Chapter I

Introduction

and

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1.0 Introduction

The observation that organisms are adapted to the environment lies at the foundation of biology. The evolutionary and ecological framework to this observation attributes to the organism a suite of adaptive responses (biochemical, cellular, physiological and behavioural), which together enable it to survive within a particular set of environmental conditions. Every plant has variability in effects and thereby variability in its response to the effects caused by both biotic (insects, bacteria, fungi and viruses) and abiotic (drought, salinity, temperature, heavy metals, radiation and inadequate/excess mineral nutrients) stresses. While all these environmental factors (Fig 1.1) can substantially reduce crop yield; drought, salinity and high temperature have been especially problematic for agricultural productivity (Zhu, 2002; Burke et al., 2008).

Fig 1.1 Biotic and abiotic stresses signals that affect plant growth and development.

1.1 Abiotic stress

Abiotic stresses trigger a wide range of plant responses, from altered gene expression and cellular metabolism to changes in growth rates and crop yields. The duration, severity, and rate at which the stress is imposed influence how a plant responds (Fig 1.2). Several adverse conditions in combination may elicit a response different from that of a single type of stress. Features of the plant, including organ or tissue identity, development age, and genotype, too influence the plant’s response to stress. Some responses clearly enable a plant to acclimatize to stress, whereas the functional role of other responses is not apparent. Therefore, identifying which responses promote or maintain plant growth and development
during stress is important for understanding the stress response process. The ability to withstand stresses frequently becomes the limiting factor for plant growth, survival and geographical distribution. The study of the behavior of plants under stress is of practical importance from the point of view of agricultural yield.

![Fig 1.2: Factors determining plant response to environmental stress. (Adapted from Bray et al., 2000).](image)

Plant adaptation to environmental stresses is dependent upon the activation of cascades of molecular networks involved in stress perception, signal transduction and the expression of specific stress-related genes and metabolites (Fig 1.3). The physiological and metabolic changes underlying these adaptations include production of osmoprotectants such as proline, glycine betaine, sugars and sugar alcohols that allow cellular osmotic adjustment for continued water uptake (Zhu, 2002; Krasensky and Jonak, 2012), alterations in the lipid composition (Testerink and Munnik, 2011), regulation of stomatal aperture (Umezawa et al., 2010), and regulation of ion homeostasis by transport proteins in various cellular membranes (Gao et al., 2007). In some plants, stress adaptation has been correlated with increased expression of genes encoding polypeptides rich in hydrophilic amino acid residues (Garay-Arroyo et al., 2000). The major gap in our understanding of stress tolerance in many plant species is our limited knowledge of stress-associated metabolism. Therefore, comprehensive profiling of stress-associated metabolites will be a key factor in molecular breeding for tolerance. One approach to improve stress tolerance in crops would be to transfer the genes for these adaptive traits from the tolerant organism to the susceptible crop (Fig 1.4). Metabolic traits, especially pathways with few enzymes, are best characterized genetically and more amendable to such manipulations than structural and developmental traits. As the plants are exposed to multiple stresses, mechanism of sensing and responding to different environmental factors appear to be overlapping (Mathur et al., 2008). When plants are subjected to abiotic stress, a number of genes are turned on while some are turned off. The
products of these genes not only function in stress tolerance but also in regulation of gene expression and signal transduction in stress response (Shinozaki and Shinozaki, 2006).

**Fig 1.3:** Stress responsive mechanisms activated by downstream signaling and transcription control (Vinocur and Altman, 2005).

**Fig 1.4:** Enhancement of acquired plant stress tolerance by manipulating stress-associated genes and proteins and by overexpression of stress associated metabolites (Vinocur and Altman, 2005).
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There is a world-wide need to understand how plants respond to environmental stress. One of the reasons is to meet the requirements of food security of the ever expanding human population. Another reason is to preserve biodiversity in the face of continuing destruction of natural habitats and intensification of farming. The impact of stress can be alleviated through crop management techniques (e.g. field irrigation scheduling) and through plant improvement using breeding and biotechnology.

1.1.1 Salt Stress

Salinity is the most important environmental constraint that causes substantial crop yield losses throughout the globe, and its menace is increasing day by day (Yadav et al., 2011). Increasing salinity reduces the average yield of major crops by more than 50 % (Bray et al., 2000), which is of great concern to countries like India, whose economies are mainly agriculture based. A study on global land use patterns by FAO Land and Nutrition Management Service (2008) reveals that 6 % of the world’s land area, amounting to 800 million hectares, has become saline. There are two main sources of salinization of soils; primary and secondary salinization. Primary or natural salinization, the more widespread of the two, results from weathering of minerals and soil derived from saline parent rocks (Rengasamy, 2006), whereas secondary salinization is caused by human interference such as irrigation, deforestation, overgrazing, or intensive cropping (Ashraf, 1994).

Salinity poses many adverse effects on plant growth and development at physiological and molecular level. These adverse effects are due to: (i) low osmotic potential of soil solution, (ii) inhibition of cell expansion and reduced biomass production (Ashraf and Mahmood, 1990), (iii) inhibition of enzymatic activities (Ashraf and Tufail., 1995), (iv) ionic imbalance (Kidambi, 1990), (v) alterations in different metabolic activities of plants (Lawlor and Cornic, 2002), (vi) disturbances in solute accumulation (Khan et al., 1999), and (vii) specific ion effects, or combination of these factors.

Salinity stress affects plant growth, as well as development processes such as germination, seedling growth and vigour, vegetative growth, flowering and fruit set (Sairam and Tyagi, 2004) by causing cytotoxicity and osmotic stress (Munns, 2002). Such growth reductions have been reported in tomato (Romero-Aranda et al., 2002), cotton (Meloni et al., 2003) and sugar beet (Ghoulam et al., 2002). Time course studies show that salinity influences relative growth, net assimilation capacity, leaf expansion rate and leaf area index (Zheng et al., 2008).
The multifarious effects of saline environments on plant physiology include osmotic stress, ion-specificity, nutritional and hormonal imbalances, and oxidative damage. Outcomes of these effects cause disorganization of cellular membranes, inhibition of photosynthesis, reduced nutrient absorption, generation of toxic metabolites and reactive oxygen species (ROS), ultimately leading to plant death (Flowers and Flowers, 2005). High salt concentration also decreases the net photosynthetic rate, stomatal conductance and pigment content (Romero-Aranda et al., 2002). Reduction in photosynthetic rate is attributed to factors like dehydration of cell membranes, ion toxicity (particularly Na\(^+\) and Cl\(^-\)), stomatal closure, reduction in CO\(_2\) fixation, changes in enzyme activities and reduced sink strength (Iyengar and Reddy, 1996). Accumulation of salts in rooting medium causes a reduction in soil water potential thereby reducing the root’s ability to extract water (Munns, 2002). This impairs cell division and enlargement resulting in inhibition of plant growth (Munns and Tester, 2008). Addition of inhibitory levels of NaCl in Arabidopsis have been shown to cause in plasticity of cell wall almost instantaneously (Wang and Li, 2008). Accompanying ion toxicity can also occur due to the excessive uptake and accumulation of certain ionic species (e.g. Na\(^+\), Cl, SO\(_4^{2-}\) and HCO\(_3^-\)). Leaf burning or drying of leaf tissues in sugarcane is a symptom of Cl\(^-\) toxicity (Wahid, 2004). In woody perennials, Na\(^+\) is retained in the roots and stem whilst Cl\(^-\) accumulates in the shoots, and appears to be more damaging to the photosynthetic capacity of plants (Flowers and Yeo, 1988). However, for many crop plants the major ion causing toxicity is Na\(^+\) (Tester and Davenport, 2003). Salinity affects the nutrient availability by competing with uptake, transport and partitioning of Na\(^+\) within plants. It also influences the uptake of K\(^+\), N, P and Ca\(^{2+}\) (Hu et al., 2007). For instance, ionic imbalances were noted in Lotus creticus (Mokhtar et al., 2006) and clover seedlings (Ben et al., 2003) due to higher amounts of Na\(^+\) and Cl\(^-\) in soil.

Salt-induced intracellular accumulation of ROS impairs plant survival by damaging cellular membranes, enzymes and DNA (Munns and Tester, 2008). Salinity affects the plant’s ability to synthesize non-enzymatic antioxidant compounds (tocopherols, carotenoids, glutathione and ascorbate) and antioxidative enzymes [superoxide dismutase, catalase, peroxidase and glutathione reductase] (Smirnoff, 2005). In several studies, salt tolerance was found to be positively correlating with more efficient oxidative system (Noctor and Foyer, 1998a; Mishra et al., 2011, Sharma et al., 2012). Substantive proofs to this argument came from the fact that a recessive deletion mutant of Arabidopsis showing higher activities of SOD and APX was salt tolerant compared with salt sensitive wild-type (Mittler et al., 2004).
In other studies, over-production of GR and APX has been found to improve oxidative stress and salt tolerance of wheat (Sairam et al., 1998). Abiotic stresses stimulate production of various secondary metabolites i.e., phenolics, flavonoids and phenyl propanoids via the phenylpropanoic acid, shikmic acid, mevalonic acid and methyl erythritol phosphate pathways (Taiz and Zeiger, 2006). Polyols also accumulate in response to salinity stress and are noted to enhance salinity tolerance (Williamson et al., 2002). Enhancement of salt tolerance in rice was attributed to the increase in contents of polyamines, such as putrescine and spermidine (Katiyer and Dubey, 1990). Salt tolerance was reported in plants accumulating nitrogen containing compounds such as amino acids, amides, imino acids and quaternary ammonium compounds etc., (Nguyen et al., 2003). Levels of soluble phenolics, anthocyanins and flavones are found to be increased under salt stress, and known to provide protection against ion induced oxidative damage by binding ions and reducing toxicity on cytoplasmic structures in sugarcane (Wahid and Ghazanfar, 2006).

Molecular and genomic approaches have been successfully employed to understand response to salinity in plants. Main responses include metabolic adaptations to salt stress at the cellular level. These responses being amenable to molecular analysis lead to the identification of a large number of genes induced by salt stress (Bray, 1997; Shinozaki et al., 1998; Xiong et al., 2002; Cheong and Yun, 2007; Hossain et al., 2012). Some examples of genes/proteins induced by salt stress include Sal 1 (Quintero et al., 1996), Bnd 22 (Reviron et al., 1992), Vitronectin and fibronectin-like proteins found in membranes and cell wall of NaCl-adapted cells (Zhu et al., 1993), Osmotin (Singh et al., 1987), and RAB21 (Mundy et al., 1990) to name a few. These inducible genes can be classified based on their physiologic or metabolic function predicted from sequence homology with known proteins; and include genes for photosynthetic enzymes, synthesis of compatible solutes and free radical-scavenging enzymes. However, by a salt-hypersensitivity assay in Arabidopsis, additional genes have been detected which led to the identification of mutations involved in K⁺ uptake, which play a critical role in salt sensitivity (Wu et al., 1996). The Arabidopsis SOS (salt overly sensitive) mutants were identified by an inability to maintain root growth under high salinity. Genetic screening of these SOS mutants revealed three genes associated with the salinity stress (Zhu, 2003). These SOS mutants did not show altered expression to drought or osmotic stress suggesting that SOS genes function specifically in coping with the ionic aspect of salt stress. Of these three gene, sos1 encodes Na⁺/H⁺ antiporter localized in the plasma membrane (Shi et al., 2006), SOS2 encodes a serine/threonine protein kinase with an amino
terminal catalytic domain and a carboxy-terminal regulatory domain (Liu et al., 2000), and SOS3 encodes a myristoylated Ca$^{2+}$ binding protein (Ishitani et al., 2000); proposing that SOS3 senses cytosolic calcium changes that are elicited by salt stress (Liu and Zhu, 1998). These SOS mutants have provided significant insights into components of signal transduction pathways that are likely to be involved in controlling plant responses to salinity (Tester and Davenport, 2003) (Fig 1.5).

