PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF DROUGHT TOLERANCE IN CHICKPEA

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ABSTRACT

PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF DROUGHT TOLERANCE IN CHICKPEA

Chickpea (*Cicer arietinum* cv. Gokce.) is an agronomically and economically significant plant for Turkey. It is successfully grown under severe drought conditions which limit the growth of other plants. It is generally affected by terminal drought which causes retardation of flowering and decreases yield in Mediterranean and sub-tropical climates. The aim of this study was to determine significant factors which can be used to identify chickpea plant tolerance to drought stress. With this objective we assessed physiological (fresh and dry weight, relative and real water content) and biochemical (enzymatic and non-enzymatic antioxidants, malondialdehyde, total protein and phytohormone contents) parameters which were used to measure the impact of drought on chickpea. To determine drought's effects, we collected stressed (drought treated) and control (non drought treated) samples from the chickpea cultivar Gokce. Results showed that both fresh and dry weights of plants increased while real and relative water contents of plants decreased under drought stress. There was an increase in both malondialdehyde (MDA) and total protein contents under drought stress. Furthermore, glutathione reductase (GR) and catalese (CAT) enzyme activity increased in drought treated plants whereas guaiacol peroxidase (POD) and superoxide dismutase (SOD) enzyme activity decreased. Moreover, contents of indole acetic acid (IAA) and abscisic acid (ABA) increased in all tissue parts while contents of salicylic acid (SA), gibberellic acid (GA) and jasmonic acid (JA) increased in specific plant tissue parts during drought treatment. In conclusion it is obvious that all of these characters play essential roles in the drought tolerance of plants.

ÖZET

NOHUTTA KURAKLIK TOLERANSININ FİZYOLOJİK VE BİYOKİMYASAL KARAKTERİZASYONU

Nohut (*Cicer arietinum cv.* Gokce), tarımsal ve ekonomik açından Türkiye için önemli bir bitkidir. Diğer bitkilerin büyümesini kısıtlayan şartlar altında başarıyla büyür. Nohut, Akdeniz ve tropikal iklimlerde bitkinin büyümesini yavaşlatan ve verimliliğini düşüren terminal kuraklıktan etkilenir. Bu çalışmanın amacı, nohutun kuraklık stresine dayanıklılığını sağlayan önemli faktörlerin belirlenmesidir. Bu amaçla, kuraklığın nohut üzerindeki etkilerini belirlemek için fizyolojik (taze ve kuru ağırlık, bağıl ve gerçek suyu miktarı) ve biyokimyasal (enzimatik ve enzimatik olmayan antioksidantların miktarı, malondialdehit, toplam protein ve bitki hormonlarının miktarları) parametreleri ölçtük. Kuraklığın etkilerini belirlemek için bir nohut çeşidi olan Gokce bitkisinin stres ve kontrol gruplarından örnekler topladık. Sonuçlar, bitkinin yaş ve kuru ağırlıkları artarken, bağıl ve gerçek su miktarlarının azaldığını göstermiştir. Kuraklık stresi altında hem malondialdehit (MDA) hem de toplam protein miktarlarında bir artış gözlenmiştir. Bunların yanı sıra, kuraklığa maruz bırakılan bitkilerde glutatyon reductaz (GR) ve katalaz (CAT) enzimlerinin aktivitesi artarken, guaiakol peroksidaz (POD) ve superoksit dismutaz (SOD) enzimilerinin aktivitelerinin azaldığı tespit edilmiştir. Bu sonuçlara ek olarak, kuraklık stresi boyunca indola asetik asit (IAA) ve absisik asit (ABA) miktarları tüm bitki dokularında artarken salisiklik asit (SA), gibberellik asit (GA) and jasmonik asit (JA) miktarlarının belli dokularda arttığı saptanmıştır. Sonuç olarak, bütün bu karakterlerin, bitkinin kuraklık toleransında önemli bir rol oynadığı belirlenmiştir.

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CHAPTER 1

INTRODUCTION

1.1. Chickpea (*Cicer arietinum* L.)

The Leguminosae family is classified into 650 genera with 18,000 species including chickpea (Varshney et al. 2009). Chickpea (*C. arietinum* L.) is a self pollinated plant with 16 chromosomes (2n=16). Its genome size is approximately 740 Mb (Arumuganathan and Earle 1991). It is the third most widely cultivated legume crop after dry bean (*Phaseolus vulgaris* L.) and field pea (*Pisum sativum* L.). Chickpea ranks fifth among legumes, and 15th among grain crops (Katerji et al. 2001). There are two types of chickpea seed. *Desi* chickpeas have brown-colored, small seeds and *kabuli* chickpeas have white or beige-colored, large seeds. The *desi* type is cultivated in 85% of global chickpea fields and is abundantly produced in South and East Asia, Iran, Ethiopia, and Australia (Varshney et al. 2011). On the other hand, the *kabuli* type is commonly cultivated in Mediterranean regions, West Asia, North Africa, and North America (Varshney et al. 2011). The *kabuli* type differs from the *desi* type in terms of the quantity and quality of seed dietary fiber components (Singh 1987). According to cytogenetic and seed protein analysis, *C. reticulatum* is the ancestor of cultivated *C. arietinum* and it is proposed that *C. reticulatum* originated in southeastern Turkey (Ladizinsky and Adler 1976).

Chickpea contains high amounts of protein (23%), dietary fiber, carbohydrates (64% total carbohydrates), and minerals such as calcium, magnesium, potassium, phosphorus, iron, zinc, and manganese, and it is assumed to be a nutraceutical plant (Thudi et al. 2011). Due to its high concentration of protein and carbohydrate, chickpea has an important place in human nutrition and plays an especially essential role in solving malnutrition problems in developing countries. Furthermore, chickpea is an essential source of fodder and it is a very significant crop for sustainable agriculture. Chickpea's roots host *Rhizobium* bacteria which are important in nitrogen fixation, so

chickpea provides enrichment of nitrogen in soil (Babaoglu 2003). Because of its N_2-fixing features, chickpea is also used as a rotation crop (Macar and Ekmekci 2009).

In 2006, chickpea was cultivated on 10.7 million hectares (Mha) around the world. India, Pakistan, and Iran grew more than 8 Mha and the rest of the cultivated area included countries in Asia, the Middle East, and Canada (FAOSTAT 2006) (Varshney et al. 2011). Although, Turkey ranks second after India in annual chickpea production with over 618 million tons (FAOSTAT 2010 Figure 1.1), there was a 31% reduction in chickpea production between 1992 and 2010. Chickpea is harvested over 446 Kha in Turkey.

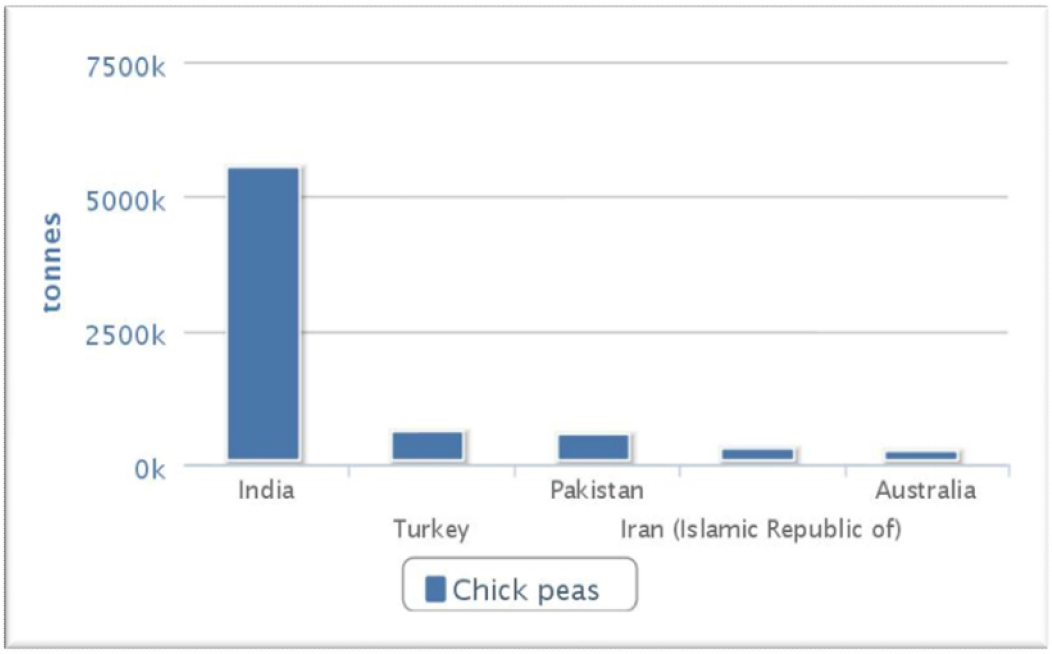

Figure 1.1. Chickpea production of top 5 producers around the world
(Source: FAOSTAT 2010, Average 1992-2010)

1.2. Drought Stress

Drought is one of the main risks associated with global warming. But there is no generally-approved, specific definition of drought. Possibly the most commonly accepted definition of drought is offered by Beran and Rodier (1985): 'drought is reduction in water availability in a specific time and on a specific region'. Thus, drought

happens when an essential water shortage expands both in time and area (Tsakiris and Vangelis 2004).

Agriculture is the major consumer of water resources in many regions of the world. Due to global warming and climate change, drought is increasing. In addition to this increase, the growing world population affects the availability of water. Therefore water will become the most important vital substance in the near future. In recent years, understanding the effects of drought on plants has became a very important matter for improvement of plant breeding and management techniques in agriculture and in determination of the fate of natural vegetation in the environment (Chaves et al. 2003).

Drought tolerance can be defined as the ability of the plant to withstand water deficit while maintaining appropriate physiological activity. However, drought tolerance is a very complex mechanism; it not only depends on features of defense mechanisms but also depends on the response to several environmental signals (Xiong and Ishitani 2006). Although suitable irrigation and agronomic practices reduce yield losses by certain amounts, drought stress' effect on yield mostly depends on the genetic structure of the crop (resistance and susceptibility) (Chaves and Oliveria 2004; Turner 2004). Under severe drought, plants activate several molecular, biochemical and physiological pathways which are divided into six classes.

1. *Drought escape* involves finishing the plant life cycle before dramatic water reduction. Early flowering in plants before severe drought conditions is an example of escape.

2. *Drought avoidance* is through increased capability for water uptake. For example, improvement of root mechanisms or saving water by reduced number of stomata are avoidance mechanisms.

3. *Drought tolerance* is mostly through development of osmotic adjustment capability and increasing cell wall flexibility to manage tissue turgidity.

4. *Drought resistance* occurs by changing metabolic pathways to survive under stress. Increases in antioxidant metabolism are an example of resistance.

5. *Drought abandon* is loss of a leaf or another part of the plant from the main body. For example, abscission of old leaves under drought stress is a type of drought abandonment.

6. *Drought adaptation* is the stimulation of biochemical and physiological traits via genetic mutations and modifications for adaption of plants to a long period of drought stress.

Plant response mechanisms against drought stress are also affected by several environmental factors (Figure 1.2) (Chaves et al. 2003).

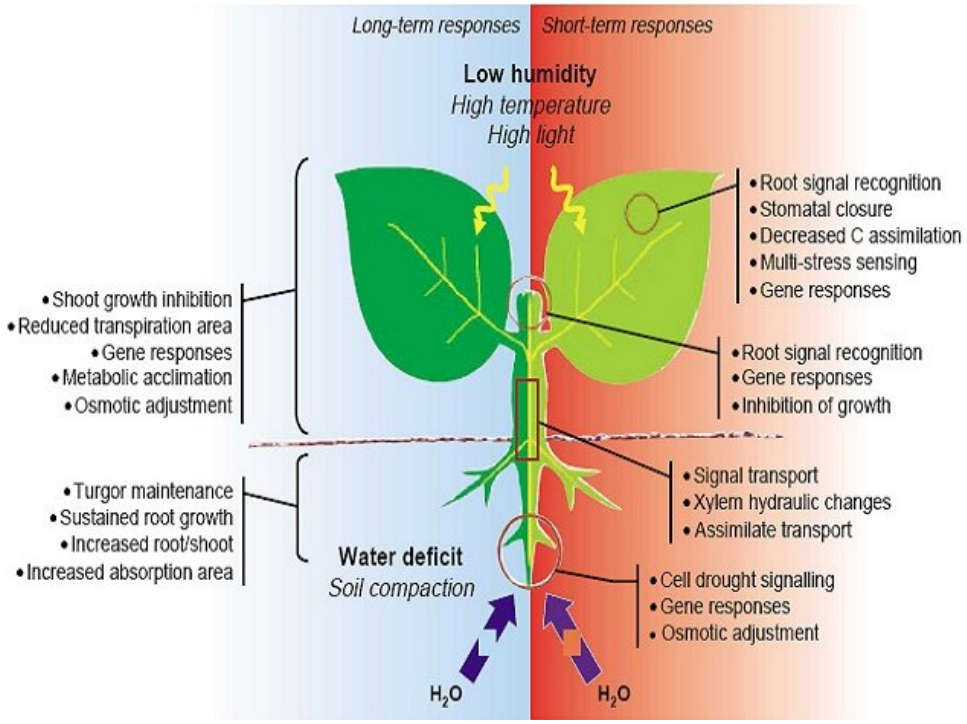

Figure 1.2. Plant adaptation mechanisms in response to decreased water availability. (Source: Chaves et al. 2003)

Drought escape can also be described as successful reproduction of the plant before the drought stress. Selection is a very common and significant method for fast phenological improvement in breeding for drought resistance in plants. Jordan et al. (1983) and Saeed and Francis (1983) indicated that late maturing genotypes adapted to wet circumstances better than early maturing genotypes whereas early maturing genotypes showed better adaptation to drought stress. However, much research has indicated that there is a positive relationship between long growth period and yield potential. So, it is assumed that some yield potential is lost for early phenological improvement under stress conditions. This may cause a significant complication in environments where plants experience unstable moisture patterns. This also may be a

disadvantage, particularly in indeterminate plants that have a potential for re-growth and productivity after recovery (Bidinger et al. 1982; Turk et al. 1980; and Villalobos-Rodruigez). Increased environment predictability can allow better optimization of crop duration. Growing more plants can also reduce the negative effect of decreased yield potential in early maturing genotypes (Blum 1970).

