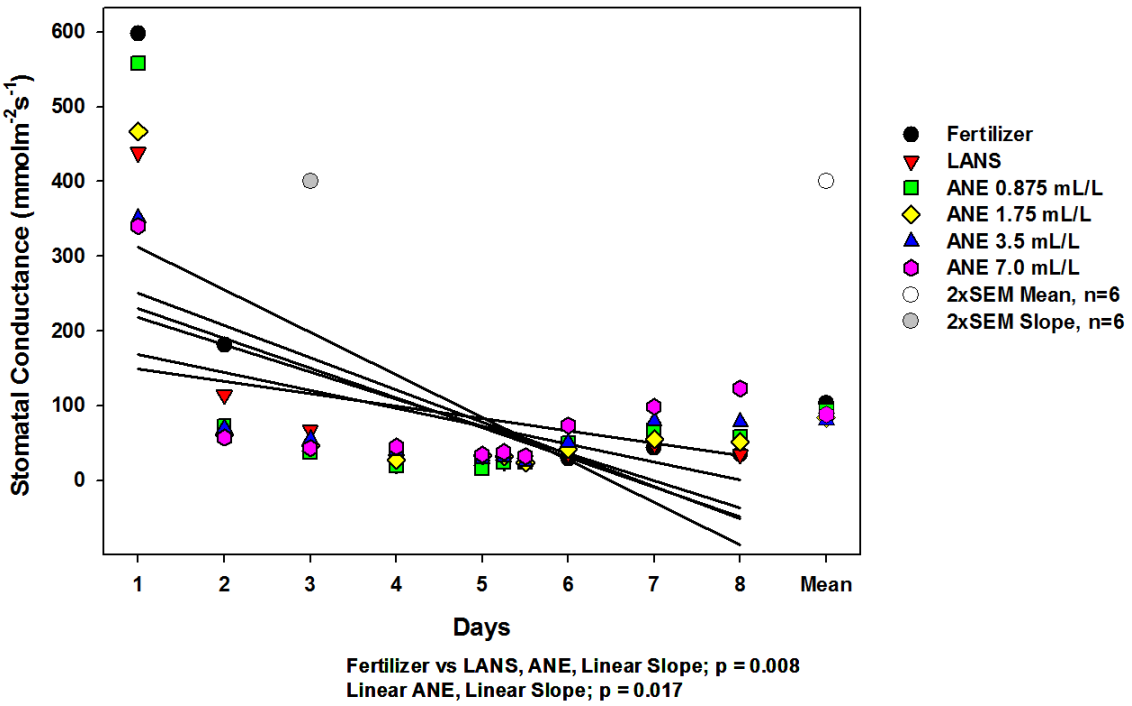

Figure 5.4. Stomatal conductance change with fertilizer (20-20-20), LANS and 4 levels of ANE (0.875 mL/L, 1.75 mL/L, 3.5 mL/L and 7.0 mL/L) treatments. Number of replicates (n) = 6. Vertical bar represents two standard errors of the mean (SEM). Significant conductance effect for treatment to days initiation (P = 0.008), with negative linear response for N addition (P = 0.035).

On Day 1, all the plants recorded higher stomatal conductance (350 – 450 mmol m^{-2} s^{-1}). On Day 2, the stomatal conductance dropped considerably in all treatments; fertilizer and LANS treated plants recorded conductance around 100 – 150 mmol m^{-2} s^{-1}, while ANE treated plants recorded a conductance of 50 mmol m^{-2} s^{-1}. From Day 3 to Day 5, all recorded conductance around 50 mmol m^{-2} s^{-1}, but 7.0 mL/L ANE treated plants showed slightly higher conductance compared to the rest of the treatments. All the treatment groups recorded the same pattern 12 h after re-watering (~ 50 mmol m^{-2} s^{-1}), but on Day 6 (24 h after re-watering), all concentrations of ANE treated plants indicated

higher conductance. Among ANE treated plants, 7.0 mL/L ANE treated group recorded higher conductance compared to the 3.5 mL/L, 1.75 mL/L and 0.875 mL/L treated groups. By Day 7, both 3.5 mL/L and 7.0 mL/L ANE treated plants' conductance was greater than the rest of the treatments. On Day 8, 7.0 mL/L ANE treated plants recorded a conductance around 100 mmol m^{-2} s^{-1} and other 3 ANE treated groups showed higher conductance compare to fertilizer and LANS treated plants. On Day 8, both fertilizer and LANS treated groups recorded similar conductance in Day 7.

5.2.3 Recovery Percentage

Plant recovery percentage was significantly different between treatments ($P < 0.0001$), and days ($P < 0.0001$) but did not show any significant difference in interaction between treatments and days ($P=0.2068$).

During the first 3 h after re-watering, none of the plants in the control groups (fertilizer or LANS) groups recovered. However, more than 50% of the plants in 3.5 mL/L ANE and 7.0 mL/L ANE treatments recovered. Two lower concentrations of ANE treatments exhibited over 20% recovery. However, six hours after re-watering, all the plants in treatment and control groups started to recover. Fertilizer displayed the lowest recovery percentage (~ 30%) and both 3.5 mL/L ANE and 7.0 mL/L ANE treated plants recorded the highest (~ 84%) recovery percentage of any treatment. Highest concentration of (7.0 mL/L) ANE treated plants recovered 100% recovery by the end of the 12h and 3.5 mL/L ANE treated group recovered completely by the end of 18h. The rest of the groups recovered over 50% by the end of 18 h. All the LANS treated plants recovered by end of 24 h. However, 72 h after re-watering, fertilizer, 0.875 mL/L ANE and 1.75 mL/L ANE treated groups did not recover completely (Figure 5.5).

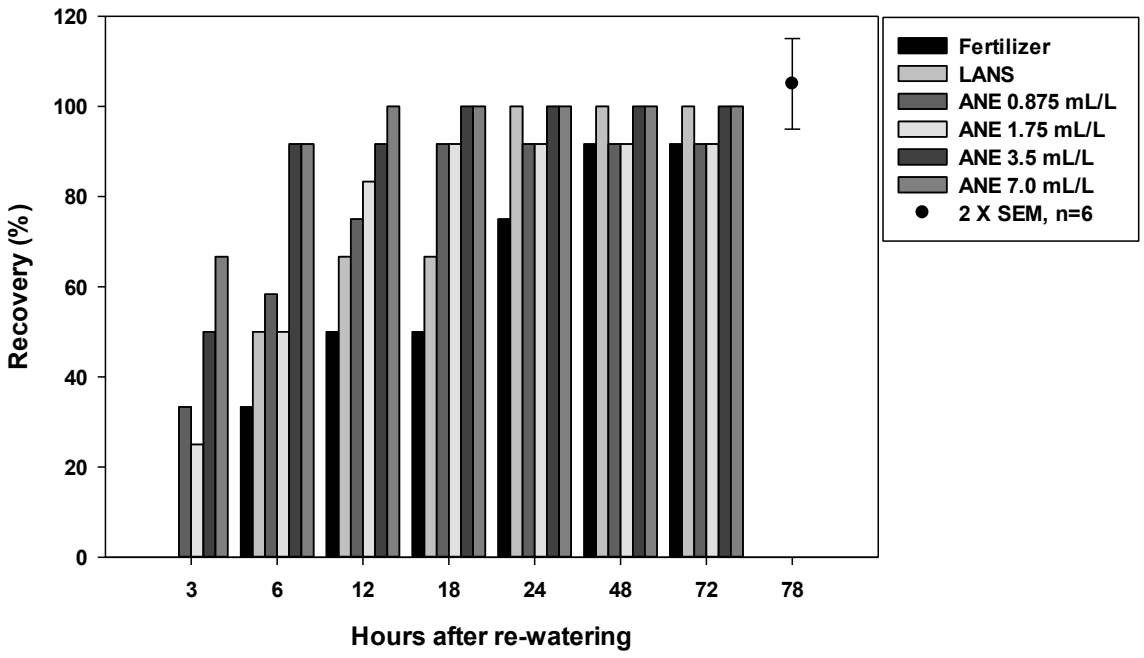

Figure 5.5. Plant recovery after re-watering in six treatments; fertilizer (20-20-20), LANS and 4 levels of ANE (0.875 mL/L, 1.75 mL/L, 3.5 mL/L and 7.0 mL/L). Number of replicates (n) = 6 and experiment was repeated 2 times. Vertical bar represents two standard errors of the mean (SEM).

5.2.4 Plant Water Potential

Plant water potential was measured to understand the effect of ANE treatments on plant water status under drought stress. Plant water potential reduced with time and it increased after re-watering (Figure 5.6). However, there was no significant differences were found between treatments (P=0.545), days (P=0.657) or interaction between treatments and days (P=1.000).

Even though, there is no significant difference between treatments or days, there is a common trend in water potential reduction in all the treatments. On Day 1, all the treatments had plant water potential around -0.2 MPa and with time, it declined. On Day

3, less water potential was observed in all the groups (0.3 – 0.5 MPa), but, highest water potential was recorded in ANE 7.0 mL/L treated group (-0.3 MPa). On Day 5, water potential reduction was greater in all treatments (~ -0.7 MPa). Twelve hours after re-watering (Day 5.5), only ANE 7.0 mL/L treated plants increased water potential, compared to Day 5 (-0.5 MPa) and other groups indicated further reduction in water potential. LANS treated plants recorded the lowest water potential (-1.3 MPa). Fertilizer, 0.875 mL/L ANE, 1.75 mL/L ANE treated plants showed a water potential around -1.0 MPa. By Day 6, highest water potentials were recorded in both 3.5 mL/L ANE and 7.0 mL/L ANE treated plants compared to rest of the groups (-0.5 MPa). Both LANS and fertilizer were the groups with lowest water potential (-1.1 MPa and -0.9 MPa respectively). On Day 7, three highest concentrations of ANE (1.75 mL/L, 3.5 mL/L and 7.0 mL/L) treated plants indicated the highest water potential (-0.5 MPa) and fertilizer treated group was the lowest (-0.9 MPa). However, no treatment group was able to gain its original water potential (-0.2 MPa) by the end of Day 7. Overall, ANE treated plants exhibit higher water potential compared to fertilizer and LANS treated groups.

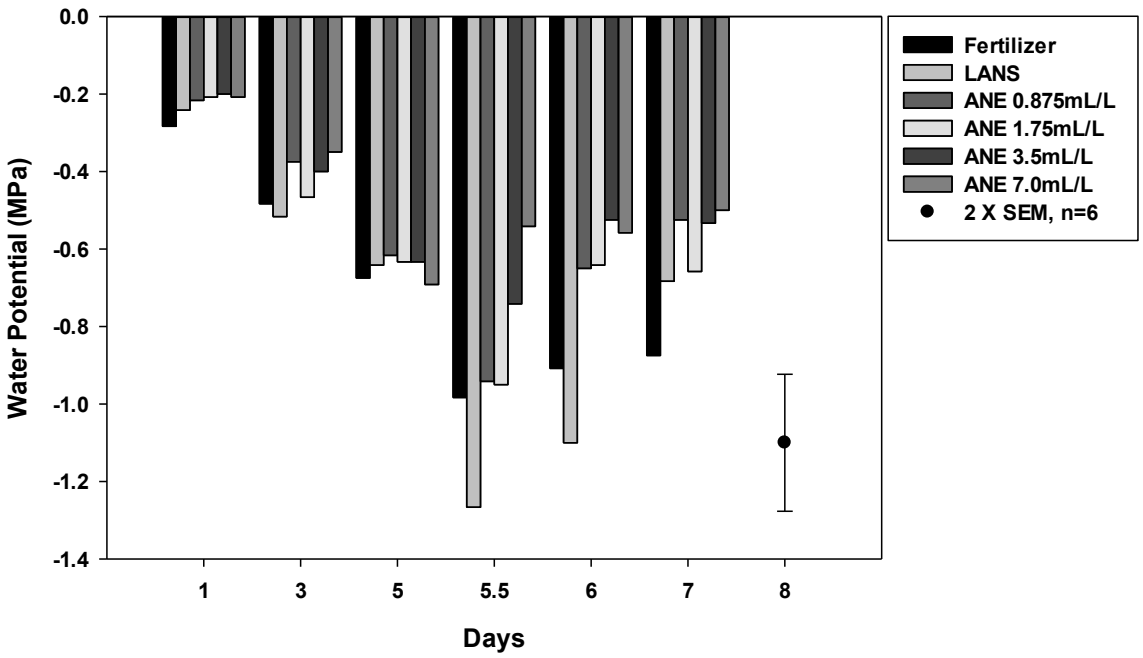

Figure 5.6. Plant water potential change with six treatments; fertilizer (20-20-20), LANS and 4 levels of ANE (0.875 mL/L, 1.75 mL/L, 3.5 mL/L and 7.0 mL/L). Number of replicates (n) = 6 and experiment was repeated 2 times. Vertical bar represents two standard errors of the mean (SEM).

5.2.5 Leaf Relative Water Content (RWC)

Leaf relative water content was reduced with the progression of stress and it increased after re-watering but did not return to the original relative water content at Day 1, by the end of Day 7 (Figure 5.7). There was a significant difference between days (P =0.003), but no significant differences were found between treatments (P = 0.955) or interaction between treatments and days (P = 0.999).

On Day 1, all the treatment groups had similar RWC (~78%), but, compared to Day 1, RWC reduced on Day 3. By Day 5, it reduced considerably in all the treatment

groups (50-60%) and it was the lowest among six days (~ 50%). Twelve hours after re-watering (Day 5.5), RWC increased in all the treatments. By Day 6, RWC reached ~70% and it remained same by Day 7. Even though, there was no significant difference between treatments, both 3.5 mL/L ANE and 7.0 mL/L ANE treated groups showed higher RWC, compared to other 4 groups. Especially, 7.0 mL/L ANE treated plants reached ~70% RWC by 12h after re-watering, and it remained unchanged until Day 7. Further, 3.5 mL/L ANE treated plants exhibited higher RWC compared to the other four treatment groups in the study.

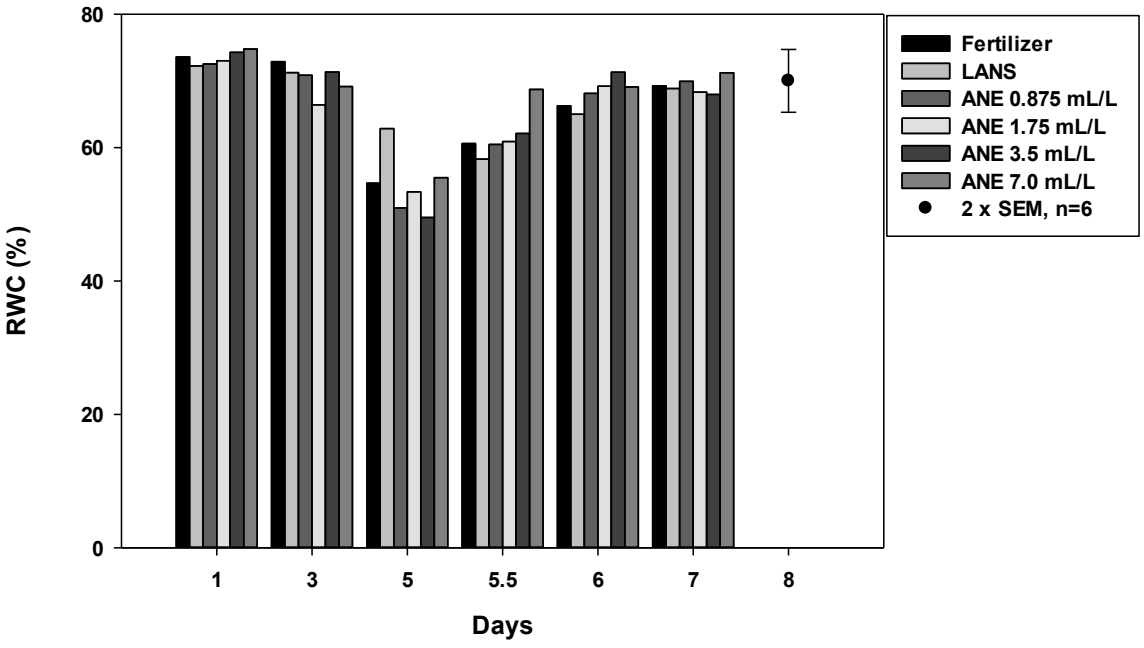

Figure 5.7. Leaf RWC change with six treatments; fertilizer (20-20-20), LANS and 4 levels of ANE (0.875 mL/L, 1.75 mL/L, 3.5 mL/L and 7.0 mL/L). Number of replicates (n) = 6 and experiment was repeated 2 times. Vertical bar represents two standard errors of the mean (SEM).

5.2.6 Plant Dry Matter Content

Plant dry matter content increased with stress over time and it reduced after re-watering (Figure 5.8). There was a significant difference between days (P =0.033) but no significant difference between treatments (P = 0.959) or interaction between treatments and days (P = 0.999).

On Day 1, all the treatment groups had dry matter of 6-7%. Dry matter content increased with the stress. On Day 3, dry matter content was around 8% and on Day 5, it reached the maximum level (~11%). Twelve hours after re-watering (Day 5.5), lower dry matter percentage was noted in all groups compared Day 5. By day 6, it reached around 8% and the level remained unchanged in Day 7.

