Chapter 1
Introduction
Stress can be defined as any environmental factor capable of inducing a potentially injurious strain in living organisms. A biological strain is any change in physiological process and functional activity of enzymes of metabolism under stress (Zlatev et al., 2012). Plants are frequently exposed to many stresses, such as, drought, low temperature, salt, flooding, heat, oxidative stress and heavy metal toxicity, while growing in nature. These stresses are of two types - biotic and abiotic. Biotic stress involves damage to plants by other living organisms, such as, bacteria, viruses, fungi, parasites, harmful insects and weeds, while, abiotic stress is a negative impact of non-living factors on plants in specific environment, such as, light, temperature, water availability, nutrients, and soil composition. Abiotic stress affects plant metabolism, physiological and biochemical processes and disrupt cellular homeostasis. Amongst abiotic stresses, osmotic stress, drought and salinity are the most severe problems in worldwide agricultural production (Saha et al., 2010).

Osmotic stress means insufficient water availability i.e. water deficit, which limits plant growth and development and hence productivity (Zhu et al., 1997). The first response of virtually all the plants to acute water deficit is the closure of stomata to prevent the transpirational water loss (Manfield et al., 1990). Closure of stomata may result from direct evaporation of water from the guard cells with no metabolic involvement. It is a major temporary adaptive change that prevents further water loss from the plants. When water deficit becomes too intense or prolonged, plants can wilt, cells can undergo shrinkage and this may lead to mechanical constraint on cellular membranes. The normal bilayer structure of membrane due to removal of water disrupts and becomes porous when desiccated. This in turn impairs the functioning of ions and transporters as well as membrane associated enzymes (Bowler, 1992). Further, due to cell shrinkage and a decline in cellular volume, cellular content becomes viscous, therefore increasing the probability of protein–protein interaction leading to their aggregation and denaturation (Mahajan et al., 2005). Increased concentration of solutes may also exceed toxic levels, which may be deleterious for the functioning of the enzymes
including the enzymes required for photosynthetic machinery (Hoekstra et al., 2001; Mahajan et al., 2005; Farooq et al., 2009).

Water deficit influences many physiological responses in plants. Thus, decrease in germination rate, relative water content, fresh weight, dry weight, root length and shoot length due to water stress have been reported in several plant systems (Sharma et al., 2004; Nayyar and Gupta, 2006 and Jamwal et al., 2012). Osmotic stress has been reported to stimulate respiration in pea protoplasts (Saradadevi and Raghavendra, 1994; Dwivedi et al., 2003). A major effect of water deficit is reduction in photosynthesis, which arises by a decrease in leaf expansion, impaired photosynthetic machinery, premature leaf senescence and thus associated with reduction in food production (Wahid and Rasul, 2005). The effects can be direct due to decreased CO₂ availability caused by diffusion limitations through the stomatal closure and the mesophyll conductance (Flexas et al., 2007) or indirect due to oxidative stress. Decline in intracellular CO₂ levels also results in the over-reduction of components within the electron transport chain and the electrons get transferred to oxygen at photosystem I. Further, the steady state levels of major photosystem II mRNAs and proteins, half lives of D1 and D2 proteins, as well as of psb-A and psb-D mRNAs have been shown to decline with increasing water stress in barley leaves (Yuan et al., 2005). Furthermore, changes in the structure of developing chloroplast by water stress which decreased the net photosynthetic rate and reduction in the contents and activity of ribulose-1,5-bisphosphate carboxylase / oxygenase by water stress have been reported (Bourque et al., 1975; Chaitanya et al., 2003; Zgalla et al., 2005; Saibo et al., 2009).