Drought avoidance protects a high level of tissue water potential. It tries to keep water potential at the same high level under soil and atmospheric water stress. Generally, the primary response of a plant to water deficiency is to avoid low tissue water potential. To protect the stability of this potential, plants raise their water intake or decrease their water loss. To decrease water loss, plants initially close their stomata. Decreased root and shoot growth are the most significant changes in crop plants which are observed during long periods of water deficiency. Drought avoidance can be enough to preserve plant performance (Kramer and Boyer 1995). There is a correlation between decreases in both carbon gain and loss of water which is maintained by stomatal control in plants. Consequently a decrease in loss of water which is controlled by stomatal closure also causes a decrease in carbon assimilation with the resulting effects on productivity.

Drought tolerance is not associated with productivity but is related to plant survival. Under significant water stress, loss of turgor and dehydration is observed in cells when plant tissue is not defended by avoidance systems. Cellular dehydration stimulates essential changes in cellular structures (Poljakoff-Mayber 1981). One of the important parts of dehydration tolerance is the capability of plant tissue to manage turgor pressure while under dramatic water stress (Hsiao 1973; Hsiao et al. 1976). Most research studies about drought tolerance traits are initially related to protection of cellular structures from the impact of dehydration. Various types of protective proteins including dehydrins and late-embryogenesis abundant (LEA) proteins are stimulated by reductions in tissue water level (Close 1997). These proteins act like chaperones and play important roles in protection of protein and membrane structure (Bravo et al. 2003; Verlag et al. 2001). In addition to these proteins, compatible solutes can take part in the protection of protein and membrane structures under dehydration (Hincha and Hagemann 2004).

Recently, many studies about reactive oxygen species (ROS) have been done to understand the role of ROS in the stress signaling pathway. ROS have a significant

function in the stress induced signaling pathway and are used to gain information about the effects of environmental changes on plants (Chen and Gallie 2004; Hung et al. 2005).

Plant hormones (phytohormones) are also affected by environmental stress; severe drought stress causes changes in the levels of some plant hormones. For example, abscisic acid (ABA) level increases under water deficiency (Yurekli et al. 2001). ABA plays a significant role in the reduction of water loss via transpiration. ABA mediates stomatal closure by many events including formation of activated oxygen species, alkalization and alterations in the flux of Ca^{2+} and K^{+} between guard cells (Bright et al. 2006; Mori et al. 2001; Pandey et al. 2007). ABA is not the only plant hormone which regulates stomatal closure; methyl jasmonate (JA), salicylic acid (SA) and ethylene are also included in this process. For example, SA mediates stomatal closure by influencing ABA signaling (Cho et al. 2008).

1.3. Reactive Oxygen Species (ROS)

Approximately 2.7 billion years ago, molecular oxygen became part of our environment through O_2-evolving photosynthetic organisms and, ever since, ROS have been unwanted products of aerobic life (Halliwell 2006). The O_2 molecule is defined as a free radical and contains two weakened electrons that possess identical spin quantum number. This spin limits the acceptance of electrons and O_2 receives its electrons one by one, initiating formation of ROS which can be harmful to cells. ROS are also generated constantly as co-products of diverse metabolic pathways that take place in different cellular compartments like the chloroplast, mitochondria, and peroxisomes (Sandalio et al. 2006; Navrot et al. 2007). Different abiotic and biotic stress factors like salinity, UV radiation, drought, heavy metals, temperature extremes, nutrient deficiency, air pollution, herbicides and pathogen attack can disturb the balance between the production and scavenging of ROS. This perturbation in balance stimulates sudden increases in intracellular amounts of ROS which can cause significant harm to cell structure. It has been predicted that 1-2% of O_2 consumption leads to generation of ROS in plant tissues (Bhattachrjee 2005). By means of many different reactions, $O_2^{\cdot-}$ stimulates generation of H_2O_2, $OH^{\cdot}$, and other ROS. The ROS containing $O_2^{\cdot-}$, H_2O_2,

$^1O_2$, $HO_2^{\cdot -}$, $OH^{\cdot}$, ROOH, $ROO^{\cdot}$, and $RO^{\cdot}$ are extremely toxic and reactive. Moreover they induce harm to proteins, lipids, carbohydrates, and DNA, eventually causing cell death (Figure 1.3).

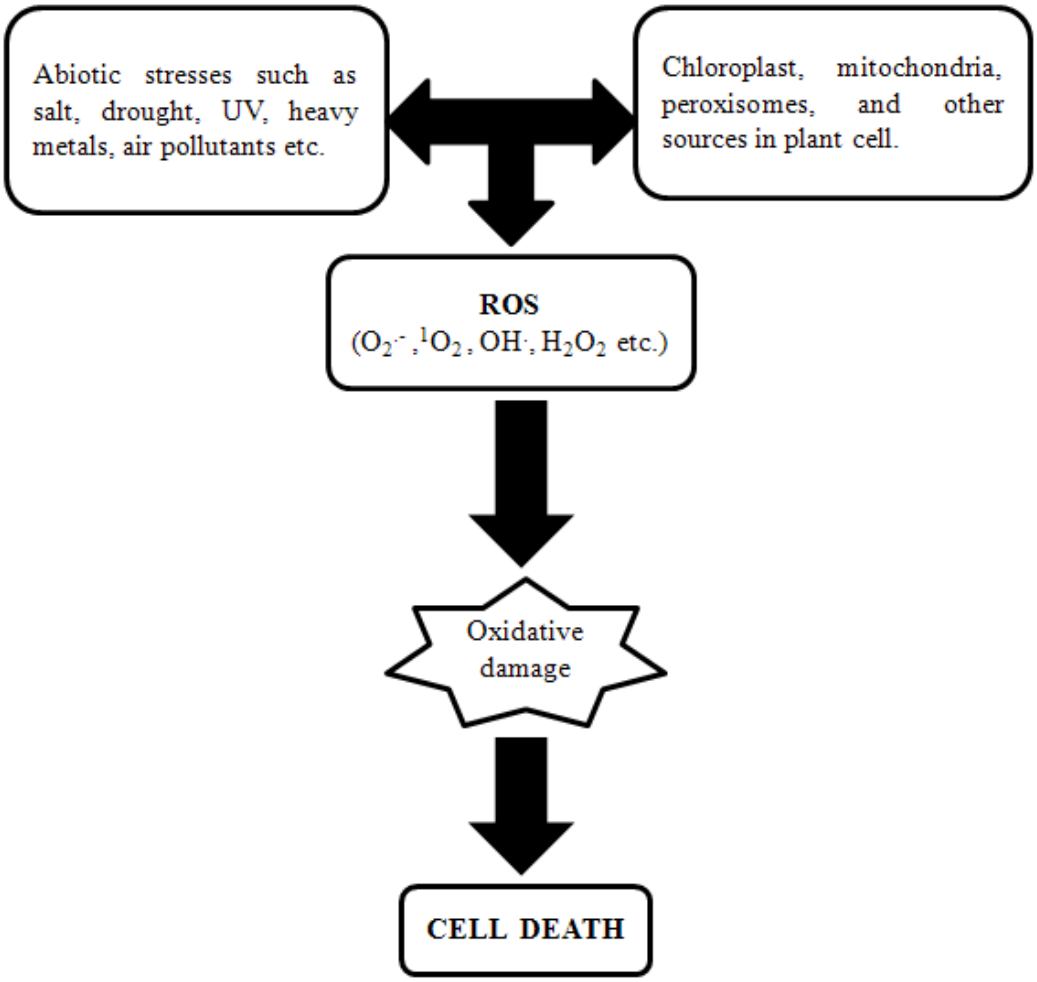

Figure 1.3. Abiotic stress induced ROS production and cell death.
(Source: Drawn from Gill and Tuteja 2010)

ROS accumulation due to various environmental stresses is a main cause of crop productivity loss around the world (Mittler 2002). Depending on the balance between ROS generation and scavenging at different locations and times, ROS can act as damage-inducing, protective or signaling factors (Gratao 2005). ROS alters peroxidation (LPO) (Foyer and Noctor 2005). ROS can damage the cell but on the other hand they can also trigger responses like new gene expression. Activation of the cell response strongly depends on several factors. Subcellular location can be a very

significant factor for formation of ROS because ROS penetrate only a very short distance before reacting with cellular molecules. Stress-induced ROS accumulation is countered by antioxidant enzyme systems that contain a variety of scavengers, like superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX), glutathione-S-transferase (GST), and catalase (CAT) and also non-enzymatic antioxidant system like ascorbate (ASH), reduced glutathione (GSH), ∞-tocopherol, carotenoids and flavonoids (Mittler et al. 2004). In addition, proline can be included in the list of non-enzymatic antioxidants which microbes, animals, and plants require to resist the inhibitory effects of ROS (Chen and Dickman 2005). Lately, it has become evident that plants continuously generate ROS which may direct varied physiological pathways like biotic and abiotic stress response, pathogen defense and systemic signaling.

1.4. Antioxidant Defense System

Negative environmental conditions like temperature extremes, heavy metals, drought, reduced water availability, air pollutants, nutrient deficiency, or salt stress can enhance the generation of ROS such as, $^1O_2$, $O_2^{\cdot-}$, H_2O_2 and $OH^{\cdot}$ in plants. Plants have developed defense mechanisms to protect themselves from these toxic molecules. Plant cells and organelles like the chloroplast, mitochondria and peroxisomes activate antioxidant defense systems (Figure 1.4) (Tuteja 2007; Khan and Singh 2008). The constituents of the antioxidant defense mechanism are divided into two classes: enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants include SOD, CAT, APX, and GR and non-enzymatic antioxidants include water soluble (GSH, ASH) and insoluble (carotenoids and tocopherols) compounds and osmolytes (proline) (Gill and Tuteja 2010). The antioxidant defense mechanism plays an important role in detoxification and removal of toxins, thereby hindering their negative effects.

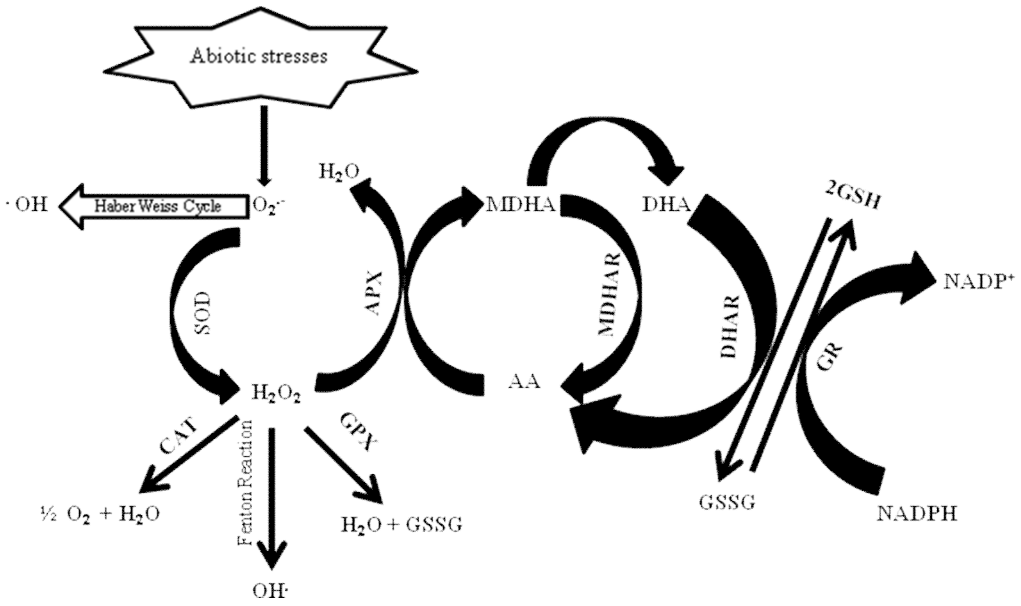

Figure 1.4. ROS and antioxidant defense mechanism.
(Source: Drawn from Gill and Tuteja 2010)

1.4.1. Enzymatic Antioxidants

Superoxide dismutase (SOD) is the most efficient intracellular enzymatic antioxidant that is found in all aerobic organisms and in all subcellular compartments subject to ROS-mediated oxidative stress. It is well known that many different environmental stresses usually cause an increase in the formation of ROS and SOD plays a significant role in this plant stress tolerance mechanism. SOD provides the first defense response against the toxic effects of high levels of ROS by converting $O_2^{\cdot-}$ to H_2O_2 and O_2 (Table 1.1). In this reaction one $O_2^{\cdot-}$ molecule is reduced to H_2O_2 and oxidized to O_2. Through a metal-catalyzed Haber-Weiss type reaction, SOD prevents the activation of $O_2^{\cdot-}$ and reduces the risk of $OH^{\cdot}$ generation. The spontaneous dismutation rate is 10,000 fold slower than this reaction rate (Gill and Tuteja 2010). SODs are divided into three classes according to their metal cofactors. These are copper/zinc (Cu/Zn-SOD), manganese (Mn-SOD), and iron (Fe-SOD) SODs, which are found in different cellular compartments (Mittler 2002). All types of SODs are nuclear-encoded and are directed to their subcellular compartments via an amino terminal targeting sequence.