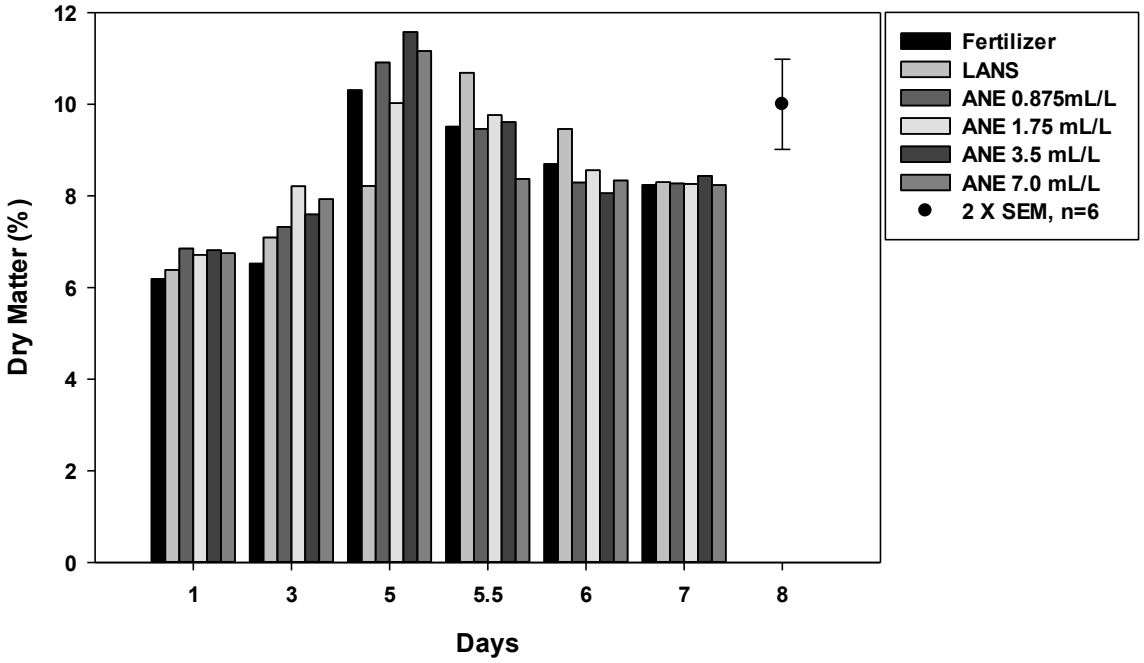

Figure 5.8. Plant dry matter percentage change with six treatments; fertilizer (20-20-20), LANS and 4 levels of ANE (0.875 mL/L, 1.75 mL/L, 3.5 mL/L and 7.0 mL/L). Number of replicates (n) = 6 and experiment was repeated 2 times. Vertical bar represents two standard errors of the mean (SEM).

5.2.7 Root Growth

It was found that ANE treated plants enhanced root generating. Both root area and root lengths of stressed and non-stressed plants were measured to understand how the rooting effected by ANE compared to control treatments.

5.2.7.1 Root Area

There was a significant difference between treatments ($P < 0.0001$) and between days ($P < 0.0001$) but no significant difference in interaction between treatments and days

(P = 0.1025). On Day 1, all the groups had root area around 6.7 cm^2, but with time, the root area increased in the unstressed groups. In stressed groups, a slight increase in root area in all three groups was evident, but the ANE treated group had the least increase compared to the other two groups (Figure 5.9).

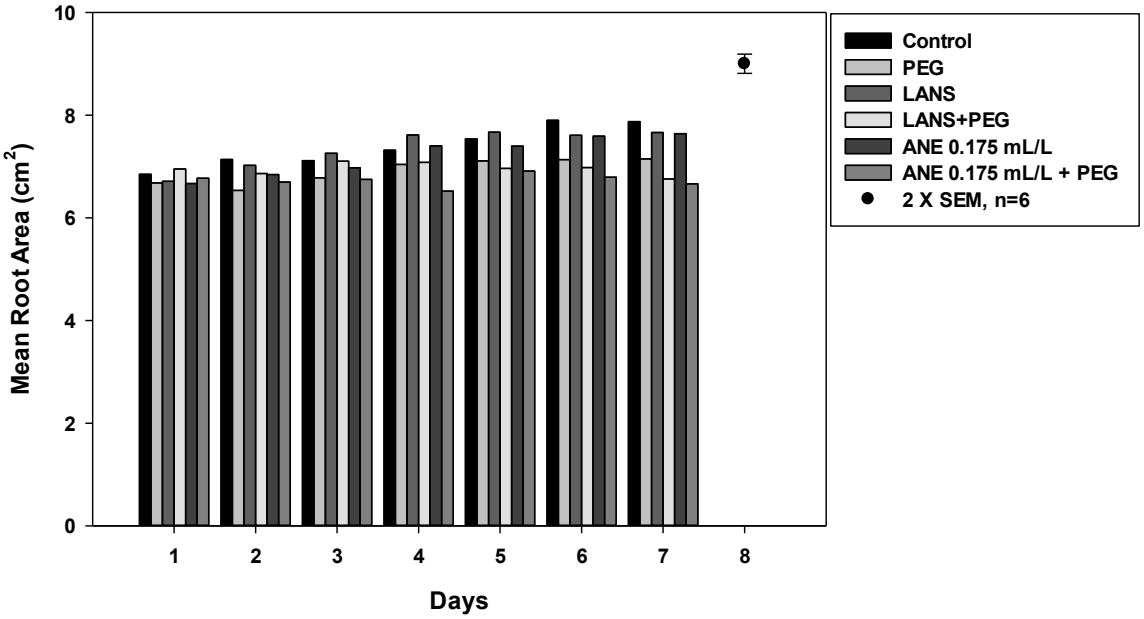

Figure 5.9. Root area change over the time with six treatments; control, PEG, LANS, LANS + PEG, ANE 0.175 mL/L and ANE 0.175 mL/L + PEG. Number of replicates (n) = 6 and experiment was repeated 2 times. Vertical bar represents two standard errors of the mean (SEM).

5.3.7.2 Root Length

There was a significant difference between treatments (P < 0.0001), days (P < 0.0001) and interaction between treatments and days (P < 0.0001). Root length increased over the time. It was evident that the root length increase was higher in all the unstressed groups compared to PEG treated groups. Among the three unstressed groups, the control

group had higher increase compared to LANS and ANE treated groups and it was clearly observed from Day 4 onwards. From the stressed groups (PEG treated), both the control and LANS treated plants showed increase compared to ANE treated group. Root length increased was very less in ANE + PEG treated group (Figure 5.10).

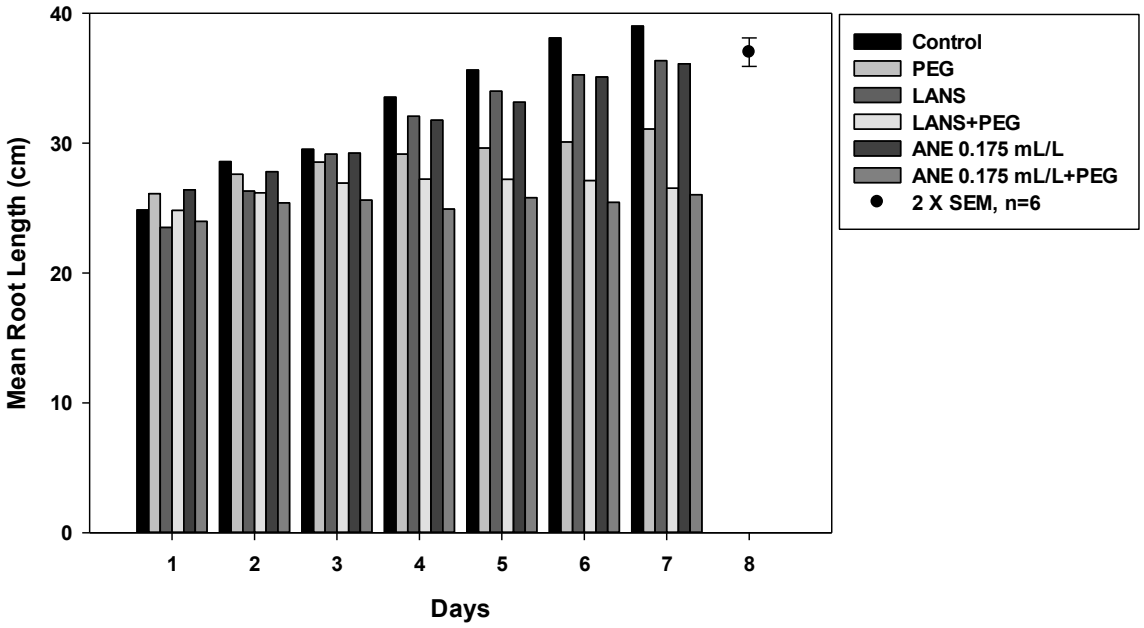

Figure 5.10. Root length change over the time with six treatments; control, PEG, LANS, LANS + PEG, ANE 0.175 mL/L and ANE 0.175 mL/L + PEG. Number of replicates (n) = 6 and experiment was repeated 2 times. Vertical bar represents two standard errors of the mean (SEM).

5.3 Experiment 2: Biochemical Analysis

Under stress conditions, plants under going various processes, such as, production of different substances like ROS, degradation of photosynthetic pigments and accumulation of substances like MDA or proline. These generated substances are either

favorable or harmful to plants. Following set of experiments were done with an objective of examine how these ROS, MDA, porline, and pigments levels were changed with drought stress in ANE, LANS and fertilizer treated plants.

5.3.1 Antioxidant Enzyme Assays

Antioxidant enzymes are the key factors to overcome oxidative stress in plants. Reactive oxygen species (ROS) production increases with the stress. Antioxidant enzyme assays were done to examine how the tomato plants maintain antioxidant levels under stress with application of ANE, LANS and fertilizer.

5.3.1.1 Superoxide Dismutase (SOD) Activity

There was no significant difference in SOD activity between treatments ($P = 0.999$), between days ($P = 0.226$) or interaction between treatments and days ($P = 0.986$). Even though, there was no significant difference observed in days, it was observed Days 1, 3 and 5 indicated similar SOD activities (80-120 U mg protein^{-1}) and SOD activity reduced after re-watering. From six hours after re-watering (Day 5.25) up to Day 6, activity remains on same range (40-80 U mg protein^{-1}) (Figure 5.11).

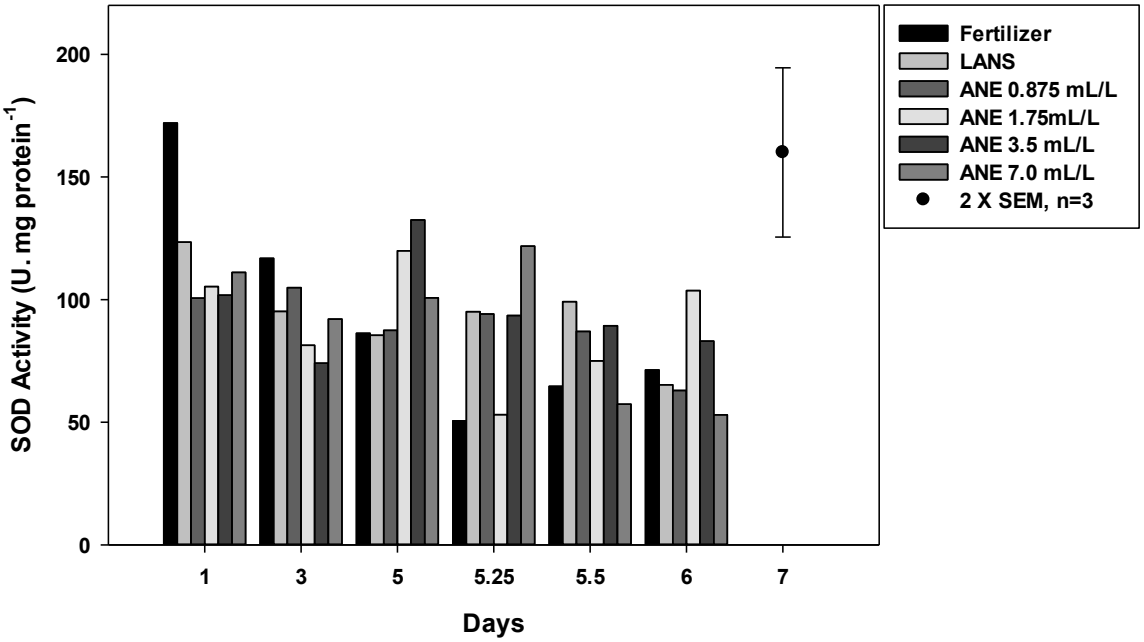

Figure 5.11. Superoxide dismutase (SOD) activity in tomato leaves from six treatments; fertilizer (20-20-20), LANS and 4 levels of ANE (0.875 mL/L, 1.75 mL/L, 3.5 mL/L and 7.0 mL/L). Number of replicates (n) = 3 and experiment was repeated 2 times. Vertical bar represents two standard errors of the mean (SEM).

5.3.1.2 Catalase (CAT) Activity

There was no significant differences in CAT activity between treatments (P = 0.744), between days (P = 0.785) or interaction between treatments and days (P = 0.899) (Figure 5.12). On average CAT activity ranges from 60 µM min^{-1}mg $protein^{-1}$ to 100 µM min^{-1}mg $protein^{-1}$.

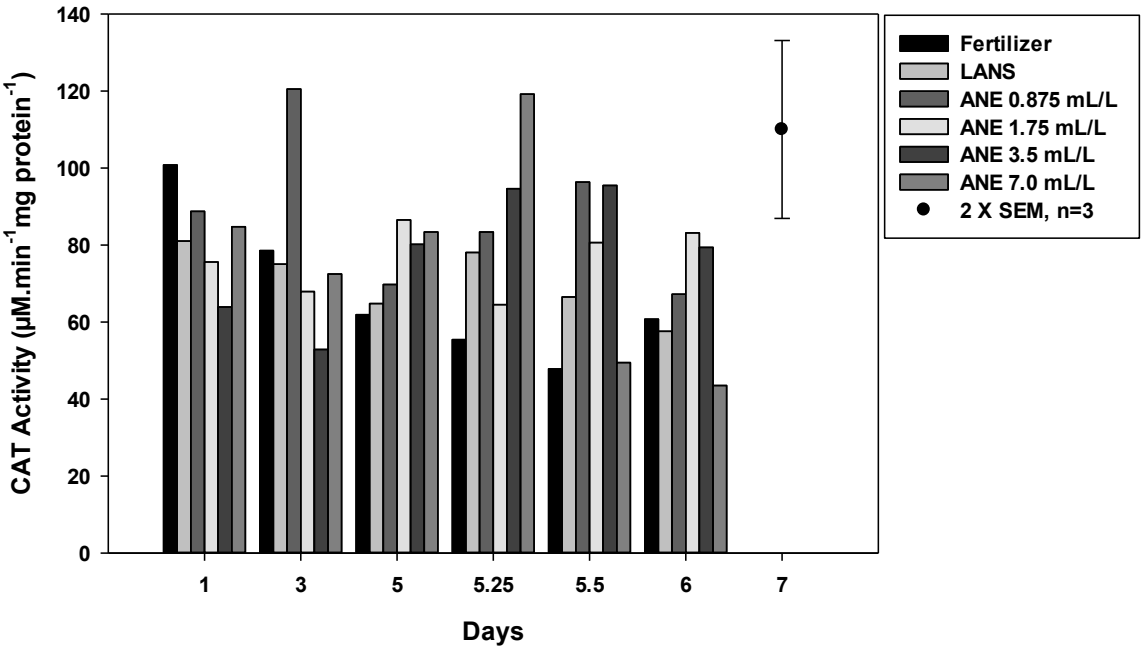

Figure 5.12. Catalase (CAT) activity in tomato leaves from six treatments; fertilizer (20-20-20), LANS and 4 levels of ANE (0.875 mL/L, 1.75 mL/L, 3.5 mL/L and 7.0 mL/L). Number of replicates (n) = 3 and experiment was repeated 2 times. Vertical bar represents two standard errors of the mean (SEM).

5.3.1.3 Guaiacol Peroxidase (GPOD) Activity

The GOPD activity was not affected by treatments (P = 0.852), days (P = 0.825) or interaction between treatments and days (P = 0.923) (Figure 5.13). On average GPOD activity ranges from 2 mM min^{-1}mg $protein^{-1}$ to 4 mM min^{-1}mg $protein^{-1}$.

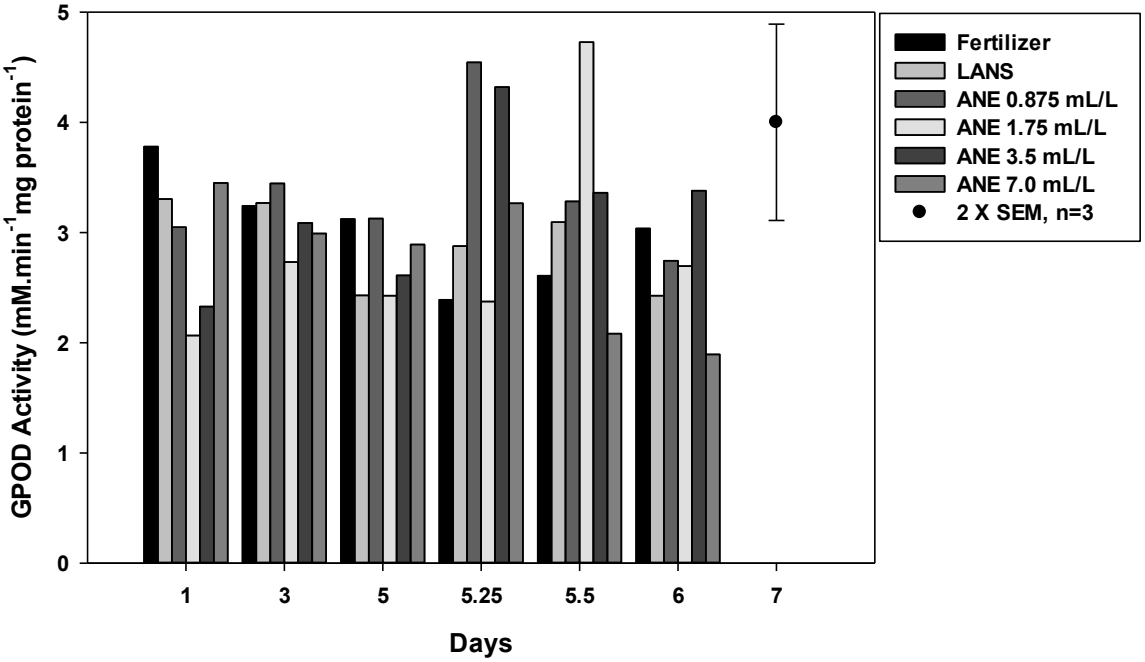

Figure 5.13. Guaiacol peroxidase (GPOD) activity in tomato leaves from six treatments; fertilizer (20-20-20), LANS and 4 levels of ANE (0.875 mL/L, 1.75 mL/L, 3.5 mL/L and 7.0 mL/L). Number of replicates (n) = 3 and experiment was repeated 2 times. Vertical bar represents two standard errors of the mean (SEM).