![Fig 1.5: Regulation of ion homeostasis by SOS signaling pathway for salt stress adaptation. Salt stress induce Ca$^{2+}$ signal that activates the SOS3/SOS2 protein kinase complex, which then phosphorylates a plasma membrane Na$^+$/H$^+$ antiporter SOS1, and regulates the expression of some genes. SOS2 also activates tonoplast Na$^+$/H$^+$ antiporter sequestering Na$^+$ into the vacuole (NHX1). ABI1 regulates the gene expression of NHX1 whereas ABI2 interacts with SOS2 and negatively regulates ion homeostasis either by inhibiting SOS2 kinase activity or the activities of SOS2 targets. CAX1 (H$^+$/Ca$^{2+}$ antiporter) is an additional target for SOS2 activity restoring cytosolic Ca$^{2+}$ homeostasis. SOS3 and SOS2 complex negatively regulate the activity of AtHKT1. SOS4 gene encodes a pyridoxal (PL) kinase that is involved in the biosynthesis of PL-5-phosphate (PLP), which contributes Na$^+$ and K$^+$ homeostasis by regulating ion channels and transporters. SOS5 is involved in the maintenance of cell expansion. Dashed arrow shows SOS3-independent and SOS2-dependent pathway (Turkan and Demiral, 2009).

Salt tolerance in plants can be improved by growing plants with desired traits obtained by wide-crossing, hybridization, and use of transgenics. In addition, exogenous (foliar and/or seed) application of osmoprotectants also enhances stress tolerance (Zhu, 2003). The latter method provided results much quicker and is of shorter duration (Wahid et
al., 2007a). Among various osmoprotectants, proline is known to maintain osmotic balance, scavenge ROS, stabilize sub-cellular membranes and proteins, and buffer cellular redox potential (Yang and Lu, 2005; Ashraf and Foolad, 2007; Wahid et al., 2007a) and also induce salt stress responsive genes (Chinnusamy et al., 2005). Transgenic tobacco plants over-expressing pyrroline-5-carboxylate synthetase (P5CS), a major enzyme in proline biosynthesis, accumulated high levels of proline and exhibited salt and drought tolerance (Kavi Kishore et al., 2005). Such growth sustenance was due to improvement in metabolism (Rana and Rana, 1996), reduced peroxidation of membrane lipids (Yazici et al., 2007) and protection from oxidative damage (Banu et al., 2009). For instance, application of proline to cultured soybean cell in saline medium increased the activities of SOD and POD, which increased salt tolerance in these cells (Hua and Guo, 2002).

1.1.2 Drought Stress

Drought stress prevails when the available water in the soil is reduced and atmospheric conditions cause continuous loss of water by transpiration or evaporation. Water deficit and salt stresses are global issues to ensure survival of agricultural crops and sustainable food production (Jaleel et al., 2007c-e; Nakayama et al., 2007). Growth rates of several plants are directly proportional to the availability of water in the soil (Kamel and Loser, 1995). Plant or cellular water deficit occur when the rate of transpiration exceeds water uptake resulting in the reduction of the relative water content (RWC), cell volume and cell turgor (Lawlor and Cornic, 2002).

Response to drought stress involves metabolic and structural modifications that improve plant function under stress (Fig 1.6). Some of these alterations include changes in root to shoot ratio, leaf anatomical changes, temporary accumulation of reserve matter in stem and petioles and alterations in carbon and nitrogen accumulation (Chartzoulakis et al., 2002). Under water limiting conditions, excitation energy harnessed by chlorophyll cannot be dissipated via photosynthesis and leads to the formation of reactive oxygen species (ROS), which if left unquenched, can cause considerable subcellular damage (Smirnoff, 1993). These ROS act as second messengers in redox signal transduction and are implicated in hormone mediated events (Foyer and Noctor, 2003). H$_2$O$_2$ signals closure of leaf stomata, acclimation of leaf to high irradiation and the induction of heat shock proteins [HSPs] (Kwak et al., 2003). The primary constituents of the plants protective mechanism to prevent the damage by free radicals include antioxidant enzymes such as SOD, CAT, POX and GR, and free radical scavengers such as carotenoids, ascorbate, tocopherols and oxidized and reduced
glutathione (Turkan et al., 2005; Contour-Ansel et al., 2006; Torres-Franklin et al., 2008). Prolonged water deficit is known to impose mechanical constraint on cellular membranes. The strain on membrane is one of the severe effects of drought imposed on a plants’ physiology as it impairs the functioning of transporters and membrane associated enzymes due to de-esterification of membrane lipids (Cruz de Carvalho, 2008). Dehydration cause decline in cellular volume, thereby increasing the probability of protein–protein interaction and causing aggregation and denaturation (Hoekstra et al., 2001).

Fig 1.6: Whole-plant responses to drought stress. Left, long-term or acclimation responses; right, short-term responses (Chaves et al., 2003).

Loss of water from cell triggers a cellular signal transduction pathway, and cellular perception of water loss activates signalling mechanisms to induce specific genes (Hirayama and Shinozaki, 2010). Some genes are induced rapidly against drought stress while the others are induced slowly following the accumulation of ABA. Water loss triggers ABA synthesis leading to induction of many, but not all drought-inducible genes. These findings indicate the existence of both ABA-independent and ABA-dependent signal transduction cascades (Umezawa et al., 2010). The enhancement of drought tolerance involves a number of stress responsive genes and their products; which have been identified and classified into two groups [Fig. 1.7] (Shinozaki and Shinozaki, 2006). The first group (Functional proteins) includes molecules such as chaperones, late embryogenesis abundant (LEA), osmotin, antifreeze proteins, mRNA-binding proteins, key enzymes for osmolyte biosynthesis, water
channel proteins, sugar and proline transporters, detoxification enzymes, and various proteases. The second group (regulatory proteins) includes various transcription factors, protein kinases, protein phosphatases, enzymes involved in phospholipid metabolism, and other signaling molecules such as calmodulin-binding proteins which are involved in further regulation of signal transduction and stress-responsive gene expression. Aquaporins, members of the major intrinsic protein (MIP) family are water selective intrinsic proteins whose expression in both plasma membrane and vacuolar membranes increase upon drought stress causing an enhancement in the osmotic hydraulic conductivity of the membrane by 10 to 20 folds (Martre et al., 2002). This augments the movement of water across the membranes.

![Drought stress proteins](image)

**Fig 1.7:** Functions of drought stress-inducible genes in stress tolerance and response (Shinozaki and Yamaguchi-Shinozaki, 2006).

Genetic engineering of the metabolic pathways for production of osmolytes such as proline, glycine betaine, mannitol, galactinol, fructans, trehalose, raffinose, and ononitol have been shown to increase the resistance to drought (Mathur et al., 2008). Proteins like LEA functioning as chaperones, number of transcription factors and protein kinases have been found useful in engineering drought resistance. 9-cis-epoxycarotenoid dioxygenase (NCED), key enzyme in ABA biosynthesis, and ABA 8'-hydroxylase, an enzyme that degrades ABA have been implicated in drought resistance by transgenic methods (Umezava, et al., 2006). Analysis of ESTs generated under salt stress, drought and ABA application have indicated a closer match between ABA and drought signaling pathways and cross-talk between salt, drought, and cold stress (Rabbani et al., 2003).
Drought stress response is found to be influenced by the ABA-independent pathway. The promoters of a drought-, high salinity-, and cold- inducible gene, RD29A/COR78/LTI78 in *Arabidopsis* contain two major cis-acting elements, ABRE (ABA-responsive element) and DRE (dehydration-responsive element)/CRT (C-Repeat), both of which are involved in stress-inducible gene expression (Shinozaki and Shinozaki, 2006). While ABRE functions in ABA-dependent expression, DRE/CRT functions in ABA-independent genes (Fig 1.8). Transcription factors, CBF/DREB and DREB2 (ERF/AP2 family) bind to DRE/CRT elements. Overexpression of CBF/DREB1 in transgenic plants increased stress tolerance to freezing, drought, and salt stresses, suggesting the role of CBF/DREB1 proteins in cold- and drought- stress tolerance. Overexpression of OsDREB1 or Arabidopsis DREB1 also improved drought and chilling tolerance in transgenic rice (Ito et al., 2006). Most of the CBF/DREB1 target genes contain the DRE motif with a conserved (A/G)CCGACNT sequence in their promoter regions. The target gene products of these proteins are consequently involved in establishing stress tolerance. The DREB2 genes on the other hand are induced by dehydration stress and may activate other genes involved in drought stress tolerance. The DREB2 protein expressed under normal growth conditions is activated by osmotic stress through post-translational modification in the early stages of the osmotic stress response.

Several drought-inducible genes including ERD1 (early responsive to dehydration1), which encodes a Clp (calpain) protease regulatory subunit, ClpD are not induced by ABA, suggesting an ABA-independent pathway of regulation. The ERD1 gene is not only induced by dehydration but is also up-regulated during natural senescence and dark-induced senescence. The promoter of ERD1 has two distinct cis-acting elements specific for dehydration stress and dark-induced senescence (Simpson et al., 2003). ABA accumulated under drought stress also regulates the expression of genes. One of the ABA-responsive gene from *Arabidopsis* has ARE elements in its promoter. In presence of ABA, ARE will be bound by two basic leucine zipper (bZIP) transcription factors, AREB/ABF to activate ABA-dependent genes (Choi et al., 2000). The ABA-dependent activation of AREB/ABF has been attributed to ABA-dependent phosphorylation. Transgenic plants expressing a phosphorylated form of AREB1 with multisite mutations were able to induce many ABA-responsive genes without exogenous ABA application (Furihata et al., 2006). The induction of drought-inducible RD22 gene from Arabidopsis is mediated by ABA and requires protein biosynthesis for its ABA-dependent expression. A MYC (AtMYC2) and a MYB (AtMYB2)
transcription factors synthesized upon endogenous accumulation of ABA are known to cooperatively activate the RD22 gene by binding to cis elements in RD22 promoter. Over expression of these transcription factors in transgenic plants have resulted in an ABA-hypersensitive phenotype with increased osmotic stress tolerance. The RD26 gene encodes a NAC transcription factor, induced by drought, high salinity, ABA, and JA. The protein is localized in the nucleus regulates transcriptional activity. ABA- and stress-inducible genes were up-regulated in the RD26-overexpressing transgenics and repressed in the RD26 repressor lines. While a number of typical ABA-inducible genes such as LEA, RD, ERD, COR, and KIN were not targets of RD26, many JA-inducible genes were found to be its targets (Fujita et al., 2005). These observations indicated the role of RD26 in cross-talk between ABA signaling and JA signaling during drought and wounding stress responses (Shinozaki and Shinozaki, 2006).

**Fig 1.8:** Transcriptional regulatory networks of abiotic stress signals and gene expression. At least six signal transduction pathways exist in drought, high salinity, and cold-stress responses: three are ABA dependent and three are ABA independent. In the ABA-dependent pathway, ABRE functions as a major ABA-responsive element. AREB/ABFs are AP2 transcription factors involved in this process. MYB2 and MYC2 function in ABA-inducible gene expression of the RD22 gene. MYC2 also functions in JA-inducible gene expression. The RD26 NAC transcription factor is involved in ABA- and JA-responsive gene expression in stress responses. These MYC2 and NAC transcription factors may function in cross-talk during abiotic-stress and wound-stress responses. In one of the ABA-independent pathways, DRE is mainly involved in the regulation of genes not only by drought and salt but also by cold stress. DREB1/CFBs are involved in cold-responsive gene expression. DREB2s are important transcription factors in dehydration and high salinity stress-responsive gene expression. Another ABA-independent pathway is controlled by drought and salt, but not by cold. The NAC and HD-ZIP transcription factors are involved in ERD1 gene expression (Shinozaki and Yamaguchi-Shinozaki, 2006).
Two principal strategies have been employed to defend dehydration damage; i) the synthesis of protective molecules during the dehydration phase to prevent damage, and ii) repair-based mechanism during rehydration to neutralize the damage. Dehydration tolerance is a trait that exists in seeds of most of the higher plants, but only in some species such as the resurrection plant, at the whole plant level (Kranner et al., 2002). Most of the information on molecular responses to dehydration and desiccation, starting from sensing and signaling to regulation of gene expression, are obtained from model plants such as *A. thaliana* and *C. plantagineum*. However, recent investigations have also shed light on the responses shown by a number of tropical plants (Turkan et al., 2005; Sharma and Dubey, 2005; Torres-Franklin et al., 2008; Cramer et al., 2011).

