CHAPTER II

ANATOMICAL

AND

PHYSIOBIOCHEMICAL ANALYSES

ANATOMICAL AND PHYSIOBIOCHEMICAL ANALYSES

2.1 Review of literature

Plant's growth and productivity are adversely affected by nature's wrath in the form of various biotic and abiotic stress factors. Water deficit or commonly known as drought is one of the major abiotic stresses, which adversely affects growth and yield of crops. Drought can be defined as the absence of adequate moisture necessary for normal plant's growth and development to complete the life cycle. Water is a fundamentally important component of the metabolism of all living organisms, facilitating many vital biological reactions by being a solvent, a transport medium and evaporative coolant. In plants, water plays the additional role of providing the energy necessary to drive photosynthesis through autolysis by yielding electrons. Hence, drought reduces plant's growth by affecting various physiological and biochemical processes, such as photosynthesis, respiration, translocation, ion uptake, carbohydrates, nutrient metabolism and growth promoters (Jaleel et al. 2009). Plants have evolved a wide range of defense mechanisms to contend with this problem. Drought is a common occurrence in rain-fed areas, brought about by inadequate rains or poor irrigations. Tea plant is a woody perennial, and as such, encounters a large number of environmental stresses throughout its long life span. Out of all, drought is the most important abiotic stress which causes around 40% crop loss (Barua 1989, Jain 1999).

2.1.1 Abiotic stress responses in plants

Plants must respond and adapt to abiotic stresses to survive in various environmental conditions. Plants have acquired various stress tolerance mechanisms, which are different processes involving physiological and biochemical changes that result in adaptive or morphological changes (Urano et al. 2010) (Fig. 2.1). Different environmental stresses to a plant may result in similar responses at the cellular and molecular level. This is due to the fact that the impacts of the stressors trigger similar strains and downstream signal transduction chains. Drought, cold and salinity are three major forms of abiotic stress that affect the water relations of a plant at

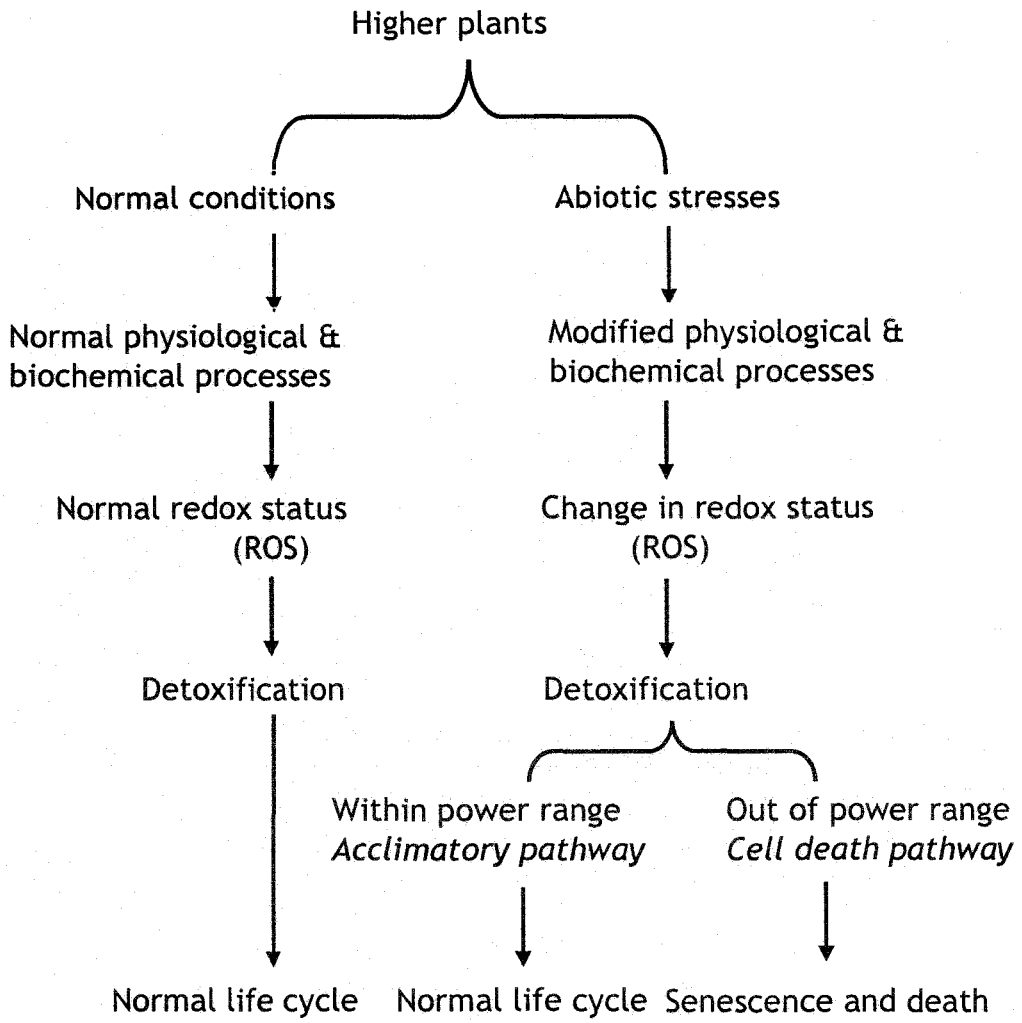

Fig. 2.1: Flowchart of main antioxidants and redox signaling processes in higher plants (modified from Hong-bo et al. 2008).

cellular and whole plant level causing specific as well as unspecific reactions, damages and adaptation reactions (Beck et al. 2007). Drought or cold stress induces various primary effects at cellular level, termed as strains (Levitt 1980), which in turn lead to uncontrolled (damage) or controlled effects (adaptation) on that level. Adaptation to stress occurs when a plant withstands the imposed stress that may arise from either tolerance or a mechanism that permits escape from the situation (Beck et al. 2007). This will have direct and indirect effects on the reduction of net photosynthesis and the overall production of ROS (Cruz de Carvalho 2008). During that period, plants maintain the internal water potential, turgor and water uptake by increasing the level of osmolytes, either by uptake of soil solutes or by synthesis of metabolic (compatible) solutes (Zhu 2002) as well as maintain the toxic oxygen species and compounds through anti-oxidative mechanisms.

2.1.2 Physiological responses to drought stress

Drought is one of the most important manifestations of abiotic stress in plants. The initial reaction to stress-induced drought is to modify stomatal conductance by reducing stomatal aperture, this minimizes the loss of water by transpiration. However, stomatal closure has complex consequences, requiring adjustment at the levels of photosynthesis and respiration, altering ion, nutrient and water fluxes and change in the allocation of carbon and nitrogen (Hsiao 1973). This response operates more or less precociously at the onset of drought period, depending on the plants' inherent strategy (and acclimation) towards drought stress (Cruz de Carvalho 2008). The phytohormones play a crucial role in triggering several molecules for performing cascades of biochemical reactions in response mechanism. For instance, ABA activates the synthesis of H_2O_2 in guard cells by a membrane bound NADPH oxidase and that H_2O_2 mediates stomatal closure by activating plasma membrane Ca^{2+} channels (Kwak et al. 2003, Pei et al. 2000). As a consequence, drought stress reduces the photosynthesis rate, transpiration rate, stomatal conductance, water use efficiency and RWC in leaf as well as enhances the ABA content and leaf water potential (Jiang and Hartung 2007). It also affects chl content, inhibits roots growth, dry matter production and severely reduces the yield and yield components (Loggini et al. 1999). Physiological responses to drought stress have been reported in numbers of crop plants for the years. For example: Pirdashti et al. (2009) reported a

significant and positive correlation between chl content and RWC and also between these traits and grain yield in four rice cultivars. Efeoğlu et al. (2009) recorded the decrease of RWC, chl a, chl b, total chl (a + b) and carotenoids content in three maize cultivars during drought stress as well as control values after recovery from stress. Bano and Yasmeen (2010) reported that drought stress resulted in maximum decrease in IAA and GA content but maximum increase of ABA and proline in wheat cultivar.

2.1.3 Responses of biochemical solutes to drought stress

Drought stress induces not only physiological but also biochemical changes that finally alter the metabolic status of the stressed plant. To counter with drought stress, many plants increase the osmotic potential of their cells by synthesizing and accumulating osmolytes (Du et al. 2004). Two hypotheses have been suggested to explain the functions of accumulating solutes (Truchet et al. 1991). In the first, accumulation leads to 'osmotic adjustment' through mass action, which results in increased water retention and/or sodium exclusion. The second hypothesis considers accumulating compounds as 'compatible solutes'. In this function they could replace water as a solute in biochemical reactions. Compatible solutes could also, or as an alternative, associate with lipids or proteins and thus prevent membrane disintegration, dissociation of protein complexes, or inactivation of enzymes. The solutes that accumulate vary with the organism and even between plant species (Bray 1997), which include elemental ions, such as K^+ and majority of organic solutes (Cruz de Carvalho 2008). The major category of organic solutes consists of simple sugars (mainly fructose and glucose), sugar alcohols (glycerol and methylated inositols) and complex sugars (trehalose, raffinose and fructans) (Bohnert and Jensen 1996). Others include quaternary amino acid derivatives (proline, glycine betaine, ß-alanine betaine, proline betaine, tertiary amines), and sulfonium compounds (choline osulfate, dimethyl sulfonium propironate) (Bray 1997).

2.1.3.1 Carbohydrates

Carbohydrates such as sugars accumulate under drought stress and play a leading role in osmoprotection, osmotic adjustment, carbon storage and radical scavenging

(Hsiao 1973). Higher accumulation of soluble carbohydrates in response to drought stress has been widely reported in various crop plants such as rice (Vajrabhaya et al. 2001), wheat (Hakimi et al. 1995, Kameli and Losel 1993), maize (Mohammadkhani and Heidari 2008a) and pea (Sánchez et al. 1998). An increase in both reducing and non-reducing sugars and a decrease in starch under drought stress have also been reported in maize (Mohammadkhani and Heidari 2008a), wheat (Nicolas et al. 1985) and coffee (Matta et al. 1997) cultivars. Trehalose, a disaccharide, also accumulates under drought conditions and protects membranes and proteins in cells exposed to stress and reduce aggregation of denatured proteins (Singer and Lindquist 1998). It has a suppressive effect on apoptotic cell death under drought (Yamada et al. 2003). Higher accumulation of trehalose in response to drought has been reported in various plant species such as rice (Garg et al. 2002) and wheat (El-Bashiti et al. 2005).