5.3.2 Lipid Peroxidation

Lipid peroxidation was significantly difference between days (P =0.016) but not affected between treatments (P = 0.505) or interaction between treatments and days (P = 0.702). From Day 1 to Day 5, MDA equivalents increased in all treatments (~ 40 nmol to 65 nmol). Day 5 and 6h after re-watering (Day 5.5), it was remained at the same level (65 nmol). However, MDA equivalents reduced in all groups by Day 5.5 and lowest MDA equivalents were recorded by Day 7 (Figure 5.14).

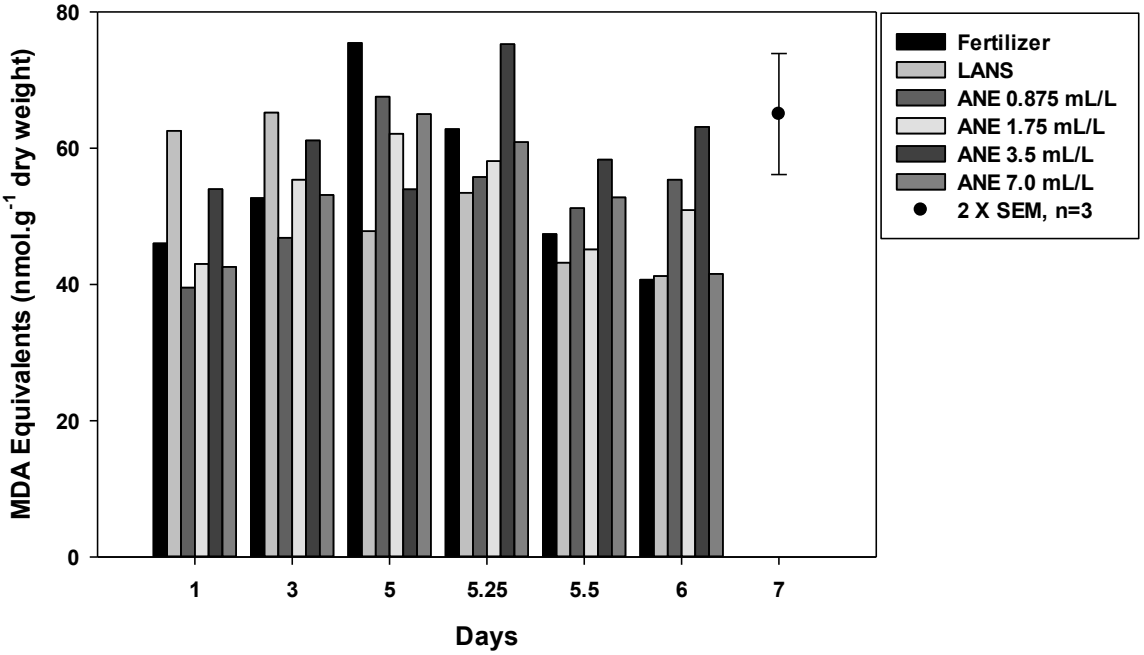

Figure 5.14. Lipid peroxidation in tomato leaves from six treatments; fertilizer (20-20-20), LANS and 4 levels of ANE (0.875 mL/L, 1.75 mL/L, 3.5 mL/L and 7.0 mL/L). Number of replicates (n) = 3 and experiment was repeated 2 times. Vertical bar represents two standard errors of the mean (SEM).

5.3.3 Proline Content

There was a significant different between days in Proline content (P =0.008) but not affected by treatments (P = 0.988) or interaction between treatments and days (P = 1.000). At Day 1, all the groups indicated the lowest proline amount (15 – 20 μM g^{-1}) in the samples and it increased up to Day 3. Days 3, 5 and 5.25 showed similar amount of proline (30 – 45 μM g^{-1}) and it reduced on 12 h after re-watering (day 5.5). By Day 6, it remained same at same level as in Day 5.5 (25 – 30 μM g^{-1}) (Figure 5.15).

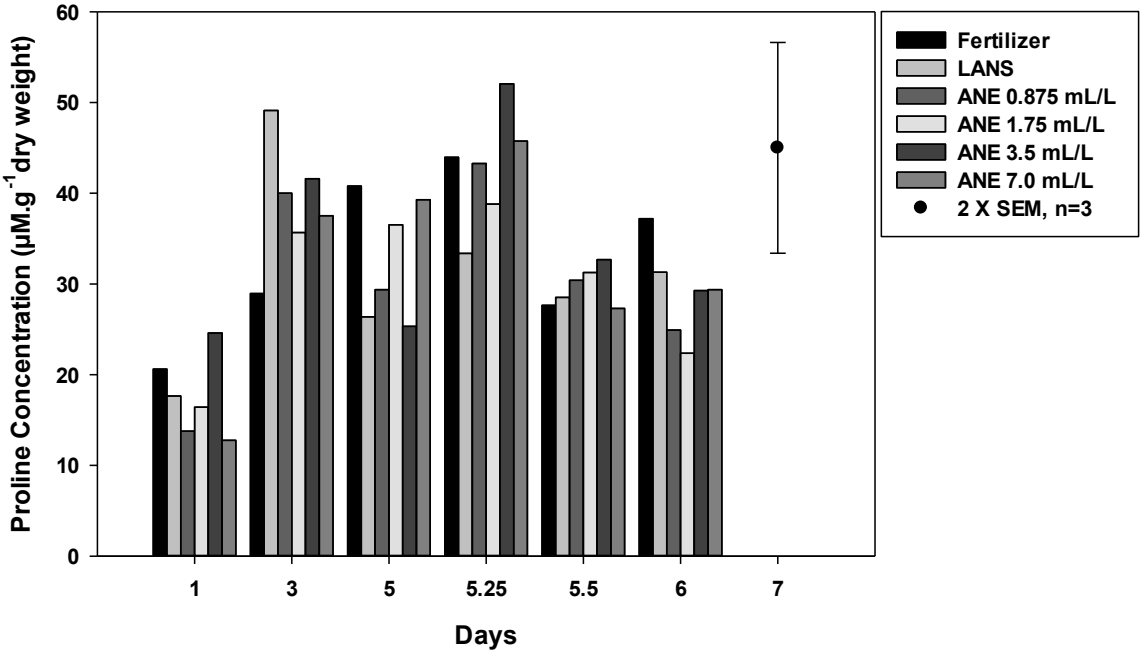

Figure 5.15. Proline content in tomato leaves from six treatments; fertilizer (20-20-20), LANS and 4 levels of ANE (0.875 mL/L, 1.75 mL/L, 3.5 mL/L and 7.0 mL/L). Number of replicates (n) = 3 and experiment was repeated 2 times. Vertical bar represents two standard errors of the mean (SEM).

5.3.4 Total Chlorophyll and Total Carotenoids

5.3.4.1 Chlorophyll *a*

Chlorophyll *a* content was not affected by treatments (P = 0.658), days (P = 0.170) or interaction between treatments and days (P = 0.923) (Figure 5.16).

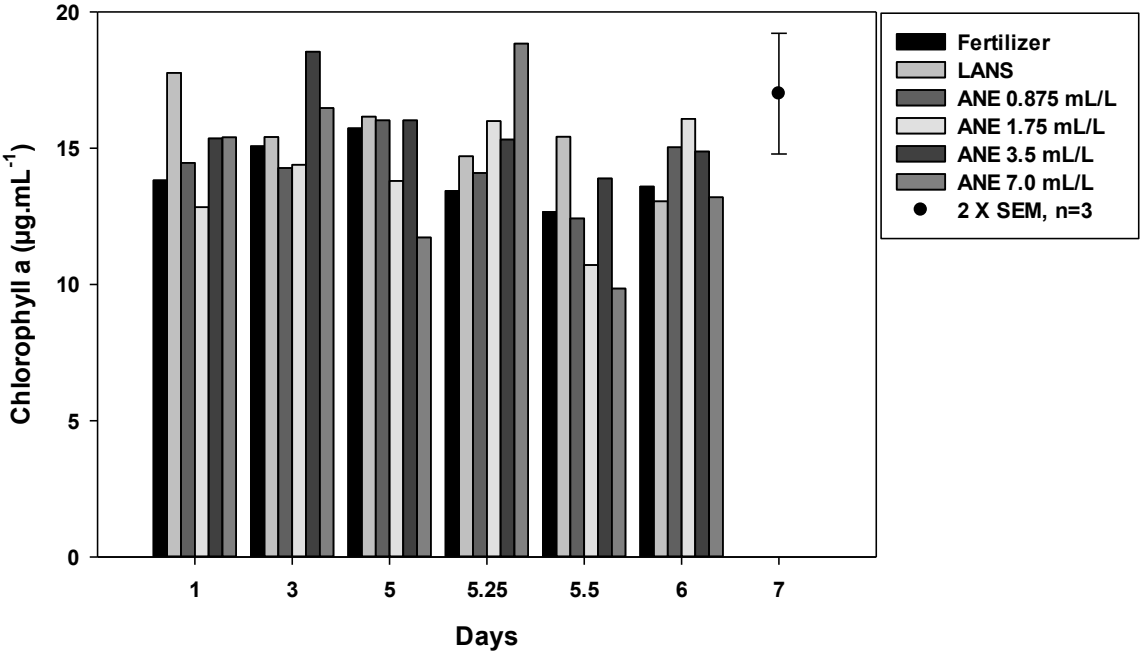

Figure 5.16. Chlorophyll *a* content in tomato leaves from six treatments; fertilizer (20-20-20), LANS and 4 levels of ANE (0.875 mL/L, 1.75 mL/L, 3.5 mL/L and 7.0 mL/L). Number of replicates (n) = 3 and experiment was repeated 2 times. Vertical bar represents two standard errors of the mean (SEM).

5.3.4.2 Chlorophyll *b*

Chlorophyll *b* content was not affected by treatments (P = 0.731), days (P = 0.360) or interaction between treatments and days (P = 0.987) (Figure 5.17).

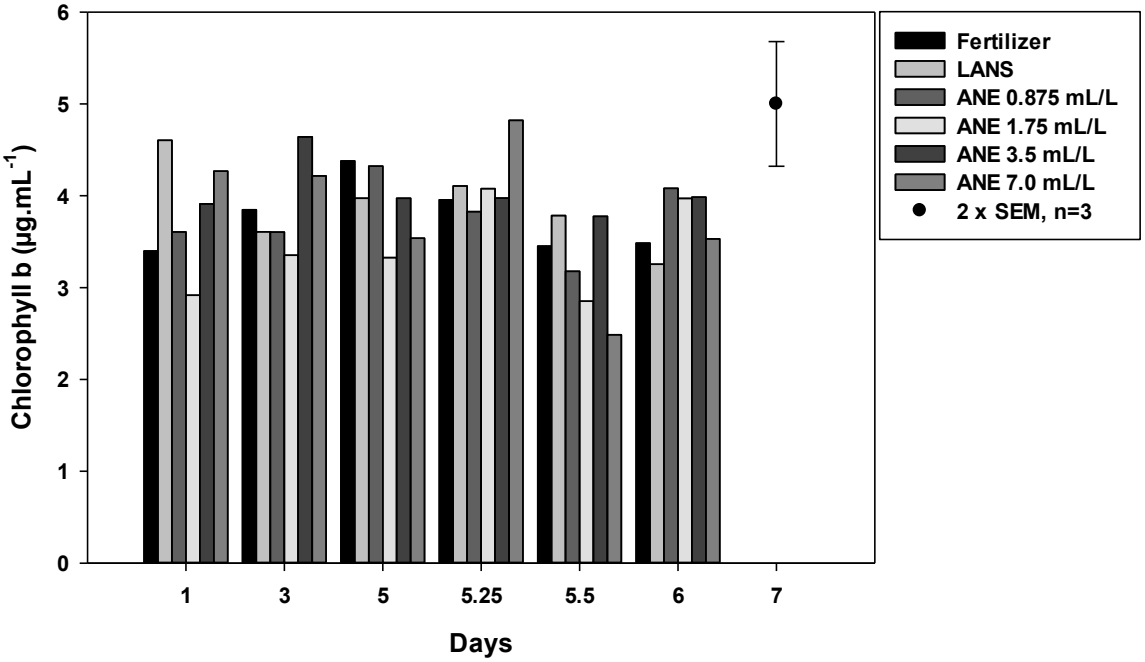

Figure 5.17. Chlorophyll *b* content in tomato leaves from six treatments; fertilizer (20-20-20), LANS and 4 levels of ANE (0.875 mL/L, 1.75 mL/L, 3.5 mL/L and 7.0 mL/L). Number of replicates (n) = 3 and experiment was repeated 2 times. Vertical bar represents two standard errors of the mean (SEM).

5.3.4.3 Total Chlorophyll

Total chlorophyll content followed the similar pattern as chlorophyll *a* and chlorophyll *b*. It was not affected by treatments (P = 0.686), by days (P = 0.194) or by the interaction between treatments and days (P = 0.940) (Figure 5.18).

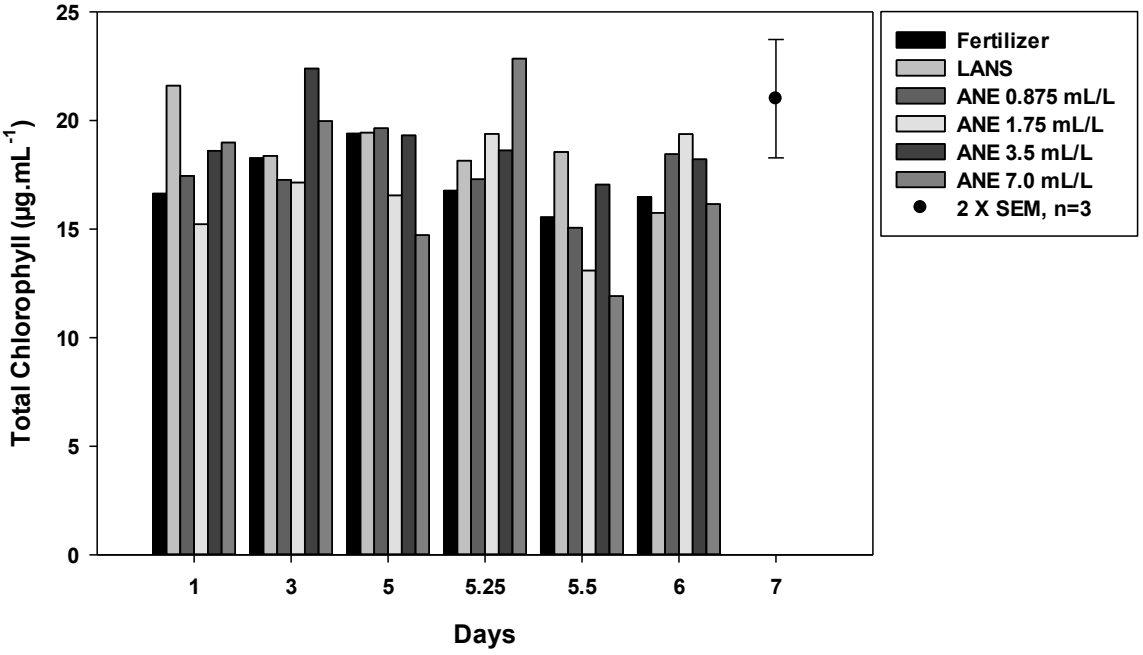

Figure 5.18. Total chlorophyll content in tomato leaves from six treatments; fertilizer (20-20-20), LANS and 4 levels of ANE (0.875 mL/L, 1.75 mL/L, 3.5 mL/L and 7.0 mL/L). Number of replicates (n) = 3 and experiment was repeated 2 times. Vertical bar represents two standard errors of the mean (SEM).

5.3.4.4 Carotenoids

Carotenoids content was not affected by treatments (P = 0.707), by days (P = 0.234) or by the interaction between treatments and days (P = 0.902) (Figure 5.19).

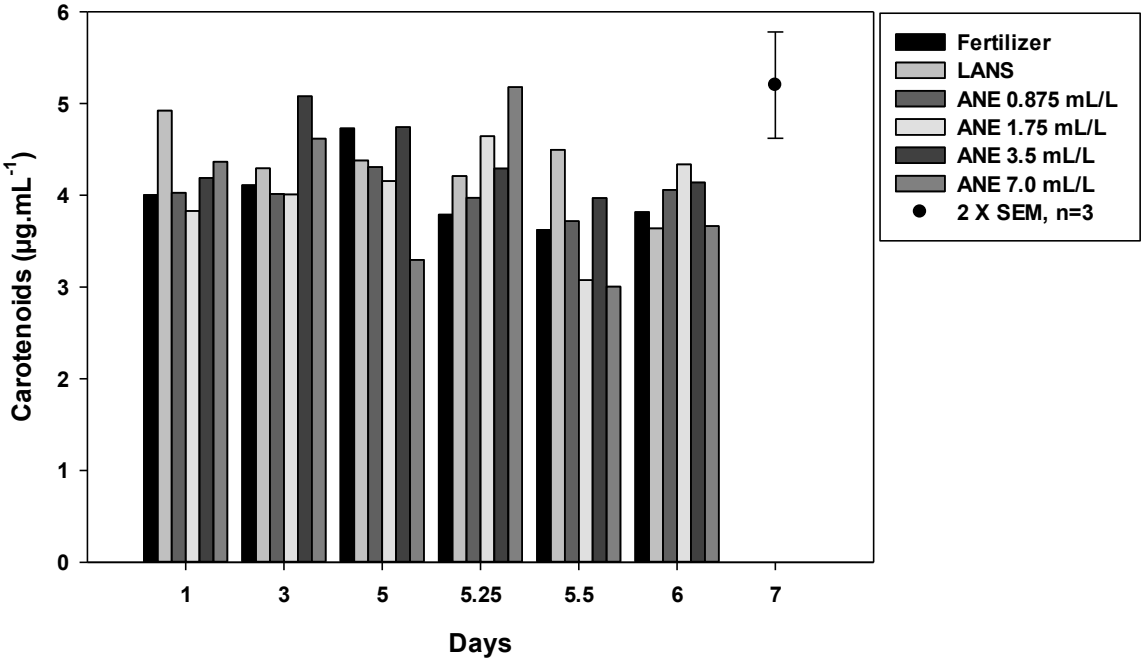

Figure 5.19. Carotenoids content in tomato leaves from six treatments; fertilizer (20-20-20), LANS and 4 levels of ANE (0.875 mL/L, 1.75 mL/L, 3.5 mL/L and 7.0 mL/L). Number of replicates (n) = 3 and experiment was repeated 2 times. Vertical bar represents two standard errors of the mean (SEM).