### 1.1.3 Heat Stress

High temperature stress is a major growth restraining factor for most crop plants. Excess radiation and elevated temperatures in the tropical areas of the world significantly change plant growth and yield responses. Long term or even a temporary exposure to high temperature can alter metabolic functions, thereby affecting various plant parts including leaves, flower buds and roots (Iwaya-Inoue et al., 2004). Main symptoms of heat stress on plants may include scorching of leaves and twigs, sunburn on branches and stems, leaf senescence and abscission (Guilioni et al., 1997; Giaveno and Ferrero, 2003; Vollenweider and Gunthardt-Goerg, 2005). High temperature causes delayed germination of seeds and loss of seed vigor (Egli et al., 2005), reduced plant emergence and patchy crop stand. Shoot dry mass, relative growth rate, net assimilation rate and productivity are significantly reduced by high temperature (Rainey and Griffiths, 2005; Wahid et al., 2007b).

At the whole plant level, heat stress causes a reduction in cell size and closure of stomata, negatively affecting leaf water status (Anon et al., 2004). It evokes the generation of ROS (Potters et al., 2007), increases plasma membrane permeability (Zhang et al., 2005), alters structural organization of thylakoids and inhibits photosynthesis (Karim et al., 1997). Tolerant plants entail a tendency of protection against the damaging effects of ROS with the synthesis of various enzymatic and non-enzymatic ROS scavenging and detoxification systems (Apel and Hirt, 2004). This phenomenon starts with the conversion of O$_2^-$ by SOD into H$_2$O$_2$, with the help of APX or CAT. It has been reported that a number of physiological processes are influenced by the overexpression of SOD in plants, including removal of H$_2$O$_2$, oxidation of toxic reductants, biosynthesis and degradation of lignin in cell walls and auxin catabolism (Scandalios, 1993). More specifically, expression and activation of APX is
related to physiological injuries caused in plants by heat stress (Mazorra et al., 2002). Plants must be protected against oxidative stress so that they can survive under high temperature. Heat acclimated turf grass showed lower production of ROS as a result of enhanced synthesis of ascorbate and glutathione (Xu et al., 2006). Temperature stress also induces production of various secondary metabolites i.e. phenolics, flavonoids and phenyl propanoids (Wahid et al., 2007b). Increased activity of phenylalanine ammonia lyase (PAL) in response to heat stress is the main acclimatory response in watermelon (Wahid et al., 2007b). Acclimation to heat stress is triggered by the biosynthesis of phenolic compounds acting as powerful antioxidants (Rivero et al, 2001; Sachray et al., 2002; Wahid and Ghazanfar, 2006).

Plants have evolved various mechanisms for thriving under higher prevailing temperatures (Fig 1.9). These include short term avoidance/acclimation mechanism or long term evolutionary adaptations. In case of sudden heat stress, short term response i.e., leaf orientation, transpirational cooling and changes in membrane lipid composition are more important for survival (Wahid et al., 2007b). Smaller yield losses due to early maturation in summer shows possible involvement of an escape mechanism in heat stress tolerance (Adams et al., 2001). Different tissues in plants show variations in terms of developmental complexity, exposure and responses towards the prevailing or applied stress types (Queitsch et al., 2000). The stress responsive mechanism is established by an initial stress signal that may be in the form of ionic and osmotic effect or changes in the membrane fluidity. This helps to re-establish homeostasis and to protect and repair damaged proteins and membranes (Vinocur and Altman, 2005). Plants lacking the ability to display rapid heat acclimation responses may be more prone to thermo-damage. Since plants have to face temperature fluctuations during day/night cycle, the acquisition of thermotolerance reflects a more general mechanism that contributes to homeostasis of metabolism on a daily basis (Hong et al., 2003). Some major mechanisms, which make plants thermotolerant include ion transporters, free radical scavengers, LEA proteins, osmoprotectants and factors involved in signaling cascades and transpirational control (Wang and Luthe, 2003). Heat stress effects are of greater concern at various levels including plasmalemma, biochemical pathways operative in the cytosol or organelles (Sung et al., 2003). Studies have revealed that first target of heat stress are the plasmalemma that shows increased fluidity of lipid bilayer (Wahid et al., 2007b). This leads to the induction of Ca$^{2+}$ influx and reorganization of cytoskeleton and eventually the upregulation of calcium dependent protein kinase (CDPK) and MAPK. Nuclear signaling of such cascades shows the synthesis of cytosolutes and antioxidants. The
cytosolutes help to maintain cellular water balance, while the antioxidants scavenge the ROS (Maestri et al., 2002).

Fig 1.9: Proposed heat-stress tolerance mechanisms in plants. MAPK, mitogen activated protein kinase; ROS, reactive oxygen species; HAMK, heat shock activated MAPK; HSE, heat shock element; CDPK, calcium dependent protein kinase; HSk, histidine kinase (Wahid et al., 2007b).

Besides their inherent ability to withstand supraoptimal temperatures for short period of time, plants are able to become tolerant to otherwise lethal high temperatures (acquired thermotolerance). This response is triggered by prior exposure to a conditioning pretreatment, which can be a short, sublethal high temperature, a gradual temperature increase, or other moderate stress treatment (Boston et al., 1996). For example, European beech when exposed to acclimation temperature of 55 °C resulted in an elevated resistance to higher heat events (Wagenbreth, 1965). The phenomenon of acquired thermotolerance enables plants growing in their natural habitat to flourish under diurnal heat conditions that would be lethal in the absence of a rapid protective response.
1.1.4 Cold Stress

Cold stress is a collective term given to chilling and freezing stress. Chilling stress results from temperatures (0 °C to 10 °C) cool enough to produce injury without ice formation in plant tissues, whereas in freezing stress, ice formation (below 0 °C) takes place in plant tissues. Chilling sensitive plants show the physical transition of cell membrane from a flexible liquid-crystalline to a solid gel phase, instantaneously increasing membrane permeability and ion leakage, thereby affecting the cellular function in a number of ways (Farooq et al., 2009a). As a consequence of abnormal metabolism, accumulation of toxic metabolites and reactive oxygen species (ROS) takes place in the injured cells. Chilling injury is a serious problem during germination in many plant species; rice seedlings showing signs of wilting, reduced leaf expansion and chlorosis (Yoshida et al., 1996). Other symptoms include surface lesions; water soaked appearance, desiccation, discoloration, tissue breakdown, accelerated senescence, shortened shelf life and faster decay due to leakage of plant metabolites (Solanke and Sharma, 2008). The reproductive development is especially sensitive to chilling temperatures in some plants. If rice plants are exposed to chilling stress at the time of anthesis (floral opening), it results in sterile flowers (Satake, 1976). Cell membrane, the primary site of injury in plants subjected to freezing stress undergoes gross structural changes due to severe dehydration linked to freezing (Thomashow, 2001). Changes in membrane fluidity arise mainly due to fatty acid unsaturation in membrane lipids, change in composition and ratios of lipid to protein in the cellular membranes (Wang and Li, 2006). As temperatures drop below 0 °C, the ice nucleation generally begins in the intracellular spaces. Presence of solutes leads to a higher freezing point for the intracellular fluid (Thomashow, 1999). The chemical potential of ice is less than that of liquid water, therefore the formation of ice results in a decrease in the water potential outside the cell. Consequently, the unfrozen water moves from a higher potential in the cell to a lower potential in the intracellular space. This water movement causes severe cellular dehydration during freezing. Growth at low temperatures increases the concentration of ROS, causing damage to membrane lipids, proteins and nucleic acids, leading to death of cells (Apel and Hirt, 2004). Reduced cellular respiration and ROS damage the photosystem II (Suzuki and Mittler, 2006). These ROS are either the signals for ROS scavenging or other protective mechanisms contributing to reduction in stress injury in plants (Zhang et al., 2008), such as signal for ABA in mediating catalase 1 (CAT1) gene expression (Guan et al., 2000), thermotolerance (Gong et al., 1998), activating Ca^{2+} channels in guard cells and stomatal...
closure (Zhang et al., 2001) as well as for ABA biosynthesis (Zhao et al., 2001). Although considered wasteful products in early research, secondary metabolites are now known to serve many roles in plant tolerance to biotic and abiotic stresses (Hadacek, 2002). Salicylic acid (SA) is an important stress signaling molecule involved for chilling tolerance in a number of plant species (Wang and Li, 2006). Exogenous application of SA improves chilling tolerance in maize (Farooq et al., 2009a). Microarray and RNA gel blot analyses have shown that free proline induces the expression of many genes, which have the proline-responsive element (PRE) in their promoters (Oono et al., 2003).

Dehydrins are believed to play a protective role during cellular dehydration by stabilizing membranes and rescuing hydrolytic enzymes under dehydrative conditions (Puhakainen et al., 2004). Members of another group of proteins called heat shock proteins (HSP) have been implicated in cold acclimation and endodormancy (López-Matas et al., 2004). Plasma membrane rigidification has been shown to induce cold responsive (COR) genes through cytosolic Ca\(^{2+}\) increase, thereby triggering amplification and phospholipid signalling (Komatsu et al., 2007). Increased expression of dehydration-responsive element-binding protein (DREB or CBFs), which binds to cis-elements in the promoters of COR genes causes upregulation of the genes under these promoters. These regulate the expression of genes involved in a wide variety of physiological phenomena e.g., phosphoinositide metabolism, transcription, ROS detoxification, hormone metabolism, signalling and many others with known or anticipated protective functions (Lee et al., 2005). Microarray analysis of transgenic Arabidopsis plants ectopically expressing CBFs revealed constitutive induction of downstream cold-responsive transcription factor genes (RAP2.1 and RAP2.7), which possibly control subregulons of the CBF regulon (Fowler and Thomashow, 2002). During cold acclimation, CBF induction in Arabidopsis is done by a MYC-type basic helix–loop–helix transcription factor via MYC recognition elements in the CBF3 promoter (Fig 1.10). Among various CBFs, constitutive over-expression of ICE1 enhanced the expression of CBF3, CBF2 and COR genes during cold acclimation, and increased freezing tolerance of transgenic Arabidopsis. ICE1 is constitutively expressed and nucleus localized, but causes CBFs expression only under cold stress, suggesting cold stress-induced post-translational modification, necessary for ICE1 to activate downstream genes in plants (Chinnusamy et al., 2007).
Fig 1.10: Schematic illustration of the cold response network in Arabidopsis. Cold sensing and signaling leads to the activation of multiple transcriptional cascades, one of which involves ICE1 and CBFs. The ubiquitin E3 ligase HOS1 negatively regulates ICE1. Metabolism, and RNA processing and export, affect cold tolerance via cold signaling and/or cold-responsive gene expression. The constitutive HOS9 and HOS10 regulons have a role in the negative regulation of CBF-target genes. MYBRS, MYB recognition sequence; MYCRS, MYC recognition sequence (Zhu et al., 2007).

1.1.5 **Heavy Metal Stress**

Heavy metals (Al, Ar, Cd, Co, Cr, Cu, Pb, Mn, Hg, Ni, Se and Zn) make significant contribution to environmental pollution as a result of anthropogenic activities such as mining, energy- and fuel production, power transmission, intensive agricultural practices, sludge and industrial effluent dumping and military operations (Orcutt and Nilsen, 2000; Pilon-Smits, 2005). A few metals, including Cu, Zn and Mn, are essential micronutrients required for a wide variety of physiological processes in plants (Reichman, 2002). Cu, for example, is a vital component of electron-transfer reactions mediated by proteins such as superoxide dismutase, cytochrome c oxidase and plastocyanin (Clemens, 2001). However, these same metals can be toxic and inhibit growth of plants when present at excessive levels (Reichman, 2002). Toxicity results from the binding of heavy metals to sulphydryl groups in proteins, leading to inhibition of activity or disruption of structure, or displacement of essential metal
ions from the active centres of enzymes (Van Assche and Clijsters, 1990). Direct effects include binding of the metal to membranes, especially after illumination, through oxygen atoms (Vierke and Struckmeier, 1997) or histidine, tryptophan and tyrosine group of polypeptides (Maksymiec, 1997). In consequence, the quinone acceptor sites of PS II, and/or electron donation from Tyr to P680+, and the electron flow through cyt b559 of PS II reaction center are disturbed (Fig 1.11). Some analyses have indicated that heavy metals can substitute Mg in Chl (Kowalewska et al., 1987). Ahmed and Tajmir-Riahi (1993) reported that Cd, Hg and Pb may interact in vivo with LHCII by binding with proteins of the complex and leading to their conformational changes. In consequence, photosynthetic activity can be decreased as a result of disturbances in assembly of their pigment protein complex in thylakoids without any significant changes due to oxidative stress (Linger et al., 2005). The indirect action of heavy metals arises as a result of heavy metal-induced mineral nutrient disturbances and their consequences on plant growth (Siedlecka, 1995). Cu stress in cucumber plants lead to decreased potassium leaf uptake and inhibition of photosynthesis (Alaoui-Sossé, 2004). A distinct decrease in the mitotic index was observed in the case of Pb (Wierzbicka, 1999), Hg (Patra et al., 2004) and Cd (Vecchia et al., 2005). Decrease in cell wall elasticity may cause inhibition of leaf elongation as noted in plants stressed by Cd (Poschenrieder et al., 1989) and Cu (Maksymiec et al., 1995). Growth inhibition may be caused by inhibition of photosynthesis as reported in runner bean plants exposed to Cd (Skórzyńska and Baszyński, 1998) and rice seedlings to Cd or Ni (Moya et al., 1993).