2.1.3.2 Amino acids, amides and proteins

Amino acids such as proline, alanine, leucine, valine, aspartate and amides such as glutamine and asparagine have also been reported to accumulate in response to drought in various crop plants. For example, drought treatment in maize seedlings caused an increase in total free amino acid content and a consistent rearrangement of the amino acid pool, with an accumulation of alanine, arginine, valine, aspartate, serine, threonine and tyrosine, as well a decrease in glutamic acid, glycine and methionine (Ranieri et al. 1989). Among the amino acids, proline is osmotically very active and contributes to membranes stability and mitigates the effect of stress on cell membrane disruption (Szekely 2004). Proline accumulation normally occurs in the cytosol where it contributes substantially to the cytoplasmic osmotic adjustment, and even at supra-optimal levels, it does not suppress enzyme activity (Ketchum et al. 1991). In higher plants, proline is synthesized from glutamate or ornithine. It may act as a signaling or regulatory molecule that may able to activate multiple responses that are component of the adaptation process (Maggio et al. 2002). Proline accumulation under drought and its role in conferring tolerance have been studied extensively from model plants like *Arabidopsis* (Szekely 2004) to crop plants such as rice (Mostajeran and Rahimi-Eichi 2009), chickpea (Najaphy et al. 2010), drought tolerant genotypes of wheat (Nayyar and Walia 2003) and potato

(Schafleitner et al. 2007). Although amides generally accumulate in environmentaly stressed plants to a lesser extent than do other nitrogen containing compounds (Mansour et al. 2002), concentration of asparagine and glutamine frequently increase in response to drought. For example, a substantial accumulation of asparagine and glutamine in different drought resistant cultivars of maize were registered under drought (Thakur and Rai 1982). Proteins that accumulate under drought may provide a storage form of nitrogen for re-utilizing later and may play a role in osmotic adjustment. A higher content of soluble proteins in response to drought has been reported in various crop plants such as broad bean (El-Tayeb 2006), mustard (Ashraf and Mehmood 1990) and cotton (Li et al. 2010), however there are also reports of decrease of the same in diverse crop species under drought such as maize (Mohammadkhani and Heidari 2008b) and grapevine (Maroco et al. 2002). In higher plants, numbers of proteins accumulate in response to drought as part of the tolerance mechanism such as molecular chaperons (heat shock proteins and dehydrins), aquaporins and antioxidative enzymes (SOD, APX etc.) (Shinozaki and Yamaguchi-Shinozaki 2007).

2.1.3.3 Quaternary ammonium compounds

Quaternary ammonium compounds such as glycine-betaine and proline-betaine are now well-known to have osmoprotective effects under drought (Cushman 2001). QACs can accumulate to high concentration which may not only increase the osmotic pressure of the cytoplasm without perturbing metabolism but also stabilize enzymes and membranes (Yancey 1994, Kavikishore et al. 2005). The synthetic pathway to glycine-betaine, the most common QAC, therefore has been the target of recent metabolic engineering efforts to improve plant's stress tolerance (McNeil et al. 1999, Rathinasabapathi et al. 2000, Sakamoto and Murata 2000). This organic compound is mainly localized in chloroplast thylakoid membranes, thereby maintaining photosynthetic efficiency and plasma membrane integrity (Huang et al. 2000). Glycine-betaine accumulates in response to drought in many crops including rice (Sawahel 2003), wheat (Wang et al. 2010a), maize (Quan et al. 2004) and sorghum (Yang et al. 2003).

2.1.3.4 Polyols

Polyols, the polyhydric alcohols, are among the compatible solutes involved in osmoregulation which are thought to play a role in plant's drought tolerance (Bohnert et al. 1995). The most common polyols in plants include acyclic forms such as mannitol, glycerol and sorbitol as well as cyclic forms such as ononitol and pinitol. Higher accumulation of different polyols in conferring drought tolerance has been reported in diverse crop plants such as mannitol in wheat (Abebe et al. 2003) and sorbitol in cherry (Ranney et al. 1991) and apple (Wang and Stutte 1992).

2.1.4 Antioxidative responses to drought

Metabolism of higher plants is highly regulated in order to allow effective integration of diverse spectrum of biosynthetic pathways. Higher plants, as other aerobic organisms, require oxygen for the efficient production of energy. During the reduction of O_2 to H_2O, ROS, namely superoxide radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$) can be formed (Hong-bo et al. 2008). Most of the cellular compartments in higher plants have the potential to become a source of ROS. In order to cope with continuous ROS production, plants have a battery of enzymatic and non-enzymatic antioxidants, which function as an extremely efficient co-operative system (Mittler 2002). Under drought stress, CO_2 fixation is reduced which consequently enhance ROS production through multiple ways and create chances of oxidative damage to cellular membranes and organelles (Cruz de Carvalho 2008). For example, the limitation on CO_2 fixation reduces $NADP^+$ regeneration through the calvin cycle, hence provoke an over reduction of the photosynthetic electron transport chain. In fact, during photosynthesis under drought stress, there is a higher leakage of electrons to O_2 by the Mehler reaction (Smirnoff 1993). Plant's defense system fights against the higher production of ROS under drought by enhancing the activities of enzymatic antioxidants such as SOD, CAT, APX, POX, GR etc. and by accumulating non-enzymatic antioxidants such as glutathione, ascorbate, tocopherol, proline and betaine etc. (Mittler 2002). In addition to crucial roles in defense system and as enzyme co-factors, antioxidants influence growth and development of higher plants by modifying processes from mitosis and cell elongation to senescence and death (Hong-bo et al. 2008).

2.1.4.1 Non-enzymatic antioxidants

A continuous oxidative assault on plants during drought stress has led to the presence of an arsenal of non-enzymatic antioxidant defenses to counter the phenomenon of oxidative stress (Du et al. 2004). AA is an important antioxidant, which reacts not only with H_2O_2, but also with $O^{\cdot}_2$, OH and lipid hydroperoxidases (Hong-bo et al. 2008). AA is water soluble and has an additional role in protecting or regenerating oxidized carotenoids or tocopherols. It also involved in the neutralization of secondary products of ROS (Mittler 2002). AA is one of the most extensively studied antioxidant and has been detected in the majority of plant cell types, organelles and apoplast (Loewus 1988). Glutathione is also takes part in the control of H_2O_2 levels. Change in the ratio of its reduced (GSH) to oxidized (GSSG) form during the degradation of H_2O_2 is important in redox signaling pathways (Mittler 2002). It has been suggested that the GSH/GSSG ratio is an indicative of the cellular redox balance which may be involved in ROS perception (Cruz de Carvalho 2008). Reduced glutathione (GSH) acts as an antioxidant and is involved directly in the reduction of most active oxygen radicals generated due to drought (Cruz de Carvalho 2008). Tocopherols, found in green parts of plants, also scavenge lipid peroxyl radicals through the concerted action of other antioxidants (Hong-bo et al. 2008). Further, they are known to protect lipids and other membrane components by physically quenching and chemically reacting with O_2 in chloroplasts, thus protecting the structure and function of photosystem II (Asada 2006). α-tocopherol interact with the polyunsaturated acyl groups of lipids, stabilize membranes and scavenge as well as quench various ROS and lipid soluble by-products of oxidative stress (Asada 2006). Singlet oxygen quenching by tocopherols is highly efficient, and it is estimated that a single α-tocopherol molecule can neutralize upto 120 singlet oxygen molecules *in vitro* before being degraded (Wu and Tang 2004). Other antioxidants such as citrulline and metallothionein are also efficient in scavenging hydroxyl radical and protecting proteins and DNA effectively from oxidative damage (Cruz de Carvalho 2008). Responses of antioxidants under drought stress have been studied in diverse crop plant species through the years, and their observations have revealed differential responses that varied among the crops (Table 2.1).

Table 2.1: Drought stress responses of major antioxidants in various crop plants

Antioxidants	Crops	Responses to drought stress	References
Ascorbic acid	Rice	decreased significantly	Sharma and Dubey 2005
	Soybean	increased highly in tolerant genotypes	Angra et al. 2010
	Wheat	decreased in high percentage	Sairam et al. 1998, Bartoli et al. 1999
	Apple	increased under moderate drought stress	Sircelj et al. 2007
Glutathione	Rice	decreased under severe drought	Sharma and Dubey 2005
	Apple	increased significantly under moderate drought stress	Sircelj et al. 2007
Tocopherol	Wheat	increased significantly	Bartoli et al. 1999, Keles and Oncel 2002
	Apple	decreased under severe drought stress	Sircelj et al. 2007
Proline	Maize	increased at different drought intensities	Mohammadkhani and Heidari 2008a
	Sorghum	increased significantly only at severe drought stress	Waldren and Teare 1974
	Soybean	increased significantly only at severe drought stress	Waldren and Teare 1974
	Olive	increased in different cultivars	Ahmed et al. 2009
SOD	Rice	increased consistently with increasing drought stress	Sharma and Dubey 2005
	Maize	increased in different cultivars	Moussa and Abdel-Aziz 2008
	Wheat	significantly increased at two different stages after anthesis	Sairam et al. 1998
	Wheat	increased or maintained at same level	Navari-Izzo et al. 1993
	Soybean	increased equally in tolerant and susceptible genotypes	Angra et al. 2010
	Oilseed Rape	increased activity in different genotypes	Abedi and Pakniyat 2010
	Olive	increased in different cultivars	Ahmed et al. 2009
	Coffee	increased in both tolerant and susceptible genotypes	Pinheiro et al. 2004

(Table 2.1 continued..)

APX	Rice	increased consistently with increasing drought stress	Sharma and Dubey 2005
	Wheat	significantly increased at two different stages after anthesis	Sairam et al. 1998
	Soybean	increased highly in tolerant genotypes	Angra et al. 2010
	Olive	increased in different cultivars	Ahmed et al. 2009
	Coffee	increased in both tolerant and susceptible genotypes	Pinheiro et al. 2004
GR	Rice	increased under different drought stress level	Sharma and Dubey 2005
	Wheat	decreased under severe drought	Selote and Khanna-Chopra 2010
	Coffee	increased in both tolerant and susceptible genotypes	Pinheiro et al. 2004
CAT	Rice	decreased consistently with increasing drought stress	Sharma and Dubey 2005
	Wheat	significantly increased at two different stages after anthesis	Sairam et al. 1998
	Wheat	increased or maintained at same level	Navari-Izzo et al. 1993
	Maize	increased in different cultivars	Moussa and Abdel-Aziz 2008
	Soybean	increased highly in tolerant genotypes	Angra et al. 2010
	Oilseed Rape	increased activity in different genotypes	Abedi and Pakniyat 2010
	Coffee	increased in both tolerant and susceptible genotypes	Pinheiro et al. 2004
	Olive	increased in different cultivars	Ahmed et al. 2009

2.1.4.2 Enzymatic antioxidants

Plants defense system for controlling ROS under drought stress includes several antioxidative enzymes that actively and efficiently participate in the process. The major enzymatic scavenging mechanisms include SOD, enzymes and metabolites from the ascorbate-glutathione cycle and CAT (Bowler et al. 1992, Noctor and Foyer 1998, Willekens et al. 1997). They are located throughout the different compartments of plant cells, with the exception of CAT that is exclusively located in peroxisomes. SOD is the front-line enzyme in ROS attack since it rapidly scavenges superoxide, one of the first ROS to be produced, dismutating it to oxygen and H_2O_2 (Bowler et al. 1992). However, this reaction only converts one ROS to another, and H_2O_2 also needs to be destroyed since it promptly attacks thiol proteins. The major enzymatic cellular scavengers of H_2O_2 are CAT and APX (Noctor and Foyer 1998, Willekens et al. 1997). They have, however, different affinities for this ROS and seem to have different cellular roles in H_2O_2 scavenging. In fact, CAT does not need a reductant to scavenge H_2O_2 making it reducing power-free, whereas APX needs a reductant, ascorbate. The GR is the last enzyme of the ascorbate/glutathione cycle which has a major role in maintaining the intracellular glutathione pool in the reduced state (GSH). GSH can function as an antioxidant either directly or indirectly as a reducing agent that recycles ascorbic acid form to its reduced form by the enzyme dehydroascorbate reductase (Loewus 1988).