5.3.4.5 Chlorophyll *a* : Chlorophyll *b*

Chlorophyll *a*:*b* ratio was not difference between treatments (P = 0.137), between days (P = 0.379) or interaction between treatments and days (P = 0.983). From Day 1 to Day 5 before re-watering and 6h after re-watering (Day 5.25) to Day 6, ratio remained same (~ 4) (Figure 5.20).

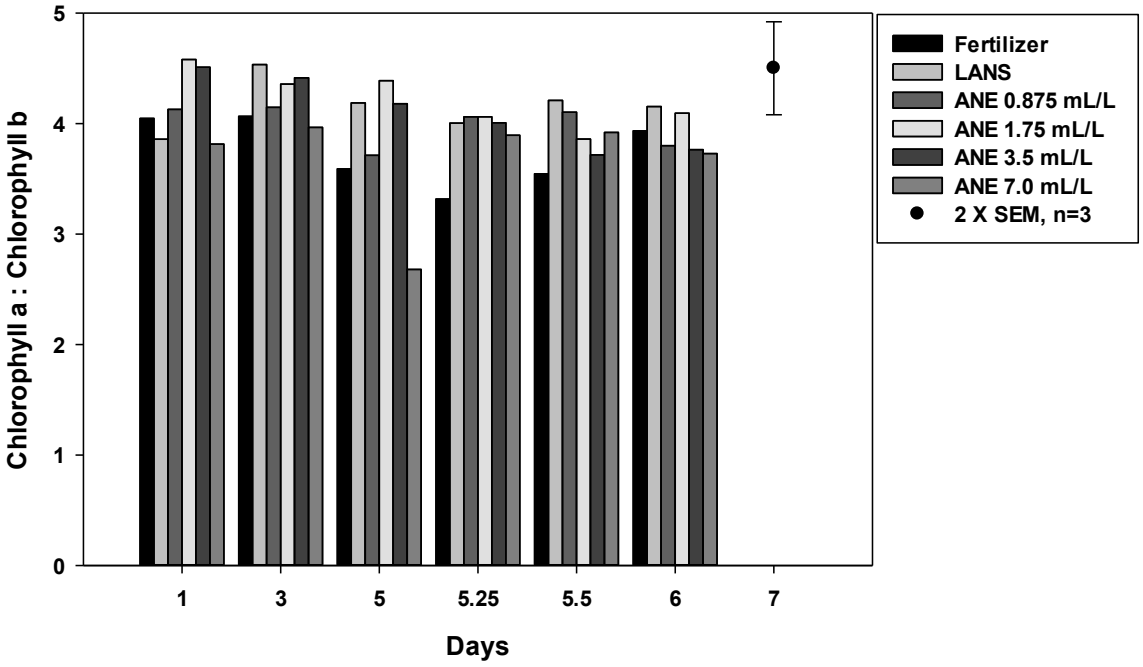

Figure 5.20. Chlorophyll *a*: chlorophyll *b* ratio in tomato leaves from six treatments; fertilizer (20-20-20), LANS and 4 levels of ANE (0.875 mL/L, 1.75 mL/L, 3.5 mL/L and 7.0 mL/L). Number of replicates (n) = 3 and experiment was repeated 2 times. Vertical bar represents two standard errors of the mean (SEM).

5.3.4.6 Carotenoids : Total Chlorophyll

The ratio of carotenoids to total chlorophyll was significant between days (P =0.034) but no significant difference was observed between treatments (P = 0.999) or interaction between treatments and days (P = 1.000). Day 1 and Day 3, ratio remained around 0.35 but it reduced to 0.2 by Day 5. Six hours (Day 5.25) after re-watering, it increased to 0.4 and same level was able to observe 12 h after re-watering. By Day 6, it was increased to 0.5 (Figure 5.21).

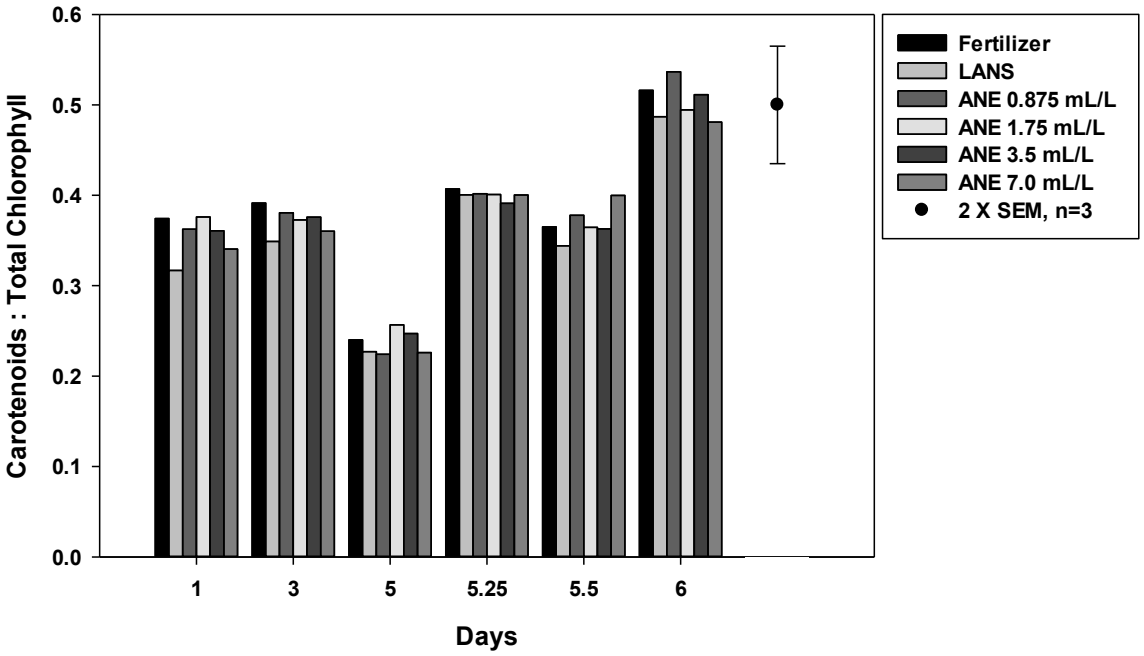

Figure 5.21. Carotenoids: total chlorophyll ratio in tomato leaves from six treatments; fertilizer (20-20-20), LANS and 4 levels of ANE (0.875 mL/L, 1.75 mL/L, 3.5 mL/L and 7.0 mL/L). Number of replicates (n) = 3 and experiment was repeated 2 times. Vertical bar represents two standard errors of the mean (SEM).

5.4 Experiment 3: Quantitative Gene Expression Analysis

Transcript levels of selected genes were analyzed with the objective of understanding how the genes regulate under drought stress with ANE, LANS and fertilizer treatments.

5.4.1 Δ-*pyrroline-5-carboxylate synthetase* (*P5CS*)

P5CS expression displayed significant difference between days (P =0.0005) but no significant differences were observed between treatments (P = 0.6280) or interaction

between treatments and days (P = 0.9972). Generally, *P5CS* expression increased with the progression of stress and after re-watering it decreased (Figure 5.22). On Day 1 and Day 3, all the treatments indicated higher expression level compared to Day 5.5 and Day 6 (~ 2 folds). Even though, there was no significant difference was evident between treatments, LANS showed lesser expression level compared to fertilizer and 3.5 mL/L ANE treated plants in all four time points.

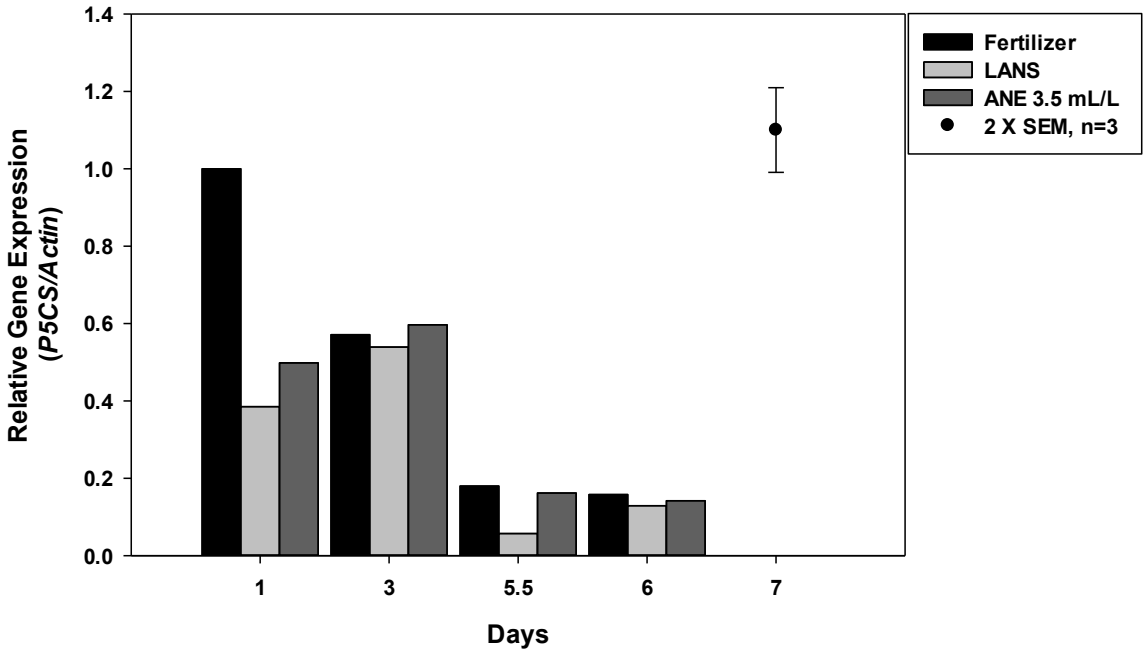

Figure 5.22. Relative expression of *P5CS* transcripts in fertilizer, LANS and 3.5 mL/L ANE treated tomato plants subjected to drought stress. Number of replicates (n) = 3; vertical bar represents two standard errors of the mean (SEM).

5.4.2 *Late embryogenesis abundant* (*LEA*)

Late embryogenesis abundant (*LEA*) expression indicated significant different between days (P < 0.0001), treatments (P < 0.0001) and interaction between treatments

and days ($P < 0.0001$). On Day 1, both LANS and 3.5 mL/L ANE treated plants indicated higher expression compared to fertilizer treated group (~ 4 folds). By day 3, expression increased in a higher degree (~10 folds). Twelve hours after re-watering (Day 5.5), all the groups showed highest level of expression. By Day 6, expression level reduced drastically in both fertilizer and 3.5 mL/L ANE treated groups (~ 20 folds and 10 folds respectively) but the expression level reduction was less in LANS (~ 0.5 folds) (Figure 5.23).

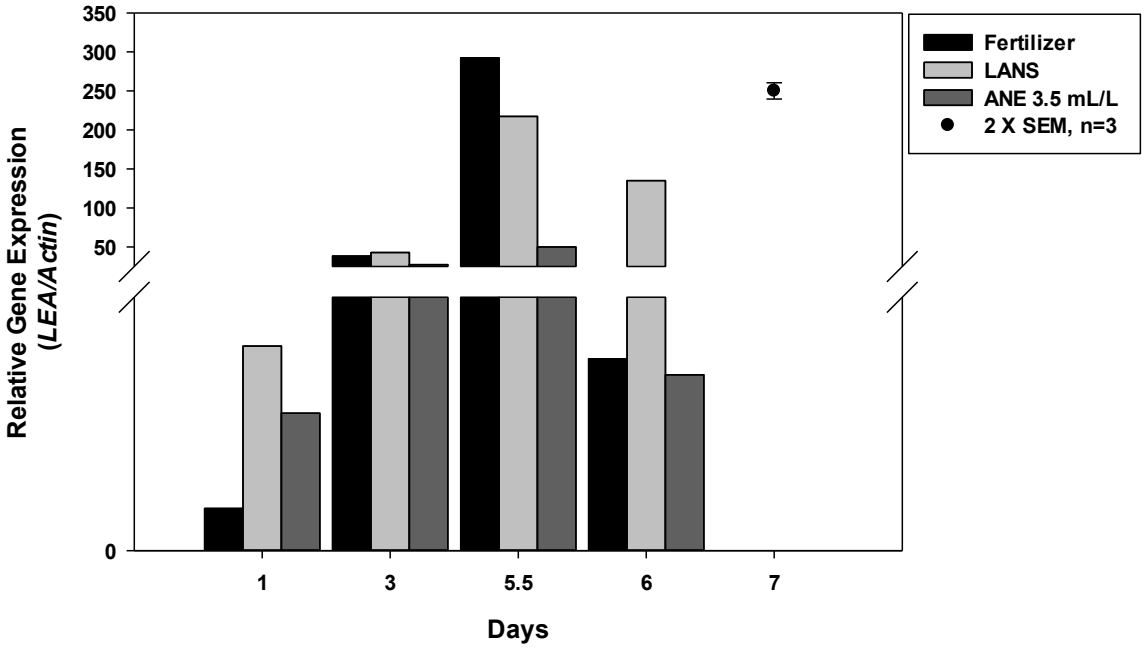

Figure 5.23. Relative expression of *LEA* transcripts in fertilizer, LANS and 3.5 mL/L ANE treated tomato plants subjected to drought stress. Number of replicates (n) = 3; vertical bar represents two standard errors of the mean (SEM).

5.4.3 *Lipid transfer protein gene 2* (*ltpg2*)

The *ltpg2* expression was affected by days ($P < 0.0001$) but not by treatments ($P = 0.1438$) or by the interaction between treatments and days ($P = 0.1212$). On Day 1,

expression level was low in all treatment groups and by Day 3, expression level increased in all groups. However, expression level reduced on Day 5.5 and further reduced by Day 6. Even though, there was no significant differences between treatments, LANS exhibited different expression pattern compared to fertilizer and 3.5 mL/L ANE treated plants on Day 3 and Day 5.5. On Day 1, all treatments showed same expression level, but on Day 3, fertilizer and 3.5 mL/L ANE treated groups showed higher expression level (~ 40 folds) compared to LANS treated group (~ 20 folds). Similar expression pattern was observed on Day 5.5 (Figure 5.24).

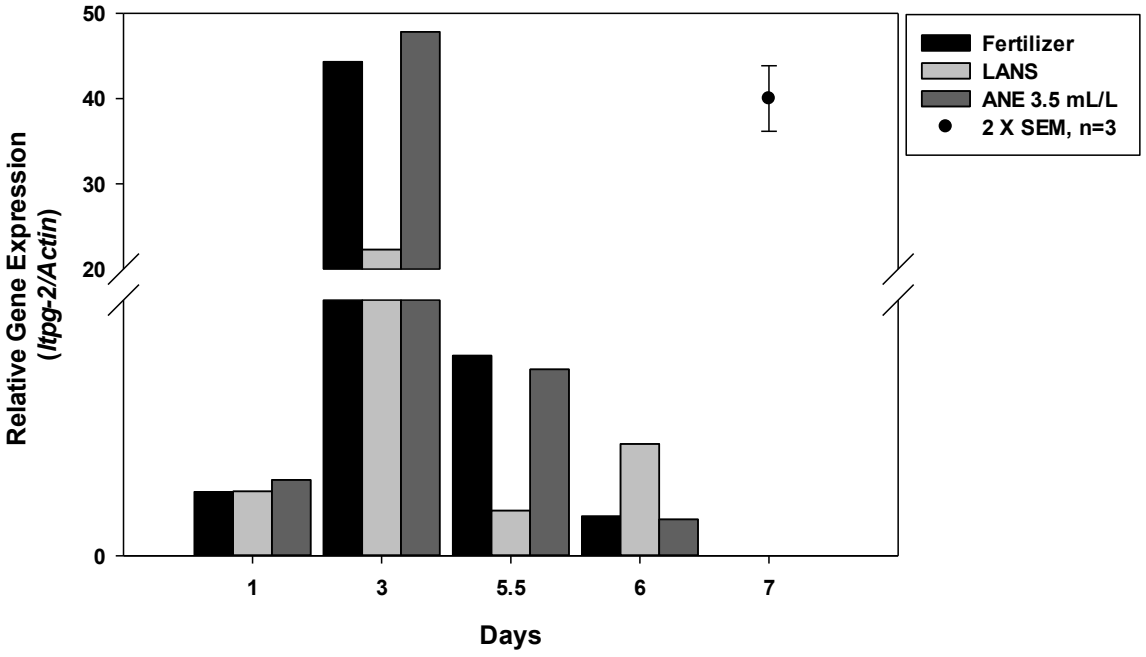

Figure 5.24. Relative expression of *ltpg-2* transcripts in fertilizer, LANS and 3.5 mL/L ANE treated tomato plants subjected to drought stress. Number of replicates (n) = 3; vertical bar represents two standard errors of the mean (SEM).