Plants, like all other organisms, in order to maintain the concentration of essential heavy metals within the physiological limits and to minimize the detrimental effects of nonessential metals, have evolved a complex network of homeostatic mechanisms that serves to control the uptake, accumulation, trafficking and detoxification of metals (Clemens, 2001). Most of the transporters described to play a role in the uptake of micronutrients are in the ZIP (ZRT, IRT-like protein) and the Nramp (natural resistance-associated macrophage protein) family (Guerinot, 2000; Williams et al., 2000). In yeast, IRT1 was shown to mediate transport of Mn$^{2+}$, Zn$^{2+}$ and possibly also Cd$^{2+}$ (Korshunova et al., 1999). ZRT1 and ZRT2 from the ZIP family were first identified in S. cerivisae and shown to represent a high-affinity and a low-affinity Zn$^{2+}$ transporter, respectively (Zhao and Eide, 1996). Other methods of maintaining physiological limits of metal ions are by binding them to chelators and chaperons. Chelators (like phytochelatins and metallothioneins) contribute to metal detoxification by buffering cytosolic metal concentrations, while chaperones specifically
deliver metal ions to organelles and metal-requiring proteins (Clemens, 2001). Sequestration of metal ions by phytochelatins is an important metal tolerance mechanism in a wide range of organisms including plants and certain fungi (Clemens and Simm, 2003). PCs are induced by a range of metals and metalloids like Cd, Zn, Cu, As (De Vos et al., 1992). Metals such as Cu, Ag, and As are detected in complexes with PCs (Maitani et al., 1996; Schmöger et al., 2000). Arabidopsis cad1-3 mutant, which is PC-deficient, is Cd$^{2+}$ hypersensitive (Howden et al., 1995) and also highly sensitive to AsO$_4^{3-}$ compared to wild type and slightly sensitive to Cu, Hg and Ag (Ha et al., 1999). On the other hand, overexpression of AtPCs1, a gene encoding PC synthase in *S. cerevisiae* cells, increased Cd, Hg and As tolerance, whereas the effect on Cu sensitivity was only small (Vatamaniuk et al., 1999).

![Fig 1.11](Image)

**Fig 1.11**: A model of the putative role of signaling pathways in heavy metal stress response in plant leaves. Heavy metal stress in excess rapidly affects all cell membranes, including thylakoids. The metals cross the plasma membrane and stimulate the production of H$_2$O$_2$ directly (*solid lines*) via NADPH oxidase and indirectly (*dashed lines*) by increasing lipid peroxidation and jasmonates (JA) level. Enhanced activity of SOD causes H$_2$O$_2$ accumulation leading to increased levels of JA, cell wall rigidity, secondary metabolites and gene transcription. Prolonged H$_2$O$_2$ results in cell disturbances and enhanced senescence processes in cooperation with JA and ethylene (Maksymiec, 2007).
Plants exposed to wide range of heavy metals generate oxidative stress via generation of toxic ROS and subsequently activating the antioxidant response (Fig 1.12). The most common method is by the transfer of electrons directly in single-electron reactions, leading to the generation of free radicals. Among heavy metals, Fe$^{2+}$ and Cu$^{2+}$ ions, react with H$_2$O$_2$ and form OH$^-$ via the Haber-Weiss reaction (Fig 1.13). Transition metals having unpaired electrons in their orbitals, accept and donate single electrons, thus promoting monoelectron transfers to O$_2$ and consequent ROS interconversion by the Fenton reaction (Fig 1.12). Increased accumulation of H$_2$O$_2$, usually connected with the cellular redox status, alerts the plant cell against environmental stresses (Foyer and Noctor, 2003), and may enhance the plant’s antioxidant response through calcium signaling (Rentel and Knight, 2004). Heavy metals are also known to inactivate the antioxidant enzymes (POX, CAT, SOD) responsible for free radical detoxification. Another method of oxidative stress generation by the metal is due to the disturbance of the thylakoid membrane by metal ions. Finally, heavy metal accumulation in plant tissue results in its sequestration by phytochelatins causing a depletion of low molecular weight antioxidants, such as glutathione (Dietz et al., 1999).

Fig 1.12: Possible biochemical and molecular mechanisms of heavy metal-mediated ROS induction and damage to the development of higher plants (Hossain et al., 2012).
1.2 Oxidative stress

The most prominent outcome of studies on the response of plants to environmental stresses being increased ROS levels, and the resistance or susceptibility of the plant has been related to the proportion and functional efficiency of a network of low molecular weight antioxidants and ROS-scavenging enzymes (Asada, 1999) produced in a plant cell against these stresses. The reactions of ROS within a cell are highly complex (Fig 1.14) due to the surface properties of membranes, electrical charges, binding properties of macromolecules, and compartmentalization of enzymes, substrates and catalysts. Thus, various sites even within a single cell differ in the nature and extent of reactions with oxygen.
1.2.1 Formation of ROS

The highly oxidizing metabolic activity and intense rate of electron flow seen in organelles such as chloroplast, mitochondria or peroxisomes make them major sources of ROS in plant cells. Phototrophs are especially at the risk of oxidative damage, because of their bioenergetic lifestyle and the abundance of photosensitizers and polyunsaturated fatty acids (PUFA) in the chloroplast envelope. Most ROS in plant cells are formed via dismutation of superoxide, which arises as a result of single electron transfer to molecular oxygen in electron transfer chains, principally during the Mehler reactions in chloroplast (Asada, 1994). Although photoreduction of oxygen is an important alternative sink for the consumption of excess energy, it is always associated with the generation of toxic ROS. If the accumulation of ROS exceeds the capacity of enzymic and non-enzymic antioxidant systems to remove them, photodynamic damage to the photosynthetic apparatus ensues, leading to cell destruction (Bhattacharjee, 2005). The ROS capable of causing oxidative damage include superoxide (O$_2^-$), perhydroxy radical (HO$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxy radical (OH$^*$), alkoxy radical (LO$^*$), peroxy radical (LOO$^*$), organic hydroperoxide (LOOH), singlet oxygen (O$_2$), excited carbonyl (RO$^*$), etc. During the reduction of O$_2$ to H$_2$O, ROS namely O$_2^-$, H$_2$O$_2$ and OH$^*$ is formed (Bhattacharjee, 2005). Protonated form of O$_2^-$, HO$_2^*$ is more reactive than superoxide itself, but in plant cells at physiological pH, a small proportion O$_2^-$ would be in this form (Eltsner, 1987). However, O$_2^-$ dismutates to H$_2$O$_2$. And much more reactive OH$^*$ can be formed from O$_2^-$ and H$_2$O$_2$ through iron catalyzed Haber–Weiss and Fenton mechanisms respectively (Fig 1.13). O$_2^-$ also reacts with NO$^*$ to give peroxynitrite (OONO$^-$) (Bhattacharjee, 2005). H$_2$O$_2$ plays a dual role in plants: at low concentrations, it acts as a signal molecule involved in acclimatory signaling triggering tolerance to various biotic and abiotic stresses, and at high concentrations, it leads to PCD (Quan et al., 2008). H$_2$O$_2$ has also been shown to act as a key regulator in a broad range of physiological processes, such as senescence (Peng et al., 2005), photorespiration and photosynthesis (Noctor and Foyer, 1998b), stomatal movement (Bright et al., 2006), cell cycle (Mittler, 2004) and growth and development (Foreman et al., 2003). It was noted that H$_2$O$_2$ increased the activities of leaf SOD, CAT, APX and GR along with the induction of related-isoform(s) under non-NaCl-stress conditions in citrus pre-treated with H$_2$O$_2$ (Tanoua et al., 2009).
1.2.2 Oxidative damage to lipids

The peroxidation of lipids is considered as the most damaging process known to occur in living organisms. It is mechanistically important from free-radical production perspective as it is one of the few examples of carbon centred radical production in plant cells (Winston, 1990). Peroxidation of lipids in plant cells appears to be initiated by a number of ROS that selectively degrade unsaturated fatty acids leading to the accumulation of aldehydes, hydrocarbons, and cross-linked products. The overall process of lipid peroxidation involves three distinct stages: initiation, progression and termination steps (Fig 1.15).

**Fig 1.15**: Formation of lipid hydroperoxides from singlet oxygen reactions result in free radical mediated lipid peroxidation processes (Buettner and Hall, 1987).

Initiation step involves transition metal complexes, especially those of Fe and Cu. The membrane peroxidation is initiated mainly by OH* that abstracts a hydrogen atom in an unsaturated fatty acyl chain of a polyunsaturated fatty acid (PUFA) residue (Gill and Tuteja, 2010). In an aerobic environment, oxygen will add to the fatty acid at the carbon-centered lipid radical to give rise to a LOO*. Once initiated, LOO* can further propagate the peroxidation chain reaction by abstracting a hydrogen atom from adjacent PUFA side chains. The resulting lipid hydroperoxide can easily decompose into several reactive species including: lipid alkoxy radicals, aldehydes (malonyldialdehyde), alkanes, lipid epoxides, and alcohols (Davies, 2001; Fam and Morrow, 2003). The overall effects of lipid peroxidation are decreased membrane fluidity, easy exchange of phospholipids between the two halves of the bilayer, increased leakiness of the membrane, damage of membrane proteins, inactivation of receptors, enzymes, and ion channels. Several aldehydes such as 4-hydroxy-2-nonenal (HNE) and MDA, as well as hydroxyl and keto fatty acids are formed as a result of PUFA
peroxidation. The aldehyde breakdown products form conjugates with DNA and proteins, for example, aldehydes formed in the mitochondria may be involved in causing cytoplasmic male sterility in maize because a restorer gene in this species encodes a mitochondrial aldehyde dehydrogenase (Moller et al., 2007).

**1.2.3 Oxidative damage to proteins**

Protein oxidation is the covalent modification of a protein induced by ROS, RNS, or byproducts of oxidative stress. It is a widespread phenomenon occurring under stress and commonly used as a diagnostic marker for oxidative stress. It has been reported extensively for proteins in mitochondria, (Ito et al., 2007; Moller et al., 2006) but until now not for the whole plant cell. Sulphur-containing amino acids (such as Cys and Met), and thiol groups are very susceptible sites for oxidation and are responsible for the metabolic redox regulation mechanism. Activated oxygen ($^{1}O_{2}$ and HO•) can abstract an H atom from cysteine residues to form a thyl radical that will cross-link to a second thyl radical to form disulphide bridges. Intra- or inter-molecular disulphide bonds can be formed between cysteine side chains and the reduced form can be regenerated by the thioredoxin or glutaredoxin systems (Moller et al., 2007). Cysteine can also form mixed disulphides with the cysteine-containing tripeptide glutathione and this might serve to protect the cysteine group against further oxidation (Ghezzi et al., 2003). Alternatively, oxygen can add to a methionine residue to form methionine sulphoxide derivatives in a reversible modification. Small HSPs in the chloroplast are known to be inactivated by methionine sulphoxidation, and reactivated by the enzyme methionine sulphoxide reductase using Trx as the reductant (Gustavsson et al., 2002). This reversible reaction is an important regulatory mechanism (Sundby et al., 2005), however, further oxidation appears to be irreversible and damaging to the protein. Apart from reactions involving the sulphur-containing amino acids, carbonylation is the most commonly occurring oxidative protein modification. The oxidation of protein amino acids – particularly Arg, His, Pro, Lys, Thr and Trp give free carbonyl groups in an irreversible reaction. Other types of protein modification under stress include oxidation of tryptophan and nitrosylation reactions (Moller et al., 2007). The oxidized proteins thus formed, aggregate to form non-degradable complexes (Davies et al., 2006) or may be degraded by autophagy (Xiong et al., 2007).
1.2.4 Oxidative damage to carbohydrates

The polysaccharides of plant cell wall are susceptible to cell wall loosening by OH• produced from ROS by cell wall-bound peroxidases under oxidative stress, releasing formic acid as the main breakdown product (Schopfer et al., 2002). HO• also reacts with free carbohydrates, such as sugars, and polyols (Smirnoff et al., 1989); reaction with mannitol removes HO• before it reacts with more vital cellular components (Shen et al., 1997). These oxidative modifications are not damaging as they are not detrimental to the modified components or the cell.