Chlorophyll (Chl) molecules play a central role in the photosynthetic apparatus by capturing light and directing the energy towards the photosystems. Chlorophyll is a tetrapyrole macro cycle containing Mg, a phytol chain, and a characteristic fifth ring. Higher plants have two chlorophyll species, chlorophyll a and b (Figure 1). Chlorophyll metabolism is a highly coordinated process that is executed via a series of cooperative reactions catalyzed by several enzymes (Beale,
Figure 1. Structure of Chlorophyll a and b
The major site of chlorophyll biosynthesis in plants is plastid, which is regulated at several steps (Masuda and Fujita, 2008; Mochizuki et al., 2010). Earlier steps of the pathway are common with biosynthesis of other tetrapyrrole derivatives like heme, phytochromes, phycobilins etc. Outline of the key steps involved in chlorophyll biosynthesis is presented in Figure 2.

The synthesis of 5-amino levulinic acid (ALA) is the first committed step of chlorophyll biosynthesis, and is therefore a key control point in the regulation of chlorophyll formation. ALA, the universal precursor contributes all the carbon and nitrogen atoms in chlorophyll molecule is synthesized by two pathways (Panek and O’Brian, 2002; Schoefs and Bertrand, 2005). In yeast, some bacteria and animals, a single enzyme ALA synthase catalyzes the condensation of glycine and succinyl Co A to form ALA through Shemin pathway (Avissar et al., 1989; Tanaka et al., 2011). In higher plants, algae and cyanobacteria the intact 5-carbon skeleton of glutamate is converted into ALA by a multienzyme system involving three steps (Kannangara et al., 1988; Bollivar, 2006). These are depicted in Figure 3 (Tanaka et al., 2011). These steps include ligation of glutamate to tRNA^Glu^ catalysed by glutamyl tRNA synthetase, the reduction of glutamate to glutamate-1-semialdehyde by glutamyl tRNA reductase (GluTR) and a final transamination step mediated by glutamate-1-semialdehyde aminotransferase (GSAT) (Kannangara et al., 1988; Tanaka et al., 2011). GluTR is the main target of regulatory mechanism modulating ALA formation and thus responding to a wide range of stimuli, whereas GSAT responds only weakly. GluTR mRNA levels correlate well with chlorophyll synthetic activity in higher plants. When etiolated seedlings are exposed to light, GluTR mRNA levels and ALA synthesis rise in parallel, while, on inhibition of GluTR mRNA production using antisense RNA, chlorophyll levels decrease in Arabidopsis (Kumar and Soll, 2000; Ujwal, 2002). On the other hand, in water-stressed Oryza sativa seedlings protein / transcript abundance of GSAT increased, while, the ALA content decreased (Mohanty et al., 2006). The authors suggested inactivation of GSAT enzyme by post-translational modification under stress condition. Further, reduced synthesis of ALA in chilled
Figure 2. Chlorophyll Biosynthetic Pathway
Figure 3. The three step reaction of 5-aminolevulenic acid (ALA) synthesis from glutamate (Reference: Tanaka et. al., 2011).
and heat stressed *Cucumis sativa* seedlings has been reported by Tewari and Tripathy (1998).

\( \delta \)-Aminolevulinic acid dehydratase (ALAD; E.C 4.2.1.24), one of the earlier enzymes of the pathway, catalyzes the asymmetric condensation of two molecules of ALA with the release of two \( H_2O \) molecules leading to the formation of the basic unit of tetrapyrroles, the porphobilinogen (PBG). In several systems the enzyme plays a major role in regulating the chlorophyll biosynthesis (Naito *et al.*, 1980; Prasad *et al.*, 1989; Padmaja *et al.*, 1990; Prasad and Prasad, 1990). ALADs from different sources are metalloenzymes that utilize a variety of divalent and monovalent cations (Jaffe, 2000). The plant enzyme requires \( Mg^{2+} \) for enzymatic activity. ALAD is a substrate modulated enzyme (Tchuanmogne *et al.*, 1989) and levulinic acid, a structural analogue of ALA, is a competitive inhibitor of the enzyme (Senior *et al.*, 1996). Decline in ALAD activity and its gene expression in water stressed rice seedlings (Dalal and Tripathy, 2012) and chill and heat-stressed *Cucumis sativa* and *Triticum aestivum* seedlings (Tewari and Tripathy, 1998; Mohanty *et al*. 2006) has been reported. Decreased availability of the substrate ALA of the enzyme could negatively regulate the gene expression in water stress conditions (Dalal and Tripathy, 2012). Inhibition of ALAD activity by cadmium, mercury and arsenic has been reported in excised greening maize leaf segments (Jain and Gadre, 2004; Sarangthem *et al.*, 2011; Gupta *et al.*, 2013).