Catalase (CAT) is an enzyme that includes a tetrameric heme and directly converts H_2O_2 into H_2O and O_2 and is unavoidable for detoxification of ROS under stress conditions (Table 1.1). CAT has the highest level of turnover among all the enzymes: one CAT molecule can change approximately 6 million H_2O_2 molecules into H_2O and O_2 per minute (Galli and Tuteja 2010). In the peroxisomes, CAT plays an essential role in the scavenging of H_2O_2 which is generated by oxidases involved in the β-oxidation of fatty acids, photorespiration and purine catabolism (Polidoros and Scandalios 1999).

Guaiacol peroxidase separates indole-3-acetic acid (IAA) into its components. In addition to this reaction, guaiacol peroxidase plays a significant role in biosynthesis of lignin and protects plants against biotic stresses by consuming H_2O_2 (Table 1.1). Guaiacol peroxidase uses aromatic electron donors like guaiacol and pyragallol. Generally, it oxidizes ascorbate at a rate of around 1% that of guaiacol (Asada 1999).

Table 1.1.Major ROS scavenging antioxidant enzymes.

Enzymatic antioxidants	**Enzyme code**	**Reaction catalyzed**
Superoxide dismutase	EC 1.15.1.1	$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightarrow 2H_2O_2 + O_2$
Catalase	EC 1.11.1.6	$H_2O_2 \rightarrow H_2O + ½\ O_2$
Ascorbate peroxidase	EC 1.11.1.11	$H_2O_2 + AA \rightarrow 2H_2O + DHA$
Guaiacol peroxidase	EC 1.11.1.7	$H_2O_2 + GSH \rightarrow H_2O + GSSG$
Glutathione reductase	EC 1.6.4.2	$GSSG + NAD(P)H \rightarrow 2GSH + NAD(P)^+$

Glutathione reductase is an enzyme involved in defense against ROS by keeping glutathione (GSH) in a reduced state and by taking part in the ASH-GSH cycle. It is mainly found in the chloroplast, but a small amount of this enzyme has also been localized in the mitochondria and cytosol (Edwards et al. 1990; Creissen et al. 1994). Glutathione reductase stimulates the reduction of glutathione, a molecule that plays a role in a large number of metabolic regulatory and antioxidative pathways in plants. Glutathione reductase induces the NADPH-dependent disulphide bond reaction of oxidized glutathione (GSSH) and so, it is essential for sustaining the glutathione (GSH) pool (Reddy and Raghavendra 2006; Rao and Reddy 2008) (Table 1.1). GSSH contains two GSH connected by a disulphide bridge which can be changed back into GSH by

glutathione reductase. GSH is involved in the cell system which includes the ASH-GSH cycle and conservation of sulfhydryl (-SH) group. In addition GSH is used as a substrate for glutathione-S transferase (Reddy and Raghavendra 2006). Glutathione reductase and glutathione-S transferase are very significant enzymes, which are used to evaluate plant tolerance to different kinds of stress.

1.4.2. Non-enzymatic Antioxidants

Ascorbic acid is the most abundant, intense and water soluble antioxidant. It plays a role in prevention or reduction of damage caused by ROS in plants (Smirnoff 2005; Athar et al. 2008). It is seen in all plant tissues; however, the level of ascorbic acid is generally higher in photosynthetic cells and meristems (and some fruits). The mitochondrion has an essential role in ascorbate (ASH) metabolism in plants. Plant mitochondria play a role in both production of ASH and reformation of oxidized forms of ASH (Szarka et al. 2007). The half-life of completely oxidized dehydroascorbic acid is very short and if it is not reduced back, it could be lost, so the formation of ascorbate is very significant. Due to its capability to donate electrons in several enzymatic and non-enzymatic reactions, ASH is thought to be the strongest ROS scavenger. It can protect membranes by removing $O_2^{\cdot -}$ and $OH^{\cdot}$ and by re-producing α-tocopherol from the tocopheroxyl radical (Smirnoff 2000).

Tripeptide glutathione (GSH) is a molecule that plays an essential role in intracellular protection from oxidative damage stimulated by ROS in plants. The reduced form of GSH is enriched in plant tissues and in all cell compartments such as the cytosol, endoplasmic reticulum, vacuole, mitochondria, chloroplasts, peroxisomes, and apoplast (Mittler and Zilinzkas 1992; Jimenez et al. 1998). GSH is involved in many physiological processes, like regulation of sulfate transport, signal transduction, conjugation of metabolites, detoxification of xenobiotics and the expression of stress responsive genes (Mullineaux and Rausch 2005). GSH is required to maintain the normal reduced state of cells, so they can resist the destructive effects of ROS-induced oxidative stress (Meyer 2008). Most ROS like $^1O_2$, H_2O_2 and $OH^{\cdot}$ are scavenged by GSH. In addition to this, GSH also reproduces the water soluble antioxidant ASH, through the ASH-GSH cycle (Foyer and Halliwell 1976). It has been observed that

when stress increases, GSH intensities generally decrease and the redox state of the cell becomes more oxidized, causing retrogression of the system (Tausz et al. 2004). Because of its role in the antioxidant defense mechanism, GSH is used as a stress marker. But, its antioxidant function depends on the amount of cellular GSH and it can vary under abiotic stresses. Moreover, studies showed that increased GSH is associated with the resistance of plants against metal-induced oxidative stress (Pietrini et al. 2003; Metwally et al. 2005; Sun et al. 2007; Molina et al. 2008).

In addition to being an osmolyte, proline (Pro) is now thought to be a powerful antioxidant and inhibits programmed cell death (PCD). As a result of this, proline can be considered as a non-enzymatic antioxidant and it is needed to decrease the negative effect of ROS in microbes, animals and plants (Chen and Dickman 2005). It was shown that there is a huge accumulation of proline after salt, drought and metal stress, and this can result from increased synthesis or reduced degradation. Free proline functions as an osmoprotectant, a secondary metabolite, a protein stabilizer, a metal chelator, an inhibitor of lipid peroxidation (LPO), and a scavenger of $OH^{\cdot}$ and $^{1}O_2$ (Ashraf and Foolad 2007; Trovato et al. 2008). Among proline, sorbitol, mannitol, and myo-inositol, proline is the most powerful $OH^{\cdot}$ scavenger (Smirnoff and Cumbes 1989). In addition to its important role in the redox signaling pathway, proline is an efficient suppressor of ROS which are induced by salt, metal and dehydration stress conditions in plants. It was proposed that the capability of proline to remove ROS and to inhibit apoptosis which is mediated by ROS may play a significant role in the response to cellular stress. Increases in the amount of proline have been associated with tolerance of plants against different kind of abiotic stresses particularly drought and salt stress. Under drought or salt stress, the formation of proline increases and it plays a role in reduction of cytoplasmic acidosis and protection of the balance between $NADP^{+}$ and NADPH (Gill and Tuteja 2010). A number of studies proposed that formation of proline has a significant role in the activity of the pentose-phosphate pathway, because of its importance as a component of antioxidative defense mechanisms, which require NADPH to obtain reduced forms of GSH and ASH (Hare and Cress 1997). Analysis of the proportion of reduced/oxidized GSH and the amounts of malondialdehyde (MDA) show that free proline rates are associated with the GSH redox form and amounts of malondialdehyde in heavy metal-treated algae. (Siripornadulsil et al. 2002).

1.5. Plant Hormones

Plants hormones (phytohormones) are defined as chemical messengers that are generated in one part of the plant and are transported to other parts. They have significant roles in activation of the plant response mechanism under stress conditions. Phytohormones are also known as plant growth regulators (Javid et al. 2011). They act as mediators of endogenous developmental programmes and regulate host responses to a wide range of biotic and abiotic stresses like pathogen challenge, insect herbivory, drought, cold and heat stress (Forcat et al. 2008). Indole acetic acid, gibberellic acid, jasmonic acid, salicylic acid and abscisic acid are the most significant plant hormones that play roles in the plant's stress response mechanism (Cho et al. 2008).

Indole acetic acid (IAA) is a member of the auxin family. Auxin plays a role in the control of most features of plant growth and development involving cell division, elongation and differentiation (Pagnussat et al. 2003). In the auxin family, it has been proven that indole acetic acid regulates root formation, apical dominance and tropic responses (Pagnussat et al. 2004).

Gibberellic acid (GA) is an essential plant hormone and plays roles in many features of plant biology, including seed germination, leaf expansion, stem and root elongation, flowering time, and fruit development (Qin et al. 2011). Gibberellic acid metabolism and signaling regulate GA homeostasis. However, this homeostatic mechanism has not been solved and still remains unclear. There is also a relationship between GA action and other hormone signaling or environmental stresses to regulate plant growth and development. Gibberellic acid accumulation quickly increases in plants under both biotic and abiotic stresses (Javid et al. 2011).

Methyl jasmonate (MeJA) and its free acid (jasmonic acid) are known as jasmonates. They are significant cellular regulators that are involved in various developmental processes, like seed germination, root growth, fertility, fruit ripening, and senescence (Wasternack and Hause 2002). Moreover, jasmonates play a role in stimulation of the plant defense mechanism in response to insect-driven wounding, different pathogens, and environmental stresses, like drought, low temperature and salinity. Jasmonic acid biosynthesis occurs primarily in leaves (Cheong and Choi 2003).

Salicylic acid (SA) plays roles in plant growth and development by regulating seed germination, vegetative growth, photosynthesis, thermogenesis, flower formation,

seed production, senescence and protection against biotic and abiotic stresses like drought. Moreover, SA can manage cellular redox homeostasis by controlling antioxidant enzyme activity and stimulation of the alternative respiratory pathway (Rivas San Vicente and Plasencia 2011). SA is also involved in the signal transduction pathway leading to systemic acquired resistance (SAR). Leaf infection by microbial pathogens induces an increase in endogenous expression of SA (Penninckx et al. 1996).

Abscisic acid (ABA) serves as a mediator in the plant's response to various stresses, including drought and salt stress. ABA is also a main internal signal allowing plants to survive under severe stress conditions (Keskın et al. 2010). The endogenous expression of ABA in vegetative plant tissues increases in response to stresses leading to plant water deficit. It is also known that there is a correlation between plant ABA amount and plant tolerance to water deficiency. ABA stimulates stomatal closure to reduce water loss and after that reduces stress damage via the expression of various stress responsive genes that increase plant stress tolerance (Aroca 2008).

1.6. Drought Stress in Chickpea

Chickpea is an essential crop for arid and semi-arid regions and is mostly cultivated in dry and rainfed areas (Varshney et al. 2009). It is a resistant, deep-rooted plant and successfully grown under conditions which limit growth of other plants. Fertilizers and pesticides are occasionally used on chickpeas (Singh and Reddy 1991). Recent chickpea production is not very high (6.5 Mt, FAO 2009) and has decreased over the past few years. This reduction in production may have been caused by several abiotic and biotic stresses which decrease yield stability and quality. *Ascochyta* blight, *Fusarium* wilt, *Helicoverpa* pod borer, *Botrytis* grey mold, drought, cold, and salt are significant biotic and abiotic stresses affecting chickpea yield and quality. It is reported that yield losses because of biotic stresses (4.8 Mt) are lower than those due to abiotic stresses (6.4 Mt) (Ryan 1997).

Among diverse environmental factors, drought is the most significant environmental stress which restricts plant growth, development and efficiency (Boyer 1982). This stress causes 40-50% decreases in chickpea yield around the world (Ahmad et al. 2005). Chickpea is especially affected by terminal drought which delays flowering

and decreases yield in Mediterranean and sub-tropical climates (Turner et al. 2001). Early maturing varieties of chickpea have been developed to escape terminal drought (Kumar and Abbo 2001). In chickpea, the capability of a plant to finish its life cycle before severe soil water reduction (drought escape), is associated with traits such as a deep root system, osmotic adjustment, high leaf water potential, high biomass, early flowering and maturity, and re-dispersion of stem and leaf dry (Katerji et al. 2001).

1.7. Aim of the Study

In this project, our aim was to identify important factors which can be used to characterize chickpea plant tolerance to drought stress. *Cicer arietinum* cv. Gokce was characterized as a drought tolerant cultivar according to data from ICARDA (Center for Agricultural Research in the Dry Areas). To evaluate drought's effect on 'Gokce', we divided the plants into two treatments: a control group (non-drought stress treated plants) and a stress group (drought stressed plants). Treatments were started when plants were three weeks old and plant leaves were collected each day during treatment. Leaf samples were used to measure the content of enzymes and other molecules related to plant drought tolerance.

With this study we tried to elucidate the plant drought tolerance system by using chickpea as a model organism. Analysis of physiological (relative water content, fresh and dry weight of leaves) and biochemical (level of antioxidants, MDA and total protein) parameters provided information about the molecules and mechanisms which play important roles in the drought tolerance of this chickpea cultivar. Identification of the molecules which are most important will help us to define the parameters that need to be phenotyped in a molecular breeding program to develop new drought tolerant chickpea cultivars. With future studies, genes controlling these parameters can be identified using molecular marker technology. The determination of genes which play a role in the drought tolerance of chickpea not only will provide drought resistant chickpea cultivars but may also allow production of other drought resistant crops and a deeper knowledge of the genetic, molecular and biochemical control of drought tolerance in plants.