Measuring of specific antioxidant enzyme activities and/or expression analysis during water stress treatments has been generally accepted as an approach to assess the involvement of the scavenging system during drought stress (Cruz de Carvalho 2008). However, contradictory results have been gathered through the years (Table 2.1). These differences might be related not only to the plants' age and their tolerance level or strategy towards water stress treatment, but also to the duration and the intensity of the stress treatment. Nevertheless, there are reports of a direct correlation between the level of the induction of antioxidants and the degree of drought tolerance in numbers of crop plants of same genus or species such as maize (Pastori and Trippi 1992), wheat (Loggini et al. 1999), rice (Guo et al. 2006) and coffee (Lima et al. 2002).

2.1.5 Brief report on works done in tea plants

Tea plant being perennial in nature can grow under diverse climatic conditions and is always subjected to various environmental stresses including drought. The effects of drought in tea plantations are well-known which severely impairs growth, crop yield and various morphological, anatomical, physiological and biochemical processes. The changes basically take part as a mechanism of drought adaptation and/or tolerance.

2.1.5.1 Growth and physiology

High temperature and dry weather are unfavorable for the growth of tea plants. Increases in temperature, soil moisture deficit, and vapor pressure deficit create a plant water deficit, which lead to growth retardation (Wijeratne 1992). Young tea plants are more vulnerable to drought stress than the mature ones due to their inefficiency of stress tolerance (Nixon et al. 2001) which show the early symptom of shoot growth retardation (Cheruiyot et al. 2007). It was reported that the clonal tea growth and yields could adversely be affected at temperatures >26°C, soil moisture deficits >30-50 mm, and saturation vapor pressure deficits >1.2 kPa (Wijeratne 1994). Burgess and Carr (1996) studied the yield response to drought and temperature of six contrasting tea clones, and concluded that annual yields decreased curvi-linearly as the maximum soil water deficit increased. A reduction of yield about 2.9 kg per ha for each mm increase in the potential soil water deficit was reported by Stephens and Carr (1991).

Dynamic states of water potential of the shoot, synthetic function and metabolic function of tea plant under the condition of different soil water content including drought were studied by Yang et al. (1987). The results showed that leaf water potential dropped under drought stress and slowed down the growth of the leaf. The photosynthesis and synthetic metabolism were also decreased; however, the electrical conductivity of the shoot was increased. Qian (1999) also reported that the net photosynthetic rate, transpiration rate and stomatal conductance under high, moderate and low soil water content were gradually decreased but intercellular CO_2 concentration was increased with the time of drought stress.

Declining of transpiration rate and stomatal conductance alleviated cellular injury in tea plants under drought which enhances the significant accumulation of phytohormones such as ABA. Dropping of net photosynthesis rate ultimately leads to retardation of the growth of the plants (Xuelan 1987). Affects of drought stress on stomatal conductance and transpiration of tea leaves are well-documented in literature (Fordham 1971, Gee et al. 1982, Saikia and Dey 1984). Clonal differences in the stomatal conductance and diffusion resistance under drought stress have also been reported (Squire 1978, Sandanam et al. 1980). Therefore, drought stress tolerance level is varied on different clones. Ngetich and Bore (1998) found that Sc 31/37 and 303/577 clones tolerated drought stress better than the other clones planted at the same time under a soil water deficit of 391 mm in rehabilitated and replanted fields.

Plants maintain high RWC in leaves as a mechanism of water stress adaptation (Farooqui et al. 2000). Wijeratne et al. (1998) found that drought stress reduced RWC and water potential, and increased diffusive resistance in leaves. In their study, the critical leaf water potential increasing diffusive resistance and reducing transpiration of drought tolerant clone (TRI 2025) was comparatively higher than that of drought susceptible clone (TRI 2023). It was found that the clones having efficient stomatal control for reducing water loss and osmotic adjustments for absorbing water from drier soils can withstand drought.

Maintenance of RWC under drought stress was also reported in TV-1, TV-20, TV-29 and TV-30 cultivars (Upadhyaya et al. 2008). Maximum decrease in RWC was observed in TV-30 (53.07%) cultivar after 20 d of stress imposition, whereas TV-1 (42.03%) showed less decrease. After rehydration, plants were recovered RWC and maintained highest content in TV-1 (91.22%). Decrease in fresh mass was also found highest in TV-1 (51.85%) whereas TV-20 (20.01%) showed least decrease over well-watered plants after 20 d of stress imposition. Declining of RWC in TV-18, TV-26, UPASI-3, UPASI-26, T-78 and HV-39 cultivars under drought were reported by Chakraborty et al. (2002), however, drought tolerant cultivars UPASI-3 and UPASI-26 maintained higher RWC in comparison to the other clones.

It is well-known that differences in rooting depth are one of the factors responsible for the variability of drought resistance in a number of plants (Begg and Turner 1976). In tea, out of three parameters such as rooting depth, roots weight and vertical distribution of roots in the soil, a positive co-relation of rooting depth with drought stress tolerance was reported (Nagarajah and Ratnasuriya 1981). In shallow rooted clones such as NL 4/2, 2026, 2024 and 2023, drought resistance was increased with the increase of rooting depth; however, in deep rooted clones such as 2025, 2027, CY 9 and DN, drought resistance was not associated to rooting depth.

2.1.5.2 Biochemical changes

There is a co-relationship between the drought injury of tea plants and the damage that is caused by ROS. A positive correlation between MDA content and leaked electrolyte level ($r=0.9938$, $p=0.01$) was reported by Lu (1992). Increased activities of SOD and CAT were also recorded at the initial stage of drought which was decreased sharply with increasing drought intensity, but POD activity was increased continuously during drought stress (Lu 1992). Yang et al. (1987) reported that drought stress decrease the activities of shoot POD and POX. Chakraborty et al. (2001) studied the biochemical responses of drought stress in TV-22, TV-23, TV-25, TV-26, TV-27 and TV-17 cultivars by withholding water for 7 and 14 d. Both total phenol and PAL activity were increased moderately in those 4 varieties following 7 d of stress imposition, while longer period of drought resulted in a sharp decline of both phenolics and PAL activity. The POX activity was increased following drought while POD activity registered a marked decline. Protein content was not significantly affected by initial stage of drought; however it was decreased significantly in extended drought period. Higher activities of PAL, POD and POX and phenol content were also reported in TV-18, TV-26, UPASI-3, UPASI-26, T-78 and HV-39 cultivars at the initial stage of drought which were decreased under severe drought condition (Chakraborty et al. 2002). Jeyaramraja et al. (2003) reported a reduction of PAL activity in drought tolerant 'Assam' cultivar UPASI-2, followed by UPASI-8 and UPASI-9. Lower PAL activity correlated well with lower synthesis of flavanols such as EGCG and ECG. Hernández et al. (2006) reported that ECQ and EGCGQ, the oxidation products of EC and EGCG, increased up to 100 and 30 fold, respectively, in tea plants exposed to 19 d of water deficit. The formation of ECQ and EGCGQ were

found negatively correlated with the extent of lipid peroxidation in leaves supporting the protective roles of these compounds under drought stress. Accumulation of proline as a protective osmolyte under drought stress in tea plants is well-documented in literature (Qian 1999, Chakraborty et al. 2001, Chakraborty et al. 2002, Upadhyaya and Panda 2004). Upadhyaya and Panda (2004) reported the decreased activities of guaiacol peroxidase and GR as well as contents of chl, carotenoids, ascorbate and glutathione after 5 d of drought imposition. Simultaneously, increased H_2O_2 and superoxide anion content and lipid peroxidation level as well as CAT and SOD activities were achieved. In another attempt, Upadhyaya et al. (2008) revealed the effect of drought stress by studying the ROS metabolism in TV-1, TV-20, TV-29 and TV-30 cultivars after 20 d of drought stress imposition followed by rehydration. The results showed non-enzymatic antioxidants like ascorbate and glutathione content were decreased with differential responses of antioxidative enzymes. Increased level of lipid peroxidation, $O_2^{\cdot}$ and H_2O_2 content were also recorded. The antioxidative efficiency and biochemical tolerance of the selected clones in response to drought stress were observed as TV-30>TV-1>TV-29>TV-20.

Cheruiyot et al. (2007) conducted an experiment to determine the association of tea polyphenols with water stress and their suitability as indicators for drought tolerance. The experiment was conducted in a 'rain-out' shelter, and consisted of six tea clones (BBK 35, TRFK 6/8, TRFK 76/1, TRFK 395/2, TRFK 31/30, and TRFK 311/287) at four different soil water levels (38, 30, 22 and 14% v/v), which were maintained for a period of 12 weeks. The results indicated that declining of soil water content reduced the content of polyphenols. Drought tolerant clones maintained a higher level of polyphenol content under stress, and also showed less fluctuation in total phenolics content. There was a significant ($P<0.001$) correlation of total polyphenol content with shoot growth and water stress of tea, and a linear relationship between soil water content and both water stress index and shoot polyphenol content. This study showed a potential use of polyphenol as an indicator for selection of drought tolerant cultivars. In another study, Cheruiyot et al. (2008) suggested specifically the potential use of tea polyphenol, EC and EGC as indicators in predicting drought tolerance. The study consisted of six tea clones (BBK 35, TRFK 6/8, TRFK 76/1, TRFK 395/2, TRFK 31/30, and TRFK 311/287) and four levels of soil

water content (38, 30, 22, and 14% v/v). Shoot contents of EC and EGC in the clones showed varied responses, with a distinct pattern in the water-stress tolerant clones (TRFK 6/8 and TRFK 31/30). Total catechins showed significant correlation with shoot growth (r=0.65, P=0.006), soil water content (r=0.54, P=0.0066), and water stress index (r=0.67, P=0.0004).

Even though some works on physiological and biochemical responses to drought stress of tea plants have been done so far, these are primarily descriptive in nature. More information is yet to be gathered at physiological, biochemical and molecular level which is essential for better understanding of the mechanism of drought stress responses and which would provide a basis for comprehending the underlying process of their tolerance.

2.2 Materials and methods

Plants response to stress is often manifested by its physiological and biochemical reactions, which can provide a basis for better understanding the stress tolerance mechanism as well as screening and selection of individual varieties and germplasms resistant to stress. In this present investigation, drought-tolerant and -susceptible tea cultivars were selected for studying their variability in drought stress responses. The study was undertaken in both leaves and roots for understanding the responses and effects of drought stress at anatomical, physiological and biochemical level.