5.4.4 *ABA-response element binding protein 1* (*SlAREB-1*)

ABA-response element binding protein 1 (*SlAREB1)* expression was significant between days ($P = 0.0177$), treatments ($P < 0.0001$) and interaction between treatments and days ($P < 0.0001$). On Day 1, 3.5 mL/L ANE treated groups showed highest expression level compared to both fertilizer and LANS treated groups. By Day 3, all the groups indicated higher expression levels compared to Day 1 (~ 30 folds) and 3.5 mL/L ANE treated group was indicated the lowest expression level among 3. Twelve hours after re-watering (Day 5.5), expression level reduced drastically and it followed the similar pattern as in Day 1. By Day 6, expression levels of both fertilizer and LANS increased compared to Day 5.5 (~ 5 folds and 30 folds respectively) but 3.5 mL/L ANE treated group showed lesser expression level (~ 6 folds) (Figure 5.25).

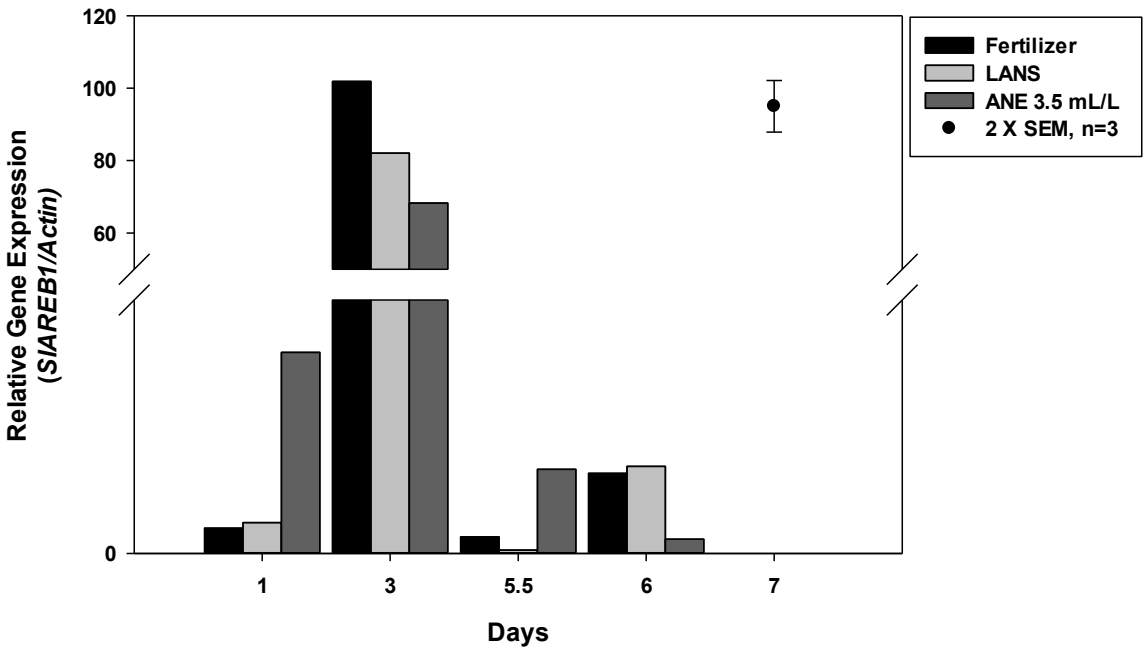

Figure 5.25. Relative expression of *SlAREB1* transcripts in fertilizer, LANS and 3.5 mL/L ANE treated tomato plants subjected to drought stress. Number of replicates (n) = 3; vertical bar represents two standard errors of the mean (SEM).

5.4.5 *Metacaspase 1* (*LeMCA1*)

Metacaspase 1(LeMCA 1) was affected significantly by treatments (P =0.0408) but not by days (P = 0.1428) or interaction between treatments and days (P = 0.8048). On Day 1, both fertilizer and 3.5 mL/L ANE treated groups indicated higher expression level compared to LANS group (~ 10 folds). By Day 3, all the treatments showed higher expression level compared to Day 1, but the increase in 3.5 mL/L ANE treated groups were much greater than the other 2 groups. At Day 5.5 (12 h after re-watering), all the groups showed reduction in expression level, but 3.5 mL/L ANE treated group indicated the highest reduction (~ 20 folds). However, by Day 6, all the groups exhibited higher

increase in expression level and fertilizer treated group showed the highest level (Figure 5.26).

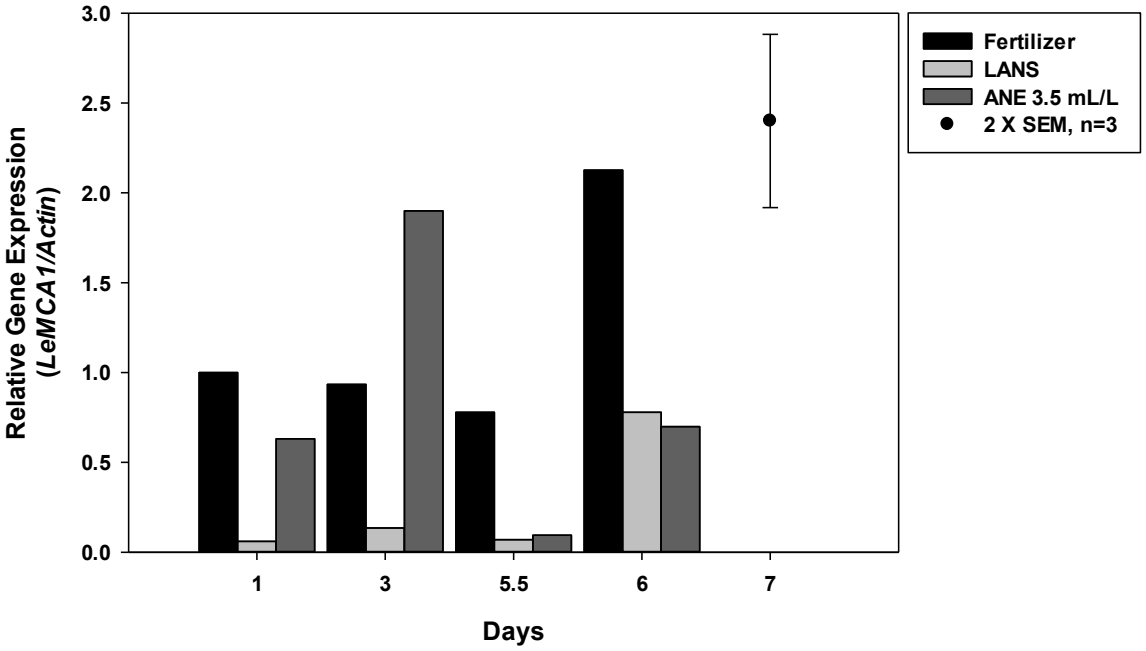

Figure 5.26. Relative expression of *LeMCA1* transcripts in fertilizer, LANS and 3.5 mL/L ANE treated tomato plants subjected to drought stress. Number of replicates (n) = 3; vertical bar represents two standard errors of the mean (SEM).

5.5 Experiment 4: Greenhouse Experiment

5.5.1 Total Number of Fruits Set

Total number of fruits set in trial 1 was greater than trial 2, but there was no significant difference in both trials between treatments (P= 0. 2837 – trial 1 and P= 0. 2945 - trial 2). Even though, there was no significant difference observed in both trials, there were some trends evident. Both 3.5 mL/L and 7.0 mL/L ANE treated groups had higher number of fruits compared to other treatments in both the trials. In trial 1, fertilizer

control group had lesser fruits compared to rest of the stressed groups but all the stressed groups had higher fruit number compared to non-stressed group. In trial 2, 1.75 mL/L ANE treated group showed lowest number of fruits compare to the rest of the treatment (Figure 5.27).

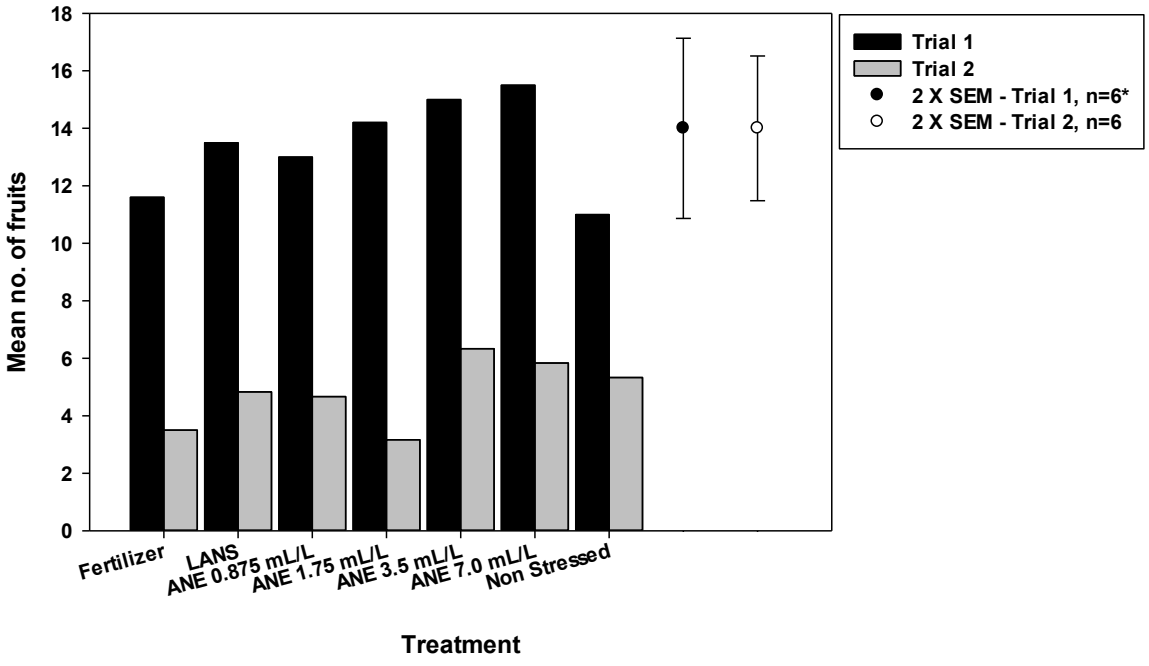

Figure 5.27. Total number of fruits set from each treatment in trial 1 and 2. Number of replicates (n) = 6 (* - in trial 1, for fertilizer, ANE 0.875 mL/L and ANE 1.75 mL/L n=5, non- stressed plants, n=3); vertical bar represents two standard errors of the mean (SEM).

5.5.2 Harvested Fruits

Total number of fruits harvested in trial 1 was greater than trial 2 but there was no significant difference between treatments in both the trials (P= 0.1302 – trial 1 and P= 0.1158 - trial 2). Even though, there was no significant difference between treatments in both trials, ANE treated plants produced higher number of fruits compared to the rest.

Especially, 1.75 mL/L and 7.0 mL/L ANE treated groups in trial 1 and 7.0 mL/L and 3.5 mL/L ANE treated groups in trial 2 produced the highest number of fruits. In both trials, fertilizer treated group showed the lowest fruit number (Figure 5.28).

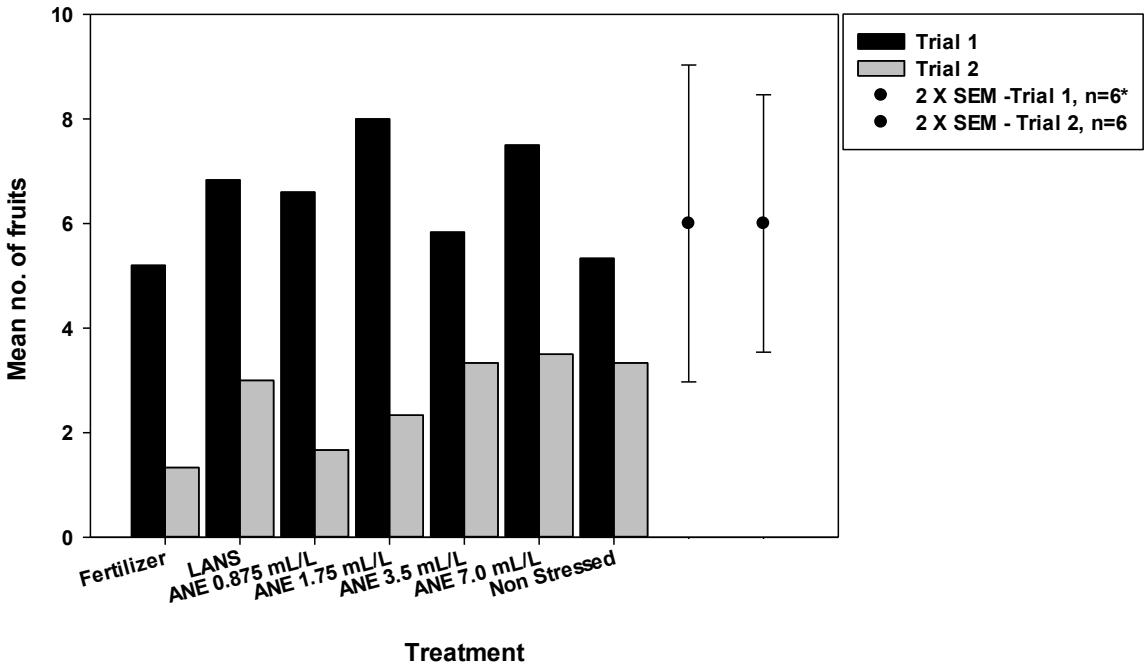

Figure 5.28. Total number of fruits harvested from each treatment in trial 1 and 2. Number of replicates (n) = 6 (* - in trial 1, for fertilizer, ANE 0.875 mL/L and ANE 1.75 mL/L n=5, non- stressed plants, n=3); vertical bar represents two standard errors of the mean (SEM).

5.5.3 Total yield per plant

Total yield from a plant in trial 1 was greater than in trial 2. There was no significant difference in trial 1 (P= 0.1501) but showed a significant difference between the treatments in trial 2 (P=0.0340). In trial 2, LANS treated plants produced the highest yield and fertilizer treated batch was the lowest. However, both 3.5 mL/L and 7.0 mL/L

ANE treated groups and non-stressed group had the second highest yield compared to the other groups (Figure 5.29).

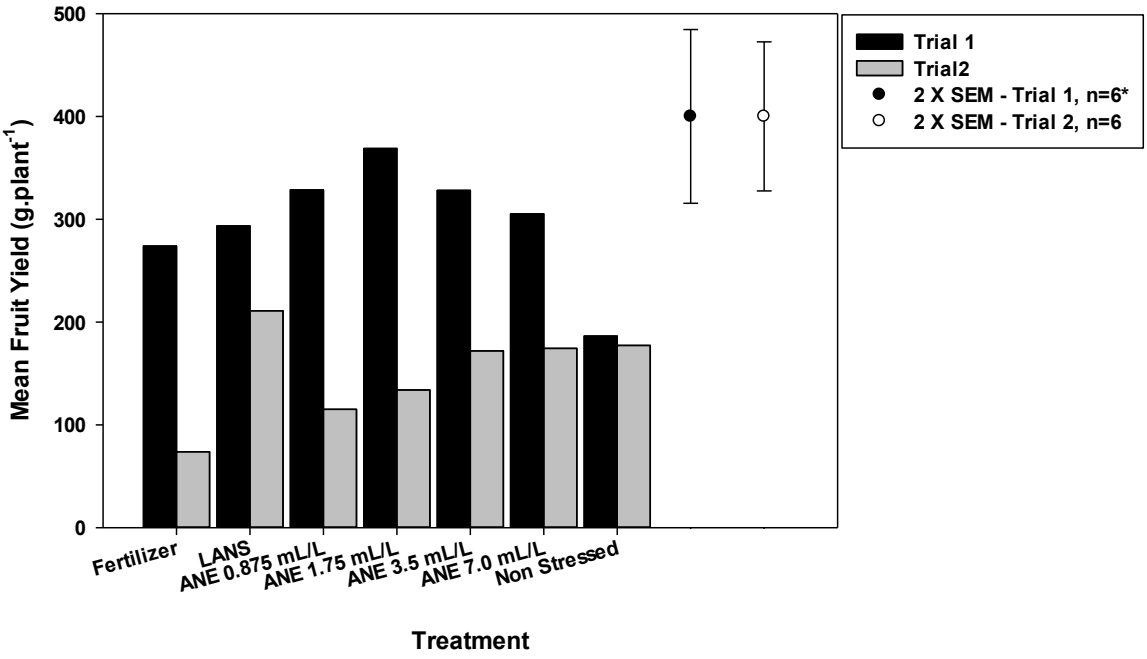

Figure 5.29. Total number of fruits harvested from each plant in trial 1 and 2. Number of replicates (n) = 6 (* - in trial 1, for fertilizer, ANE 0.875 mL/L and ANE 1.75 mL/L n=5, non- stressed plants, n=3); vertical bar represents two standard errors of the mean (SEM).

5.5.4. Mean fruit weight

Fruit weights were almost similar in all the treatments in both trials. Fruit weigh range from 40 g to 55 g in both occasions. There was a significant difference in trial 1 ($P< 0.0001$) but did not show significant difference between the treatments in trial 2 ($P=0.0530$). However, in trial 2, it is almost reaching significance (0.05 vs 0.053) in treatments (Figure 5.30).