Porphobilinogen deaminase (PBGD; EC 2.5.1.61) condenses four PBG molecules to form the first tetrapyrrole, hydroxymethylbilane, which further forms uroporphyrinogen III through the nonphysiological product uroporphyrinogen I (Battersby *et al.*, 1979). The enzyme is found to be highly specific for PBG and requires thiol compounds for activity (Bogorad, 1958). Decline in the rate of synthesis of protochlorophyllide (Pchlide) and Chlorophyllide (Chlide) due to loss of ALAD and PBGD activity has been suggested in senescent barley leaves (Hukmani and Tripathy, 1994). Inhibition of PBGD activity by chill and heat stress has been demonstrated in etiolated *T. aestivum* seedlings (Tewari and Tripathy, 1998). Down regulation of transcript abundance is suggested as the cause
of reduced PBGD activity in water-stressed *O. sativa* seedlings (Dalal and Tripathy, 2012). In subsequent steps urogen III is converted to Protoporphyrin IX, which chelates with Mg\(^{2+}\) to form Mg Protoporphyrin IX, which is then converted to Pchlide. Pchlide is converted to Chlide and then to chlorophylls. Changes in chlorophyll a/b ratio and carotenoids due to drought stress have been reported in *Hordeum vulgare* L. (Anjum et. al., 2003) and *O. sativa* (Sikuku et. al., 2010). Reduction in chlorophyll content due to drought has been shown in *Helianthus annuus* L. (Kiani et. al., 2008), *Vaccinium myrtillus* L. (Tahkokorpi et. al., 2007), *Gossypium hirsutum* L. (Massacci et. al., 2008), *Catharanthus roseus* L. (Jaleel et. al., 2008), *Oryza sativa* (Chutia and Borah, 2012). Regulation of the levels of chlorophyll branch intermediates is extremely important since most of these molecules are strong photosensitizers. When present in excess they produce reactive oxygen species causing oxidative damage or cell death (Mock et. al., 2002).

Plants have many adaptive mechanisms contributing to water stress resistance. One of them is osmotic adjustment (OA), which involves the net accumulation of solutes in cells in response to a fall in the water potential of the environment (Zhang et. al., 1999). Organic compounds that function as solutes (osmoprotectants / osmolytes) include amino acids, such as, proline, sugar alcohols, such as, mannitol, and quarternary ammonium compounds, such as, glycine-betanine (GB). Accumulation of proline (Viegas and Silvera, 1997 and Kumar et. al., 2012) and glycine-betaine (Pollard and Wyn Jones, 1979; Sairam et. al., 2002) has been reported in several plant systems as a result of osmotic stress. Overproduction of proline in response to salinity and osmotic stress has also been shown in a genetically modified cyanobacterium, *Nostoc muscorum* (Bhargava, 2006). It influences protein solvation and preserves the quarternary structure of complex proteins, maintains membrane integrity under dehydration stress and reduces oxidation of lipid membranes or photoinhibition (Demiral and Turkan, 2004). Furthermore, it also contributes in stabilizing sub-cellular structures, scavenging free radicals, and buffering cellular redox potential during stress conditions (Ashraf and Foolad, 2007; Anjum et. al., 2011). Besides proline,
abundant levels of other amino acids, such as, asparagine, glutamate and γ-amino benzoic acid have also been reported in response to drought (Alia et al., 2001; Diaz et al., 2005; Asharaf et al., 2007; Chutia and Borah, 2012).