2.2.1 Plant materials, stress induction and sample collection

Two year-old vegetatively propagated well-rooted tea seedlings (~36 inch height) of S.3/A3 (drought-susceptible) and TV-23 cultivars (drought-tolerant) (Konwar 2004) were planted in earthen pots (12 inch dia) under controlled greenhouse conditions at a light intensity of 300 μmol m^{-2} s^{-1} and 25 ± 2°C with relative humidity of 65-70%. A total of 24 seedlings of each cultivar were collected from Nagrakata Experimental Station, Tea Research Association, Jalpaiguri, West Bengal, India. Drought sensitivities of the cultivars were confirmed by breeders and commercially established on the basis of yield stability in drought years. Initially, all the seedlings were watered regularly for 2 months to establish new growth, and subsequently withheld water in half numbers of seedlings in each cultivar. The period of severe drought stress was determined on the basis of soil moisture content and physiological status of the plants. Soil moisture content and physiological status of the plants were evaluated in every 2 d interval from the d of withholding water for confirmation of drought stress induction. On 21st d of stress induction, at average 7% of soil moisture content (-1.2 Mpa ± 0.20), 8.73 μmol m^{-2} s^{-1} of photosynthesis rate and 0.42 mmol m^{-2} s^{-1} of stomatal conductance, roots and leaves were collected for various experiments. The 3rd mature leaves from top of the shoots and young white fibrous roots (100 mg) of control and drought induced plants were taken and subsequently performed various experiments.

2.2.2 Determination of soil moisture content, photosynthesis rate, transpiration rate and stomatal conductance

Soil moisture content was measured following a gravimetric method according to Black (1965). Photosynthesis (Pn) and transpiration rate (E) as well as stomatal conductance (SC) were recorded for all the treatments independently using an intelligent portable photosynthesis system (LCpro+, ADC, UK) between 9:00 to 10:00 h.

2.2.3 Determination of relative water content

Five mature leaves were considered for estimating RWC. Fresh leaves were weighed (FW) and left saturated in water for three hours followed by measuring their turgid weights (TW). The samples were then dried in an oven at 80^0C for 24 h and weighed again (DW). RWC (%) was determined according to Barr and Weatherley (1992) as follows: RWC (%) = (FW-DW)/(TW-DW) x 100.

2.2.4 Anatomical studies using light and transmission electron microscopy

In a potato block, semi-thin sections of tissues such as leaves, petioles and roots from drought stressed and well-watered (control) plants were made by sharp blades for light microscopic observations. Sections were stained with 0.1% of safranin solution (v/v) for 5 min and visualized at 20X resolution under a compound microscope (Leica, DM1000). An impression of stomata was also taken on a piece of transparent cello tap from the abaxial side of leaves with the help of nitrocellulose solution, and subsequently visualized under light microscope. For the study of ultra-structures, 1-2 mm sections of fresh leaves and roots were made after washing with 50 mM potassium phosphate buffer (pH 7). Samples were fixed with 2.5% of glutaraldehyde solution prepared in 50 mM potassium phosphate buffer (pH 6.9) for overnight followed by 3 times of washing for 15 min each with 100 mM sodium cacodylate buffer (pH 6.9) (Sandalio et al. 2001). Samples were then dehydrated with gradual changes of acetone concentration as 30%, 50%, 70%, 80%, 90%, 95% and 100% each of 15 min duration with 2 changes. It was followed by drying with liquid carbon dioxide at its critical point i.e 31.5^0C at 1100 psi. Dried specimens were

mounted on brass and coated with gold at 35 nm thickness. Thereafter, clearing was carried out using propylene oxide at room temperature for 15 min with two consecutive changes. The specimens were then infiltrated with propylene oxide and embedding medium in 3:1 ratio for overnight and afterwards transferred into an embedding moulds or BEEM capsules in proper orientation. Pure embedding medium was then poured into the mould or capsule and transferred to an embedding oven at 50^0C for overnight. Oven temperature was raised later on to 60^0C and kept for another 24 h for polymerization. Thick sections of specimens were made (0.5-1.0 µm) and stained with toluidine blue for 2-3 min. Sections were observed under microscope for precise location of making ultra-thin sections at 60-90 nm thick (silver-yellow color). The ultrathin sections were collected onto grids and dried for overnight. The grids were stained with uranyl acetate for 15 min and lead citrate for 5 min and observed under TEM (JEM 100C x II, Jeol) at an accelerating voltage of 80 kV.

2.2.5 Estimation of TSS, RS, starch, pigments, phenols, proline, abscorbic acids and ABA contents

Total soluble sugar was estimated using anthrone reagent (Devi 2007). For estimation of TSS, the samples were hydrolyzed by keeping in a boiling water bath for 3 h with 5 ml of 2.5 N HCl and cooled at room temperature. The reaction was neutralized with solid sodium carbonate and made up the final vol to 10 ml. After centrifugation at 10000 rpm for 10 min, the supernatant was collected and diluted to 10 times. For analysis, 200 and 600 µl aliquots of the diluted sample were taken and made the final vol to 1 ml with distilled water. In 1 ml vol of sample, 4 ml of anthrone reagent was added and heated in a boiling water bath for 8 min followed by rapid cooling. It produces a green colour solution and absorbance was read at 630 nm using a UV-VIS spectrophotometer (lamda 25, perkinelmer). The amount of soluble sugar was calculated comparing with a standard curve of glucose by the following formula and expressed as mg in per g FW of sample.

Amount of TSS (% mg) = [Sugar value from graph (mg)/ sample used (ml)] x [total vol of extract (ml)/ weight of sample (mg)] x 100

Estimation of reducing sugar (RS) was done by the method of Miller (1972). Five hundred mg of sample was treated with 10.0 ml of 80% ethyl alcohol. In 3.0 ml of alcoholic extract, 3.0 ml of DNS reagent was added. The mixture was heated for 5 min in a boiling water bath. After the colour had developed 1.0 ml of 40% rochelle salt was added when the contents of the tubes were still warm. The tubes were cooled under running tap water. Absorbance was recorded using spectrophotometer at 515 nm. Amount of reducing sugar was calculated using standard curve prepared from glucose. The quantity of reducing sugar was expressed as mg in per g FW of sample.

Starch content was measured as liberated glucose using anthrone reagent following hydrolysis of the extracted powders with perchloric acid (MacRae et al. 1974). Samples were ground with 1 ml of hot 80% ethanol and centrifuged at 14000 rpm for 15 min at room temperature. Pellet was taken and washed several times with hot 80% ethanol till become colourless. It was centrifuged again, collected the pellet and dried with speed vacuum. Pellet was dissolved in 1 ml of distilled water and 1.3 ml of 52% perchloric acid (w/v) in a cool condition by incubating for 20 min. Then, it was centrifuged at 14000 rpm for 15 min at room temperature and collected the supernatant finally to adjust 25 ml of vol with 52% perchloric acid. Further, 100 µl of diluted sample was taken and adjusted to 1 ml with distilled water followed by addition of 4 ml of anthrone reagent. The solution was heated for 8 min and cooled rapidly in ice. The absorbance was read at 630 nm, and the amount of starch was calculated from a standard curve of glucose and expressed as mg in per g FW of sample.

Pigments such as chl a, chl b and carotenoids were assayed according to the protocol of Lichtenthaller (1987) with minor modifications. Samples were grounded with 2 ml of 80% acetone and centrifuged at 5000 rpm for 5 min at room temperature. Supernatant was collected and the residual amount was grounded again with 2 ml of 80% acetone. Volume of the collected supernatant was finally adjusted to 10 ml with 80% acetone. Absorbance was read at 470, 645 and 663 nm. The amount of chl a, chl b and carotenoids were estimated by the following formula and expressed as µg in per g FW of sample.

Chl a (µg/ml) = (12.21 x A_{663}) - (2.81 x A_{646})

Chl b (µg/ml) = (20.13 x A_{646})-(5.03xA_{663})

Total carotenoids (µg/ml) = (1000 x A_{470})- (3.27 x chl a) - (104 x chl b)/198

For converting µg/ml into µg per g of FW = µg/ml x final vol made/weight of tissue taken in g

Total phenol was estimated using FCR reagent (Malick and Singh 1980). Samples were grounded in liquid nitrogen and transferred the powder to 1 ml of 80% ethanol. Homogenates were centrifuged at 10000 rpm for 20 min at 4^0C. Supernatant was discarded and re-extracted the residue with 80% ethanol in five times of the initial vol, followed by centrifugation and collected the pellet. The residual supernatant was evaporated using a rotary evaporator at 25^0C and finally dissolved the pellet in 1 ml of distilled water. The dissolved sample was taken in 0.3 ml and 0.7 ml of aliquots and adjusted the vol to 3 ml with distilled water followed by addition of 0.5 ml of FCR reagent (diluted with distilled water in 1:1 ratio). After incubation for 3 min, 2 ml of 20% sodium carbonate (w/v) solution was added and mixed thoroughly. The reaction was performed exactly for one min by incubating the samples in a boiling water bath. The solution was cooled down and read the absorbance at 650 nm. Total phenol was estimated comparing the absorbance value with known catechol amount in a standard curve and expressed as mg phenol in per g FW of sample.

Proline content was estimated using acid ninhydrin (Bates et al. 1973). Sample homogenates were prepared in 2 ml of 3% sulphosalicylic acid (w/v) and centrifuged at 14000 rpm for 15 min at 4^0C. The supernatant was collected and used as sample. In 2 ml of sample, glacial acetic acid and acid ninhydrin were added in 2 ml each. The solution was heated in a boiling water bath for 1 h followed by incubation in ice for few min. Finally, 4 ml of toluene was added and mixed by vortexing. Upper pink layer was collected and absorbance was taken at 520 nm. Proline content was estimated from a standard curve of known proline and expressed as µmole in per g FW of sample.

Amount of AA was measured following the modified protocol of Oser (1979). Samples were ground in liquid nitrogen and extracted with 5 ml of 6% TCA. The suspension was centrifuged at 7000 rpm at 4^0C for 15 min and collected the supernatant. In 4 ml of supernatant, 2 ml of 2% DNPH was added followed by mixing of 1 drop of thiourea. The solution was heated for 15 min in a boiling water bath. After cooling at room temperature, 5 ml 80% sulphuric acid was added keeping the tubes in an ice bath. Absorbance was read at 530 nm and estimated the content of AA using a standard curve of pure AA.

For ABA determination, tissues were homogenized separately in 2 ml of chilled 80% methanol containing BHT (100 mg per litre). Homogenates were stored for 24 h in dark at 4°C followed by centrifugation at 10000 rpm for 10 min at 4°C. The supernatant was filter sterilized and dried at 35°C *in vaccuo* in a rotary shaker. Pellet was dissolved in 4 ml of 0.1 M potassium phosphate buffer (pH 8.0), the pH of which was adjusted at 2.5 and extracted for 3 times in an equal vol of diethyl ether. Subsequently, the ether phase was collected, evaporated *in vaccuo* and dissolved in 1 ml of methanol. It was filtered through a 0.45 µm filter (Millipore) and finally 20 µl of filtered sample was injected into a RP 18 (10 µm) column protected by a guard column. Elution was carried out with methanol/water (8/2, v/v) with 1% acetic acid at a flow rate of 1 ml per min. Solvents were also filtered through a 0.45 µm filter (Millipore). The column was monitored by a UV detector at 254 nm in a HPLC system (Waters, US) and ABA was measured by referring to an authentic (±) standard ABA (Sigma) (Nagar and Kumar 1996).