1.2.5 Oxidative damage to DNA

The susceptibility of DNA to free radical attack is due to its effective binding to metals involved in Fenton reactions, inducing numerous lesions in DNA that cause deletions, mutations and other lethal genetic effects. Characterization of this damage to DNA has indicated that both the sugar and the base moieties are susceptible to oxidation, causing base degradation, single strand breakage, and cross-linking to protein (Imlay and Linn, 1986). OH• is the most reactive, 1O2 primarily attacks guanine, and H2O2 and O2• do not react at all (Wiseman et al., 1996). 8-Hydroxyguanine is the most commonly observed modification (Moller et al., 2007). Cross-linking of DNA to protein is a consequence of OH• attack on either DNA or its associated proteins (Oleinick et al., 1986). The major sites of ROS production are the chloroplast and mitochondria; the DNA of these organelles is therefore more prone to oxidative damage (Thorslund et al., 2002), especially at specialized regions called hot spots (Halliwell et al., 1999). However, the presence of multiple copies of mtDNA and ctDNA enables the cell to select against negative mutations. The indirect effects involve creation of an extra ring in guanine by conjugation with MDA (Jeong et al., 2005) and changes in the regulation of gene expression by methylation of cytosines (Halliwell, 2006). As repair mechanisms such as; direct reversal of damage, replacement of base, and replacement of the whole nucleotide (Tuteja et al., 2001) are operative in plant system, the damages of moderate level are considered non-toxic.

1.3 Role of antioxidant system in plant defense

The ability of higher plants to scavenge active oxygen seems to be very important determinant of their tolerance to these stresses. Antioxidants are the first line of defence against free radical damage. There are several antioxidant enzymes and metabolites involved
in the scavenging of ROS in plants, and their activation are known to increase upon exposure to oxidative stress (Tanaka, 1994).

### 1.3.1 ROS scavenging antioxidant enzymes

Even under optimal conditions, many metabolic processes, including the chloroplastic, mitochondrial and plasma membrane-linked electron transport systems of higher plants, produce ROS. Furthermore, the imposition of biotic and abiotic stress conditions can give rise to excess ROS, resulting in oxidative damage at cellular level. Therefore, antioxidants and antioxidant enzymes function to interrupt the cascades of uncontrolled oxidation of each organelle (Fig 1.16). Data on antioxidant levels and the activity of antioxidant regenerating enzymes are somewhat contradictory, both decreases and increases in antioxidative capacity of the tissues have been reported (Gill and Tuteja, 2010). Such diversification partly arises from the response specificity of a particular plant species and from different experimental conditions (stress treatment, duration of stress, assay procedure and parameters measured). Plants control the ROS under stress with the help of several antioxidant enzymes such as SOD (E.C. No. 1.15.1.1), ascorbate peroxidase (E.C. No. 1.11.1.11), glutathione reductase (E.C. No. 1.6.4.2), catalase (E.C. No. 1.11.1.6) and peroxidase (E.C. No. 1.11.1.7).

![Scavenging system of ROS in higher plants](image)
1.3.1.1 Superoxide dismutase (SOD)

Metalloenzyme SOD is the most effective intracellular enzymatic antioxidant which is ubiquitous in all aerobic organisms and located in subcellular compartments prone to ROS mediated oxidative stress. The SOD family is composed of metalloprotein that catalyze the dismutation of $O_2^-$ radical to $O_2$ and $H_2O_2$. Three classes of SODs are known in plants, depending on the active site metal cofactor (Mn, Fe, or Cu/Zn). The Mn-SODs and Fe-SODs are structurally related, whereas Cu/Zn-SODs show no structural relationship to the other two. The Mn-SOD is resistant to both inhibitors KCN and $H_2O_2$, Cu/Zn-SOD is sensitive to both inhibitors whereas; Fe-SOD is resistant to KCN and sensitive to $H_2O_2$. The Mn-SOD is found in the mitochondria of eukaryotic cells and in peroxisomes; some Cu/Zn-SOD isozymes are found in the cytosolic fractions, and also in chloroplasts of higher plants (del Rio et al., 2002). The Fe-SOD isozymes are usually associated with the chloroplast compartment when present (Alscher et al., 2002). Peroxisomes and glyoxysomes of *Citrus vulgaris* have been shown to contain both Cu/Zn- and Mn-SOD activity (Sandalio et al., 1988). The upregulation of SODs is implicated in combating oxidative stress and are found to play a critical role in the survival of plants under stresses. Significant increase in SOD activity under salt stress has been observed in various plants viz. mulberry (Harinasut et al., 2004), *C. arietinum* (Kukreja et al., 2005) and *Lycopersicum esculentum* (Gapinska et al., 2008). Increased SOD activity has also been detected following Cd treatment in *Hordeum vulgare* (Guo et al., 2004), *A. thaliana* (Skorzynska-Polit et al., 2004), *O. sativa* (Hsu et al., 2004), *Triticum aestivum* (Khan et al., 2007) and *Vigna mungo* (Singh et al., 2008). Increase in SOD activity following drought stress was noted in three cultivars of *P. vulgaris* (Zlatev et al., 2006), *Alternanthera philoxeroides* (Wang et al., 2008) and *O. sativa* (Sharma et al., 2005). Yang et al., (2008) have shown combined effect of drought and low light in *Picea asperata* seedlings grown at two watering regimes and found that under high light condition, drought significantly increased the SOD activity in comparison to low light. Transgenic rice plants overexpressing OsMT1a (*Oryza sativa* metallothionein Type 1) have demonstrated enhanced drought tolerance (Yang et al., 2009). Protoplasts with Mn-SOD overexpression showed less oxidative damage, higher $H_2O_2$ content and significant increase in SOD and GR activities under photooxidative stress (Melchiorre et al., 2009). Overexpression of a Mn-SOD in transgenic Arabidopsis plants also showed increased salt tolerance (Wang et al., 2004). Furthermore, they showed that Mn-SOD activity as well as the activities of Cu/Zn-SOD, Fe-
SOD, CAT and POX was significantly higher in transgenic Arabidopsis plants than control (Wang et al., 2004). Further, the combined expression of Cu/Zn-SOD and APX in transgenic Festuca arundinacea plants led to increased tolerance to H2O2, Cu, Cd and As (Lee et al., 2007).

1.3.1.2 Catalase (CAT)

CATs are tetrameric heme containing enzymes with the potential to directly dismutate H2O2 into H2O and O2 and are indispensable for ROS detoxification during stressed conditions (Garg and Manchanda, 2009). CAT has one of the highest turnover rate of 6x10^6 conversions/mole/min. CAT is important in the removal of H2O2 generated in peroxisomes by oxidases involved in β-oxidation of fatty acids, photorespiration and purine catabolism. Apart from reaction with H2O2, CAT also reacts with hydroperoxides such as methyl hydrogen peroxide (Ali et al., 2006). A variable response has been observed under metal stress. Its activity was found to decline in Glycine max (Balestrasse et al., 2001), Capsicum annuum (Leon et al., 2002) and A. thaliana (Cho et al., 2005) whereas, its activity increased in O. sativa (Hsu et al., 2004), T. aestivum (Khan et al., 2007), and V. mungo roots (Singh et al., 2008) under Cd stress. Hsu and Kao (2007) have reported that pre-treatment of rice seedlings with H2O2 under non-heat shock conditions resulted in an increase in CAT activity and protected rice seedlings from subsequent Cd stress. An increase in CAT activity under salt treatment was seen in C. arietinum (Eyidogan and Oz, 2005) while a decrease was reported in A. doliolum (Srivastava et al., 2005). Simova-Stoilova et al. (2010) have reported increased CAT activity in wheat under drought stress but it was higher especially in sensitive varieties. In another study, Sharma and Dubey (2005) reported a decrease in CAT activity in rice seedlings following drought stress. Transgenic rice plants overexpressing OsMT1a showed increase in CAT activity and thus enhanced tolerance to drought (Yang et al., 2009). CAT activity of transgenic plants was approximately two-fold higher than that of WT which correlated with enhanced tolerance under Cd stress (Guan et al., 2009). There have been many reports on CAT producing abiotic stress tolerant transgenic plants (Table 1).

1.3.1.3 Guaiacol peroxidase (POX)

POX is a heme-containing protein, which oxidizes certain substrates at the expense of H2O2, and rids the cell of excess peroxide produced by metabolism under both normal and stress conditions. POX decomposes indole-3-acetic acid (IAA) and has a role in the lignin biosynthesis and defence against biotic stresses by consuming H2O2 in the cytosol, vacuole,
and cell wall as well as in extracellular space. POX utilizes aromatic electron donors such as guaiacol and pyragallol resulting in the oxidation of ascorbate (Asada, 2000; Jebara et al., 2005).

**Table 1.1**: ROS scavenging enzymatic and non-enzymatic antioxidants and their role in transgenic plants for abiotic stress tolerance.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Source</th>
<th>Target transgenic</th>
<th>Response in transgenic plants</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Superoxide dismutase (SOD)</td>
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<tr>
<td>Cu/Zn-SOD</td>
<td><em>Oryza sativa</em> L.</td>
<td><em>Nicotiana tabacum</em></td>
<td>Enhanced tolerance to salt, water, PEG stresses and enhancement in chloroplast antioxidant system</td>
<td>Badawi et al., 2004</td>
</tr>
<tr>
<td>Mn-SOD</td>
<td><em>Arabidopsis</em></td>
<td><em>Arabidopsis</em> ecotype</td>
<td>Salt tolerance, Increased Mn-SOD, Cu/Zn-SOD, Fe-SOD, CAT and POX under salt stress</td>
<td>Wang et al., 2004</td>
</tr>
<tr>
<td>Mn-SOD + APX</td>
<td><em>Nicotiana tabacum</em></td>
<td><em>Festuca arundinacea</em></td>
<td>H$_2$O$_2$, and Cu, Cd and As tolerance, Low MDA, ion leakage and chlorophyll degradation and increase in DOS</td>
<td>Lee et al., 2007</td>
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<tr>
<td>Catalase (CAT)</td>
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<tr>
<td>CAT</td>
<td><em>Triticum aestivum</em> L.</td>
<td><em>Oryza sativa</em> cv. Yuukara or Matsumae</td>
<td>Low temperature stress tolerance due to effective detoxification of H$_2$O$_2$ by CAT</td>
<td>Matsumura et al., 2002</td>
</tr>
<tr>
<td>CAT3</td>
<td><em>Brassica juncea</em></td>
<td><em>Nicotiana tabacum</em></td>
<td>Cd stress tolerance, better seedling growth and longer roots</td>
<td>Gichner et al., 2004</td>
</tr>
<tr>
<td>Ascorbate peroxidase (APX)</td>
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<tr>
<td>cAPX</td>
<td><em>Pisum sativum</em></td>
<td><em>Lycopersicon esculentum</em></td>
<td>Enhanced tolerance to UV-B, heat, drought and chilling stresses, increase in APX activity</td>
<td>Wang et al., 2006</td>
</tr>
<tr>
<td>APX3</td>
<td><em>Arabidopsis thaliana</em></td>
<td><em>Nicotiana tabacum</em> cv. Xanthi</td>
<td>Water deficit tolerance with higher photosynthesis</td>
<td>Yan et al., 2003</td>
</tr>
<tr>
<td>APX1</td>
<td><em>Hordeum vulgare</em> L.</td>
<td><em>Arabidopsis thaliana</em></td>
<td>Salt tolerance due to higher APX, SOD, CAT and GR and low H$_2$O$_2$ and MDA content</td>
<td>Xu et al., 2008</td>
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<td>Glutathione reductase (GR)</td>
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<tr>
<td>GR</td>
<td><em>Escherichia coli</em></td>
<td><em>Triticum aestivum</em>, cv.</td>
<td>GR protoplast Higher GSH content and GSH/GSSG ratio than control, no increase in SOD and GR activities</td>
<td>Melchiorre et al., 2009</td>
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<td></td>
<td></td>
<td><em>Oasis</em></td>
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<td></td>
<td></td>
<td><em>Gossypium hirsutum</em> L.</td>
<td>Chilling stress tolerance and photoprotection</td>
<td>Kornyeyev et al., 2003</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>cv. Coker 312</em></td>
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<td>Dehydroascorbate reductase (DHAR)</td>
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<td>DHAR</td>
<td><em>Arabidopsis thaliana</em></td>
<td><em>Nicotiana tabacum</em></td>
<td>Drought and salt tolerance with higher DHAR activity and reduced ASC content</td>
<td>Eltayeb et al., 2007</td>
</tr>
<tr>
<td>DHAR</td>
<td><em>Oryza sativa</em></td>
<td><em>Arabidopsis thaliana</em> L.</td>
<td>Salt tolerance due to slight increase in DHAR activity and ASC</td>
<td>Chen and Gallie, 2005</td>
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<td></td>
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<td>(ecotype Wassilewskija)</td>
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<td>Proline (Pro)</td>
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<td>P5CS</td>
<td><em>Vigna aconitifolia</em> L.</td>
<td><em>Triticum aestivum</em> L.</td>
<td>Drought tolerance due protection mechanisms against oxidative stress</td>
<td>Vendruscolo et al., 2007</td>
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<td><em>D200126</em></td>
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<tr>
<td>P5CR</td>
<td><em>Triticum aestivum</em></td>
<td><em>Arabidopsis thaliana</em> L.</td>
<td>Salt tolerance</td>
<td>Ma et al., 2008</td>
</tr>
</tbody>
</table>