CHAPTER 2

MATERIALS AND METHODS

2.1. Plant Material and Growth

2.1.1. Plant Material

In this project, a chickpea cultivar was used for physiological and biochemical analysis. It was reported that 'Gokce' survived under severe drought condition in mid-Anatolia in 2007 (ICARDA press release 2007). Thus, previous studies indicated that this chickpea cultivar is resistant to drought stress. It is believed that *C. arietinum* cv. Gokce can easily adapt to changing environmental conditions because of its large seed size and tolerance to drought and *Ascochyta* blight.

2.1.2. Plant Growth Conditions and Drought Treatment

Four chickpea seeds were planted in 18 cm, 1.5 lt PVC pots, containing air-dried soil. Plants were grown in the growth chamber at 25 ^{o}C, 50% relative humidity and 16 hours of light (5000 lux) (270 mol/m^2 s). Plants were watered with 100 ml tap water every day and the relative humidity of the soil was kept at 30%. After the third week, water was withheld from plants in the stress group for seven days whereas plants in the control group were watered normally. Leaf samples were collected from each group every 24 hrs during the treatment period. Samples were frozen with liquid nitrogen and kept at -80^{o}C.

2.2. Methods

2.2.1. Determination of Fresh and Dry Weight

Samples were collected from both groups and their fresh weights were measured. Then samples were put in an oven (Binder, FD115 oven Forced Air, USA) for 72 hours at 80 ^{o}C. After 72 hours, leaves were weighed to determine their dry weight.

2.2.2. Determination of Relative and Real Water Content

Relative water content was measured according to Smart and Bingham (1974) and real water content was determined according to Ahmed et al. (2007). First, the fresh weights of harvested leaves (FW) were measured and then the tissue was put in a petri dish with distilled water for 5 hours. The turgor weights (TW) of plants were measured and tissue was put into an oven for 48 hours at 70^{o}C. Lastly, dry weights (DW) were measured. Relative and real water contents were then calculated.

$$\text{Relative water content (\%)} = (FW-DW) / (TW-DW)*100 \quad (2.1)$$

$$\text{Real water content (\%)} = (FW-DW) / FW*100 \quad (2.2)$$

2.2.3. Determination of Malondialdehyde (MDA) Content

MDA is the most abundant individual aldehydic breakdown product. By measuring the level of MDA, we can determine the amount of lipid peroxidation in leaves. MDA was measured according to Heath and Packer (1968). In this method, 0.5 g plant samples were put into centrifuge tubes and homogenized with 3 ml 0.25 % thiobarbituric acid (TBA) which was dissolved in 10 % trichloroacetic acid (TCA). After that, samples were put into an oven at 95^{o}C for 30 minutes and samples were cooled on ice for at least 5 minutes. Samples were centrifuged at 20000 x g with a high

speed centrifuge (Beckman Coulter, Avanti J-E centrifuge, the USA) for 25 minutes. Lastly, 2 ml supernatant for each sample was monitored at 532 nm and 600 nm by spectrophotometer (Shimadzu, 1700 UV Visible Spectrophotometer, Japan) and MDA content was determined using an 156 $mM^{-1}cm^{-1}$ extinction coefficient.

$$\text{MDA content} = [(A_{532}-A_{600}) / 156] \times 10^3 \times \text{dilution ratio} \quad (2.3)$$

2.2.4. Bradford Protein Assay for Total Protein Determination

The determination of total protein content used the Bradford protein assay method which is a spectroscopic analytical method (Bradford 1976). Bovine serum albumin (BSA) functioned as a protein standard. It was used for comparison of protein sample concentrations.

- Preparation of Coomassie Reagent: In 50 ml of 95 % ethanol, 100 mg of CBB G-250 was dissolved. After that, 100 ml of 85 % phosphoric acid was added and then ultra pure water added to a final volume of 1 L. The solution was passed through filter paper. At the end of this process, the solution was stored in an amber bottle at 4 ^oC.
- Preparation of 2 µg/µl stock BSA solution: 20 mg BSA was dissolved in distilled water and the solution was brought to 1 ml. After that mixture was diluted 1/10.

As outlined in Table 2.1, BSA standards, blank and protein samples were prepared. Sample solutions were incubated for 10 minutes at room temperature. At the end of the incubation, the absorbances of all samples were quantified at 595 nm by UV-visible spectrophotometer.

Table 2.1. Preparation of BSA standards from 0.2 mg/ml BSA and test sample for the Bradford Protein Assay.

Test sample	**Sample Volume (µl)**	**1 N HCl Volume (µl)**	**Water Volume (µl)**	**Rehydration Buffer Volume (µl)**	**Coomassie Reagent Volume (ml)**
Blank	0	10	80	10	5
BSA standard-5µg	2.5	10	77.5	10	5
BSA standard-10µg	5	10	75	10	5
BSA standard-20µg	10	10	70	10	5
BSA standard-40µg	20	10	60	10	5
BSA standard-50µg	25	10	55	10	5
BSA standard-75µg	37.5	10	42.5	10	5
BSA standard-100µg	50	10	30	10	5
Protein Sample	10	10	70	10	5

The standard curve was obtained by plotting the absorbance at 595 nm against µg of protein in BSA standard samples. As expected, we obtained a linear graph. The equation of the line was calculated using Microsoft Excel software. With this equation, we calculated the amount of protein in the unknown samples. Table 2.2 indicates absorbance values for the different BSA standards while Figure 2.1 shows the standard curve.

Table 2.2. Absorbance values for BSA standards.

Concentration (µg)	**Absorbance at 595 nm**
20	0.711
40	0.849
50	0.933
100	1.173
150	1.361
200	1.531
250	1.642

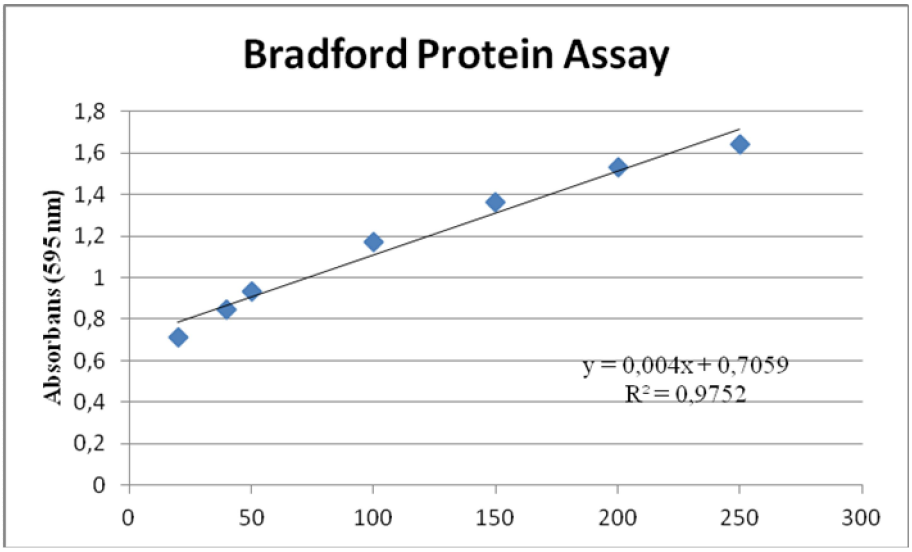

Figure 2.1. Standard curve for BSA

2.2.5. Determination of Antioxidant Enzyme Activity

2.2.5.1. Preparation of Enzyme Extracts

For the antioxidant enzyme activity measurements, 2 gram leaf samples were collected from each treatment group. The leaf material was ground with liquid nitrogen and then the samples were suspended in 8 ml extraction buffer. The homogenates were centrifuged at 20000 x g and 4^{o}C for 20 minutes using a high-speed centrifuge. Lastly, supernatants of stress and control group samples were transferred to 1.5 ml eppendorf tubes and stored at -80 ^{o}C.

Preparation of extraction buffer (100 ml):

- 1 mM EDTA solution: For 100 ml extraction buffer; 0.0292 gram of EDTA was dissolved in potassium phosphate buffer.
- 1% Polyvinylpyrrolidone (PVP): 1 gram of PVP was dissolved in potassium buffer.
- 1 mM Phenylmethanesulfonylfluoride (PMSF): For extraction buffer; 500 µl of 200 mM stock PMSF was used.
- 0.2 Triton X-100: 200 µl of Triton-X was added to reaction buffer.

- 50 mM pH 7.5 potassium phosphate buffer: Potassium phosphate was dissolved in distilled water and its pH adjusted to 7.5.
- 1 mM DTT: 0.0154 gram of DTT was added to phosphate buffer.

2.2.5.2. Determination of Guaiacol Peroxidase Enzyme Activity

Guaiacol peroxidase enzyme activity was determined according to Birecka (1973). For the reaction, the substrate (30% H_2O_2) was prepared and the absorbance of H_2O_2 was measured at 240 nm. H_2O_2 was diluted with distilled water and adjusted to absorbance of 0.4-0.41. For samples, reaction buffer was prepared (Table 2.3.).

- Preparation of 30% H_2O_2: 0.1 ml 30% H_2O_2 was dissolved in 120 ml distilled water.
- Preparation of 0.018 M guaiacol solution: 19.7 µl of 0.018 M guaiacol stock was used for the reaction mixture.
- Preparation of pH 7.8 potassium phosphate buffer: 0.68 gram of potassium phosphate was dissolved in distilled water and its pH was adjusted to 7.8.

Table 2.3. Volume of blank and sample reaction mixtures.

	Sample Reaction Mixture (ml)	**Blank Reaction Mixture (ml)**
pH=7,5 potassium phosphate buffer	2.8 ml	2.9 ml
0.018 M guaiacol solution	50 µl	50 µl
83% H_2O_2 solution	50 µl	50 µl
Enzyme Extract	100 µl	-
Total Volume	3 ml	3 ml

After preparation of the reaction mixtures, samples were measured using a UV-spectrophotometer. The change in absorbance value at 436 nm was observed for 240 seconds and DA/minute value was calculated for each sample. Using the following equation, guaiacol peroxidase (POD) enzyme activity was calculated.

POD enzyme activity = [(ΔA/dk x 4 x Vt x dilution factor)/25.5 x Vs] (2.4)

(V_t= Total Volume; V_s= Sample Volume; DA= Changes in absorbance)

2.2.5.3. Determination of Catalase Enzyme Activity

Catalase (CAT) enzyme activity was determined using the method of Aebi et al. (1984). For this method, 0.059 M H_2O_2 solution was prepared and used to dilute enzyme in 0.1 M phosphate buffer (pH 7) to obtain a rate of 0.03-0.07 DA/min (Table 2.4.). Changes in the absorbance value of the sample reaction mixture at 240 nm were observed for 2 minutes.

Preparation of 0.059 M H_2O_2 (30%): 180 µl of 0.059 M H_2O_2 was dissolved in 100 ml 0.1 M potassium phosphate buffer (pH 7).

Table 2.4. Volume of blank and sample reaction mixtures.

	Sample Reaction Mixture (ml)	**Blank Reaction Mixture (ml)**
Distilled water	1.9 ml	2 ml
0.059 M H_2O_2	1 ml	1 ml
Enzyme Extract	100 µl	-
Total Volume	3 ml	3 ml

Following that DA/min. value was calculated and then the catalase enzyme activity of the samples was determined using the following equation.

CAT enzyme activity= [(ΔA/dk x Vt x 1000*dilution factor)/43.6 x Vs] (2.5)

(V_t= Total Volume; V_s= Sample Volume; DA= Changes in absorbance)

2.2.5.4. Determination of Glutathione Reductase Enzyme Activity

The activity of glutathione reductase (GR) was determined by following the decrease in absorbance due to NADPH oxidation (Sgherri et al. 1996). For the reaction mixture, 100 mM potassium phosphate buffer (pH 7.5), 1 mM oxidized glutathione, 0.75 mM DTNB, and 10 mM NADPH were prepared (Table 2.5.). After that, decreases in absorbance at 412 nm were followed and DA/min. values were quantified. Lastly, glutathione reductase enzyme activity was identified via the following equation.

$$\text{GR enzyme activity} = [(\Delta A/dk \times Vt \times 1000 * \text{dilution factor})/14.15 \times Vs] \quad (2.6)$$

(V_t= Total Volume; V_s= Sample Volume; DA= Changes in absorbance)

Table 2.5. Volumes of blank and sample reaction mixtures.

	Sample Reaction Mixture (ml)	**Blank Reaction Mixture (ml)**
100 mM Potassium phosphate buffer	1.9 ml	2.1 ml
1 mM oxide glutathione	300 µl	300 µl
10 mM NADPH	300 µl	300 µl
0,75 mM DTNB	300 µl	300 µl
Enzyme Extract	200 µl	0 µl
Total Volume	3 ml	3 ml

Preparation of reaction mixture:

- Preparation of 100 mM potassium phosphate buffer (pH 7.5): 0,68 gram of potassium phosphate was used for reaction buffer and dissolved in 100 ml distilled water.
- Preparation of 1 mM oxidized glutathione (GSSG): 0.061 gram 10 mM stock GSSG was dissolved in 10 ml potassium buffer.
- Preparation of 0.75 mM DTNB: 0.0297 gram of DTNB was dissolved in 10 ml potassium buffer.
- Preparation of 10 mM NADPH: 0.083 gram of NADPH was dissolved in 10 ml potassium buffer.

2.2.5.5. Determination of Superoxide Dismutase Enzyme Activity

The activity of superoxide dismutase (SOD) was assayed as described by Beauchamp and Fridovich (1971). One unit of SOD activity was defined as the amount required to cause 50% inhibition of NBT (nitroblue tetrazolium) photoreduction. The reaction mixture (0.05 M pH 7.5 potassium phosphate buffer, 570 µM NBT, 99 mM L-methionine, 0.25 % Triton-X 100, and 0.044% riboflavin) was prepared and distributed to tubes which contained enzyme extract. One tube was prepared as a blank and contained only the reaction mixture (Table 2.6.). After addition of the reaction mixture, tubes were kept in the light (12000 lux) for 10 minutes and absorbance values at 560 nm were determined using the UV-spectrophotometer.