2.2.6 Determination of ROS, lipid peroxidation, hydrogen peroxide and membrane stability levels

Estimation of superoxide anion was done by monitoring the nitrate formation from hydroxylamine (Jordan and DeVay 1990). Samples were chopped and transferred to 3 ml of reaction mixture containing 50 mM potassium phosphate buffer (pH 7.8) and 2.5 mM NBT (dissolved in 70% N, N-dimethyl formamide). Samples were vacuum infiltrated and boiled, each step of 30 min duration and followed by centrifugation at 15000 rpm for 15 min at 25^0C. The supernatant was taken and adjusted the final vol to 3 ml by N, N dimethyl formamide. Absorbance was read at 580 nm.

Level of lipid peroxidation was measured in terms of malondialdehyde (MDA) according to the method of Kazuhiro et al. (1996) with some modifications. Samples were homogenized by the addition of 2 ml of 0.1% TCA solution in ice cold condition. Homogenates were centrifuged at 10000 rpm for 20 min at 4 ^{0}C. In 1.5 ml of supernatant, 1.5 ml of 0.5% TBA (w/v) were added and incubated at 95^{0}C for 30 min followed by rapid cooling in an ice bath. Solution was centrifuged again at 3000 rpm for 5 min and collected the supernatant. Absorbance of the supernatant was read at 532 and 600 nm. The MDA concentration was determined from the difference of absorbance (A_{532} - A_{600}) by dividing with molar extinction coefficient (155/mM/cm) and expressed as µmol in per g FW of sample.

Hydrogen peroxide was estimated following the protocol of Bernt (1974) with some minor modifications. Samples were made powder with liquid nitrogen and homogenized with 2 ml of cold 5% TCA (w/v). Homogenates were centrifuged at 15000 rpm at 4^{0}C for 10 min. In 1.6 ml of supernatant, 0.4 ml of 50% TCA (w/v) and 0.4 ml of 10 mM ferrous ammonium sulphate were added. Solution was mixed well and added 0.2 ml of 2.5 M potassium thiocynate for colour development. The absorbance was read at 436 nm and estimated the hydrogen peroxide content by the following formula. Molecular extinction co-efficient of H_2O_2 at 436 nm is used as 39.4/mM/cm for calculation and subsequently expressed as nmol in per g FW of sample.

$$H_2O_2 \text{ (nmol/g FW)} = (A_{436} \times \text{vol of assay solution} \times 100)/(39.4 \times \text{incubation time})$$

Degree of membrane integrity was assessed by the percent of electrolyte leakage. Samples were immersed in 30 ml of distilled water and incubated at 25°C for 2 h in a water bath. Suspension medium was measured for the initial electrical conductivity (EC1). Samples were then autoclaved for 10 min to release all the electrolytes, cooled and the final EC2 was measured at 15 mV using an electrometer (keithley). Percent leakage of electrolytes was calculated using the formula as: (EC1/EC2) × 100% (Bernt 1974).

2.2.7 Extractions and assays of antioxidative enzymes

For measuring the activity of different antioxidant enzymes, the tissue was ground with liquid nitrogen and suspended in 1.0 ml buffer, containing 50 mM Tris-HCl buffer (pH 7.8), fortified with 1% PVP (w/v). Homogenates were centrifuged at 12000 rpm for 20 min at 4^0C and collected the supernatant. Supernatant was used for assaying SOD, POX and CAT enzymes. However, for APX, the extraction buffer was consisting of 50 mM phosphate buffer (pH 7.0), 0.5 mM EDTA, 1 mM AA and 1% of PVP. The SOD was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) (Upadhyaya et al. 2008). Assay buffer in a 3 ml final vol containing 50 mM potassium phosphate buffer (pH 7.8), 10 mM methionine, 2 µM riboflavin, 0.1 mM EDTA and 75 µM NBT was used for measuring the activity by 10 µl of crude enzyme extract. Reaction was started by exposing the solution under the light of 400 W bulbs for 15 min. Assay buffer without enzyme and without enzyme as well as NBT was used as reference control and blank, respectively. Absorbance was read immediately at 560 nm. Fifty percent inhibition of the reaction between riboflavin and NBT in the presence of methionine was considered as 1 unit of SOD activity.

Ascorbate peroxidase assay was followed according to Nakano and Asada (1981) with some modifications using hydrogen peroxide as substrate. By 3 ml assay solution consisting of 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM AA, 0.1 mM EDTA and 1 mM freshly diluted hydrogen peroxide, reaction was started adding 20 µl of crude enzyme extract. Decrease in absorbency due to AA was recorded for 3 min at 290 nm. The APX activity was calculated as concentration of AA oxidized (initial reading-final reading = quantity of AA oxidised) by the following formula and expressed as µmole in per min of per mg FW. Extinction co-efficient of ascorbate was used as 2.8 per mM of per cm at 290 nm.

$(A_{290}$ x vol of assay buffer x 1000)/(2.8 x incubation time x sample used in mg)

Catalase activity was also assayed using hydrogen peroxide as substrate following the modified protocol of Chance and Maehly (1954). Reaction was started by adding 50 µl of enzyme extract in an assay buffer of 3 ml vol containing 50 mM potassium

phosphate buffer (pH 7.0) and 200 mM H_2O_2. Decrease in absorbency was recorded at 240 nm for 5 min duration. Decrease of hydrogen peroxide concentration (initial reading-final reading) was finally used for calculating the CAT activity by the following formula and expressed as µmole per min per mg of FW. Extinction coefficient of H_2O_2 was used as 40 per mM of per cm at 240 nm.

$(A_{240}$ x vol of assay buffer x 1000)/(40 x incubation time x sample used in mg)

Activity of POX was estimated using pyrogallol as a substrate following the modified protocol of Chance and Maehly (1955). Assay was performed in 3 ml buffer containing 50 mM potassium phosphate buffer (pH 7), 1 mM pyrogallol and 10 mM H_2O_2 with 50 µl of crude enzyme extract by incubating at 25^0C for 5 min. Absorbance of purpurogallin formed in the reaction was read immediately at 430 nm. Extinction co-efficient of purpurogallin is used as 2.47 per mM of per cm at 430 nm for the calculation POX activity and expressed as µmole in per min of mg FW as follows:

$(A_{430}$ x vol of assay buffer x 1000)/(2.47 x incubation time x sample used in mg)

2.2.8 Statistical analyses

Experimental design was a completely randomized block with 12 replicates of each treatment. All quantitative data were subjected to one way analysis of variance for each parameter. The mean differences were evaluated for Least Significance Difference at probability level 0.05 through INDOSTAT statistical package (Indostat Services, Hyderabad). Data were expressed as the means ± standard error for three independent experiments in each analysis.

2.3 Results

Tea plants exhibited clear and obvious changes in anatomical and physiobiochemical states under drought stress. The changes were occurred in responses to plant's inherent capability of stress adaptation and/or tolerance.

2.3.1 Stress induction and plants growth

Plants subjected to drought stress were observed clear wilting symptoms on 21st d onwards of withholding water at which soil moisture content was recorded as 7% (Fig. 2.2, Table 2.2). Rate of photosynthesis, transpiration and stomatal conductance were reduced under stress, however, the reduction was rapid in susceptible cultivar [Fig. 2.3 (A), Table 2.2]. Similarly, chl a, b and carotenoids content were also affected by drought (Table 2.2). These pigments showed a proportionate reduction in each cultivar in comparison to control plants which was comparatively more rapid in S.3/A3. The chl/carotenoids ratio was also enhanced in both the cultivars and it was higher in TV-23. It was 0.64 and 0.52 in control plants whereas 0.72 and 0.57 in drought stressed plants of TV-23 and S.3/A3, respectively. Further, a uniform decrease in RWC was observed as compared to control for both the cultivars, though the rate of reduction was higher in case of susceptible one (Table 2.2). A higher roots length of drought stressed plants of tolerant cultivar was also noted in comparison to control plant [Fig. 2.3 (B), (C) and (D)]. The 25th d of water withholding was found as critical point for reviving the plants by rehydration; and after 30th d, even the tolerant cultivar was wilted permanently (Fig. 2.2).

2.3.2 Anatomical studies

Most of the stomata were partially closed in TV-23 whereas it was vice-versa in S.3/A3 under drought [Fig. 2.4 (image nos 1 & 2)]. There were no remarkable differences of petiole anatomy in between the cultivars under drought [Fig. 2.4 (image nos 3 & 4)]. Structural deformation of vacuoles, chloroplasts and mitochondria were also observed under drought whereas intact organelles were found in normal growth (Fig. 2.5). In each organelle, less membrane integrity and higher structural damage was observed in S.3/A3. It was observed that pore

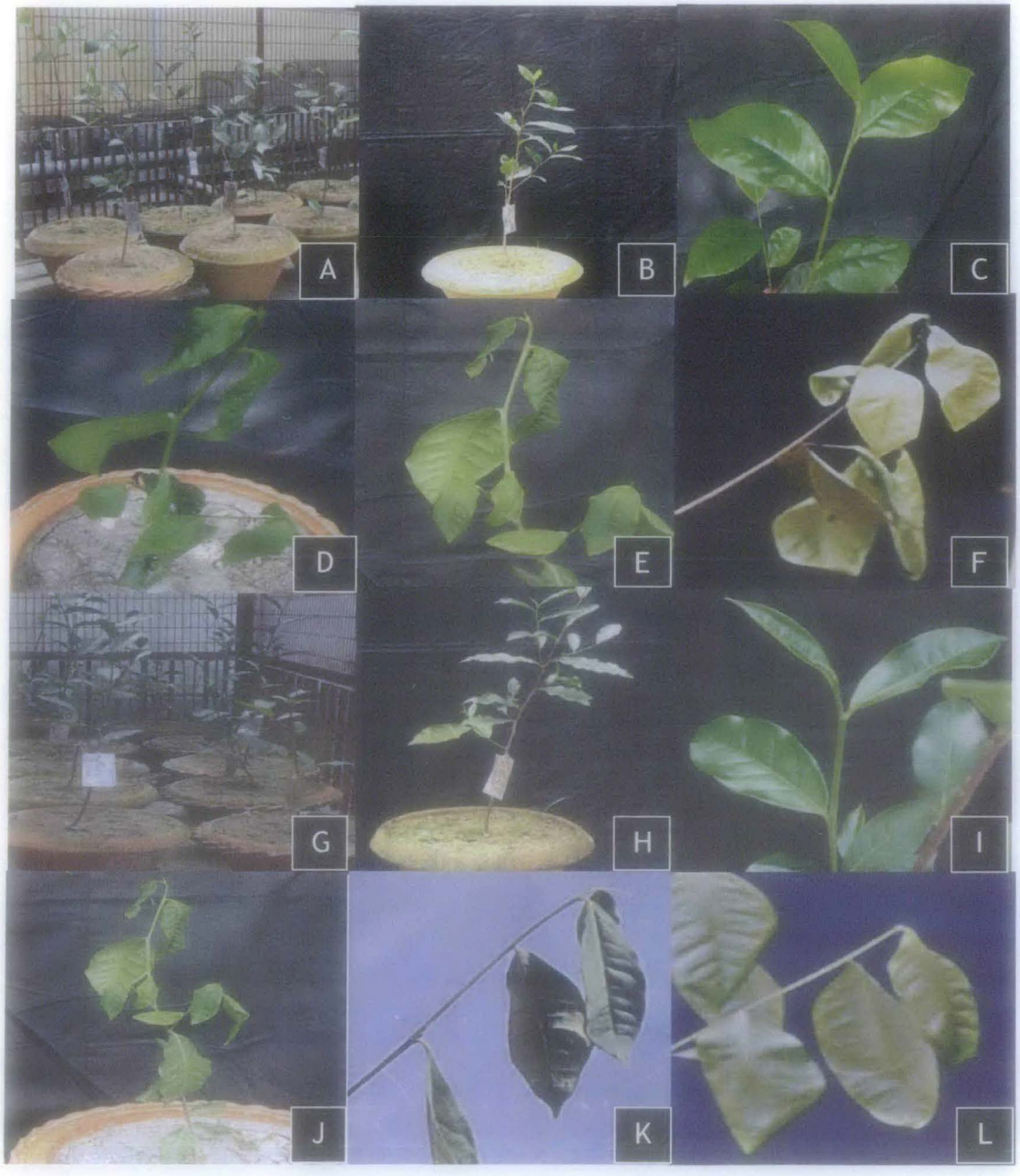

Fig. 2.2: Drought induction experiment of tea seedlings: (1) potted seedlings of A. S.3/A3 and G. TV-23 (2) control plants of B. S.3/A3 and H. TV-23 (3) shoots of control plants of C. S.3/A3 and I. TV-23 (4) drought induced plants of D. S.3/A3 and J. TV-23 (before wilting, 21st d) (5) shoots of drought induced plants of E. S.3/A3 and K. TV-23 (on 25th day) (6) shoots of drought induced plants of F. S.3/A3 and L. TV-23 (wilted permanently).