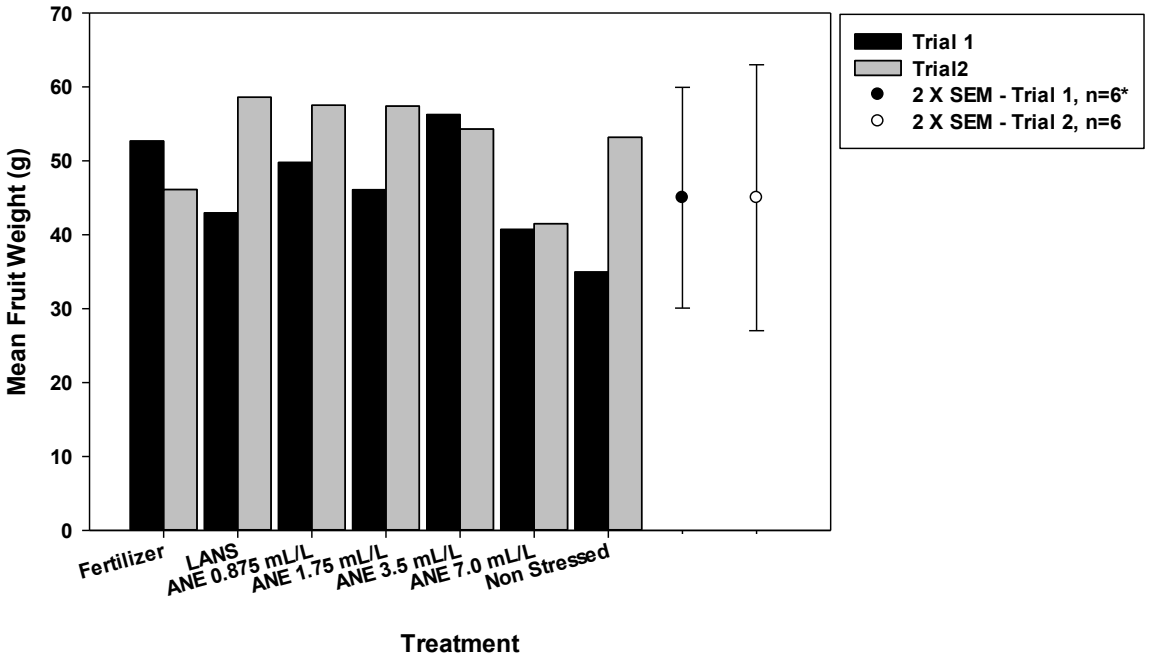

Figure 5.30. Mean fruit weight from harvested fruits from each treatment in trial 1 and 2. Number of replicates (n) = 6 (* - in trial 1, for fertilizer, ANE 0.875 mL/L and ANE 1.75 mL/L n=5, non- stressed plants, n=3); vertical bar represents two standard errors of the mean (SEM).

CHAPTER 6

DISCUSSION

6.1 Treatments

Six different treatments were used in the study. They were fertilizer (20-20-20, 1g/L), Long-Ashton Nutrient Solution (LANS) modified (100 mL/L + 20-20-20) and four concentrations of ANE (0.875 mL/L, 1.75 mL/L, 3.5 mL/L and 7.0 mL/L with 20-20-20). Fertilizer was used as a control in the study. LANS was used as an inorganic nutrient solution in various studies and it was used against plant stress during last three decades (Breuninger *et al.,* 2004; Kerton *et* al., 2008; Matimati *et* al., 2014). Modified LANS solution was equivalent to the inorganic fraction of 3.5 mL/L ANE and it was used alone with ANE in various studies (Wally *et al.,* 2013). Four different concentrations of ANE were used. The 3.5 mL/L was the recommended concentration and other three were fractions (x ¼, x ½ and x 2) based on it. During experiments, it was evident that 3.5 mL/L and 7.0 mL/L ANE concentrations were more effective than two lower concentrations. These six treatment combination was ideal in our study to check the effects of ANE over fertilizer and LANS.

In *in-vitro* rooting experiment, these concentrations were modified. The ANE rate was modified to 0.175 mL/L (x 1/20 of 3.5 mL/L) for *in-vitro* rooting experiment, where the original recommended rate (3.5 mL/L) was too strong in *in-vitro* studies and create toxic effects on plants. According to that, LANS concentration was also modified into 5 mL/L. These concentrations were used with and without PEG in *in-vitro* rooting assay.

6.2 Volumetric Water Content

Volumetric water content was reduced in all treatments with the progression of stress over time and significant differences were observed in treatments and days. However, 3.5 mL/L and 7.0 mL/L ANE groups indicated relatively high moisture content before re-watering. This indicated that the two highest concentrations of ANE treated pots lost moisture at a slower rate compared to rest of the treatments. In contrast, the LANS and 1.75 mL/L ANE treated groups lost soil moisture at a higher rate. It was reported that seaweed extracts improve soil moisture holding capacity and improve the soil structure (Dhargalkar and Pereira, 2005; Eyras *et al*., 2009). Further, it was reported that alginate, a polysaccharide extracted from brown seaweeds, improves soil structure and soil water retention (Galán-Marín *et al.,* 2010; Tang *et al.,* 2011). It has been proven that ANE also improves the soil moisture holding capacity, where alginate binds with ions in soil to form a high molecular weight structures to retain soil moisture (Khan *et al*., 2009). Therefore, alginate could be the main ingredient which helped in reducing soil moisture loss in 3.5 mL/L (recommended rate) and 7.0 mL/L ANE treated pots. Re-watering was used to bring the pots back to original moisture level. It was proven that re-watering helped to regain the soil moisture level (Yao *et al.,* 2012; Gilgen and Feller, 2013). Even though we used the same amount of water to bring the pots back to the original moisture level, none of the groups reached up to the original moisture level after re-watering. The gap between the original moisture level and the moisture level after re-watering may be due to two main reasons: it may be due to the initial moisture level of the potting media (PRO-MIX BX MYCORRHIZAE^{TM}) before adding the respective treatments, which did not consider at the beginning of the experiment or the changes

occur in potting media structure after the treatments. However, continuous water supply for a considerable time can minimize the error in future experiments.

6.3 Visual Ratings, Stomatal Conductance and Recovery Percentage

Wilting is a good indicator to understand the status of a plant under drought stress (Waterland *et al.,* 2010). Wilting was rated from one to five depending on the percentage of leaves that wilted. All the treatment groups exhibited a common pattern in wilting. With stress, plants wilt more and the rating increased (Figure 5.2). After re-watering, wilting was reduced. For each treatment, there was a quadratic relationship between the wilting ratings and days. On Day 1 and Day 2, none of the plants showed any wilting symptoms. By Day 3, all of the treatment groups started showing wilting symptoms (Figure 5.3). This indicated that the moisture level was enough for three days survival for plants. The wilting intensity was less in 3.5 mL/L and 7.0 mL/L ANE treated groups, and they were able to recover quickly after being re-watered on Day 5. By Day 8, except fertilizer treated group (wilting rating ~2), the rest of the plants had recovered (wilting rating ~1). It was recorded that the plants treated with nutrient solutions recover well over untreated plants (Perez-Perez *et al*., 2007; Khan and Panda, 2008). ANE also carries an inorganic salts in it and helped in recovery.

A negative linear relationship between days and stomatal conductance was seen in all the treatments (Figure 5.4). It was evident that stomatal conductance decrease with stress (Xu *et al.,* 2010). Stomatal conductance decreased from Day 1 to Day 5 and increased slightly after re-watering. Among the six treatments, both 3.5 mL/L ANE and 7.0 mL/L ANE treated groups showed relatively higher stomatal conductance compared to the rest of the treatments. None of the treatments were able to reach the original

conductance level by the end of Day 8. The decrease of stomatal conductance from Day 1 to Day 2 was greatest, and it remained depressed until re-watering, where marginal improvement was noted after re-watering. Sarker *et al*. (2005) studied the effects of drought stress on stomatal conductance in eggplant. They experienced a massive drop in stomatal conductance from Day 1 to Day 2, which supports the trend in this experiment.

The plant's recovery after re-watering increased over time in all the treatment groups (Figure 5.6). The rate of recover depends on the severity of the stress (Xu *et al.,* 2010). During the first three hours after re-watering, none of the plants from the fertilizer or LANS treated groups recovered. Though fertilizer and LANS treated pots absorb water rapidly into potting media, plants' water absorption was not high during first six hours. By six hours after re-watering, the majority of both 3.5 mL/L ANE and 7.0 mL/L ANE treated groups had recovered. Within 18 hours after re-watering, both groups reached 100% recovery. Except the fertilizer treated control group, the rest of the groups had mostly recovered within 24 hours after re-watering; however, the fertilizer, 0.875 mL/L ANE and 1.75 mL/L ANE treated groups did not recover 100% by the end of 72 hours. Lower concentrations of ANE (0.875 mL/L and 1.75 mL/L) and fertilizer treatments may not be effective as the rest of the treatments. Further, higher inorganic salts in LANS and higher concentrations of ANE (3.5 mL/L and 7.0 mL/L) favor on plant recovery over the rest of the treatments. It may be the reason for low recovery percentage during experiment period.

6.4 Plant Water Potential, Relative Water Content and Dry Matter Percentage

Both plant water potential and relative water content were reduced with drought stress (Porcel and Ruiz-Lozano, 2004; Turkan *et al.,* 2005; Shao *et al*., 2008), but dry matter percentage increased since the total biomass increase (Sarker *et al.,* 2005). Even though there was no significant difference between treatments or days, plant water potential exhibited a common trend in all the treatments. As expected, with the progression of the stress, plant water potential reduced and increased with re-watering.

On Day 1, all the treatment groups indicated high relative water content and low total dry matter percentage. With the progression of drought stress, relative water content was reduced and the dry matter percentage increased. After re-watering, the dry matter content was reduced and relative water content increased. However, by the end of Day 6, none of the parameters were able to reach the original levels as indicated on Day 1. Despite the fact that there was no significance difference between treatments in both relative water content and dry matter percentage, the ANE treated plants showed rapid increases in relative water content and reduction in dry matter percentage after re–watering (by Day 6) compared to the rest of the groups. Further studies have shown that ANE treated plants perform well under both biotic and abiotic stresses (Khan *et al.,* 2009; Rayirath *et al*., 2009).

6.5 Rooting

Rooting is naturally induced under dought stress (Turkan *et al.,* 2005). Abscisic acid maintains root growth under drought stress (Sharp *et al.,* 2004). Other than available water, root growth under drought stress is affected by several factors, such as physical, chemical and biological properties of soil and cell wall strength of the root (Bengough *et*

al., 2011). Both the root area and root length increased in the unstressed groups (no PEG). By the end of Day 7, the control group showed the highest root area and length among the unstressed groups. The LANS and 0.175 mL/L ANE treated groups followed respectively after the control group. Among the stressed groups (PEG treated groups), LANS + PEG and PEG treated groups exhibited higher root growth compared to 0.175 mL/L ANE + PEG treated group.

It was reported that MS media created a slight osmotic potential of approximately -0.25 MPa in square plates (Verslues *et al.,* 2006). Therefore, all the treatment groups experience the slight stress and on top of it PEG creates stress on PEG treated plants (-0.7 MPa). The ANE treated plants grew roots well under the no stress conditions. In both the stressed and unstressed groups, the 0.175 mL/L ANE treated plants showed less root growth compared to the rest. This indicates that the 0.175 mL/L ANE treated plants were not as stressed as the other groups. If the PEG treated ANE plants were stressed, then the root length and area should have been similar or close to the LANS and control groups in the study. It had been recorded that ANE treated plants perform well under stress conditions, and it may be the reason for decreased rooting in these ANE treated groups (Khan *et al*., 2009; Craigie, 2011). Sharp *et al.* (2004) observed root growth supressed due to elevated ROS levels under drought stress. High ROS levels damage DNA and modify functional proteins to reduce root growth. However, Khan *et* al. (2009) reported that ANE is capable of up-regulate stress resistance metabolome and minimize ROS levels to facilitate normal functioning of plants under drought stress and Cytokinin compounds in seaweed extracts reduce ROS in plants. It may be the possible reason for ANE treated plants not stressed under low water potentials in this study. Further,

substance such as betains (gamma-aminobutyric acid betaine, 6-aminovaleric acid betaine, and glycine betaine) and sterols present in ANE help to overcome drought stress in plants since low-molecular osmolytes like betains can minimize cellular dehydration (Beck *et al.,* 2007; Khan *et al*., 2009).

6.6 Antioxidant Enzymes

Naturally there is a balance between antioxidant enzymes and reactive oxygen species (ROS) in a system. Any stress can disturb the balance which leads to an increase in the ROS amount, causing oxidative stress. Antioxidant enzyme levels increase to overcome ROS damage and bring cellular homeostasis back (Lee *et al*., 2007; Gill and Tuteja, 2010). Superoxide dismutase (SOD) is the first enzyme that reacts on ROS and it breaks into H_2O_2 and O_2. The produced H_2O_2 further breaks down into harmless H_2O and O_2 with the aid of catalase (CAT), guaiacol peroxidase (GPOD), and ascorbate peroxidase like enzymes (Mittler *et al.,* 2004). This experiment focused on SOD, CAT and GPOD.

Despite the fact that the activities of SOD, CAT and GPOD were not significantly different between treatments, days or interaction between treatments and days, there are some trends in those enzyme levels in the study. The SOD activity was highest before re-watering, and even after re-watering it was reduced by a small degree in all of the treatments. Both CAT and GPOD activities remained constant throughout the experiment. On Day 1, it was expected that the antioxidant enzyme levels should be lower than Day 3 and Day 5. In contrast, the levels were almost the same as Day 3. It was proven that an application of N (30 mg/L) and P (30-50 mg/L) fertilizer can increase antioxidant levels in the plants (Ahn *et al.,* 2005; Nimptsch and Pflugmacher, 2007). This

may be the reason that high levels of antioxidant enzymes were detected on Day 1, where the application rate of 20-20-20 was 1g/L in our study. On Day 5.25 (6h after re-watering), the fertilizer treated group indicated the lowest activity and the ANE treated ones showed the highest level (especially the 3.5 mL/L and 7.0 mL/L ANE treated groups). The ANE treated groups showed a reduction in activity and the fertilizer treated group showed the opposite. By Day 6, the 7.0 mL/L ANE treated groups showed the lowest activity compared to the rest and it was evident, that seaweed extracts can reduce antioxidant activities inside the plants (Zhang *et* al., 2003; Dring, 2006; Khan *et al.,* 2009). The LANS treated group showed a reduction in enzyme activities from Day 1 to Day 5, and the activity increased 6 h after re-watering. It increased up to 12 h after re-watering and was reduced by Day 6. Yang *et al*. (2009) showed that increased inorganic mineral uptake helps to avoid antioxidant stress in plants. This may be the reason for showing a reducing trend in the LANS treated group during stress. The drying process also effects nutrient uptake in plants. Re-watering can increase nutrient uptake, but at the same time, excessive nutrients can cause oxidative stress. This could be the reason the LANS treated plants showed an increase in enzyme activity during the first 12 h after re-watering. Long Ashton nutrient solution contains Ca^{2+} and Mn^{2+}, which are essential for plant function. These macronutrients could be the reason for low enzymatic activity before re-watering in the LANS treated group. Experimental error was considerably high in all three enzyme assays. This may mainly due to the enzyme level difference in biological replicates in same treatment group since almost all the other factors remain constant throughout the experiments.

6.7 Lipid Peroxidation

Lipid oxidation occurs in three ways. It can be either enzymatic oxidation, non-enzymatic free radical-mediated oxidation, or non-enzymatic, non-radical oxidation (Niki *et al.,* 2005). Lipid peroxidation occurs in both cellular and organelle membranes. Increased ROS levels, beyond the threshold level due to oxidative stress, cause non-enzymatic free radical-mediated oxidation. This can disturb the normal functioning of the cell (Sharma *et al*., 2012). Normally, lipid peroxidation increases in the cells with the progression of stress (Turkan *et al.*, 2005).

In this experiment, lipid peroxidation increased with stress, and after re-watering, it was reduced. As described by Turkan *et al.* (2005), this trend was expected since the progression of the stress increases the lipid peroxidation. Even though there was no significant different between treatments, fertilizer and LANS treatments exhibited high MDA levels before re-watering, but after re-watering, both treatments showed comparatively lesser MDA levels than the ANE treated groups. The 7.0 mL/L ANE treated group expressed the lowest MDA levels among all of the ANE treated groups. Seaweed extracts reduce MDA production under drought stress and MDA levels were also lowered with an increase in ANE concentration (Ruizhi *et al*., 2009). This evidence supports the results obtained from this experiment.

6.8 Proline Content

Proline is one of the main electrolytes that accumulate during biotic and abiotic stress (Backor *et al*., 2004, Koca *et al.,* 2007). Proline levels increase with the increase of the stress (Turkan *et al.*, 2005).

In this experiment, low proline levels were observed on Day 1. With the progression of stress, it increased, and even 6h after re-watering, the proline levels were high in all of the groups. But 12 h after re-watering, proline levels were reduced and remained at the same level until Day 6. Though there was no significance difference observed between treatments, a prominent trend was evident in treatments. Lower proline levels were observed in ANE treated plants compared to the fertilizer and LANS treated groups. Renuka and Rathinavel (2006) proved that the application of seaweed extracts lowers the proline accumulation under stress. This could be the probable reason for lower proline levels in ANE treated groups. The LANS treated plants showed higher proline levels, especially on Day 3 and Day 6. The introduction of inorganic ions increases the proline accumulation in plants under the stress since the ion concentration increases in the plants (Heuer, 2003). LANS is an inorganic ion solution and it increased the ion concentration during the dehydration process in the pots. It lead to higher proline accumulation in the LANS treated plants compared to the ANE treated plants.

6.9 Photosynthetic Pigments

Chlorophyll and carotenoids are the major photosynthetic pigments in higher plants. With drought stress, chlorophyll and carotenoids levels are reduced in the plants (Sairam and Saxena, 2000; Rong-hual *et al*., 2006; Guerfel *et al*., 2009). This experiment measured chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoid level fluctuation under drought stress. There was no significant difference between treatments, day or interaction between treatments and days. However, there were expected patterns in pigment levels were able to observe in this experiment. All the pigment levels remained in a constant range from Day 1 to Day 5.25. They reduced slightly by Day 5.5, and

increased slightly by Day 6. Dordas and Sioulas (2008) proved that the addition of nitrogenous fertilizer can increase the chlorophyll content in safflower. An application of seaweed extracts also increases the chlorophyll content in the plants (Khan *et al*., 2009; Spinelli *et al*., 2010). All experimental units were treated with nitrogen fertilizer and the four groups with ANE. All treatments were under drought stress and it may be the reason for constant pigment levels in all treatments.