- Preparation of 0.05 M potassium phosphate buffer: 0.68 gram of potassium phosphate was dissolved in 100 ml distilled water and its pH was adjusted to pH 7.5.
- Preparation of 99 mM L-methionine: 0.148 gram of L-methionine was dissolved in 10 ml potassium buffer.
- Preparation of 570 µM NBT: 0.0046 gram NBT was dissolved in 10 ml potassium buffer.
- Preparation of 25% Triton-X 100: 25 µl Triton-X 100 was added into 10 ml potassium buffer.
- Preparation of 0,044% riboflavin: 0.0044 gram riboflavin was dissolved in 10 ml potassium buffer.

Table 2.6. Volumes of blank and samples for reaction mixtures.

	Sample Reaction Mixture (ml)	**Blank Reaction Mixture (ml)**
0.05 M potassium phosphate buffer	1600 µl	1800 µl
570 µM NBT	300 µl	300 µl
99 mM L-methionine	300 µl	300 µl
0.25% Triton-X 100	300 µl	300 µl
0.044 Riboflavin	300 µl	300 µl
Enzyme Extract	200 µl	-
Total Volume	3 ml	3 ml

2.2.6. Purification of IAA, GA, JA, SA and ABA

Indole acetic acid (IAA), gibberellic acid (GA), jasmonic acid (JA), salicylic acid (SA) and abscisic acid (ABA) contents in the plants were determined using the method of Yurekli et al. (2001) and Nefedieva (2003). In this method, 1 gram plant tissue was homogenized with 100 ml 90% methanol and 20 mg butylated hydroxytoluene (BHT) which was used as antioxidant. The homogenized plant tissue was stored at 4^{o}C for 12 hrs. At the end of 12 hrs, homogenized plant tissue was filtered with Whatman No.1 filter paper using a Buchner funnel. After that, supernatant was extracted with 15 ml ethyl acetate (pH 2.5 and 7) three times. During this extraction, ethyl acetate and supernatant were shaken three or four times in the Buchner funnel, so hormones in the water phase could pass into the ethyl acetate phase. After incubation, ethyl acetate and water were separated from each other using the Buchner funnel. The water phase was transferred into a beaker and the ethyl acetate phase which contained free IAA, GA, JA, SA and ABA hormones was put into an extraction flask. Following that, ethyl acetate was evaporated from the extracts using an evaporator at 45 ^{o}C. Then the residue was collected from the evaporator with 1 ml methanol. Using 20 µl of extract, the amounts of IAA, GA, JA, SA and ABA hormones were determined by reverse phase LC18 HPLC column.

CHAPTER 3

RESULTS AND DISCUSSION

In this study, we analyzed physiological (dry and fresh leaf weights; real and relative water contents) and biochemical (enzymatic and non enzymatic antioxidants contents, malondialdehyde, total protein and hormones levels) parameters related to drought tolerance. For this purpose *C. arietinum* cv. Gokce was used as plant material. To elucidate drought's effect on 'Gokce', we separated plants into two treatments: a control group (non-drought stress treated plants) and a stress group (drought stressed plants). Treatments were begun when plants were three weeks old and plant leaves were collected each day of treatment. Leaf samples were used to determine the content of enzymes and other molecules associated with drought tolerance of plants. Leaf, stem and root samples were used to determine hormone contents.

3.1. Determination of Fresh and Dry Weight

In the control group, fresh leaf weight increased until the third day of treatment and did not change until the sixth day after which a decrease occurred in fresh weight (Figure 3.1). It was observed that the control group's fresh weight increased by 0.17 g by the end of the experiment. Fresh leaf weight of the stress group increased until the fourth day of treatment and then decreased. The fresh weight of the stress group increased by 0.16 g during the treatment. Thus the increase in stress group fresh weight was only slightly less than the control group. Fresh weight of the drought treated plants only exceeded that of the control plants on the fourth day of the experiment.

In our study we observed that there was an increase in the fresh weight of control and stress groups (Figure 3.1). Previous studies indicated a reduction in plant height and fresh weight in almond and *Catharanthus roseus* under drought conditions and indicated that this reduction may be due to suppression of cell expansion and cell growth, as a result of low turgor pressure (Rouhi et al. 2007; Jaleel et al. 2008). Our

results suggest that chickpea is more resistant to drought than these two species and was able to continue to grow even under drought stress.

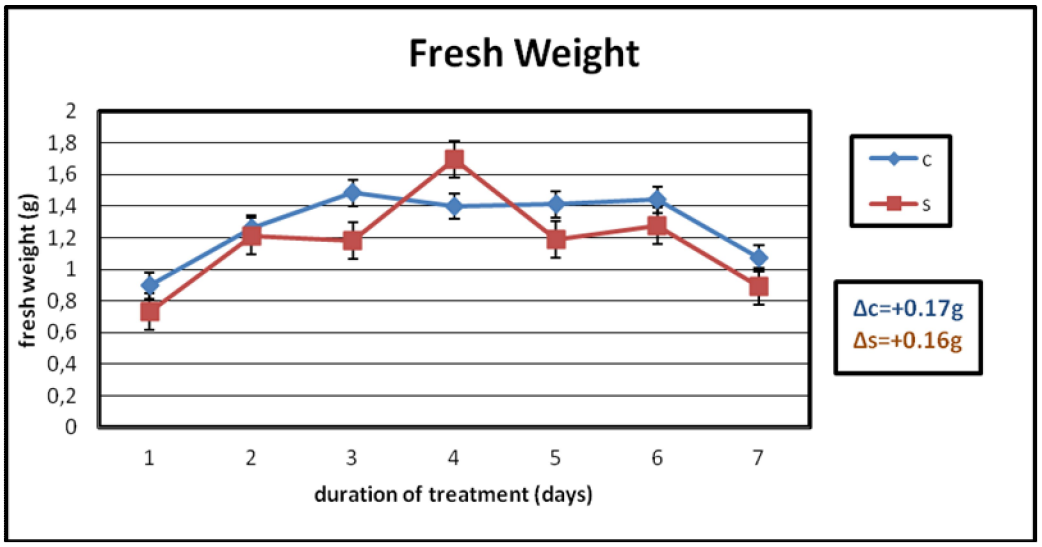

Figure 3.1. Changes in fresh weight of stress and control group plants.
(c = control group; s = stress group)
(Δc = net change in control group; Δs = net change in stress group)

In the control group, dry leaf weight increased until the sixth day of treatment and then a decline was seen in dry weight (Figure 3.2.). During the treatment, the control group's dry weight increased by 0.045 g. Dry weight of the stress group continuously increased until the sixth day of treatment after which a decrease occurred. The stress group's dry weight increased by 0.044 g. Thus, during the treatment, the increase in dry weight of the stress group was nearly the same as in the control group. As with wet weight, on the fourth day the stress group plants' dry weight exceeded that of the control group.

Like fresh weight, we observed an increase in the dry weight of control and stress groups (Figure 3.2.). Overall, the differences between net increase in dry weight of control and stress groups were insignificant. Again, these results may reflect the drought tolerance of 'Gokce' because it had the ability to continue growing even under stress.

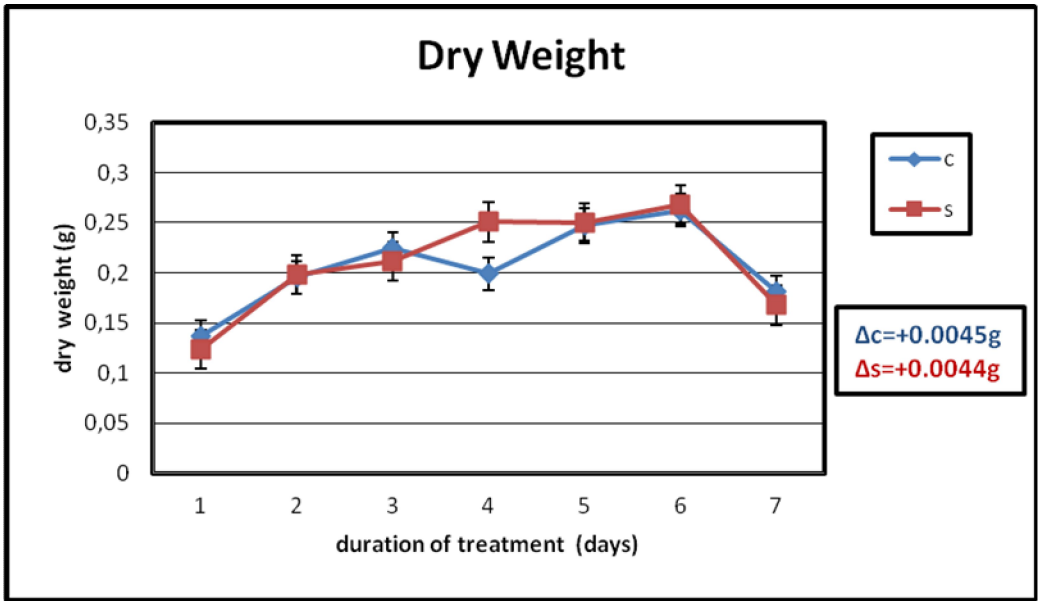

Figure 3.2. Change in dry weight of stress and control group plants.

3.2. Determination of Relative and Real Water Content

In the control group, relative water content decreased until the third day of treatment after which an increase occurred. On the fifth day of treatment, relative water content again decreased and then an increase was seen on the sixth day of treatment (Figure 3.3). An overall 0.19% reduction in relative water content was observed in the control group at the end of treatment. In the stress group, relative water content decreased until the fourth day of treatment after which an increase occurred. On the fifth day, relative water content started to decrease but again increased by the end of the experiment. A 3.75% decrease in relative water content was seen during the treatment. The reduction in relative water content in the stress group was 3.57% higher than for the control group.

Plant tolerance to drought stress may be associated with different systems involving the capability to maintain high relative water content (RWC) (Oukarroum et al. 2007). In our study, we observed a decline in the RWC of both control and stress groups (Figure 3.3.). We expected to see a reduction in the stress group's RWC. Previous studies indicated that plants decrease the RWC of leaves to adjust osmotic pressure under severe drought conditions. (Xu et al. 2010; Macar and Ekmekci 2009).

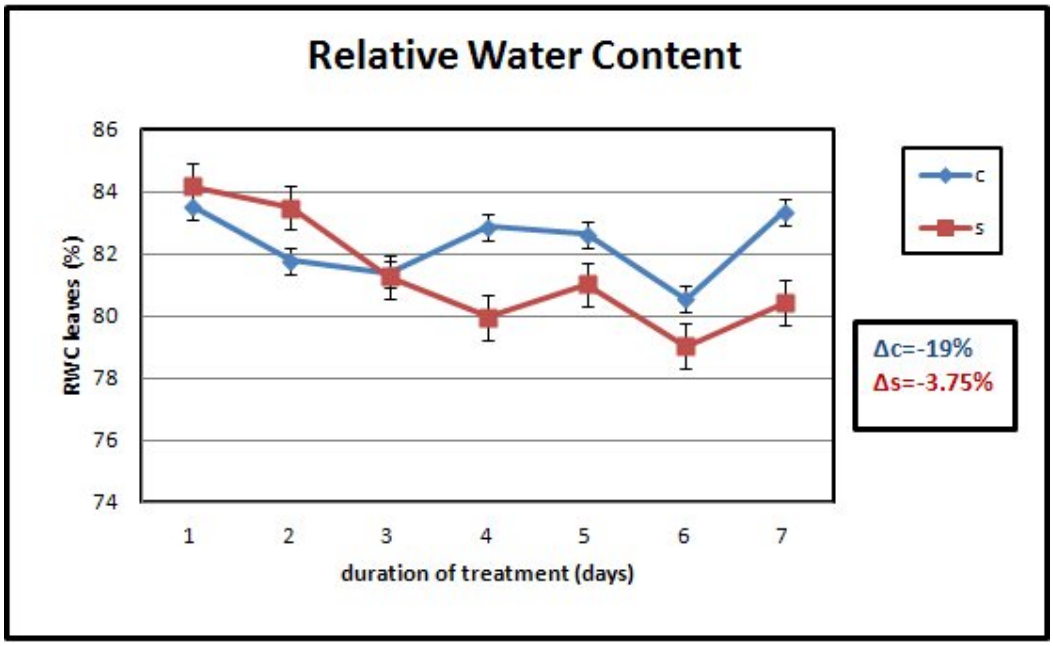

Figure 3.3. Change in relative water content of stress and control group plants.

The real water content of the control group decreased until the third day. After that a slight increase was seen in the control group. On the fifth day of treatment, a decrease was observed (Figure 3.4.). A 4.31% reduction occurred in real water content of the control group during the treatment. In the stress group, real water content decreased until the third day after which a slight increase was observed. On the fifth day, the real water content started to decrease until the end of treatment. A 5.35% decline was seen in the stress group. When we compared the control and stress groups, we observed that reduction in the real water content of the control group was 1.04% lower than for the stress group.

There were decreases in the real water content of leaves for both the control and stress groups by the end of the experiment (Figure 3.4.). We expected to see a decrease in the real water content of the stress group (Xu et al. 2010). Ahmed et al. (2007) reported that real water content in olive leaves decreased due to decreased soil moisture. But in our study, both groups followed a similar path during the experiment. We concluded that this similarity may be because chickpea is a drought tolerant plant. So this may be why the real water content of the drought stressed plants did not significantly differ from that of the control plants.