One of the most common responses of plants to water deficit leads to enhanced generation of reactive oxygen species (ROS), such as, H$_2$O$_2$, superoxide (O$_2^-$) and hydroxyl (OH$^-$) radicals due to disruption of cellular homeostasis (Mittler, 2002; Hu et al., 2008; Han et al., 2009; Mishra et al., 2011; Srivastava et al., 2011). All ROS are extremely harmful to organisms at high concentrations. When the level of ROS exceeds the defense mechanisms, a cell is said to be in a state of “oxidative stress.” The enhanced production of ROS during environmental stresses can pose a threat to cells by causing peroxidation of lipids, oxidation of proteins, damage to nucleic acids, enzyme inhibition, activation of programmed cell death (PCD) pathway and ultimately leading to death of the cells (Mittler, 2002; Meriga et al., 2004; Maheshwari and Dubey, 2009; Mishra et al., 2011).

Drought-induced overproduction of ROS increases the content of malondialdehyde (MDA) due to membrane lipid peroxidation. Thus, the content of MDA has been considered an indicator of oxidative damage (Moller et al., 2007). H$_2$O$_2$ is generated in normal metabolism via the Mehler reaction in chloroplasts, electron transport chain in mitochondria and photorespiration in peroxisomes. Increment in MDA and H$_2$O$_2$ concentrations in the water stressed cuttings of *Populus cathayana* L., *P. kangdingensis* has been shown (Yang and Miao, 2010). Further, in *Pisum sativum* L. levels of lipid peroxidation increased two to four fold with an increase in the drought stress, and this was highly correlated with protein oxidation peroxidation (Moran et al., 1994). The ability of higher plants to scavenge the toxic effects of ROS seems to be very important determinant of their tolerance to these stresses. Production and scavenging of ROS in plants is depicted in Figure 4, (da Silva et al., 2013). Antioxidants are the first line of defense against free radical damage. They are critical for maintaining optimum health of plants cells. There are several antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), dehydroascorbate reductase
Figure 4. Production and scavenging of ROS in plants (reference: da Silva et. al., 2013)
(DHAR) and glutathione reductase (GR), and non enzymatic antioxidants, such as, ascorbate (ASC), glutathione (GSH) which are effectively involved in scavenging of ROS in plants.

Superoxide dismutase (SOD; EC 1.15.1.1) is the first enzyme in the ROS detoxifying process, which catalyzes the dismutation of superoxide radical (O$^{\cdot-}$) into oxygen and hydrogen peroxide (H$_2$O$_2$).

\[
2O^{\cdot-} + 2O^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2
\]

Three isozymes of SOD copper/zinc SOD (Cu/Zn-SOD), manganese SOD (Mn-SOD), and iron SOD (Fe-SOD) are reported in plants (Fridovich, 1989; Racchi et al., 2001). All forms of SOD are nuclear encoded and targeted to their respective sub cellular compartments by an amino terminal targeting sequence (Bowler et al., 1992). Fe-SOD is localized in chloroplast where the major site of O$_2^{\cdot-}$ production is the thylakoid membrane-bound primary electron acceptor of PSI. SOD activity has been reported to increase in several plants exposed to various stresses, such as, salinity and heavy metals (Kukreja et al., 2005; Zlatev et al., 2006; Wang et al., 2008; Mishra et al., 2011).

Catalase (CAT, EC 1.11.1.6) was the first enzyme to be discovered and characterized in antioxidant enzymes. CAT is a tetrameric heme containing enzyme with the potential to directly dismutase H$_2$O$_2$ into H$_2$O and O$_2$ and is indispensable for ROS detoxification during stressed conditions (Garg and Manchanda, 2009).

\[
2H_2O_2 \rightarrow O_2 + 2H_2O
\]

The enzyme function is to remove the H$_2$O$_2$ generated in peroxisomes by oxidases involved in β-oxidation of fatty acids, photorespiration, purine catabolism and during oxidative stress (Mittler, 2002; Vellosillo et al., 2010). Increase in CAT activity is an adaptive trait possibly helping to overcome the damage to tissue metabolism by reducing toxic levels of H$_2$O$_2$. Increased CAT activity in T.
*aestivum* (Simova-Stoilova *et al.*, 2010) and in *P. asperata* (Yang *et al.*, 2008) has been shown under drought stress.