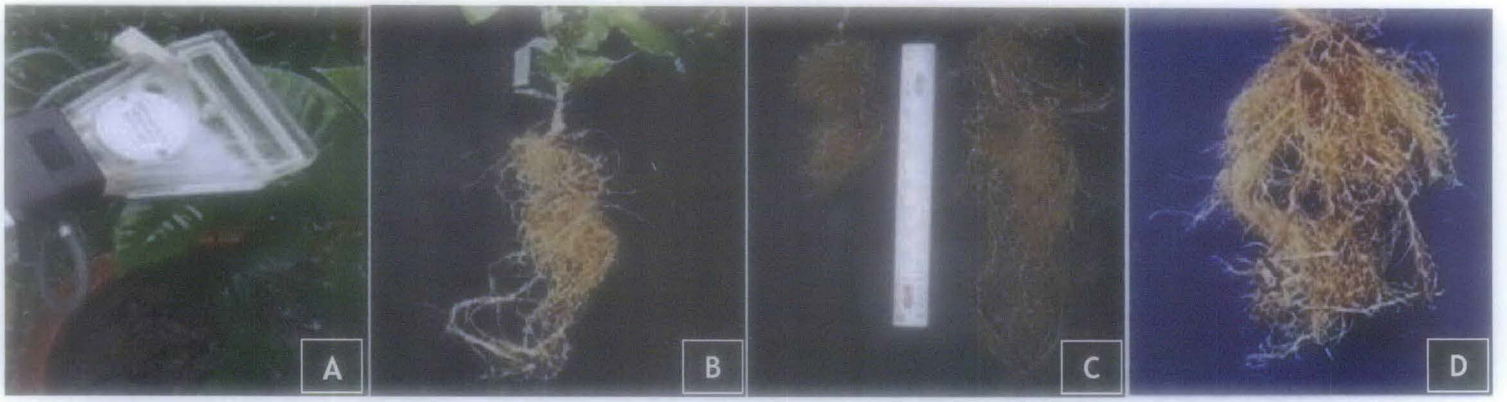

Fig. 2.3: Photographs of (A) photosynthesis rate, transpiration rate and stomatal conductance measurement using a photosynthesis system, (B) an uprooted plant, (C) root length measurement of control (left) and drought induced (right) plants of TV-23 and (D) roots for various experiments.

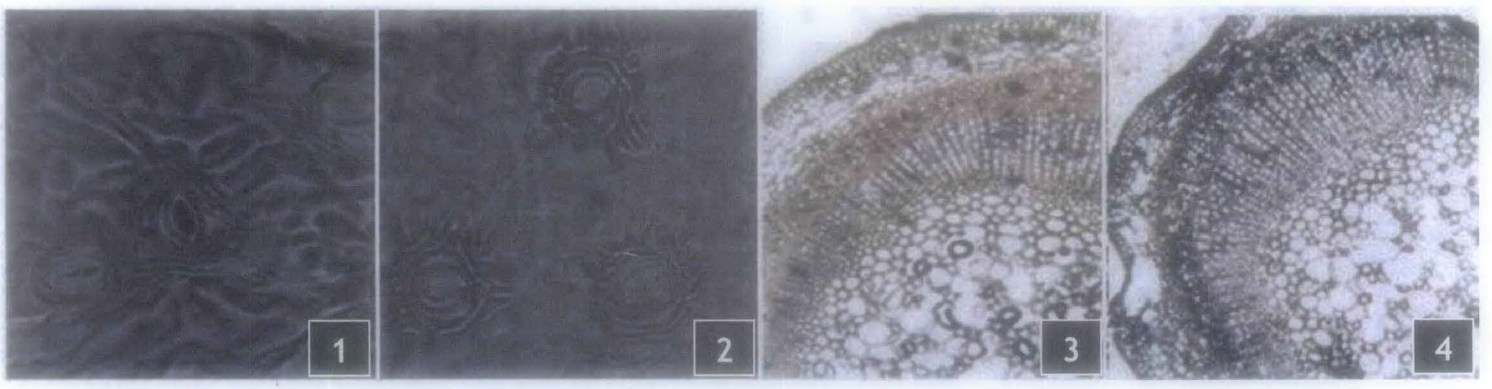

Fig. 2.4: Light microscopic images of stomata: (1) TV-23 and (2) S.3/A3 plants as well as transverse section of petioles: (3) TV-23 and (4) S.3/A3 plants on 21st d of drought stress induction.

Table 2.2: Effect of drought stress on RWC, photosynthesis rate (Pn), transpiration rate (E), stomatal conductance (SC) and pigment content of tea leaves*

Cultivars	Treatment	Soil moisture (%)	RWC (%)	Pn ($\mu mol\ m^{-2}\ s^{-1}$)	E ($mmol\ m^{-2}\ s^{-1}$)	SC ($mmol\ m^{-2}\ s^{-1}$)	Chl a ($mg\ g^{-1}$ FW)	Chl b ($\mu g\ g^{-1}$ FW)	Carotenoids ($\mu g\ g^{-1}$ FW)
TV-23	Control	35.33 ± 1.33a	92.14 ± 1.78a	14.38 ± 0.42a	4.45 ± 0.04a	0.56 ± 0.06a	2.38 ± 0.08a	399 ± 0.00ab	630 ± 0.02a
	Exp.	07.03 ± 1.50b	63.71 ± 5.81b	10.32 ± 0.41b	3.47 ± 0.07ab	0.52 ± 0.09a	1.85 ± 0.05b	389 ± 0.01ab	540 ± 0.01b
S.3/A3	Control	34.67 ± 1.33a	91.08 ± 2.55a	14.61 ± 0.36a	3.77 ± 0.06ab	0.56 ± 0.07a	2.24 ± 0.02a	430 ± 0.02a	639 ± 0.00a
	Exp.	07.21 ± 0.55b	59.87 ± 5.40b	07.14 ± 0.52c	3.04 ± 0.02c	0.31 ± 0.02b	1.77 ± 0.04b	373 ± 0.01c	534 ± 0.01b

Table 2.3: Effect of drought stress on ROS, lipid peroxidation (measured as MDA content) and level of membrane stability*

Cultivars	Treatment	Tissue	MDA ($nmol\ g^{-1}$ FW)	Electrolyte leakage (%)	Superoxide anion (A_{580})	H_2O_2 ($nmol\ g^{-1}$ FW)
TV-23	Control	Leaf	29.36 ± 0.05b	08.55 ± 0.35c	0.81 ± 0.03c	06.78 ± 0.95c
		Root	23.81 ± 0.05ab	18.31 ± 0.20b	0.69 ± 0.48b	42.44 ± 0.59c
	Exp.	Leaf	36.24 ± 0.19a	15.41 ± 0.71b	1.53 ± 0.09b	12.78 ± 0.91b
		Root	27.86 ± 0.03ab	18.75 ± 0.15b	1.02 ± 0.72a	78.40 ± 0.22b
S.3/A3	Control	Leaf	23.19 ± 0.00c	08.95 ± 0.25c	0.89 ± 0.02c	06.50 ± 1.79c
		Root	17.59 ± 0.07c	18.20 ± 0.70b	0.72 ± 0.51b	42.37 ± 0.06c
	Exp.	Leaf	31.49 ± 0.05ab	32.70 ± 0.00a	1.82 ± 0.05a	26.12 ± 2.00a
		Root	27.84 ± 0.07a	31.60 ± 2.10a	1.16 ± 0.82a	85.32 ± 0.87a

(*Alphabets indicate least significant differences)

formation or disruption of vacuolar membrane, disorientation of lamellar system or deposition of starch granules in chloroplast, shrinkage and destroyed crista in mitochondria were the consequences of ultra structures under drought (Fig. 2.5).

2.3.3 ROS, lipid peroxidation and membrane stability

Measurement of ROS, lipid peroxidation and membrane stability level were given in Table 2.3. For lipid peroxidation, MDA content in roots and leaves of both the cultivars were determined and found that MDA content was increased in both leaves and roots of each cultivar. However, the rate of increment was more in roots of each cultivar and it was comparatively higher in S.3/A3. Electrolyte leakage was also found high in leaves of each cultivar; however, the rate of increment was more in S.3/A3. Similarly, superoxide anion content was higher in each cultivar and the rate of formation was marginally more in roots of S.3/A3. H_2O_2 content was also found significantly enhanced in both roots and leaves of each cultivar and the enhancement was remarkably higher in roots and comparatively more in S.3/A3.

2.3.4 TSS, RS, starch, protein, proline, phenolics, ascorbic acids and ABA content estimation

Changes of different biochemical under drought stress were given in Table 2.4. Phenolics, TSS, RS and ABA content were increased in each cultivar and the increment was more in roots of TV-23. Protein, AA and proline content were found more enhanced in leaves of each cultivar, however, the enhancement were more and rapid in TV-23. Starch content was found in decreasing trend both in leaves and roots of each cultivar. The decrement of starch was found significant and more pronounced in roots of TV-23.