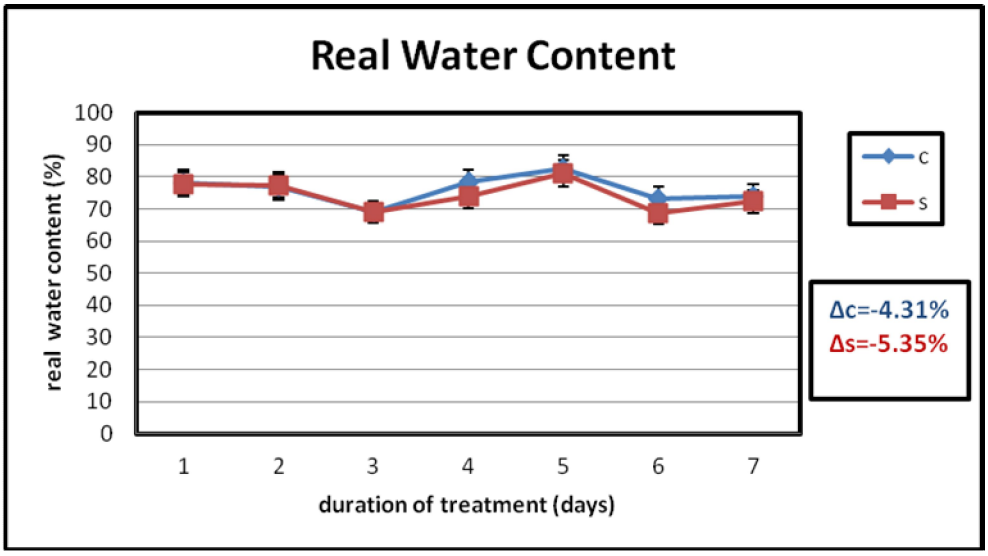

Figure 3.4. Change in real water content of stress and control group plants.

3.3. Determination of Malondialdehyde (MDA) Content

In the control group, the content of MDA decreased until the third day of treatment after which an increase occurred (Figure 3.5). There was a 0.789 nmol/g increase in MDA content by the end of the experiment. In the stress group, the content of MDA increased until the second day after which a decline was observed. On the third day, an increase occurred until the seventh day of treatment. Overall, there was a 1.491 nmol/g increase in the amount of MDA. The level of increase in MDA content for the stress group was 0.702 nmol/g higher than for the control group.

In this experiment, we measured malondialdehyde (MDA) content to determine the lipid peroxidation level because MDA is a by-product of lipid peroxidation. The data indicated that there was an increase in the MDA content of both control and stress groups. When we closely examined the data we found that the difference between the MDA content of the stress and the control groups was insignificant (Figure 3.5.).If the chickpea plants were under drought stress, we expected to see a dramatic increase in the stress group as compared to the control group (Nikolaeva et al. 2010). Macar and Ekmekci (2009) reported that the MDA content of the stress group was much higher than the control group during drought treatment in chickpea and they concluded that the increase in the MDA content of the control group resulted from aging, not because of

drought stress. But in our study the difference between the MDA contents of both groups was not significant. This may be due to aging of both treatment groups rather than a result of drought stress.

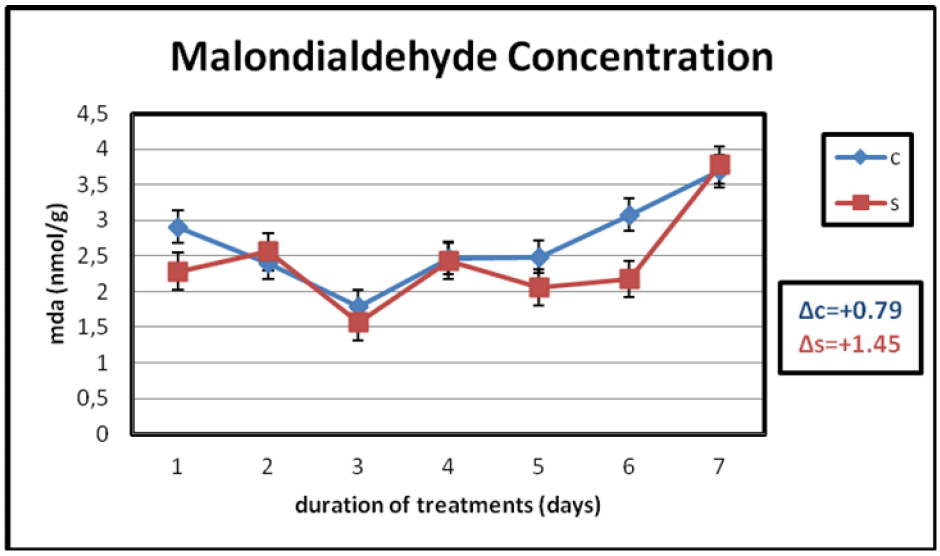

Figure 3.5. Change in MDA content of stress and control group plants.

3.4. Determination of Total Protein Content

The total protein content of the control group increased until the second day of treatment and after that a decrease occurred (Figure 3.6). On the fifth day, an increase was observed which continued until the seventh day of treatment. Overall, a 0.04 mg/g increase was observed in total protein content of the control group. In the stress group, total protein content followed a similar path as the control group. It increased until the second day and then a decline occurred. On the fifth day of treatment, total protein content again increased. There was a 0.136 mg/g increase in total protein content of the stress group by the end of the experiment. Total protein content of the stress group was 0.096 mg/g higher than for the control group.Thus the data showed that there was no significant difference in the content of total protein for the stress and control groups.

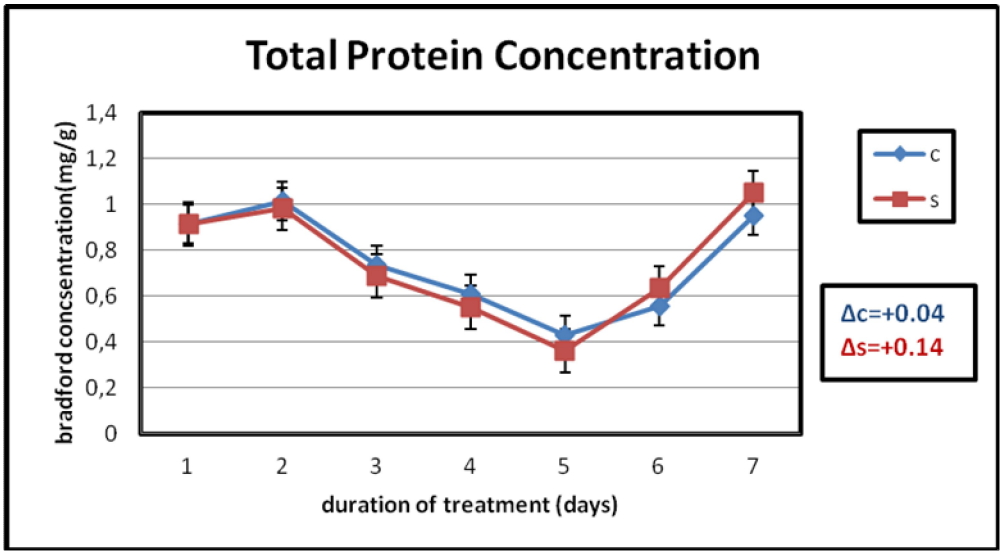

Figure 3.6. Change in total protein content of stress and control group plants.

3.5. Determination of Antioxidant Enzyme Activity

3.5.1. Determination of Guaiacol Peroxidase Enzyme Activity

The guaiacol peroxidase (POD) activity of the control group increased until the second day after which a decrease occurred (Figure 3.7.). On the sixth day, another slight increase was observed. There was a 0.129 U/mg reduction in the POD activity of the control group by the end of the experiment. In the stress group, the activity of POD decreased until the third day after which an increase was observed. On the fifth day, the POD activity again decreased until the seventh day. There was a 0.355 U/mg decline in the POD activity of the stress group. The reduction in the stress group was 0.226 U/mg higher than the reduction in the control group.

By the end of the experiment, the data showed that there was a significantly greater reduction ($p=0.027$) in the guaiacol peroxidase (POD) content in the stress group as compared to the control group (Figure 3.7.). These findings are not consistent with previous studies. Ge et al. (2006) reported that POD activity increased in maize during the water deficit period and this increase indicated that there was cooperation between POD and other H_2O_2 scavenging enzymes. The differences may be due to the different drought tolerances of maize and chickpea. As indicated before, Gokce chickpea is

known as a drought tolerant plant, so the POD content of chickpea may not have reacted to the stress conditions. Alternatively chickpea may produce higher levels of POD which is immediately used when the ROS level increases in cells.

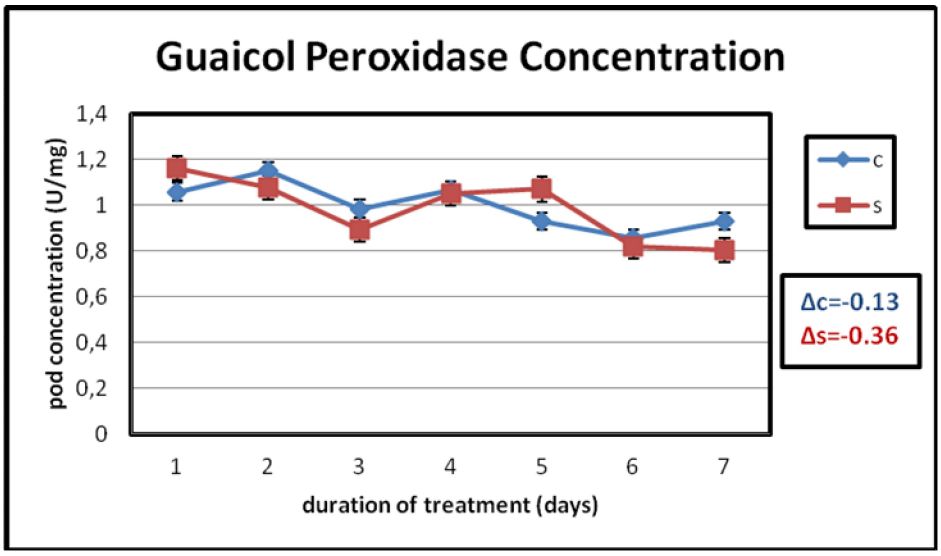

Figure.3.7. Change in POD activity of stress and control group plants.

3.5.2. Determination of Catalase Enzyme Activity

The catalase (CAT) enzyme activity of the control group increased until the third day after which a decrease occurred (Figure 3.8.). On the fourth day, CAT activity increased until the seventh day of treatment. Overall, there was a 51.376 U/mg increase in CAT activity of the control group. In the stress group, CAT activity increased until the second day after which it did not change. On the fifth day, an increase occurred following which a decrease was observed. A 36.835 U/mg increase was seen in CAT activity of the stress group by the end of the experiment. The level of increase in CAT activity of the control group was 14.541 U/mg higher than the stress group.

The results indicated that there was a dramatic and significant increase in catalase (CAT) enzyme activity in both groups (Figure 3.8.). They followed similar path during the treatment, however, the increase in control catalase activity by the end of the experiment was significantly higher ($p=0.013$) than that for the stress group. The similarity in both treatment groups may reflect the drought tolerance of ‘Gokce’ as the

stressed plants did not have a different response than control plants. Luna et al. (2005) reported that the activity of CAT was dramatically increased in wheat *(Triticum aestivum* L.) under severe drought conditions.

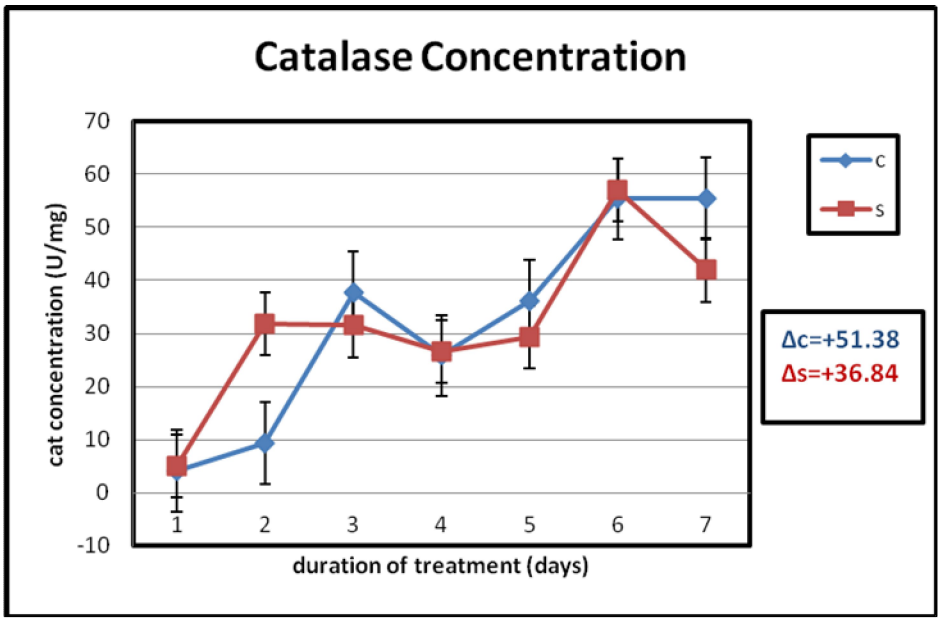

Figure 3.8. Change in CAT activity of stress and control group plants.