The activity of POX varies considerably depending upon plant species and stress condition. Increased POX has been reported in Cd-exposed plants of *T. aestivum* (Milone et al., 2003), *A. thaliana* (Cho and Seo, 2005) and *C. demersum* (Arvind and Prasad, 2003). An increase in POX activity under drought stress was reported in liquorice (Pan et al., 2006),
sunflower (Gunes et al., 2008), and polar (Xiao et al., 2008). An increase in POX activity in both the leaf and root tissues of *Vigna radiata* (Panda and Das, 2001), *O. sativa* (Koji et al., 2009) and *Phaseolus vulgaris* (Jebara et al., 2005) have also been reported under salinity stress. Under sublethal salinity conditions, level of POX activity has been used as a potential biomarker to evaluate the intensity of stress. An intrinsic role has been suggested for POX in resisting oxidative damage in plants (Cavalcanti et al., 2007; Koji et al., 2009).

1.3.1.4 Glutathione reductase (GR)

GR, a flavo-protein oxidoreductase found in both prokaryotes and eukaryotes (Romero-Puertas et al., 2006) is a potential enzyme of the ASC-GSH cycle and plays an essential role in defence system against ROS by sustaining the reduced status of GSH. It is localized predominantly in the chloroplasts, but can also been found in mitochondria and cytosol (Edwards et al., 1990; Creissen et al., 1994). It catalyzes the reduction of the oxidized form of glutathione (GSSG) utilizing NADPH, and is thus important for maintaining the GSH pool (Reddy and Raghavendra, 2006; Chalapathi and Reddy, 2008). The tripeptide GSH acts as a disulphide reductant to protect the thiol groups of enzymes, regenerate ascorbate, and react with $^1$O$_2$ and OH$^*$. GSH detoxifies herbicides by conjugation, either spontaneously or by the activity of a glutathione-S-transferase (GST), and regulates gene expression in response to environmental stress and pathogen attack. GSH also participates in the regeneration of ascorbate from DHA via the enzyme DHAR (Noctor and Foyer, 1998a). The role of GSH and GR in H$_2$O$_2$ scavenging has been well established in the Halliwell-Asada pathway (Noctor and Foyer, 1998a; Asada, 2000). GR catalyses the rate-limiting last step of the Halliwell-Asada pathway. GR activity was found to be increased in the presence of Cd in *C. annuum* (Leon et al., 2002), *A. thaliana* (Skorzynska-Polit et al., 2004), *V. mungo* (Singh et al., 2008), and *T. aestivum* (Khan et al., 2007). Eyidogan and Oz (2005) have reported increased GR activity in the leaf tissue of *C. arietinum* L. cv. Gokce under salt stress, whereas, Kukreja et al., (2005) have noted increased GR activity in *C. arietinum* roots following salt stress. Srivastava et al., (2005) have reported contrasting profile of the enzyme, wherein it declined in *A. doliolum* under Cu$^{2+}$ stress, and increased under salt stress. Sharma and Dubey (2005) have noted a significant increase in GR activity in drought stressed *O. sativa* seedlings. Bashir et al., (2007) while studying the expression patterns and enzyme activities of GR in graminaceous plants under Fe-sufficient and Fe-deficient conditions by isolating cDNA clones for chloroplastic GR (HvGR1) and cytosolic GR (HvGR2) from barley have shown that in vitro GR activity and the specific activity of HvGR1 was 3X higher
than HvGR2. Transgenic plants with less GR activity have shown enhanced sensitivity to oxidative stress. It was suggested that GR plays an important role in the regeneration of GSH and thus protects against oxidative stress also by maintaining the ASH pool (Ding et al., 2009). Also, transgenic plants that produce GR have been found to be abiotic stress tolerant (Table 1).

### 1.3.1.5 Ascorbate peroxidase (APX)

While CAT reduces H$_2$O$_2$ levels in peroxisomes, APX performs this function in chloroplast and cytosol of plant cells. APX uses ascorbate as a hydrogen donor to break down H$_2$O$_2$ to H$_2$O and monodehydroascorbate (MDHA) (Asada, 2000). APX has a higher affinity for H$_2$O$_2$ (µM range) than CAT and POX (mM range) and it may have a more crucial role in the management of ROS during stress (Gill and Tuteja, 2010). APX has two cytosolic forms, with defensive roles; and a membrane-bound form, that scavenges H$_2$O$_2$, modulates quantum efficiency and controls electron transport in conjunction with the ascorbate-glutathione (AsA-GSH) cycle (Foyer and Noctor, 2005). In chloroplasts, SOD and APX enzymes exist in both soluble and thylakoid-bound forms (Asada, 2000). O$_2$•¯ generated at the membrane surface can thus be trapped and converted immediately to H$_2$O$_2$, which in turn, is scavenged by membrane-bound APX (Asada, 2000). The mRNA of cytosolic APX is known to be up-regulated during drought stress in the alfalfa nodule (Naya et al., 2007). Hossain et al. (2009a) have noted a significant increase in APX activity in citrus plants that during water logging. Transgenic Arabidopsis plants overexpressing cytosolic APXs exhibited increased tolerance to salt stress compared to wild-type plants (Li et al., 2009). Giacomelli et al., (2007) have observed that A. thaliana deficient in two chloroplast APXs (stromal APX and thylakoid APX) show accelerated light-induced necrosis under cellular ASC. Simultaneous over expression of Cu/Zn-SOD and APX genes in chloroplasts of transgenic tall fescue plants have exhibited tolerance to abiotic stresses (Lee et al., 2007).

### 1.3.1.6 Polyphenol oxidase (PPO)

Polyphenol oxidases or tyrosinases (PPO) enzymes with a dinuclear copper centre are able to insert oxygen in an ortho-position to an existing hydroxyl group in an aromatic ring, followed by the oxidation of the diphenol to the corresponding quinone. A major focus of research in PPO has been its potential role in defense mechanism in plants especially to biotic stresses. It has been possible to manipulate levels of PPO expression using a variety of modern techniques. Thipyapong et al., (2004) have introduced antisense PPO cDNA into
tomato plants and examined the resistance of the plants to the pathogen *Pseudomonas syringae*. Although down regulation of PPO did not affect the growth and development, the plants had increased susceptibility to the pathogen. On the other hand, over-expression of PPO in tomato plants (Li and Steffens, 2002) showed enhanced resistance to the same pathogen. The involvement of PPO in imparting resistance of pearl millet to downy mildew has been demonstrated by Raj et al., (2006). Polyphenol oxidases (PPOs) have been proposed to function in the Mehler reaction, photoreduction of molecular oxygen by PSI (Thipyapong et al., 2004). Under drought stress where photosynthesis is reduced, the Mehler reaction may provide a non-destructive sink for absorbed light energy not used in photochemistry. If so, water-stressed plants with suppressed PPO are expected to exhibit photooxidative damage and plants with elevated PPO may show increased stress tolerance.

### 1.3.2 ROS scavenging non-enzymatic antioxidants

#### 1.3.2.1 Reduced glutathione (GSH)

GSH (γ-glutamylcysteinglycine) is an abundant tripeptide in plant tissues, occurring in virtually all cellular components (Noctor and Foyer, 1998a); and plays a central role in several physiological processes including regulation of sulfate transport, signal transduction, conjugation of metabolites, detoxification of xenobiotics (Xiang et al., 2001), expression of stress-responsive genes (Mullineaux et al., 2005), cell differentiation, cell death and senescence, pathogen resistance and enzymatic regulation (Rausch et al., 2005). In combination with its oxidized form (GSSG), GSH maintain redox equilibrium in the cellular compartments. This latter function is of great biological importance because it allows for the fine-tuning of the cellular redox environment under normal conditions, and upon the onset of stress, provides the basis for GSH stress signaling (Wang et al., 2008). A central nucleophilic cysteine residue is responsible for the high reductive potential of GSH, which scavenges cytotoxic H$_2$O$_2$ and reacts non-enzymatically with other ROS, such as $^1$O$_2$, O$_2$$^*$, and OH$^-$ (Noctor and Foyer, 1998a; Wang et al., 2008). The central role of GSH in the antioxidative defense is due to its ability to regenerate another powerful water-soluble antioxidant, ASC, via the ASC-GSH cycle [Fig 1.16] (Noctor and Foyer, 1998a; Halliwell, 2006). The role of GSH in the antioxidant defense system provides a strong basis for its use as a stress marker. GSH is a precursor of phytochelatins (PCs), which plays an important role in controlling cellular heavy metal concentration. Increased concentration of GSH has been observed with
the increasing Cd concentration in *P. sativum* (Metwally et al., 2005), *Sedum alfredii* (Sun et al., 2007), and *V. mungo* (Molina et al., 2008). Xiang et al., (2001) observed that plants with low levels of GSH were highly sensitive to even low levels of Cd$^{2+}$ due to limited PC synthesis. It has been reported that when the intensity of a stress increases, GSH concentrations usually decline and redox state becomes more oxidized, leading to deterioration of the system (Tausz T., et al., 2004).