The chlorophyll *a:b* ratio remained the same throughout the experiment. Nikolaeva *et al*. (2010) proved that the chlorophyll *a:b* ratio remains unchanged with the increase of drought stress conditions in three wheat varieties, which matches the obtained results. Chlorophyll *a* and *b* convert into each other depending on the necessity, and this may be the main reason for a constant chlorophyll *a:b* ratio in all the treatments (Tanaka and Tanaka, 2011). The carotenoids: total chlorophyll ratio decreased with stress, and by Day 5 it was lesser than Day 3. After re-watering, the ratio increased in all of the treatments. It was expected that there would be a reduction in the carotenoids:total chlorophyll ratio with drought stress (El-Tayeb, 2006; Xu *et al.,* 2008).

6.10 Quantitative Gene Expression Analysis

Δ-pyrroline-5-carboxylate synthetase (P5CS)

Proline, glycine betaine, mannitol and trehalose are some of the main osmolites that accumulate during abiotic stress in plants (Zhuang *et al*., 2012). During abiotic stress, the proline content increases with the intensity of the stress and the duration (Turkan *et al*., 2005; Zarei *et al*., 2012). Genes such as *P5CS, P5CR* and *ProDH* are expressed in proline biosynthesis and degradation (Yooyongwech *et al*., 2012). Further, it

was proven that expression of *P5CS* increased and *ProDH* reduced under cold stress. It confirmed that proline synthesis is encouraged under stress (Nair *et al.,* 2012). *P5CS* is one of the main target genes for proline level determination (Dobra *et al*., 2011). A significant difference was observed between days, but not between treatments or interaction between treatments and days. However, there was a common expression pattern was observed in treatment groups in all four time points. In all time points, fertilizer treated group exhibited higher expression level and LANS showed the lowest. On Day 1, the fertilizer treated group had higher expression levels compared to the LANS and 3.5 mL/L ANE treated groups. Nimptsch and Pflugmacher (2007) proved that an application of fertilizer (especially nitrogen) can cause plant stress; this could be a possible reason for having higher expression levels in all groups on Day 1. As expected, on Day 3, slightly higher expression levels were observed compared to Day 1 in LANS and 3.5 mL/L ANE treatments. However, 12 h after re-watering (Day 5.5), *P5CS* levels were lower, and reduced further by Day 6. After re-watering, *P5CS* expression levels were relatively less since re-watering helped the plants recover. However, after re-watering both LANS and 3.5 mL/L ANE treated plants indicated lower expression level compared to the fertilizer treated group. It had been proven that alginate polysaccharides able to reduce *P5CS* expression under drought stress (Liu *et al.,* 2013). Alginates are one of the major components in ANE and it could aid to lower the expression in ANE treated plants. Further, LANS treated groups exhibited relatively less expression level in all four time points and it could be due to inorganic salts' ability to overcome stress in plants (Perez-Perez *et al*., 2007). Proline levels increased with stress and were reduced after re-watering. However, the *P5CS* gene expression in both Day 1 and Day 3 was high, and it

was reduced after re-watering. This could be due to *P5CS* expression happening prior to proline synthesis in plants.

Late embryogenesis abundant (LEA)

LEA proteins are responsible for stress responses such as drought and salinity in plants (Xiao *et al.,* 2007). These proteins are low molecular weight proteins (10-30 kDa) and mainly involve in drought tolerance (Goyal *et al.*, 2005). There are different *LEA* genes and there is no specific tissue to express these genes. They can be expressed either in leaves, roots or stems or even in cotyledons (Hong-Bo *et al.*, 2005). Generally, *LEA* genes are expressed under stress, and depending on the intensity, its expression level may vary (Lu *et al.,* 2010). In this study, significant differences were found between treatments, days and interaction between treatments and days. The expression level increased in all of the treatments with the progression of stress. Maximum expression level was observed 12 h after re-watering, and by Day 6, the expression level was reduced. This trend was expected as the stress progress the expression levels increase, and after re-watering it reduces. Since we experienced the highest level of expression at 12 h after re-watering, it proved that plant recovery takes more time than expected. However, 12 h after re-watering, the 3.5 mL/L ANE treated group had lower expression level compared to the other two groups. Liu *et al.* (2013) proved that the expression of *LEA* in alginate polysaccharides applied rice was low under drought stress compared to the controls. Since alginate is a main component of ANE, it can be the main reason for low expression of ANE under stress and after re-watering.

Lipid transfer protein gene 2 (ltpg2)

Non-specific lipid transfer proteins (LTPs) are a specific protein group in plants. These LTPs are involved in stress defense, lipid recycling and plant reproduction (Edstam *et al*., 2013). In tomatoes, there are different LTPs as LTPG1 and LTPG2 (Baykal and Zhang, 2010). In this experiment, *ltpg2* expression increased with stress and it was reduced with re-watering. The highest expression levels were observed on Day 3 and expression level was decreased after re-watering. Lu *et al.* (2010) proved *ltpg2* expression increases with stress and this supports the trend in this experiment. Even thought, there was no significant difference between treatments, LANS treated group express in a different way compared to fertilizer and 3.5 mL/L ANE treated groups. Both fertilizer and 3.5 mL/L ANE treated groups indicated gradual decrease in expression, but LANS treated group had drastic reduction in expression 12 h after re-watering and it was increased by Day 6. LANS treated pots were not able to hold more moisture after re-watering compared to other groups. LANS treated pots had the lowest volumetric water content after re-watering and it indicated that LANS treated pots were unable to hold more moisture. Low moisture levels in LANS treated pots causing stress and that could be the reason to observe higher expression level on Day 6 compared to fertilizer and 3.5 mL/L ANE treated groups.

ABA-response element binding protein 1 (AREB1)

Abscisic acid is the major messenger molecule for stress response in plants under biotic or abiotic stress (Raghavendra *et al*., 2010). There are different genes in different plant species involved in ABA signaling under drought stress (Fujita *et al.,* 2005;

Raghavendra *et al*., 2010; Bastıas *et al.,* 2011). *ABRE* genes are one of the major ABA inducible genes and they are expressed under osmotic stress (Yamaguchi-Shinozaki and Shinozaki, 2006). AREB1 and AREB2 proteins are regulatory proteins (Shinozaki and Yamaguchi-Shinozaki, 2007). They are the major transcription factors in drought stress response (Yoshida *et al*., 2010). The tomato genome consists of several ABA responsive genes; *SlAREB1* and *SlAREB2* are two of the major ABA responsive genes (Bastias *et al.,* 2011). Both of these genes are expressed more often in leaves compared to other tissues such as roots, flowers or fruits (Bastias *et al.,* 2011). In this study, emphasis was put on the *SlAREB1* gene. The *SlAREB1* gene expression was significant between days, treatments and interaction between days and treatments. With the progression of stress, the expression of the *SlAREB1* gene increased approximately 30 folds by Day 3. However, after re-watering, expression levels were reduced in all groups. With stress progression, it was expected that the *AREB* gene expression would increase (Narusaka *et al*., 2003). On Day 5.5, both the fertilizer and LANS treated groups had low expression levels compared to the treated group (~3 folds). However, by Day 6, the 3.5 mL/L ANE treated group indicated a further reduction in expression level, and higher expression levels were reported in both the fertilizer and LANS groups (~3 folds). This may be due to rapid water absorption during re-watering in both the fertilizer and LANS treated groups compared to the ANE treated group. Further, the 3.5 mL/L ANE treated group gradually reduced the expression levels after re-watering as expected. It is evident that ANE can lower the *ABRE* expression under stress and it confirms the obtained result for 3.5 mL/L ANE (Takezawa *et al.,* 2011).

Metacaspase 1 (LeMCA1)

Metacaspases are deviations of caspases and they play an important role in programmed cell death (PCD) in all organisms (Carmona-Gutierrez *et al*., 2010). Two types of matacaspases found in nature: type I and type II. Type I consists of an N-terminal extension which is an important component of the pro-domain in initiator caspases and inflammatory caspases. Type II metacaspases do not consist of a pro-domain but contain a linker region between the putative large and small subunits. Both types can be found in the plants (Vercamme *et al.,* 2007). *MCA1* gene is a type II metacaspase gene which can be found in different plant species such as *Arabidopsis*, tomato and tobacco (Hoeberichts *et al*., 2003; Hao *et al.,* 2007; Kurusu *et al.,* 2012; Kurusu *et al.,* 2013). PCD is induced in the plants under stress (Reape *et al.,* 2008). Drought stress induces the *LeMCA1* gene expression in tomatoes and it leads to PCD in the plant (Hoeberichts *et al*., 2003). A significant difference was evident between treatments but not between days or interaction between days and treatment. Fertilizer treated groups' expression level was similar before re-watering and it was increased after-re-watering. Both LANS and 3.5 mL/L ANE treated groups increased with the stress and after re-watering it exhibit a reduction, but increased by Day 6. Our results indicate that drought stress induces PCD, especially in the fertilizer treated plants. Re-watering did not have much effect on the fertilizer treated plants since the *LeMCA1* expression increased by Day 6. Even though there was no significant difference between days, it was evident that all groups increased their expression level from Day 5.5 to Day 6. However, both LANS and 3.5 mL/L ANE treated groups had relatively lower levels of expression. Though, 3.5 mL/L ANE treated plants showed the highest expression level by Day 3, by

Day 6, it had the lowest level of expression. Enhanced stress tolerance in plants treated with seaweed extracts can be the reason for lower levels of expression by Day 6 in the 3.5 mL/L ANE treated group (Khan *et al.,* 2009). Higher expression levels on Day 6 compared to Day 5.5 suggests that re-watering had an immediate effect on plants, but as time progresses, it could accelerate the PCD process again. Kranner *et al*., (2006) proved that oxidative stress leads to PCD. This could be the most probable reason why an increase in antioxidant enzyme levels was observed under increased stress in this study.

In general, all the genes indicated low expression levels after re-watering in 3.5 mL/L ANE treated group compared to the fertilizer and LANS treated groups. It may be due to rapid recovery in ANE treated plants. The expression of one gene under drought stress can trigger the expression of another gene. For example, *LEA* gene regulation leads to the expression of the *AREB1* gene under osmotic stress (Hong-Bo *et al.,* 2005). This was confirmed in this study since both *LEA* and *AREB1* genes showed a similar expression pattern. Further, *ltpg2* expression was controlled by *LEA* expression (Lu *et al.*, 2010).

6.11 Greenhouse Experiment

Two greenhouse trials were carried out in 2012. Thermal and relative humidity control was very poor in the green house and it directly affected the results. Compared to trial one, in trial two, the number of fruits set and harvested was less. Mainly, this was due to seasonal changes. The first trial was conducted during the summer and it extended into early fall. The second trial was conducted from mid-fall until mid-winter. Due to the

cold climate and shorter days in mid-fall, pollinating agents such as bees were less abundant in the greenhouse. This affected fruit set and yield. However, the ANE treated groups showed higher fruit set in both trials. Especially, the 3.5 mL/L ANE and 7.0 mL/L ANE treated groups showed a higher number of fruits in both trials. It has been proven that the seaweed extract treated plants produce higher yields (Rathore *et al.,* 2009). It was reported that seaweed extract treated plants show earlier flowering compared to normally fertilized plants and later it improves the yield in those plants (Arthur *et al*., 2003; Craigie 2011). This is the most probable reason for the high number of fruit set in ANE treated plants in both trials. In both trials, the LANS treated plants had a higher number of fruit compared to the fertilizer treated group. It was expected since LANS contain more nutrients than fertilizer. In trial one, the unstressed plants had the lowest number of fruit, but in trial two, unstressed plants had a comparable number of fruits compared to the other treatments. During the first trial, all of the unstressed plants were exposed to direct sunlight and high temperatures at the beginning, but the climate was favorable for unstressed plants in trial 2. Those plants had to recover from these initially adverse conditions. This delayed maturity and reaching the reproductive stage which resulted in low fruit production. The ANE treated plants in both trials yielded well. In trial 1, both the 3.5 mL/L and 7.0 mL/L ANE treated plants produced the highest yield, and the unstressed group produced the lowest. In trial 2, the LANS treated plants produced the highest yield and the 3.5 mL/L ANE, the 7.0 mL/L ANE and the unstressed groups followed sequentially. The fertilizer treated group showed the lowest yield in trial 2. The average fruit weight was similar in both trials for all of the treatments. It has been proven that ANE treated plants increase the vegetative growth and lead to the production of more

flowers and fruit at the reproductive stage in different crop species (Norrie and Keathley 2005; Abdel-Mawgoud *et al.,* 2010).

Overall, the ANE treated plants performed better under drought stress compared to the LANS and fertilizer treated plants. Although there was no statistical significant between the treatments in most of the experiments, there was a trend which expressed the performance superiority of the ANE treated plants. Among the four different ANE treatments, the 3.5 mL/L and 7.0 mL/L treatments were the most effective. In some experiments, the error was considerably high. It was due to the variation between biological replicates. Even though the uniformity of the plant population was maintained (only in plant age and height), there were times that biological replicates were different from each other (composition of biochemical substances). It was the major reason for high error in antioxidant enzyme level determination. However, the dominance of the ANE treated plants was clearly identified in the physiological parameters and gene expression studies compared to biochemical analysis. It is important to focus on the various aspects of drought stress since it gives a broader idea of how tomato plants endure stress better when treated with ANE, compared to LANS and fertilizer.

CHAPTER 7

CONCLUSIONS

7.1 Objective of the study

Drought stress is one of main factors that determine performances of plants specially the yield. There are different approaches used to overcome the problem such as mulching, rain water harvesting, using short duration or resistant varieties, but not much success with all of them. Application of ANE is one of the best approaches used so far in the modern day agriculture.

Ascophyllum nodosm (L.) Le Jol, is the most commonly using seaweed among different seaweed species. The overall objective was to investigate the effect of ANE on the plant performances under drought stress, where tried to understand the potential ability of ANE to overcome under water deficit conditions.

7.2 Drought stress and ANE application

It is clear that application of ANE aids to overcome drought stress in tomato plants. There were four different doses used as 0.875 mL/L, 1.75 mL/L, 3.5 mL/L and 7.0 mL/L. Both 3.5 mL/L (recommended dose) and 7.0 mL/L were more effective on plant stress tolerance than the 0.875 mL/L and 1.75 mL/L. Alone with ANE both LANS and fertilizer were used. ANE treated plants less wilted under water deficiency and also their physiological parameters such as stomatal conductance, plant water potential were better than the rest. Though they did not show statistical significance, both biochemical and some of gene expressions studies, confirmed the results obtained from physiological studies. ANE treated plants exhibited rapid changes in MDA, proline levels, as well as

the expression profiles. It was effective when the plants having good water supply since the minerals are readily available for the plants. However, both fertilizer and LANS treatments were not the ideal candidates to overcome drought stress.

7.3 Recommendations for future research

It was proven the superiority of the performance of ANE treated plants over the LANS and fertilizer treatments. However, there are some more areas to be considered for further confirmation on these findings. It is better to examine on how the photosynthesis can affect with drought stress and application of ANE. Further, examine on chlorophyll fluorescence with different treatments able to understand how oxidative stress can affect on chloroplasts and impact on photosynthesis.

It is good to focus on how the plant anatomy affect with the drought stress; especially observing on the changes in leaf anatomy can derive more information on drought stress. Not only leaf anatomy but also root system and vascular bundle are the other main aspects to be considered. Moreover, consider about total antioxidants and analyzing the ABA concentrations of leaves could give better understanding on drought stress. Further, it is better to conduct these experiments in an actual field conditions rather than controlled environmental conditions for few seasons, to study how plants perform in real situations.

It is better to use drought resistant such as transgenic *CBF1* tomato (Hsieh *et al.,* 2002), and drought susceptible mutants such as IFE 1 and Fireball (Babalola and Fawusi, 1980) alone with normal tomato plants to study further on mechanisms of drought stress tolerance with ANE application. Moreover, changing the application times and modes (foliar vs. root drench) can be beneficial to identify the effective mode of application and

effective time of application. All the information derived from these studies can be asset in farming using ANE.

Overall, ANE helps to mitigate drought stress in tomato during early vegetative stage and during reproductive stage it leads to higher yields compared to the rest. It is better to apply ANE at the beginning of planting rather than middle of the stress. It aids plants to perform high and overcome stresses.