2.3.5 Activity of antioxidative enzymes

Activities of different antioxidative enzymes such as APX, SOD, POX and CAT were depicted in Fig. 2.6. There was an overall high activity of APX which was increased 237.02% in leaves of S.3/A3 whereas it was 221.74% in TV-23. Although, APX activity was also showed a similar trend in roots, the enhancement was comparatively more

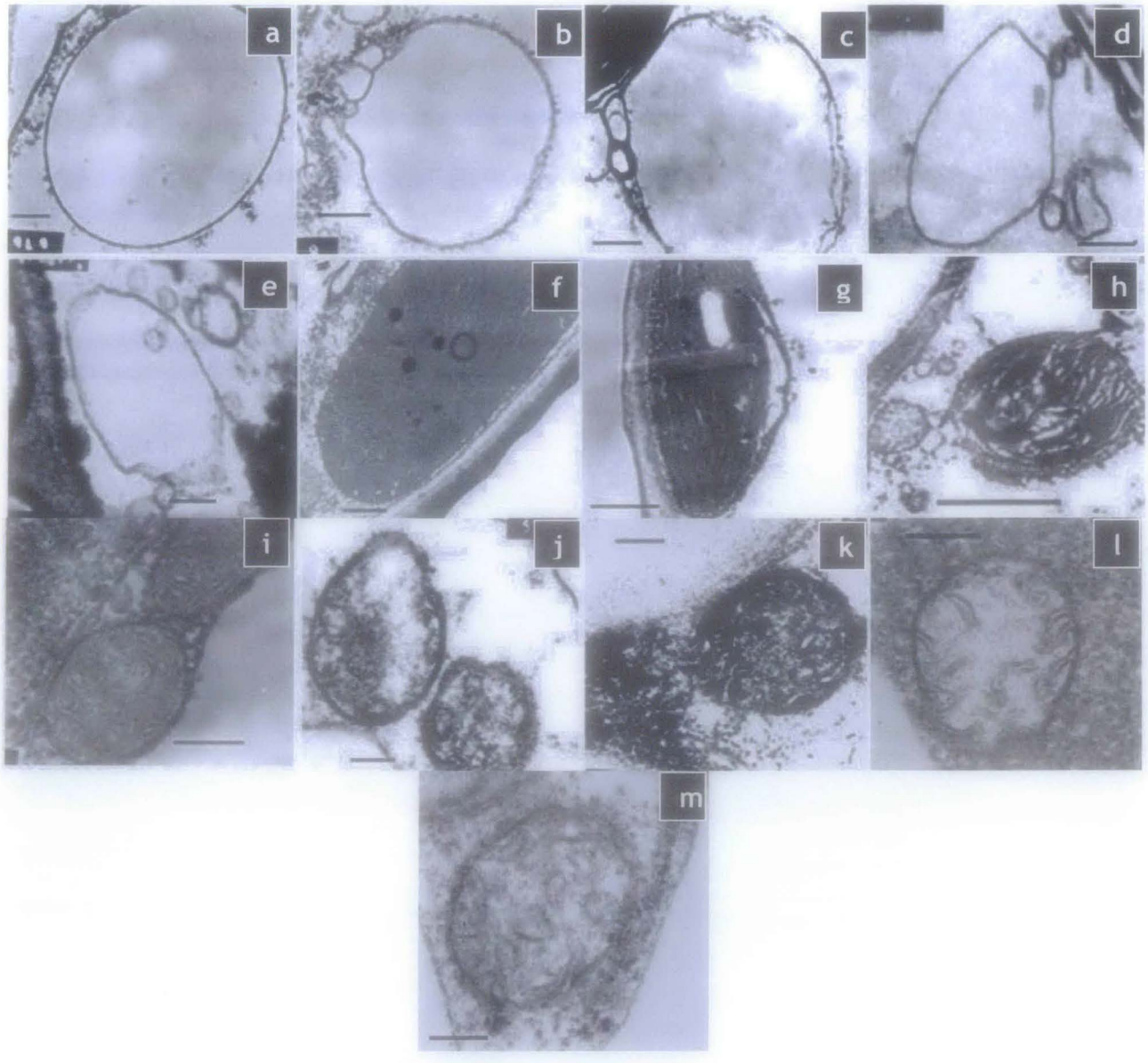

Fig. 2.5: Ultra-structural images of TEM: 1. **vacuoles** of [**a**] control plants (1.25 cm = 0.37 µm); [**b-e**] drought stressed plants: [**b**] TV-23 leaf (1.25 cm = 1 µm) [**c**] S.3/A3 leaf (1.25 cm = 0.37 µm) [**d**] TV-23 root (1.25 cm=0.37 µm) [**e**] S.3/A3 root (1.25 cm = 1 µm); 2. **chloroplast** of [**f**] control plants (1 cm=0.71 µm); [**g, h**] drought stressed plants: [**g**] TV-23 (1.25 cm=1 µm) [**h**] S.3/A3 (1.25 cm = 2.5 µm) 3. **mitochondria** of [**i**] control plants (1.25 cm = 0.25 µm) [**j-m**] drought stressed plants: [**j**] TV-23 leaf (1 cm = 0.2 µm) [**k**] S.3/A3 leaf (1 cm = 0.2 µm) [**l**] TV-23 root (1 cm = 0.25 µm) [**m**] S.3/A3 root (1 cm = 0.2 µm).

Table 2.4: Effect of drought stress on different biochemicals

Cultivars	Treat-ment	Tissue	Protein (mg g^{-1} FW)	Proline (µmol g^{-1} FW)	Starch (mg g^{-1} FW)	TSS (mg g^{-1} FW)	RS (mg g^{-1} FW)	Phenolics (mg g^{-1} FW)	Ascorbic acid (µmol g^{-1} FW)	ABA (mg g^{-1} FW)
TV-23	Control	Leaf	15.50 ± 0.10b	5.61 ± 0.32b	27.30 ± 0.08a	31.83 ± 1.02ab	12.33 ± 0.21d	4.48 ± 0.05d	16.31 ± 0.61bc	90.52±0.26b
		Root	06.60 ± 0.40a	2.10 ± 0.04c	29.13 ±0.68a	34.48 ± 0.20c	09.70 ± 0.13d	1.78 ± 0.10c	03.15 ± 0.01c	79.17±0.65b
	Exp.	Leaf	17.20 ± 0.20a	9.34 ± 0.26a	11.61 ± 0.24c	40.57 ± 3.63a	14.76 ± 0.09b	5.76 ± 0.07b	28.78 ± 1.22a	96.31±0.53a
		Root	06.90 ± 0.10a	3.96 ± 0.06a	09.54 ±1.41c	49.66 ± 1.04a	13.11 ± 0.12b	3.43 ± 0.18a	03.64 ± 0.04a	89.35±0.85a
S.3/A3	Control	Leaf	12.00 ± 0.00d	2.38 ± 0.14b	20.49 ± 0.53b	27.80 ± 1.44c	13.28 ± 0.15c	4.10 ± 0.01c	10.49 ± 1.12bc	79.35±0.85c
		Root	06.10 ± 0.10a	2.39 ± 0.13c	25.46 ± 0.82b	34.31 ± 0.37c	11.73 ± 0.00c	1.58 ± 0.14c	02.97 ± 0.02d	75.15±1.20c
	Exp.	Leaf	13.30 ± 0.10c	4.68 ± 0.38c	09.67 ± 0.17d	38.78 ± 2.54a	16.21 ± 0.18a	5.33 ± 0.03a	19.53 ± 2.57b	80.04±0.50c
		Root	06.30 ± 0.10a	2.88 ± 0.07b	07.56 ± 0.54c	42.91 ± 0.16b	15.03 ± 0.06a	2.85 ± 0.13b	03.31 ± 0.00b	79.05±0.89b

(TSS=total soluble sugar, RS=reducing sugar, ABA=abscisic acids, alphabets indicate least significant differences)

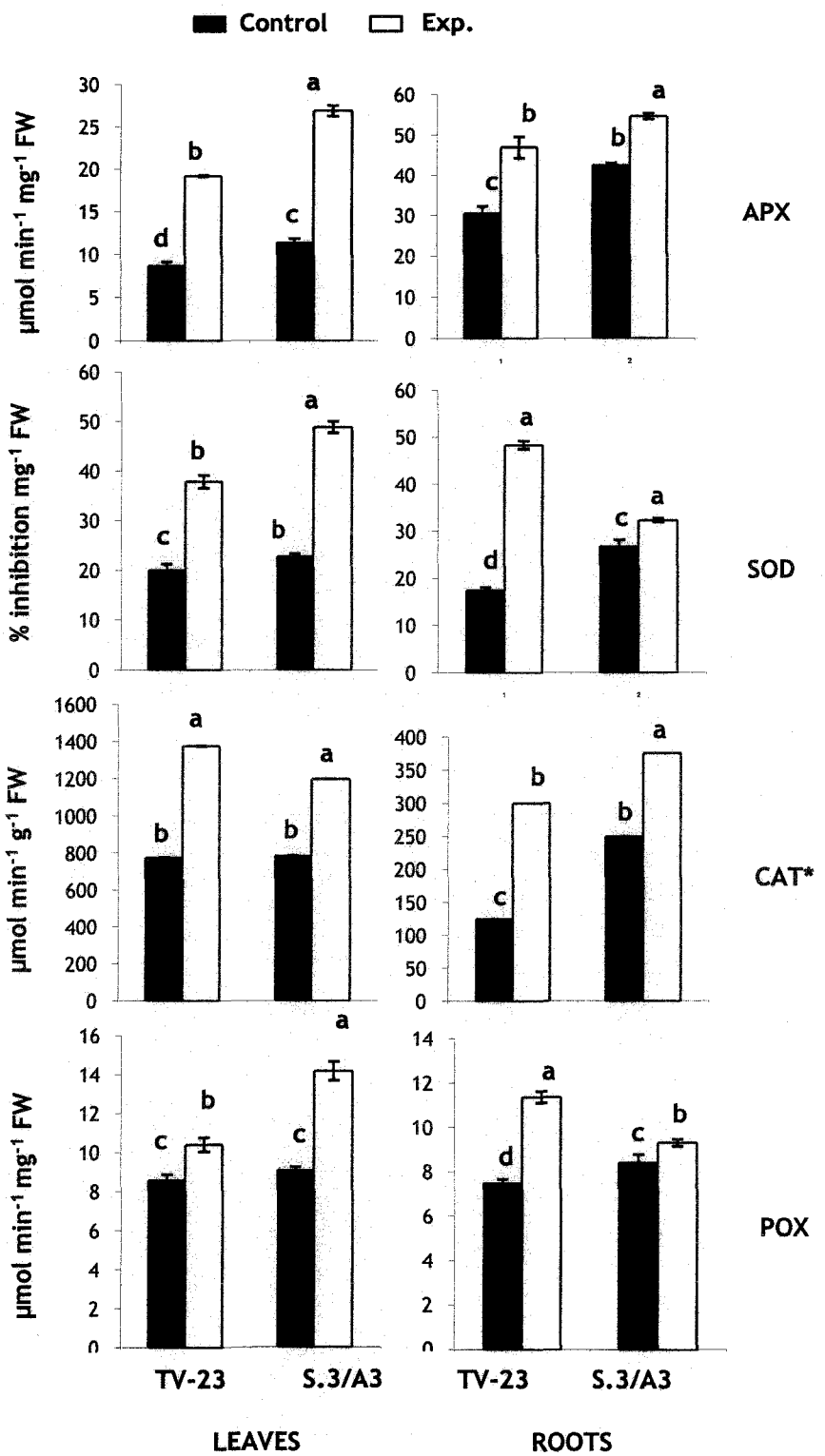

Fig. 2.6: Estimation of different antioxidative enzymes activity of leaves and roots of drought stressed and control tea plants (letters indicate least significant differences; *standard error was negligible).

in leaves. Similarly, SOD activity was found more in leaves of S.3/A3 (214.21 %) than to TV-23 (188.81%), however, a reverse trend was registered in roots where it was 275.84% in TV-23 and 120.49% in S.3/A3. The CAT activity was also enhanced in each cultivar for both types of tissues, but the increment was higher in TV-23. It was increased 177% and 153% in leaves as well as 240% and 150% in roots of TV-23 and S.3/A3, respectively. Overall, CAT activity was found very high in leaves than to roots. Activity of POX registered a different trend where it was more in leaves of S.3/A3 (158%) in comparison to TV-23 (121%), however, in roots it was higher in TV-23 (151%) than to S.3/A3 (111%).