1.3.2.2 Ascorbate (ASC)

ASC is one of the most powerful antioxidants and is present in most plant cell types, usually being higher in photosynthetic cells and meristems (and some fruits). Its concentration is reported to be highest in mature leaves with fully developed chloroplast and highest chlorophyll (Smirnoff, 2005). ASC donates electrons in a wide range of enzymatic and non-enzymatic reactions making it the main ROS-detoxifying compound in the aqueous phase (Noctor and Foyer, 1998a). It provides protection to cellular organelles by directly scavenging O$_2$•$^-$ and OH$,^*$, regenerating α-tocopherol from tocopheroxyl radical, and by preserving activities of transition-metal containing enzymes (Noctor and Foyer, 1998a; Smirnoff, 2005). The ASC redox system consisting of L-ascorbic acid and its unstable oxidized forms, monodehydroascorbate (MDHA) and dehydroascorbate (DHA) are involved in the regeneration of reduced ASC. Support for the actual role of DHAR, GSH and GR in maintaining the foliar ASC pool has come from transformed plants overexpressing GR (Foyer et al., 1995). The regeneration of reduced ASC is extremely important, because fully oxidized DHA has a short half-life and would be lost unless it is reduced back. Improved tolerance to oxidative stress in *N. tabacum* and *Populus x Canescens* plants has been found to be associated with higher foliar concentration of ASC. Elevated levels of ASC, DHA and GSH have also been reported under UV-B stress in *C. auriculata* seedlings (Agarwal, 2007). Yang et al., (2008) have reported that high light condition and drought significantly increased the ASC content in *P. asperata* seedlings. Agarwal (2007) has reported that the ASC and DHA content as well as the GSH/GSSG content and GSH:GSSG was significantly increased in *C. auriculata*. On the other hand, a decrease in the ASC in the roots and nodules of *Glycine max* has been observed under Cd stress (Leon et al., 2002). Similar decline in ASC under heavy metal stress has been reported in the leaves of *A. thaliana* (Skorzynska-Polit et al., 2004) and *P. sativum* (Romero-Puertas et al., 2007).
1.3.2.3 Phenolic compounds

Phenolics are diverse secondary metabolites (flavonoids, tannins, hydroxycinnamate esters and lignin) abundant in plant tissues (Grace and Logan, 2000). Polyphenols possess ideal structural chemistry for free radical scavenging activity. Antioxidative properties of polyphenols arise from their high reactivity as hydrogen or electron donors, and from the ability of the polyphenol-derived radical to stabilize and delocalize the unpaired electron (chain-breaking function), and from their ability to chelate transition metal ions (termination of the Fenton reaction) (Rice-Evans et al., 1997). Another mechanism underlying the antioxidative properties of phenolics is the ability of flavonoids to alter peroxidation kinetics by modification of the lipid packing order and to decrease fluidity of the membranes (Arora et al., 2000). These changes could sterically hinder diffusion of free radicals and restrict peroxidative reactions. Moreover, it has been shown recently that phenolic compounds can be involved in the $\text{H}_2\text{O}_2$ scavenging cascade in plant cells (Takahama and Oniki, 1997).

\textit{α-Tocopherol (Vitamin E):}

Tocopherols are essential components of biological membranes where they have antioxidant and non-antioxidant functions (Kagan, 1989). Out of four isomers of tocopherols ($α$-, $β$-, $γ$-, $δ$-) found in plants, $α$-tocopherol has the highest antioxidative activity due to the presence of three methyl groups in its molecular structure (Kamal-Eldin, 1996). It has been shown to prevent the chain propagation step in lipid autooxidation making it an effective free radical trap (Fryer, 1992). Additionally, it has been estimated that one molecule of $α$-tocopherol can scavenge up to 120 molecules of $^1\text{O}_2$ by resonance energy transfer (Munne-Bosch, 2005). $α$-tocopherol donates $\text{H}^+$ to PUFA oxidation products like alkoxy ($\text{LO}^\bullet$), lipid peroxyl ($\text{LOO}^\bullet$) and alkyl ($\text{L}^\bullet$) radicals at the membrane-water interphase with consequent tocopheroxyl radical ($\text{TOH}^\bullet$) formation (Kamal-Eldin, 1996; Buettner, 1993). Regeneration of the $\text{TOH}^\bullet$ back to its reduced form can be achieved by ASC, GSH (Serbinova et al., 1994) or coenzyme Q (Kagan, 1989). In addition to antioxidant functions $α$-tocopherol has several non-antioxidant functions in membranes; such as, stabilization of membrane structures, modulation of membrane fluidity, and membrane permeability to small ions and molecules (Fryer et al., 1992). There is also recent evidence of interaction between PS II with $α$-tocopherol and $α$-tocopherol quinone (Kruk et al., 2000). Oxidative stress is known to activate the expression of genes responsible for the synthesis of tocopherols in higher plants (Wu et al., 2007). Increased levels of $α$-tocopherol and ASC have been found in tomato following trizole treatment which may help in protecting membranes from oxidative damage.
and thus chilling tolerance in tomato plants (Shao et al., 2007). Increase in α-tocopherol during water stress in plants has also been reported by many workers (Wu et al., 2007; Shao et al., 2007). Srivastava et al., (2005) reported a general induction in α-tocopherol content in *A. doliolum* under NaCl and Cu$^{2+}$ stress.

**Flavonoids:**

Flavonoids occur widely in the plant kingdom and can be classified into flavonols, flavones, isoflavones, and anthocyanins based on their structure. They have many functions like pigmentation, protection against UV light, defence against phytopathogens, role in plant fertility and germination of pollen; and acting as signal molecules in plant-microbe interactions (Olsen et al., 2010). Flavonoids are among the most bioactive plant secondary metabolites outperforming well-known antioxidants, such as ASC and α-tocopherol in scavenging ROS formed under adverse environmental conditions (Lovdal et al., 2010). Mutant plants (tt4, deficient in chalcone synthase; and tt5, deficient in chalcone isomerase), unable to accumulate flavonoids were found to be more sensitive to UV light (Filkowski et al, 2004). The most abundant flavonoids, flavonols accumulate in their glycosylated form after an inductive light treatment and absorb UV-B light in the 280-320 nm region and are therefore regarded as effective UV filters (Solovchenko et al., 2003). It has been found that there is considerable increase in flavonoid levels following biotic and abiotic stresses, such as wounding, drought, metal toxicity and nutrient deprivation (Winkel-Shirley, 2002). Flavonoids are involved in the resistance to pathogens and by acting as feeding deterrents (Gould and Lister, 2006).

**Phenolic acids and phenols:**

Acidic compounds incorporating phenolic groups have been repeatedly implicated as active antioxidants (Taiz and Zeiger, 1991). Caffeic acid, chlorogenic acid and its isomers including 4-*o*-caffeoylquinic acid were isolated from sweet potatoes. Chlorogenic acid was found to be the most abundant phenolic acid in the plant extract and also the most active antioxidant (Taiz and Zeiger, 1991). Fatty acids of the seed *Phalaris canariensis* were predominantly unsaturated indicating that the presence of large amounts of esters of caffeic acid present was acting to protect them from oxidation (Fengel and Wegener, 1984). Higher amounts of phenolic acid are known to cause lignification of the water transporting tissues, enabling the colonization of primitive plants on dry land (Taiz and Zeiger, 1991). The antioxidant activity of rosmaridiphenol, a diterpene derivative isolated from rosemary, a
drought tolerant species has antioxidant activity exceeding that of the synthetic antioxidants, BHA and BHT. Related phenolic diterpenes with antioxidant activity have also been isolated from this plant (Munne-Bosch and Alegre, 2000). Carnosic acid displays high antioxidant activity and is responsible for protection of biological membranes and chloroplast from oxidative stress (Haraguchi et al., 1995). Curcumin, an antioxidant from turmeric functioning by metal ion chelation has greater antioxidant potency than α-tocopherol. Polyhydroxylated chalcones such as butein which are biosynthetic intermediates between cinnamic acids and flavonoids, also show considerable antioxidant activity for lard (Haraguchi et al., 1995).

1.3.2.4 Nitrogenous compounds

Alkaloids:

Increasing evidence from a variety of sources indicates that the basic nitrogen compounds of higher plants include many representatives that are potent inhibitors of various oxidative stresses. These include the alkaloids cepharanthine and caffeine which possess antioxidative activity comparable to BHA and BHT (Vaneil et al., 1980). Several other alkaloids, such as strychnine and brucine physically quench ⁠\(^1\)⁠O\(_2\) without chemically destroying it. Thus, in principle, they could inactivate many molecules of singlet oxygen per molecule of alkaloid. Alkaloids of quinolizidine type, for example sparteine have been found to be stored principally in the epidermal cells of lipinus suggesting an antifeedant, antioxidant and UV light filtering role (Ahmed et al., 1989).

Proline (Pro):

In addition to being an osmolyte, Pro is considered a potent antioxidant and potential inhibitor of PCD. The synthesis of L-Pro from L-glutamic acid via \(\Delta^1\)-pyrroline-5-carboxylate (P5C) is catalyzed by the activities of the enzymes \(\Delta^1\)-pyrroline-5-carboxylate synthetase (P5CS) and \(\Delta^1\)-pyrroline-5-carboxylate reductase (P5CR) in plants (Verbruggen and Hermans, 2008). On the other hand, mitochondrial enzymes Pro dehydrogenase (oxidase) (ProDH) and P5C dehydrogenase (P5CDH) metabolize L-Pro into L-Glu via P5C.

It is well documented that following salt, drought and metal stress, the dramatic accumulation of Pro may be due to increased synthesis or decreased degradation (Ashraf and Foolad, 2007; Trovato et al., 2008; Chen and Dickman, 2005). Free Pro has been proposed to act as an osmoprotectant, a protein stabilizer, a metal chelator, an inhibitor of LPO, and OH\(^*\) and \(\cdot\)O\(_2\) scavenger (Ashraf and Foolad, 2007; Trovato et al., 2008). In an interesting study Chen and
Dickman, (2005) have reported that addition of Pro to DARas mutant C. trifolii cells effectively quenched ROS levels and prevented cell death. Furthermore, Pro also protected wild type C. trifolii cells from UV light, salt, heat and H$_2$O$_2$ stress. The ability of Pro to scavenge ROS and inhibit ROS-mediated apoptosis can be an important function in response to cellular stress. Enhanced synthesis of Pro under drought or salt stress has been implicated as a mechanism to alleviate cytoplasmic acidosis and maintain NADP$^+$:NADPH at values compatible with metabolism (Hare and Cress, 1997). An additional advantage of the refilling of NADP$^+$ supply by Pro synthesis may be to support redox cycling, which is especially important in plant antioxidant defense mechanisms during stress (Babiychuk et al., 1995). It has also been noted that salt stress increased the accumulation of Pro in the leaves of two rice cultivars differing in salt tolerance (Demiral and Türkan, 2005). Several lines of evidence suggest the important role for Pro synthesis in potentiating pentose-phosphate pathway activity (Hare and Cress, 1997) for the production of NADPH needed to maintain GSH and ASC in the reduced state for antioxidative defense reactions. There are reports of proline levels correlating with ratio of GSH:GSSG and MDA in metal treated algae (Siripornadulsil et al., 2002). Gajewska and Sklodowska (2005) while studying the effect of Ni on pea plants have found that stimulation of GST activity and accumulation of Pro in the tissues rather than antioxidative enzymes are involved in response of pea plants to Ni stress. Transgenic tobacco cells (silenced at their tobacco Pro dehydrogenase(NtProDH) gene) accumulated more Pro than WT cells and showed enhanced osmotolerance (Tateishi et al., 2005). Also, Pro is known to ameliorate the inhibition of growth of BY-2 cells under salt stress. BY-2 cells showed significant decrease in the activities of SOD, CAT and POX under salt stress but exogenously applied Pro alleviated the reduction in CAT and POX activities under salt stress (Hoque et al., 2007). The potato transgenic plants overexpressing P5CS cDNA from A. thaliana showed significant increase in Pro levels under salt stress and showed less altered tuber yield in comparison to control plants (Hmida-Sayari et al., 2005). Constitutive and stress induced expression of P5CS cDNA in transgenic O. sativa have been shown to confer higher tolerance against salt stress and water deficiency (Su and Wu, 2004). Similarly, overexpression of Vigna aconitifolia P5CS cDNA under the control of a stress-induced promoter complex-AIPC resulted in enhanced Pro accumulation under water deficit. It has also been found that overexpression of Pro biosynthetic pathway genes enhance the abiotic stress tolerance in transgenic plants (Table 1).

Chlorophyll:
Chlorophyll is one of the major chloroplast components for photosynthesis, and relative chlorophyll content has a positive relationship with photosynthetic rate. The decrease in chlorophyll content under drought stress has been considered a typical symptom of oxidative stress and may be the result of pigment photo-oxidation and chlorophyll degradation. Photosynthetic pigments are important to plants mainly for harvesting light and production of reducing powers. Both the chlorophyll a and b are prone to water deficit (Farooq et al., 2009b). Drought stress is known to cause a large decline in the chlorophyll a-, b- and total chlorophyll content in different sunflower varieties (Manivannan et al., 2007). Exposure of two olive cultivars to reduced irrigation led to lower Chl (a + b) contents. Loss of chlorophyll contents under water stress is considered to be the main cause of inactivation of photosynthesis. Furthermore, water deficit induced reduction in chlorophyll content has been ascribed to loss of chloroplast membranes, excessive swelling, distortion of the lamellae vesiculation, and the appearance of lipid droplets (Kaiser et al., 1981). From a physiological perspective, leaf chlorophyll content is a parameter of significant interest in its own right, on majority of chlorophyll loss in plants in response to water deficit occurs in the mesophyll cells with a lesser amount being lost from the bundle sheath cells.