3.5.3. Determination of Glutathione Reductase Enzyme Activity

The glutathione reductase (GR) activity of the control group increased until the fifth day of treatment after which it did not change (Figure 3.9.). The GR activity of the control group increased 679.859 U/mg during the experiment. In the stress group, GR activity increased until the second day after which a decrease occurred. On the third day, GR activity again started to increase. On the sixth day a decrease was observed in GR activity. There was a 499.117 U/mg increase in GR activity of the stress group by the end of the experiment. When we compared control and stress groups' GR activity, we observed that the increase in GR activity of the control group was 180.742 U/mg higher than for the stress group. This difference was statistically significant (p=0.005).

Like CAT activity, glutathione reductase (GR) enzyme activity significantly increased in both groups during the experiment (Figure 3.9.). Previous studies showed that GR is part of ASH-GSH cycle which provides efficient protection against the

negative effects of ROS in sub-cellular organelles of plant cells (Möller 2001; Nikolaeva et al 2010; Tuteja and Galli 2010), so as we expected, the stress group's GR enzyme activity increased with drought to defend the plant against the drought stress.

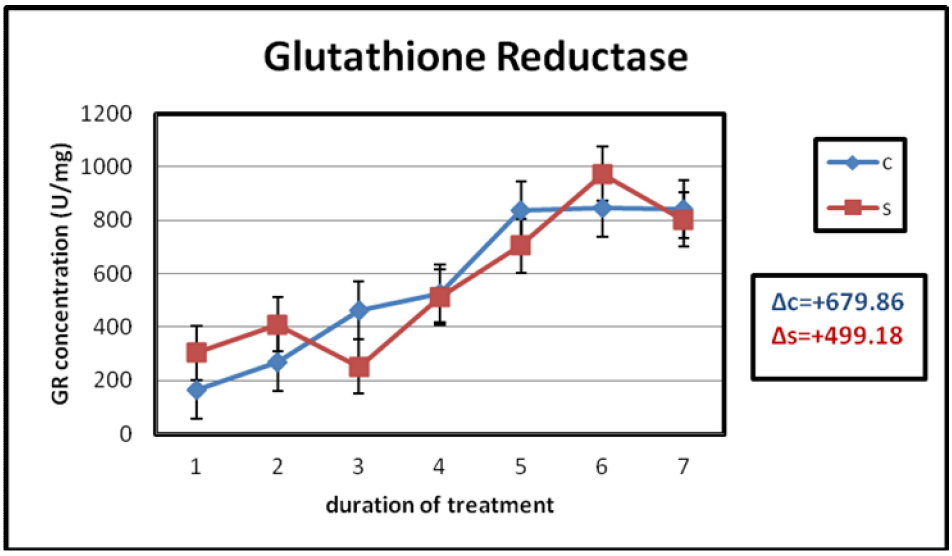

Figure 3.9. Change in GR activity of stress and control group plants.

3.5.4. Determination of Superoxide Dismutase Enzyme Activity

In the control group, the activity of superoxide dismutase (SOD) decreased until the fourth day. An increase was observed in SOD activity on the fourth day after which activity did not change (Figure 3.10.). The SOD activity of the control group increased by 0.007 U/mg during the experiment. In the stress group, SOD activity followed a similar trend as the control group. SOD activity decreased until the fourth day of treatment after which an increase occurred. On the fifth day, SOD activity became stable and did not change until the end of the experiment. There was a 0.017 U/mg decrease in SOD activity of the stress group. Overall, SOD activity of the control group was only 0.012 U/mg less than SOD activity of the stress group.

In our study, we observed that the difference between the superoxide dismutase (SOD) activity of the control and stress groups was not significant (Figure 3.10.). Several previous studies indicated that there was a dramatic increase in SOD enzyme activity under drought stress (Xu et al. 2010; Filippou et al. 2011). Pitcher et al. (1991)

reported that for efficient protection against oxidative stress, there must be increase in both SOD activity and H_2O_2 detoxification systems in transgenic tobacco plant. But in our experiment we did not observe any dramatic increase in SOD activity of the stress group. Perhaps the constitutive level of SOD was high enough for the plant to deal with the drought stress conditions.

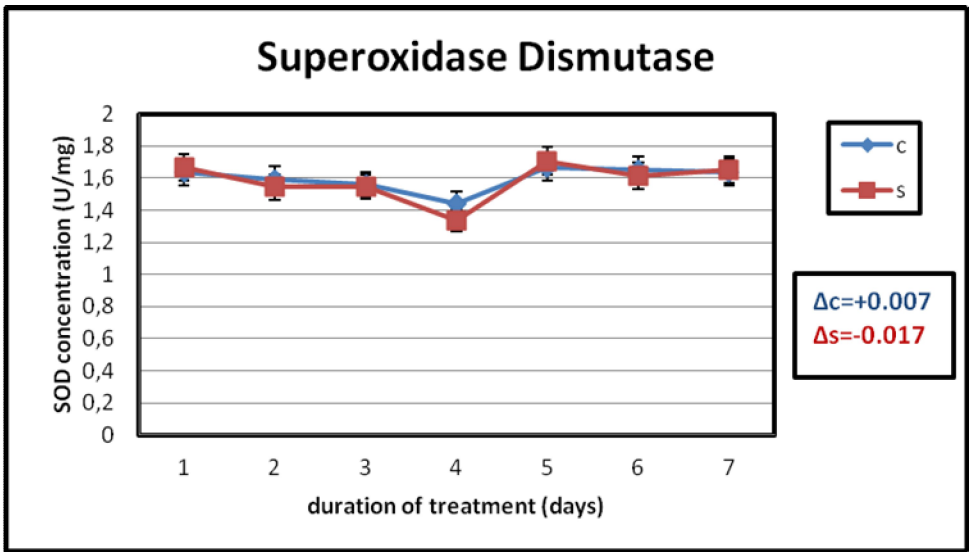

Figure 3.10. Change in SOD activity of stress and control group plants.

3.6. Purification of IAA, GA, JA, SA and ABA

To evaluate drought's effects on plant hormones we collected root, stem and leaf tissues after seven days of treatment.

3.6.1. Purification of Indole Acetic Acid

We observed that, for the control tissues, leaves had the highest indole acetic acid content with 0.209 ppb. The stem tissue ranked second with 0.164 ppb. The content of indole acetic acid of stem tissue was 0.033 ppb higher than the root tissue (Figure 3.11.). In the stress group, the root tissue had the highest content of indole acetic acid with 0.369 ppb. The content of indole acetic acid of leaf tissue ranked second with

0.281 ppb which was 0.046 ppb higher than for stem tissue. Overall, plant tissues in the stress group had more indole acetic acid than tissues in the control group with a statistically significant difference seen in roots (p=0.036).

In our present study, we found that the indole acetic acid (IAA) content of the stress group was higher than for the control group (Figure 3.11.). As we expected, the results showed that IAA plays a role in the improvement of defense responses to drought stress. Pustovoitova et al. (2004) reported that IAA content in cucumber leaves, flower buds, and flowers increased during long periods of drought stress. In their experiment, the results indicated that there was a relationship between drought resistance and IAA content. This relationship is also confirmed by the presence of the most IAA in chickpea roots, the part of the plant which is first subjected to drought stress. Overall our results suggest that the drought tolerance of 'Gokce' may be related to its increased production of IAA under stress.

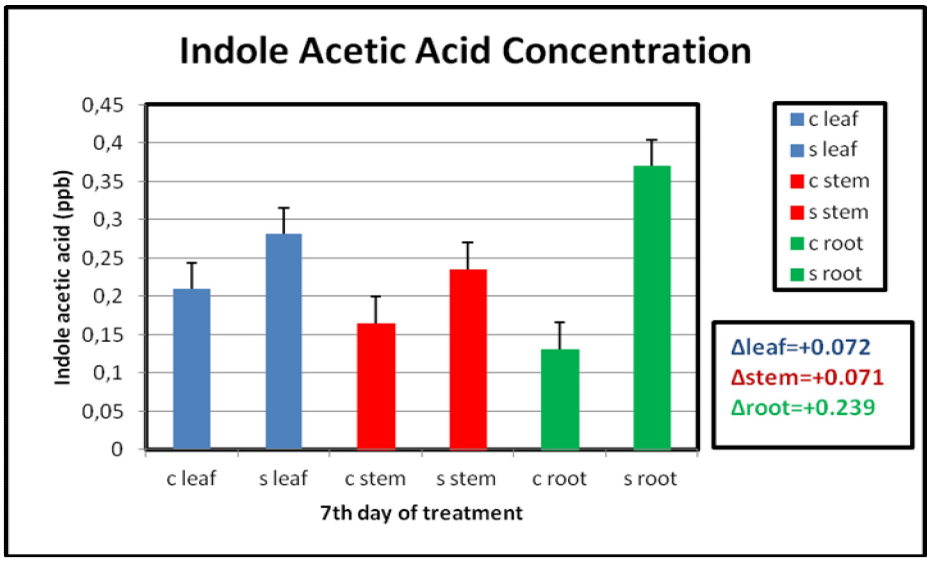

Figure 3.11. Amounts of indole acetic acid in stress and control group tissues. (Δleaf=net changes in leaf; Δstem=net changes in stem; Δroot=net changes in root)

3.6.2. Purification of Gibberellic Acid

In the control group, the plant's stem had the highest content of gibberellic acid with 0.356 ppb. Root tissue ranked second with 0.256 ppb. The amount of gibberellic

acid in the root was 0.184 ppb higher than in the leaf (Figure 3.12). In the stress group, plant roots had the highest gibberellic acid content with 0.426 ppb. Leaf tissue ranked second with 0.337 ppb which was 0.081 ppb higher than stem tissue. The stress group had more gibberellic acid than the control group. The only exception was seen for stem tissue in the stress group.

The experimental results showed that the stress group's gibberellic acid (GA) content was usually higher than for the control group. The root tissue of the stress group contained an especially high amount of GA perhaps because this is the first tissue which is exposed to drought stress (Figure 3.12.). However, previous studies indicated that the content of GA and cytokinin decreased while the content of abscisic acid increased in tobacco plants under stress conditions (Boucaud and Unger 1976). The increase in gibberellic acid may be caused by the drought response mechanism of chickpea.

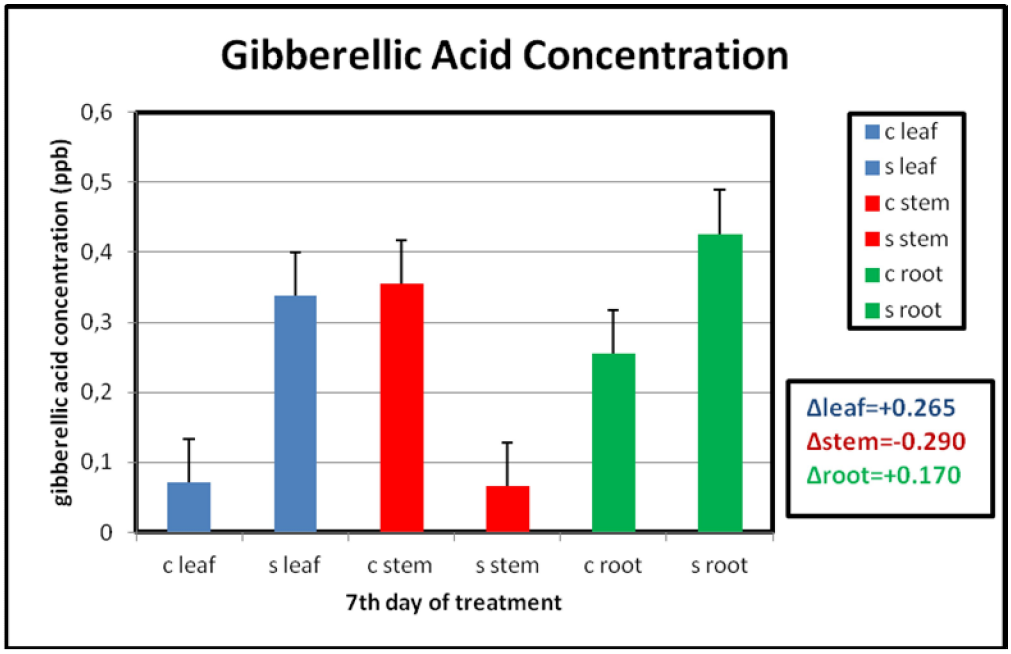

Figure 3.12. Gibberellic acid content of stress and control group plant tissues.

3.6.3. Purification of Jasmonic Acid

When we compared plant tissues in the control group, we observed that stem tissue had the highest amount of jasmonic acid with 7.203 ppb (Figure 3.13). After stem

tissue, leaf tissue ranked second with 1.813 ppb and the content of jasmonic acid in leaf tissue was 0.829 ppb higher than the content in root tissue. In the stress group, the stem tissue had the highest jasmonic acid content with 4.086 ppb. Leaf tissue came after stem tissue with 2.1 ppb of jasmonic acid. Root tissue had the lowest jasmonic acid content for the stress group with 0.562 ppb. Plant tissues in the control group had higher jasmonic acid content compared to the stress group. The only exception was observed in leaf tissue. The jasmonic acid content of leaf tissue in the stress group was 0.27 ppb higher than for the control group.

In our study, we observed that the jasmonic acid (JA) content in the control group was usually higher than in the stress group. The only exception was seen in the leaf tissue. Previous studies indicated that JA cooperated with absicisic acid and salicyclic acid in the stress response mechanism (Huang et al. 2008). It was observed that the content of JA increased under abiotic stress condition and also played significant roles in biotic stress and wounding responses (Adie et al. 2007; Walia et al. 2007 and Huang et al. 2008).