2.4 Discussion

Drought is one of the important environmental factors, which induces significant alterations in plant physiology and biochemistry. Changes occur as part of the tolerance mechanism which is an inherent capacity of the plants that enables to maximize water absorption and minimize transpiration during drought. A clear understanding of tolerance mechanism at cellular level provides us the scope for deciphering the lying mechanism at molecular level. Owing to the fast climatic changes and water limitation, understanding of drought tolerance mechanism for selecting and/or developing drought tolerant cultivar with quality crop is an urgent need in modern agriculture.

2.4.1 Plants growth and anatomical studies

A drastic change in plant's morphology and growth were observed under drought. Fresh and dry mass were also decreased more rapidly in susceptible cultivar. Perhaps, it was due to the fact that restriction of water supply from the soil in relation to genotype reduced the plants biomass (Ogbonnaya et al. 2003). Length of roots in tolerant cultivar was increased under drought may be due to facilitating maximum water absorption from water deficit soil. This finding was in accordance with the previous results of various crop plants including tea (Grzesiak et al. 1997, Burgess and Carr 1996). Moreover, Nagarajah and Ratnasuriya (1981) reported that rooting depth confers drought resistance in tea. The rate of CO_2 assimilation in leaf decreases at leaf water deficits or even before leaf water status changes in response to a drop in soil water potential (Gollan et al. 1986). Relative water content of leaves decreased in each cultivar due to drought but it was least in TV-23. Maintenance of high RWC in drought-tolerant cultivar has been reported as an adaptation to water stress in several crop species including tea (Valentovič et al. 2006, Nautiyal et al. 1995, Upadhyaya et al. 2008). Stomatal control of water loss has been identified as an early event in plant responses to water deficit leading to limitation of carbon uptake by the leaves (Chaves 1991). It occurs in response to either a decline in leaf turgor and/or water potential, or to a low-humidity atmosphere (Maroco et al. 1997). As a rule, stomatal responses are more closely linked to soil moisture content than to leaf water status. This suggests that stomata

are responding to chemical signals (e.g. ABA) produced by dehydrating roots (Davies and Zhang 1991). The TV-23 cultivar being a drought-tolerant cultivar restricts stomatal function under stress and improves water use efficiency. Pigment content is an indicator of photosynthetic activity. Pigment loss is associated with environmental stress and the variation in total chl/carotenoids ratio may be a signal of stress in plants (Ünyayar et al. 2005). Present results showed that pigment such as chl-a, chl-b and carotenoids were decreased under drought but the reduction was more and significant in S.3/A3. Chl/carotenoid ratios were also found more increased in S.3/A3 under drought stress. It may be an indication of higher pigment degration in susceptible cultivar.

Cell division and enlargement as well as intercellular spaces are reduced by water deficit, which contribute to the survival of plants under dry conditions (Levitt 1980). Changes in water regime can strongly modify ultra structural cell characteristics if water stress is severe enough (Ciamporova 1987). Water stress can disrupt the ultra structural features of chloroplasts, mitochondria and vacuoles and alter the nucleus and cell membranes in general (Crevecoeur et al. 1987). Due to higher amount of compatible solutes in vacuole such as sugar and other derivatives for osmotic adjustment, it gets swelled. In increasing stress level, the vacuolar membrane faces higher pressure and gradually, it gets damaged (Blokhina et al. 2003). A high resistance of vacuolar membranes was observed in tolerant cultivar. Mitochondria are the site of ROS production through its electron transport chain (Chen et al. 2003). Under stress condition, higher production of ROS and their accumulation causes destruction of its own inner membranous structures. In susceptible cultivar, the mitochondrial structure was found almost destroyed due to its comparatively higher production and accumulation of ROS and its less capability of detoxification. Due to production, transportation and detoxification of ROS, the inner structures of chloroplast such as thylakoids and lamella were observed as disoriented; however the distortion was severe in susceptible cultivar. The main damage to the chloroplast caused by water stress also reported earlier as excessive swelling, distortion of thylakoids, and the appearance of lipid droplets (Vieira 1976, Berlin et al. 1982). Increase in chloroplast starch accumulation under drought has also been observed in cotton plants (Ackerson and Hebert 1981). The destroyed chloroplast in susceptible cultivar proved its inefficiency in drought tolerance. Changes in water

supply seem to affect the structural characteristics of chloroplasts differentially in various cultivars such as bean, maize, cotton (Stoyanova and Yordanov 1999, Ristic and Cass 1991, Utrillas and Alegre 1997). Hence, observation of the damage to cell membranes, such as plasmalemma and thylakoid membranes, provides valuable information for the ability of a plant to withstand stress (Utrillas and Alegre 1997). Shrinking of cells leads to loss of turgor, osmotic stress and a potential change of membrane potentials. Upon severe water loss from the cells, membrane disintegration and abolition of metabolic processes occur (Mahajan and Tuteja 2006).

2.4.2 Determination of ROS, lipid peroxidation and membrane stability levels

Reactive oxygen species inactivate enzymes and damage important cellular components. The increased production of toxic oxygen derivatives is considered to be a universal or common feature of stress conditions (Arora et al. 2002). ROS are responsible for stress-dependent peroxidation of membrane lipids (Ratnayaka et al. 2003). Lipid peroxidation is often used as an indicator of increased oxidative damage (Cornic and Briantais 1991). The plant cell membranes are considered as a primary site of stress injury and the membrane destabilization is frequently attributed to lipid peroxidation (Singh et al. 2006). Increased production of MDA by environmental stress exposure has been well-documented in various crop plants such as sunflower, wheat, maize etc. including tea (Gallego et al. 1996, Hong-Bo et al. 2005, Moussa and Abdel-Aziz 2008, Upadhyaya et al. 2008). It has been suggested that plasma membrane stability may be a reliable index of drought and heat resistance in plants (Blokhina et al. 2003). Dehydration in sensitive plants is often accompanied by cytoplasmic membrane injury, resulting in the leakage of solutes. The increase level of electrolyte leakage under drought has been reported in several crops (Panda et al. 2003, Mohammadkhani and Heidari 2007, Pandey et al. 2010). In present study, there was a significant increase in the level of both MDA content and electrolyte leakage in leaves under drought stress. It indicates that leaves are more vulnerable to drought stress than roots. Higher level of lipid peroxidation observed here was probably due to the harmful effect of excessive levels of H_2O_2 or its ROS derivatives in the cellular compartments (Bowler et al. 1992). Excessive levels of ROS may have resulted in damage to cell organelles including the photosynthetic apparatus,

ultimately leading to severe cellular damage and chlorosis of the leaves (Blokhina et al. 2003). Lower level of lipid peroxidation in leaves and roots of TV-23 suggest that, this cultivar is better protected from oxidative damage under drought stress than S.3/A3. This result is in agreement with the results of tea (Upadhyaya et al. 2008) and wheat (Sairam et al. 2005) where the tolerant cultivar showed lower membrane damage under drought stress.

2.4.3 Determination of TSS, RS, starch, protein, proline, phenolics, ascorbic acids and ABA contents

Sugars are special in that they allow the removal of closely associated water from protein without leading to conformational changes and loss of enzymatic function. According to the water replacement hypothesis, sugars act as a water substitute by satisfying the hydrogen-bonding requirement of polar groups of dried protein surface (Blokhina et al. 2003). Accumulation of soluble and reducing sugars may occur perhaps due to degradation of sub-cellular component affected by drought stress. It was found that starch was decreased under drought in both the cultivars. It indicates that due to less water potential raised soluble sugar fraction was accompanied by a significant decrease in the starch fraction. Similar result was also observed in rice, maize and sugar beet (Vajrabhaya et al. 2001, Mohammadkhani and Heidari 2008a, Hoffmann 2010).

Osmotic adjustment involves an active accumulation of cellular solutes such as proline and soluble proteins within the plant in response to lowering of the soil water potential and reducing the harmful effects of water deficit (Hong-bo et al. 2008). Proline is known to accumulate in response to dehydration treatment which is considered as compatible osmolyte as well as scavenger against ROS (Matysik et al. 2002). In present study, higher proline content in TV-23 confirmed that this cultivar is better protected under drought stress as it helps in water relation; prevent membrane distortion and acts as a hydroxyl radical scavenger. High proline accumulation in response to drought stress was also reported in several crop plants including tea (Upadhyaya et al. 2008). Increased in total phenol and ascorbate contents in TV-23 in response to drought stress suggested the gradual protection of seedling to overcome a drought-induced oxidative damage as reported in numbers of

plant species including tea (Dixon and Steele 1999, Chakraborty et al. 2002, Cheruiyot et al. 2007). Ascorbate is a key substance in the network of antioxidants that includes ascorbate, glutathione, α-tocopherol, and a series of antioxidative enzymes. Ascorbate has also been shown to play multiple roles in plants growth, such as in cell division, cell wall expansion, and other developmental processes (Smirnoff and Wheeler 2000). Phytohormone ABA plays a key role in plant's adaptation to adverse environmental conditions including drought. Numerous studies have shown that ABA accumulation is a key factor in controlling downstream responses essential for adaptation to stresses (Ramagopal 1987, Shinozaki and Yamaguchi-Shinozaki 1996). In present study also higher accumulation of ABA was found under drought, and it was comparatively high in the tolerant cultivar. It proves that TV-23 cultivar is better in adaptation to the adverse environmental conditions.

2.4.4 Activities of antioxidative enzymes

To keep the levels of active oxygen species under control, plants have non-enzymatic and enzymatic antioxidant systems to protect cells from oxidative damage (Mittler 2002). Antioxidant enzymes play an important role in defense system of plants against oxidative stress such as SOD, APX, CAT, POX and GR (Hong-bo et al. 2008). The balance between ROS production and activities of antioxidative enzymes determines whether oxidative signalling and/or damage will occur. The capability of scavenging ROS and reducing their damaging effects may correlate with the drought tolerance of plants. There are many reports in literature that underline the intimate relationship between enhanced or constitutive antioxidant enzyme activities and increased resistance to drought stress in several plant species, such as rice (Guo et al. 2006), wheat (Khanna-Chopra and Selote 2007), common bean (Turkan et al. 2005) and barley (Acar et al. 2001). In present investigation, activity of POX was enhanced with drought which has also been reported in various plant species including tea (Chakraborty et al. 2002); suggesting POX plays an important protective role against drought stress. Enzyme, SOD is an essential component of plant antioxidation system as it dismutates superoxide radicals to H_2O_2 and O_2 in the cytosol, mitochondria and chloroplast (Salin 1988). Activity of SOD was also found up-regulated by drought stress in numbers of plant species including tea

(Chakraborty et al. 2002, Upadhyaya et al. 2008). Increased level of APX and CAT activities in response to drought are well-established in literature (Srivalli et al. 2003, Upadhyaya and Panda 2004).