Guaiacol Peroxidase (Gu-POX, E.C. 1.11.1.7), a heme containing protein, preferably oxidizes aromatic electron donor, such as guaiacol and pyragallol at the expense of H$_2$O$_2$. Many isoenzymes of Gu-POX exist in plant tissues localized in vacuoles, the cell wall, and the cytosol (Asada, 1992). It is involved in large number of biological and physiological processes including plant growth and differentiation, cell elongation, cross linking of cell wall polysaccharides, lignifications, wound healing, phenolic oxidation, flavonoids degradation and defense against pathogen. It can function as effective quencher of reactive intermediary forms of O$_2$ and peroxo radicals under stressed conditions (Vangronsveld *et al.*, 1994). Increased Gu-POX activity under drought stress conditions in various plants, like *Glycyrrhiza uralensis* L. (Pan *et al.*, 2006), *Helianthus* cultivar (Gunes *et al.*, 2008) and *P. cathayana* L. (Xiao *et al.*, 2008) has been reported.

Ascorbate peroxidase (APX, E.C. 1.1.11.1) is a central component of ascorbate – glutathione cycle (ASC-GSH cycle), and plays an essential role in the control of intracellular ROS levels.

\[
\text{ASC} + \text{H}_2\text{O}_2 \leftrightarrow 2\text{DHA} + 2\text{H}_2\text{O}
\]

It is a member of Class I super family of heme peroxidases and is regulated by redox signals and H$_2$O$_2$. Five distinct isoenzymes of APX have been found in cytosolic, stromal, thylakoidal, mitochondrial and peroxisomal locations (Nakano and Asada, 1987; Jimenez *et al.*, 1997; Madhusudan *et al.*, 2003). These isoforms respond differently to metabolic and environmental signals (Kubo *et al.*, 1995). Significant increase in APX activity was noted under water stress in three cultivars of *Phaseolus vulgaris* L. (Zaltev *et al.*, 2006) and *P. asperata* L. (Yang *et al.*, 2008).
Glutathione reductase (GR, E.C. 1.6.4.2), a NADPH-dependent enzyme catalyzes the reduction of GSSG to GSH and thus, maintains high cellular GSH/GSSG ratio.

\[
\text{NADPH + GSSG} \rightleftharpoons \text{NADP}^+ + 2\text{GSH}
\]

GR belongs to a group of flavoenzymes and contains an essential disulfide group (Ghisla and Massey, 1989). It is located in the chloroplasts, cytosol, mitochondria, and peroxisomes (Edwards et al., 1990). In chloroplast, GSH and GR are involved in detoxification of \(\text{H}_2\text{O}_2\) generated by Mehler reaction. Eyidogan and Oz (2005) reported increased GR activity in the leaf tissue of \(\text{Cicer arietinum}\) L. under salt stress.