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APPENDIX A

Mineral Composition of Modified Long Ashton Nutrient Solution (LANS)

Compound	Stock Concentration (g/ 100mL)	LANS-M (pH = 8) (mL/L)
KNO_3	2.02	2
$Ca(NO_3)_2.4H_2O$	4.72	0.4
$MgSO_4.7H_2O$	1.84	2
$NaH_2PO_4.H_2O$	1.84	15
Fe-citrate H_2O	0.25	0.5
$MnSO_4.H_2O$	0.034	0.5
$CuSO_4.5H_2O$	0.0125	0.1
$ZnSO_4.7H_2O$	0.058	0.1
H_3BO_3	0.186	0.1
$Na_2MoO_4.2H_2O$	0.0121	0.1
NaCl	0.585	0.1
$CoSO_4.7H_2O$	0.0053	0.1
KCl	6.24	2
KOH	4.72	2

Source: Acadian Seaplants Limited, Dartmouth, NS, Canada

APPENDIX B

ANOVA – Wilting

Source	D.F	Mean Visual rating, Wilting (1-5)		Linear Visual rating, Wilting (1-5)		Quadratic Visual rating, Wilting (1-5)		Cubic Visual rating, Wilting (1-5)	
		P	Mean Square	*P*	Mean Square	*P*	Mean Square	*P*	Mean Square
Rep stratum	2		0.7286		0.04388		0.002974		0.0006304
Rep.mPlot stratum									
Fert	5	**<0.001**	0.8537	**0.058**	0.02676	**<0.001**	0.007262	**0.059**	0.0002548
. Fert vs Rest	1	**<0.001**	1.974	**0.016**	0.07204	**0.011**	0.005758	0.993	0.000000006004
. LANS vs ANE	1	**0.025**	0.5135	0.845	0.000342	**<0.001**	0.013	0.777	0.000006924
. linANE	1	**<0.001**	1.581	**0.036**	0.04986	**<0.001**	0.01576	**0.006**	0.0009807
. quadANE	1	0.288	0.09361	0.689	0.001447	0.112	0.001793	0.228	0.0001345
. Deviations	1	0.258	0.1069	0.301	0.01011	0.926	0.000005374	0.202	0.0001519
Residual	10		0.07436		0.008506		0.0005887		0.00008147
Rep.mPlot.Samp.fU stratum	18		0.08674		0.01058		0.0009944		0.0001167
Total	35								

APPENDIX C

ANOVA – Stomatal Conductance

Source	D.F	Mean Visual rating, Wilting (1-5)		Linear Visual rating, Wilting (1-5)		Quadratic Visual rating, Wilting (1-5)		Cubic Visual rating, Wilting (1-5)	
		P	**Mean Square**	***P***	**Mean Square**	***P***	**Mean Square**	***P***	**Mean Square**
Rep stratum	2		28.5		518.8		27.84		33.09
Rep.mPlot stratum									
Fert	5	0.137	416.5	**0.024**	1238	0.132	76.06	0.153	19.83
. Fert vs Rest	1	**0.018**	1537	**0.008**	3117	**0.054**	162.4	0.199	18.14
. LANS vs ANE	1	0.630	47.04	0.231	470.4	0.727	4.395	0.714	1.364
. linANE	1	0.869	5.422	**0.017**	2365	0.110	104.8	**0.036**	55.89
. quadANE	1	0.152	459.2	0.388	235.3	0.112	103.5	0.147	23.72
. Deviations	1	0.681	34.08	0.925	2.707	0.703	5.261	0.949	0.04114
Residual	10		190.7		289.3		34.15		9.574
Rep.mPlot.Samp.fU stratum	18		138.6		91.2		15.23		4.796
Total	35								

APPENDIX D

ANOVA – Recovery%

Effect	DF	*F*	*Pr > F*
Treatment	5	13.64	**<0.0001**
Time	6	34.76	**<0.0001**
Treatment * Time	30	1.31	0.2068

** Significant at $\alpha = 0.05$

APPENDIX E

ANOVA – Water Potential, RWC and Dry Matter Content

Source	DF	Water Potential		RWC		Dry Matter %	
		P	Mean Square	*P*	Mean Square	*P*	Mean Square
Rep stratum	2		0.1111		172.6		10.23
Rep.mPlot stratum							
Treatment	5	0.545	0.1523	0.955	28.8	0.959	1.191
. Fert vs Rest	1	0.214	0.295	0.881	2.996	0.626	1.392
. LANS vs ANE	1	0.857	0.006125	0.444	79.25	0.534	2.274
. lin ANE	1	0.309	0.1969	0.876	3.252	0.774	0.4849
. quad ANE	1	0.306	0.1991	0.581	41.12	0.587	1.733
. Deviations	1	0.560	0.06436	0.719	17.36	0.913	0.06961
Time	5	0.657	0.1233	**0.003**	523.3	**0.033**	15.08
.. lin Time	1	0.157	0.3847	0.711	18.52	0.482	2.905
.. quad Time	1	0.346	0.1686	**<0.001**	2188	**0.001**	66.37
.. Deviations	3	0.953	0.02101	0.387	136.7	0.788	2.043
Treatment*Time	25	1.000	0.0392	0.999	42.2	0.999	1.78
.. Fert vs Rest.linTime	1	0.959	0.0005035	0.896	2.3	0.564	1.955
.. LANS vs ANE.linTime	1	0.729	0.02276	0.893	2.414	0.915	0.06654
.. Fert vs Rest.quadTime	1	0.588	0.05546	0.641	29.31	0.472	3.034
.. linANE.linTime	1	0.458	0.1045	0.674	23.79	0.690	0.9294
.. LANS vs ANE.quadTime	1	0.912	0.002299	0.477	68.33	0.815	0.3215
.. Fert vs Rest.Dev	3	0.985	0.00938	0.680	67.5	0.619	3.47
.. quadANE.linTime	1	0.300	0.2043	0.859	4.248	0.715	0.7813
.. linANE.quadTime	1	0.671	0.03405	0.823	6.697	0.808	0.3462
.. LANS vs ANE.Dev	3	0.882	0.04122	0.749	54.29	0.734	2.481
.. Dev.linTime	1	0.583	0.05705	0.694	20.85	0.874	0.148
.. quadANE.quadTime	1	0.840	0.007744	0.817	7.207	0.870	0.1556
.. linANE.Dev	3	0.821	0.05737	0.664	70.56	0.665	3.063
.. Dev.quadTime	1	0.936	0.001216	0.642	29.02	0.971	0.007822
.. quadANE.Dev	3	0.909	0.0339	0.836	38.11	0.872	1.363
.. Deviations	3	0.951	0.02147	0.737	56.47	0.809	1.878
Residual	70		0.1876		133.5		5.812
Rep.mPlot.Samp.fU stratum	108		0.01096		22.49		1.107
Total	215						

APPENDIX F

ANOVA – Root Growth

Root Length

Effect	DF	*F*	*Pr > F*
Treatment	5	49.78	**<0.0001**
Time	6	37.96	**<0.0001**
Treatment * Time	30	3.88	**<0.0001**

** Significant at α = 0.05

Root Area

Effect	DF	*F*	*Pr > F*
Treatment	5	14.25	**<.0001**
Time	6	8.61	**<.0001**
Treatment * Time	30	1.35	0.1025

** Significant at α = 0.05

APPENDIX G

ANOVA – Antioxidant Enzymes

Source	DF	SOD		CAT		GPOD	
		P	Mean Square	*P*	Mean Square	*P*	Mean Square
Rep stratum	2		2921		1504		1.76
Rep.mPlot.Samp.fU stratum							
Treatment	5	0.999	139	0.744	867.3	0.852	0.9341
. Fert vs Rest	1	0.900	57.21	0.337	1499	0.958	0.006593
. LANS vs ANE	1	0.857	117.7	0.407	1116	0.739	0.2672
. lin ANE	1	0.996	0.09202	0.497	747.5	0.391	1.776
. quad ANE	1	0.732	424.2	0.626	383.4	0.977	0.001987
. Deviations	1	0.871	95.68	0.546	590.5	0.298	2.619
Time	5	0.226	5106	0.785	781	0.825	1.027
.. lin Time	1	0.024	19186	0.314	1647	0.837	0.101
.. quad Time	1	0.931	26.91	0.655	322.9	0.590	0.6985
.. Deviations	3	0.625	2106	0.752	644.9	0.612	1.444
Treatment * Time	25	0.986	1602	0.899	1011	0.923	1.404
.. Fert vs Rest.linTime	1	0.051	14108	0.143	3528	0.322	2.365
.. LANS vs ANE.linTime	1	0.651	739	0.574	512.8	0.364	1.983
.. Fert vs Rest.quadTime	1	0.630	836.6	0.629	377	0.606	0.6374
.. linANE.linTime	1	0.786	267	0.921	16	0.204	3.909
.. LANS vs ANE.quadTime	1	0.871	95.65	0.924	14.72	0.877	0.05767
.. Fert vs Rest.Dev	3	0.855	923.3	0.843	440.9	0.616	1.433
.. quadANE.linTime	1	0.510	1567	0.103	4377	0.221	3.637
.. linANE.quadTime	1	0.767	315.6	0.879	37.61	0.986	0.0007449
.. LANS vs ANE.Dev	3	0.844	977.8	0.998	21.43	0.973	0.1808
.. Dev.linTime	1	0.995	0.1465	0.927	13.4	0.809	0.1407
.. quadANE.quadTime	1	0.592	1037	0.223	2427	0.737	0.2696
.. linANE.Dev	3	0.661	1908	0.128	3155	0.739	1
.. Dev.quadTime	1	0.588	1059	0.580	494.7	0.764	0.2163
.. quadANE.Dev	3	0.751	1445	0.919	266.4	0.885	0.5133
.. Deviations	3	0.755	1423	0.766	611.9	0.105	5.553
Residual	65		3577		1603		2.377
Total	102						

APPENDIX H

ANOVA – Lipid Peroxidation and Proline Content

Source	DF	Lipid Peroxidation		Proline Content	
		P	Mean Square	*P*	Mean Square
Rep stratum	2		116.9		896.6
Rep.mPlot.Samp.fU stratum					
Treatment	5	0.505	206	0.988	48.61
. Fert vs Rest	1	0.995	0.009743	0.753	40.32
. LANS vs ANE	1	0.548	86.31	0.907	5.581
. lin ANE	1	0.907	3.268	0.741	44.77
. quad ANE	1	0.085	720.6	0.608	107.8
. Deviations	1	0.338	219.9	0.741	44.55
Time	5	**0.016**	714.5	**0.008**	1389
.. lin Time	1	0.306	251.5	0.044	1711
.. quad Time	1	**0.009**	1721	**0.004**	3655
.. Deviations	3	0.090	533.2	0.282	525.8
Treatment * Time	25	0.702	194.1	1.000	99.21
.. Fert vs Rest.linTime	1	0.888	4.693	0.645	86.65
.. LANS vs ANE.linTime	1	0.005	1946	0.647	85.88
.. Fert vs Rest.quadTime	1	0.398	170.6	0.363	339.3
.. linANE.linTime	1	0.635	53.61	0.741	44.76
.. LANS vs ANE.quadTime	1	0.933	1.706	0.916	4.495
.. Fert vs Rest.Dev	3	0.349	263.6	0.808	131.5
.. quadANE.linTime	1	0.629	55.65	0.627	96.35
.. linANE.quadTime	1	0.531	93.88	0.995	0.01604
.. LANS vs ANE.Dev	3	0.829	69.67	0.662	215.8
.. Dev.linTime	1	0.615	60.2	0.915	4.671
.. quadANE.quadTime	1	0.570	77.01	0.669	74.49
.. linANE.Dev	3	0.808	76.64	0.957	42.77
.. Dev.quadTime	1	0.486	116.2	0.906	5.642
.. quadANE.Dev	3	0.257	325.7	0.926	63.06
.. Deviations	3	0.963	22.33	0.817	126.3
Residual	65		236.3		405.3
Total	102				

APPENDIX I

ANOVA – Photosynthetic Pigments

Source	DF	Chlorophyll *a*		Chlorophyll *b*		Total Chlorophyll		Carotenoids		Chlorophyll *a/b*		Carotenoids /Tot Chlorophyll	
		P	Mean Square	*P*	Mean Square	*P*	Mean Square	*P*	Mean Square	*P*	Mean Square	*P*	Mean Square
Rep stratum	2		1.808		0.3716		3.421		0.09574		0.4202		0.003486
Rep.mPlot.Samp.fU stratum													
Treatment	5	0.658	9.644	0.731	0.7746	0.686	13.79	0.707	0.5951	0.137	0.9225	0.999	0.001986
. Fert vs Rest	1	0.490	7.084	0.915	0.01588	0.568	7.345	0.585	0.304	0.144	1.159	0.848	0.001919
. LANS vs ANE	1	0.404	10.38	0.680	0.2379	0.449	12.92	0.426	0.6463	0.400	0.3804	0.725	0.006449
. lin ANE	1	0.924	0.1365	0.566	0.4612	0.834	0.9858	0.918	0.01081	0.060	1.939	0.863	0.001549
. quad ANE	1	0.277	17.62	0.570	0.4521	0.321	22.24	0.219	1.553	0.207	0.8614	0.994	0.000002632
. Deviations	1	0.350	12.99	0.167	2.706	0.289	25.45	0.501	0.4615	0.475	0.2732	0.987	0.0000132
Time	5	0.170	23.63	0.360	1.545	0.194	33.92	0.234	1.414	0.379	0.5725	0.034	0.1327
.. lin Time	1	0.239	20.76	0.705	0.2003	0.307	23.57	0.244	1.394	0.066	1.843	0.363	0.04329
.. quad Time	1	0.253	19.52	0.456	0.7776	0.281	26.27	0.215	1.58	0.657	0.1054	0.090	0.1528
.. Deviations	3	0.162	25.96	0.191	2.25	0.157	39.92	0.264	1.366	0.633	0.3048	0.036	0.1559
Treatment * Time	25	0.923	8.822	0.987	0.6129	0.940	12.77	0.902	0.6333	0.983	0.2456	1.000	0.0006367
.. Fert vs Rest.linTime	1	0.804	0.9166	0.624	0.3362	0.756	2.17	0.796	0.06764	0.770	0.04548	0.900	0.0008261
.. LANS vs ANE.linTime	1	0.419	9.723	0.396	1.01	0.403	15.79	0.459	0.5599	0.294	0.5924	0.881	0.001168
.. Fert vs Rest.quadTime	1	0.821	0.7596	0.557	0.4815	0.752	2.237	0.901	0.01586	0.656	0.1058	0.998	0.0000003905
.. linANE.linTime	1	0.276	17.73	0.170	2.667	0.238	31.63	0.281	1.19	0.873	0.01367	0.923	0.0004809
.. LANS vs ANE.quadTime	1	0.716	1.956	0.649	0.2891	0.694	3.473	0.642	0.2201	0.480	0.2669	0.928	0.0004299
.. Fert vs Rest.Dev	3	0.790	5.125	0.883	0.3032	0.809	7.191	0.590	0.6486	0.763	0.2048	1.000	0.0002491
.. quadANE.linTime	1	0.982	0.007323	0.945	0.006731	0.999	0.00004755	0.850	0.03653	0.393	0.3914	0.923	0.0004866
.. linANE.quadTime	1	0.558	5.094	0.674	0.2465	0.575	7.083	0.595	0.2869	0.706	0.07612	0.921	0.0005141
.. LANS vs ANE.Dev	3	0.292	18.67	0.528	1.034	0.329	26	0.309	1.23	0.897	0.105	0.999	0.0004554
.. Dev.linTime	1	0.542	5.529	0.552	0.4935	0.535	8.675	0.724	0.1268	0.951	0.002037	0.914	0.0006035
.. quadANE.quadTime	1	0.521	6.111	0.712	0.1896	0.553	7.918	0.454	0.5717	0.634	0.1209	0.960	0.0001307
.. linANE.Dev	3	0.254	20.39	0.642	0.7768	0.312	27.03	0.293	1.276	0.350	0.589	0.994	0.001329
.. Dev.quadTime	1	0.689	2.378	0.795	0.0945	0.706	3.199	0.672	0.1828	0.822	0.02691	0.939	0.0003097
.. quadANE.Dev	3	0.841	4.1	0.729	0.6017	0.819	6.873	0.830	0.2955	0.411	0.515	0.997	0.0007816
.. Deviations	3	0.631	8.499	0.806	0.4531	0.661	11.9	0.534	0.7417	0.922	0.08538	0.997	0.0008402
Residual	65		14.71		1.384		22.29		1.008		0.5298		0.0517
Total	102												

APPENDIX J

ANOVA – Gene Expression Study

P5CS

Effect	DF	*F*	*Pr > F*
Treatment	2	0.47	0.6280
Time	3	7.58	**0.0005**
Treatment * Time	6	0.09	0.9972

** Significant at α = 0.05

LEA

Effect	DF	*F*	*Pr > F*
Treatment	2	36.60	**<0.0001**
Time	3	110.86	**<0.0001**
Treatment * Time	6	25.41	**<0.0001**

** Significant at α = 0.05

ltpg-2

Effect	DF	*F*	*Pr > F*
Treatment	2	2.09	0.1438
Time	3	40.50	**<0.0001**
Treatment * Time	6	1.89	0.1212

** Significant at α = 0.05

AREB1

Effect	DF	*F*	*Pr > F*
Treatment	2	4.70	**0.0177**
Time	3	88.40	**<0.0001**
Treatment * Time	6	10.39	**<0.0001**

** Significant at α = 0.05

MCA1

Effect	DF	*F*	*Pr > F*
Treatment	2	3.67	**0.0408**
Time	3	1.99	0.1428
Treatment * Time	6	0.50	0.8048

** Significant at α = 0.05

APPENDIX K

ANOVA – Green House Experiment

Total Number of Fruits Set - Trial 1

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Treatment	6	77.3055556	12.8842593	1.31	0.2837
Error	29	285.0000000	9.8275862		
Total	35	362.3055556			

** Significant at α = 0.05

Total Number of Fruits Set - Trial 2

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Treatment	6	48.4761905	8.0793651	1.27	0.2945
Error	29	222.0000000	6.3428571		
Total	35	270.4761905			

** Significant at α = 0.05

Total Number of Fruits Harvested – Trial 1

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Treatment	6	33.0555556	5.5092593	1.82	0.1302
Error	29	87.8333333	3.0287356		
Total	35	120.8888889			

** Significant at α = 0.05

Total Number of Fruits Harvested – Trial 2

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Treatment	6	27.4761905	4.5793651	1.86	0.1158
Error	29	86.1666667	2.4619048		
Total	35	113.6428571			

** Significant at α = 0.05

Total Yield – Trial 1

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Treatment	6	74097.7668	12349.6278	1.73	0.1501
Error	29	207303.8010	7148.4069		
Total	35	281401.5678			

** Significant at α = 0.05

Total Yield – Trial 2

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Treatment	6	83366.6222	13894.4370	2.64	**0.0340**
Error	29	168371.9038	5261.6220		
Total	35	251738.5260			

** Significant at α = 0.05

Mean Fruit Weight – Trial 1

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Treatment	6	8833.95200	1472.32533	6.60	**<0.0001**
Error	29	51100.54976	223.14651		
Total	35	59934.50177			

** Significant at α = 0.05

Mean Fruit Weight – Trial 2

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Treatment	6	4188.16099	698.02683	2.16	**0.0530**
Error	29	33305.37820	323.35319		
Total	35	37493.53919			

** Significant at α = 0.05