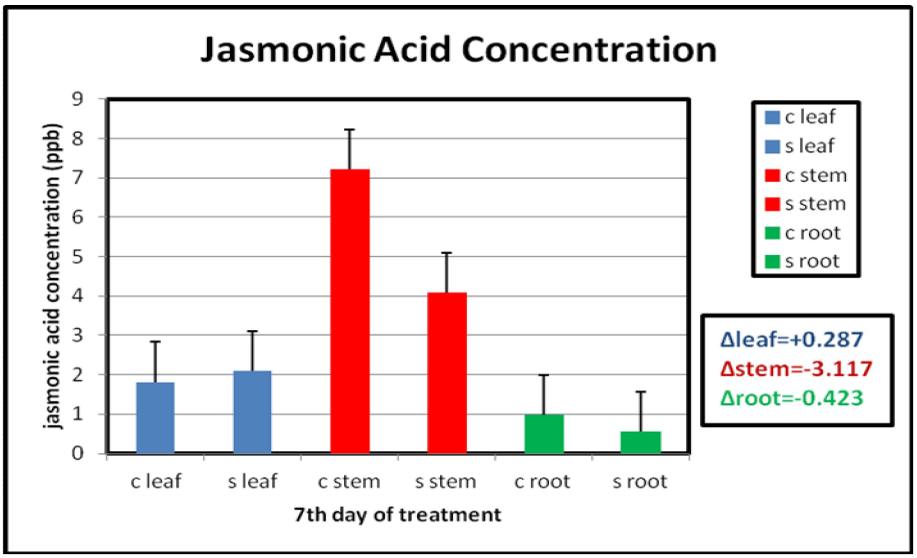

Figure 3.13. Jasmonic acid content of stress and control group plant tissues.

3.6.4. Purification of Salicylic Acid

In the control group, plant roots ranked first for salicylic acid content with 2.756 ppb (Figure 3.14.). Leaf tissue ranked second with 1.69 ppb salicylic acid content. Stem tissue had the lowest salicylic acid content with 1.35 ppb. In the stress group, root tissue ranked first with 15.2 ppb salicylic acid. Following the root tissue, stem tissue ranked second with 2.046 ppb salicylic acid. Leaf tissue had the lowest content of salicylic acid with 1.105 ppb. The stress group had higher salicylic content than the control group. The only exception was seen in the leaf tissue. For this tissue, the salicylic acid content of the stress group was 0.585 ppb less than for the control group.

The salicylic acid (SA) content of the stress roots was significantly higher than for the control roots ($p=0.0011$) (Figure 3.14.). Previous studies revealed that SA level increased under biotic and abiotic stresses (Huang et al. 2008; Cho et al. 2008). It was shown that there is a positive relationship between the antioxidant defense system and SA content in the plant. However this positive relation depends on the level of SA in the plant. If the endogenous expression of SA is very high, SA cooperates with ROS and causes cell death. However, if the SA concentration is at an appropriate level, it works with the antioxidant defense mechanism to neutralize the negative effects of ROS (Hayat et al. 2012). The dramatic increase of SA in the root indicates that roots were first to respond to drought stress by producing high SA.

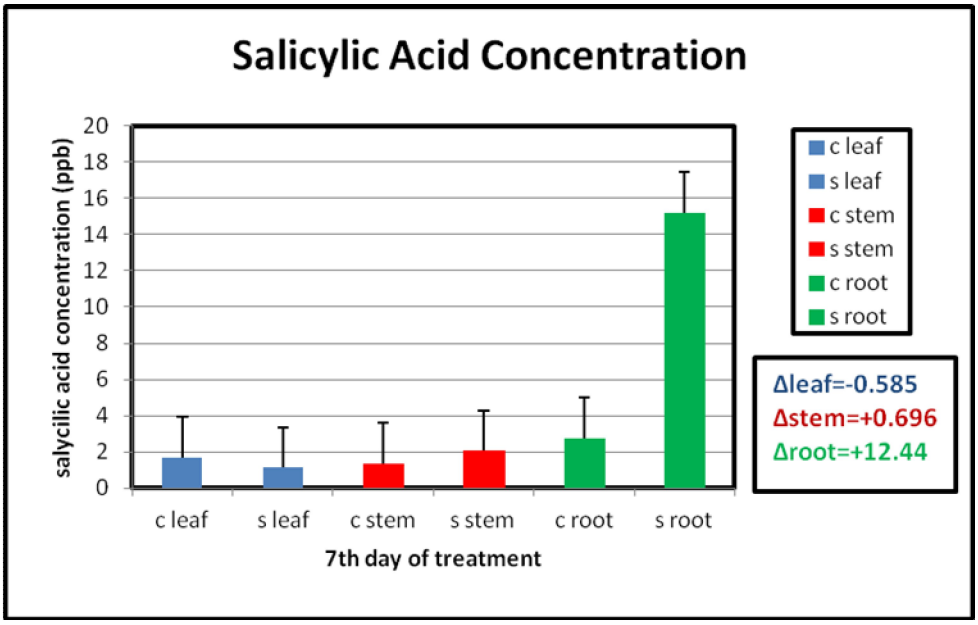

Figure 3.14. Salicylic acid content of stress and control group plant tissues.

3.6.5. Purification of Abscisic Acid

In the control group, leaf tissue had the most abscisic acid with 0.0064 ppb. The stem tissue ranked second with 0.0059 ppb abscisic acid (Figure 3.15.). Root tissue had the lowest abscisic acid content with 0.0024 ppb. In the stress group, the root tissue ranked first with 0.029 ppb abscisic acid. Leaf tissue came after root tissue with 0.021 ppb abscisic acid. Stem tissue contained 0.018 ppb abscisic acid and had the lowest amount of abscisic acid among the stress group. Overall, the stress group had higher abscisic acid content than the control group.

In our experiment, the ABA content increased in all tissue parts of the stress group by the end of the experiment (Figure 3.15.). Previous studies revealed that ABA plays an important role in the drought tolerance mechanism. The level of ABA increased under drought stress and also ABA stimulated the activation of other phytohormones during long periods of water deficiency (Pustovoitova et al. 2003; Huang et al. 2008; Javid et al. 2011; Kar 2011). Our results were consistent with previous studies. ABA is especially important in the roots of chickpea. These results suggest that chickpea increases the level of ABA to deal with drought stress.

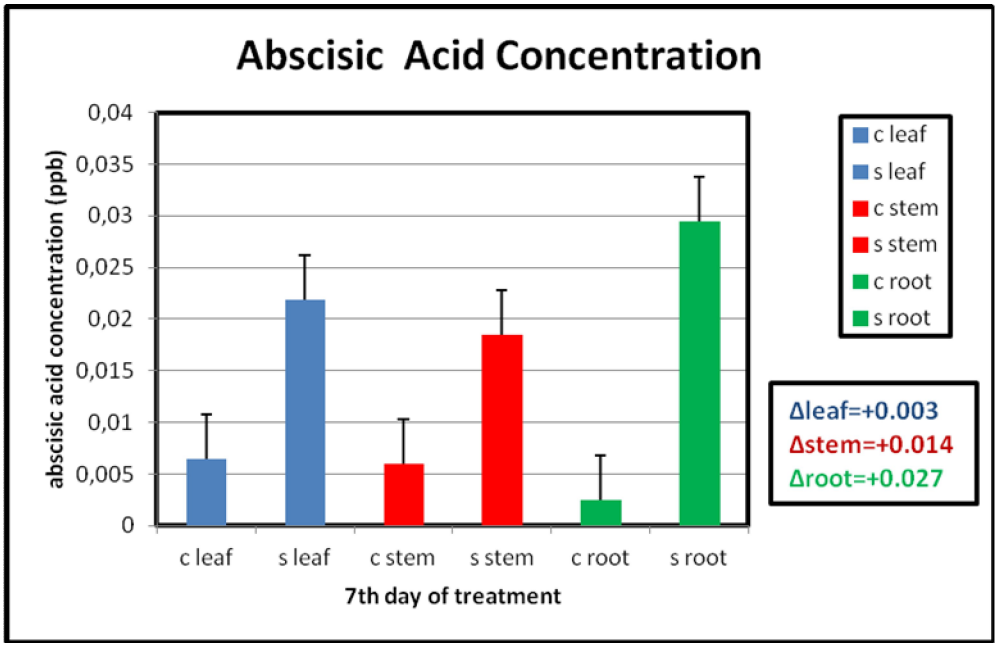

Figure 3.15. Abscisic acid content of stress and control group plant tissues.

3.7. Correlations Between Traits

Table 3.1 indicates the correlation values between control and stress values for each trait. These results show a strong negative correlation between the control and stress groups for fresh weight, real water content, salicylic acid content in root tissue, and abscisic acid content in stem tissue. On the other hand, there were strong positive correlations between the control and stress groups for relative water content, catalase enzyme activity, glutathione reductase enzyme activity, superoxide dismutase enzyme activity, indole acetic acid content in stem tissue, gibberellic acid content in leaf and root tissue, and abscisic acid content in root tissue. For these traits, performance of the plant under control conditions was a good indicator of its performance under drought stress.

Correlation analysis was also performed between the different measured parameters for the drought treated plants (data not shown). As expected we observed a very strong positive correlation (r=0.89) between dry and fresh weight traits. Relative and real water content traits also showed positive correlation (r=0.93) as expected.

When antioxidant enzymes were considered, we observed a positive relationship (r=0.97) between POD and CAT enzyme activities. In addition to these two enzymes, GR and SOD enzyme activity also had a positive correlation (r=0.96). Such results were expected because these enzymes must work cooperatively with each other to defend the plant against the negative effects of ROS.

We also examined the relationship between antioxidant enzyme activities and water content in plant. As we expected, there was a negative correlation between all water characteristics (dry-fresh weight and real -relative water content) of the plants and GR and SOD enzymes activities under stress condition. Therefore, reduced water content due to drought stress was associated with increased activity of these antioxidant enzymes.

When we checked the correlation between hormones we observed that there was a positive correlation between GA and SA contents in leaf tissue. On the other hand we saw a negative correlation between IAA and JA contents. We also found that there were positive correlations among the hormones in stem tissue. As we expected, SA had positive correlations with both JA and ABA. In root tissue, we observed that there was a negative correlation between GA and SA contents. On the other hand, GA had positive correlations with both IAA and ABA. We also observed that SA had negative correlations with both IAA and ABA. As previously indicated, depending on its amount in the plant, SA can work with ROS or other plant hormones.

We also examined the relationship between water characteristics and hormones under drought stress. We observed that plant dry weight had positive correlations with GA and ABA in leaf tissue. On the other hand, dry weight had negative correlations with IAA and JA in stem tissue. We also saw that there was a negative relationship between RWC and JA in root tissue. On the other hand, RWC had positive correlations with GA in leaf tissue and JA in leaf and stem tissue. Overall the correlation results show that drought response in chickpea is a complex process and involves many interrelated biochemical and physiological changes.

Table 3.1. Correlation values of control and stress group for each trait.

Traits	**Correlations**
Fresh weight	-0.92
Dry weight	-0.57
Real water content	-0.99
Relative water content	0.93
Malondialdehyde content	0.77
Total protein content	0.52
Guaiacol peroxidase enzyme activity	-0.75
Catalase enzyme activity	0.99
Glutathione reductase enzyme activity	0.99
Superoxide dismutas enzyme activity	0.89
Indole acetic acid content in leaf	-0.32
Indole acetic acid content in stem	0.94
Indole acetic acid content in root	-0.52
Gibberellic acid content in leaf	0.99
Gibberellic acid content in stem	0.45
Gibberellic acid content in root	0.99
Jasmonic acid content in leaf	0.28
Jasmonic acid content in stem	-0.61
Jasmonic acid content in root	-0.15
Salicylic acid content in leaf	-0.76
Salicylic acid content in stem	-0.62
Salicylic acid content in root	-0.98
Abscisic acid content in leaf	0.11
Abscisic acid content in stem	-0.96
Abscisic acid content in root	0.95

CHAPTER 4

CONCLUSION

Chickpea (*C. arietinum* L.) is an agronomically significant plant which has an essential role in the economy and human diet especially in developing countries. Chickpea is successfully grown under conditions which limit growth of other plants. It is especially affected by terminal drought which delays flowering and decreases yield in Mediterranean and sub-tropical climates. The aim of this study was to determine parameters which can be used to identify chickpea plant tolerance to drought stress. With this purpose we measured physiological (fresh and dry weight, relative and real water content) and biochemical (enzymatic antioxidants, malondialdehyde content, total protein content and phytohormone contents) parameters which were used to determine drought's effects on chickpea. To evaluate the effect of drought, we divided the plants into two groups: a control group (non drought stress plants) and a stress group (drought stressed plants). Treatments were started when plants were three weeks old and plant leaves were collected each day of treatment. Leaf samples were used to determine the content of enzymes and other molecules associated with drought tolerance of plants.

By the end of the experiment, results indicated that both fresh and dry weights of plants increased while real and relative water contents of plants decreased. In addition to these results we found that there was an increase in both MDA and total protein contents under drought stress. Moreover GR and CAT enzymes activities increased in plants because of the stress whereas POD and SOD enzymes activities decreased. The results also showed that contents of ABA and IAA increased in all tissue parts while JA, SA and GA increased in the specific plant' tissue parts during the drought treatment. In conclusion it is evident that all of these parameters play significant roles in the drought tolerance of plants. For example, RWC is necessary to adjust to osmotic pressure in the cell under drought stress. Moreover, CAT and GR enzymes are correlated with each other to defend the plants against stress conditions. ABA and IAA are also important members of the defense mechanism. Levels of both ABA and IAA increase under severe drought to protect the plant. These parameters can be phenotyped in molecular breeding programs to develop new drought tolerant chickpea cultivars. Determination of

the genes which control these parameters, may also allow development of other drought resistant crops.

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