Nonenzymic components of the antioxidative defense system include the major cellular redox buffers, ascorbate and glutathione (\(\gamma\)-glutamyl-cysteinyl-glycine) as well as tocopherol, carotenoids, and phenolic compounds. They perform multiple roles in defense and as enzyme cofactor, thus, regulate plant growth and development by modulating processes from mitosis and cell elongation to senescence and cell death (Pinto and Gara, 2004). Ascorbate is the most abundant, low molecular weight antioxidant that reacts with \(\text{OH}^-, \text{O}_2^{*−},\) and \(\text{\textsuperscript{1}}\text{O}_2\) and plays a key role in defense against oxidative stress (Chen and Gallie, 2003). Ascorbate removes \(\text{H}_2\text{O}_2\) via ASC-GSH cycle (Pinto et al., 2004), where two molecules of ASC are utilized to reduce \(\text{H}_2\text{O}_2\) to water with concomitant generation of monodehydro ascorbate (MDHA). MDHA is a radical with a short life time and can spontaneously dismutate into dehydroascorbate (DHA) and ASC or is reduced to ASC by NADPH dependent enzyme monodehydro ascorbate reductase (MDHAR) (Miyake and Asada, 1994). ASC level has been reported to alter in response to various stresses, such as, water deficit, low or high temperature and metal stress (Sharma and Dubey, 2005; Radyuk et al., 2009; Maheshwari and Dubey, 2009; Mishra et al., 2011; Srivastava et al., 2011).
Reduced glutathione occurs abundantly in plant tissues and is localized in all cell compartments like cytosol, endoplasmic reticulum, vacuoles, mitochondria, chloroplasts, peroxisomes as well as in apoplast (Jimenez et al., 1997). GSH reacts chemically with $O_2^-$, $OH^-$, $H_2O_2$ and, therefore, can function directly as a free radical scavenger. It can protect macromolecules (i.e. proteins, lipids, DNA) either by the formation of adducts directly with reactive electrophiles (glutathiolation) or by acting as proton donor in the presence of ROS or organic free radicals, yielding oxidized glutathione (GSSG) (Asada et al., 1994). It can participate in regeneration of another potential antioxidant ASC, via the ASC-GSH cycle. GSH recycles ASC from its oxidized to reduced form by the enzyme dehydroascorbate reductase (DHAR) (Loewus, 1988). The role of GSH in the antioxidative defense system provides a rationale for its use as a stress marker. Srivastava et al. (2005) reported an appreciable decline in glutathione reductase (GR) activity and GSH pool under Cu stress and significant increase under salt stress.

Sorbitol is a low molecular weight 6 carbon alditol which has been used to impose osmotic stress on leaf slices, protoplast or chloroplast. It is a direct product of photosynthesis in mature leaves, in parallel with sucrose, and both serve similar functions, such as, translocation of carbon skeletons and energy between sources and sink organs. Increased transport of polyols, both in the xylem and phloem occurs frequently as a result of salt or drought stress (Noiraud et al., 2001). In addition to sorbitol, polyethylene glycol (PEG), a neutral osmotically active polymer, is most frequently used in plant water deficit studies to induce dehydration by decreasing the water potential of nutrient solution. PEG 6000 cannot enter the pores of plant cells and thus, cause cytorrhysis rather than plasmolysis (Oertli, 1985); therefore, PEG solutions mimic dry soil more closely (Verslues et al., 1998).

The present study aimed at analyzing the effect of osmotic stress induced by sorbitol and PEG-6000 on overall growth and biochemical parameters, related to growth and stress response, enzymes of chlorophyll biosynthesis, such as, ALA synthesizing activity, ALAD as well as non- enzymatic and enzymatic components
of antioxidative system in dark and light grown maize leaf segments with a view to elucidate the mechanistic details. The study was planned with the following objectives:

- To analyze the effect of sorbitol and PEG-6000 on overall growth and biochemical parameters, such as, RWC, total protein, total RNA, DNA, proline, chlorophylls and carotenoids.

- To analyze the effect of sorbitol and PEG-6000 on δ-amino levulinic acid content and δ-aminolevulinic acid synthesizing activity, a key step controlling chlorophyll biosynthesis.

- To analyze the effect of sorbitol and PEG-6000 on δ-amino levulinic acid dehydratase activity, one of the earlier enzymes of chlorophyll biosynthesis.

- To analyze the effect of sorbitol and PEG-6000 on enzymatic antioxidants, such as, superoxide dismutase, catalase, guaiacol peroxidase, ascorbate peroxidase and glutathione reductase.

- To analyze the effect of sorbitol and PEG-6000 on non enzymatic antioxidants, such as, total ascorbate and GSH.

It is envisaged that such a study will provide useful information about the effect of osmotic stress induced by sorbitol and PEG-6000 on key steps of chlorophyll biosynthesis and antioxidative system for further advanced studies.