Carotenoids:

Plants have evolved several mechanisms for getting rid of excess energy in photosynthetic membranes, some of which involve isoprenoid compounds. In all photosynthetic organisms, the carotenoids β-carotene and zeaxanthin serve important photoprotective role, either by dissipating excess excitation energy as heat or by scavenging ROS and suppressing LPO (Gill and Tuteja, 2010). The lipid soluble antioxidants carry out three major functions in plants; (i) absorb light at wavelength between 400 and 550 nm and transfer it to the Chl (an accessory light harvesting role) (Sieferman-Harms, 1987). (ii) protect the photosynthetic apparatus by quenching a triplet sensitizer (Chl3), 1O2 and other harmful free radicals which are naturally formed during photosynthesis (an antioxidant function) (Collins, 2001). (iii) are important for the PSI assembly and the stability of light harvesting complex proteins as well as thylakoid membrane stabilization (a structural role) (Niyogi et al., 2001). An increase in carotenoid content has been reported following Cd stress in (Foyer and Harbinson, 1994). It is believed that some isoprenoids (including several carotenoids and tocopherols) play an effective role in photoprotection (Peñuelas et al., 2005). Furthermore, it has been proved that monoterpene improved thermotolerance at elevated temperatures and that monoterpene had a protecting role against oxidative stress (Loreto et
al., 2004). Thus, altering levels of carotenoids could contribute to alleviation of oxidative stress.

**Polyamines:**

Polyamines, mainly putrescine (Put), spermidine (Spd) and spermine (Spm), are polycationic compounds of low molecular weight which accumulate under a variety of abiotic stress conditions (Krishnamurthy and Bhagwat, 1989). They are regarded as plant growth regulators that are involved in a broad spectrum of physiological processes, such as embryogenesis, cell division, morphogenesis, and development (Liu et al., 2006a). They play an integral part in plant stress response either in free or conjugated form (Alcázar et al., 2006; Bouchereau et al., 1999). An increase in spermidine synthase activity and spermidine content in leaves of *Arabidopsis* has been found to be associated with tolerance to chilling, freezing, salinity, hyperosmosis, drought and paraquat toxicity (Kasukabe et al., 2004). Under stress, plants show specific reaction response in terms of polyamine fluctuation. Thus, changes in cellular polyamines under stress provide clues on its possible implication in stress response, however there is no evidence for its role in counteracting stress.

**1.3.2.5 Total soluble sugars (TSS)**

Small soluble sugars and the enzymes associated with their metabolic pathways are widely believed to be connected to oxidative stress and ROS signaling (Takahashi and Murata, 2008; Suzuki and Mittler, 2006). Endogenous sugar availability is known to feed the oxidative pentose phosphate pathway (Debnam et al., 2004), creating reducing power for glutathione (GSH) production, contributing to \( \text{H}_2\text{O}_2 \) scavenging. The longer water-soluble oligo- and polysaccharides, such as fructans, are believed to be effective candidates for capturing ROS in tissues exposed to a wide range of environmental stresses (Van den and Valluru, 2009). Fructans are known to protrude deep between the head groups of the membranes to stabilize them (Vereyken et al., 2003) by temporarily scavenging \( \text{OH}^* \) and \( \text{OOH}^* \) radicals produced in the vicinity of these membranes by peroxidases during the hydroxylic cycle of these enzymes (Dunand et al., 2007). In this process, the fructans (other sugars/sugar-like compounds) are converted into (less harmful) fructan radicals. It has been proposed that such sugar radicals could be recycled back into sugars with the help of phenolic compounds or anthocyanins (Van den and Valluru, 2009). Interestingly, the synthesis of phenolic compounds and anthocyanins can also be stimulated by sugar-mediated signalling.
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and metabolic pathways. Among a range of TSS tested, sucrose has shown the strongest antioxidant capacity in vitro (Nishizawa et al., 2008). At low concentrations, sucrose might serve as a substrate or signal for stress-induced modifications, whereas at higher concentrations it may function directly as a protective agent co-operating cytoplasmic antioxidant systems (Van den and Valluru, 2009). Similarly, sugar alcohols (mannitol, inositol, sorbitol), also possess ROS scavenging capacities. In tobacco, mannitol is believed to protect thioredoxin, ferredoxin, GSH and the thiol-regulated enzyme phosphoribulokinase against OH$^-$ radicals. The targeting of mannitol biosynthesis to chloroplasts in transgenic tobacco plants is found to cause an increased resistance to methyl viologen-induced oxidative stress (Shen et al., 1997).

TSS do not only function as antioxidants but also act as metabolic resources, structural constituents of cells, and regulators of various processes associated with plant growth and development (Jang and Sheen; 1997; Loreti et al., 2001). Sugar signalling pathways interact with stress pathways into a complex network to modulate metabolic plant responses (Tran et al., 2007; Ho et al., 2001). TSS may either act directly as negative signals or as modulators of plant sensitivity and thus, they can also play important roles in cell responses to stress-induced remote signals. TSS act as primary messengers and regulate signals that control the expression of different genes involved in plant growth and metabolism (Chen et al., 2006). They regulate the growth and metabolism by modulation of gene expression and enzymes activities in both sugar exporting (source) and importing (sink) tissues. This ensures optimal synthesis and use of carbon and energy resources (Coruzzi and Bush, 2001). Differential source-sink effects on metabolism induced by unfavourable environmental factors lead to a differential expression of several proteins related to carbohydrate metabolism e.g., enzymes related to starch biosynthesis and sucrose metabolism (Stitt and Hurry, 2002). In addition, genes whose products are involved in another metabolic pathways and cellular functions are also positively regulated by TSS (Kim et al., 1991). In contrast, many genes are negatively regulated by sugars; for example, TSS represses expression of α-amylase genes in suspension cells and germinating embryos of rice (Yu et al., 1996). Relevant physiological studies have shown that TSS significantly contributes to the mechanisms of adaptation to salt stress by osmoregulation (Parida et al., 2002).
1.4 Metabolic enzymes

1.4.1 β-Amylase (AMY)

AMY is an exoamylase that hydrolyzes α-1,4 glycosidic linkages of polyglucan chains at the nonreducing end to produce maltose. The primary function of AMY is involvement in starch breakdown in plants (Kossmann and Lloyd, 2000). Based on in vitro studies, β-AMY plays a major role in transitory starch breakdown (Scheidig et al., 2002). Down-regulation of a chloroplast localized AMY using antisense methods resulted in a starch excess phenotype in potato (*Solanum tuberosum*) leaves compared to wildtype plants (Scheidig et al., 2002). Additionally, the product of AMY is translocated to the cytosol by a maltose translocator to be metabolized to glucose units by cytosolic glucosyltransferases during transitory starch degradation (Lu and Sharkey, 2004). AMY exhibits a complex regulation. Expression and activity are regulated by light (Chandler et al., 2001), sugars (Maeo et al., 2001), phytohormones (Wang et al., 1996), proteolytic cleavage (Sopanen and Lauriere, 1989), and abiotic stresses such as osmotic stress (Dreier et al., 1995; Datta et al., 1999), salt stress (Dreier et al., 1995; Datta et al., 1999), cold (Seki et al., 2001; Sung, 2001), and heat stress (Dreier et al., 1995).

1.4.2 Acid phosphatase (AP)

APs form a group of enzymes catalyzing hydrolysis of a variety of phosphate esters in the acidic environments. They are believed to increase orthophosphate (Pi) availability under phosphorous deficient conditions (Vance et al., 2003). Organic P comprises 30-80% of the total P in the soil (Dalal, 1978). For organic P sources in the soil to be used, they must be first hydrolyzed by APs. Free soluble Pi play a vital role in many biological processes including photosynthesis, respiration, enzyme regulation, energy transfer, metabolic regulation, important structural constituents of biomolecules like phytin bodies in the ungerminated seeds, protein and nucleotide phosphorylation (Fincher, 1989; Ehsanpour and Amini, 2003). Although, there are many controversial issues with AP accumulation and stress resistance, it is believed that high levels can be beneficial to the stressed plant (Ehsanpour and Amini, 2003). Salt, water, and osmotic stresses have also been reported to increase AP activity (Barrett-Lennard et al., 1982; Szabo-Nagy et al., 1992). It has been demonstrated that drought induced an increase in AP activity without exerting significant changes in the Pi level (Barrett-Lennard et al., 1982). Szabo-Nagy et al. (1992) also demonstrated that the induction of AP under osmotic and salt stresses was not accompanied...
by a decrease in Pi level. However, AP does play an adaptive role under stress conditions (Ehsanpour and Amini, 2003).

1.4.3 Invertase (INV)

INV catalyses the hydrolytic cleavage of the transport sugar sucrose released into the apoplast. This mechanism contributes to long-distance assimilate transport, provides the substrate to sustain heterotrophic growth and generates metabolic signals known to affect various processes of primary metabolism and defence responses (Roitsch et al., 2003). The regulation of INV by all classes of phytohormones indicates an essential link between the molecular mechanism of phytohormone action and primary metabolism under stress. The up-regulation of INV appears to be a common response to various biotic and abiotic stress-related stimuli such as pathogen infection and salt stress, in addition to specific stress-related reactions. Partitioning of assimilates and its effect on dry matter distribution is influenced by several environmental factors such as temperature, drought, salinity, and nutrient availability (Wardlaw, 1990). This dry matter redistribution is closely associated with carbohydrate allocation to the roots (Cakmak et al., 1994). Abiotic stress causes a reduction in sink enzyme activities, leading to an increase in sucrose in source leaves with a decrease in photosynthesis rate by feedback inhibition (Poljakoff-Mayber and Lerner, 1994). The growth capacity of tomato plants under salinity have been related to the increase in sink activity of young leaves and roots by the induction of vacuolar INV and sucrose synthase activities (Balibrea et al., 2000). This activity was much higher in the roots of the salt-tolerant wild species *Lycopersicon pennellii* than in those of the domestic *L. esculentum* plants. It has also been shown that induction of male sterility in wheat by meiotic stage water deficit is preceded by a decline in vacuolar INV (Dorion et al., 1996). In vegetative sink and source organs of water-stressed maize plants, the organ-specific induction of acid invertase activity is found to correlate with an increase in the Ivr2 gene transcripts and in the vacuolar invertase proteins (Kim et al., 2000). Cold-induced stalk elongation in tulip is mediated by the induction of INV expression (Balk and de Boer, 1999). Low oxygen stress decreases INV expression (Ivr1 and Ivr2), in maize root tips (Zeng et al., 1999). These responses have an important implication in acclimation to low oxygen stress by the conservation of sucrose and ATP and reducing the hexose-based sugar-signalling system.
1.5 Field bean

*Dolichos lablab* (Field bean) a member of *Fabaceae*, is an ancient crop and has been documented by archaeo-botanical finds in India prior to 1500 BC at Hallur, India’s earliest Iron Age site in Karnataka (Fuller, 2003). Despite its label as ‘underutilized’, substantial cultivation of Field bean is seen in certain tropical regions, either as a sole crop or in mixed production systems. It’s popularity may also be demonstrated by the more than 150 local names reported by various authors and recorded in databases (MMPND, 2005).

Field bean has been noted for being one of the most agro-morphologically diverse (Islam, 2008) and versatile tropical legume species through its roles as pulse (*dhal*), vegetable (green bean, pod, leaf), forage/green manure, herbal medicine, and even ornamental (Adebisi and Bosch 2004; NRC 2006). More recently, Morris (2009) has reviewed its bio-functional properties for use as pharmaceutical or nutraceutical. Remarkable morphological variations have also been reported throughout India (Yadav et al., 2003; Sankaran et al., 2007). It also has considerable physiological diversity. A range of adaptation to acidity, low soil phosphorous, and drought has been reported for the plant (Mugwira and Haque, 1993; Karachi, 1997).

Drought is an important production constraint in the arid and semi-arid regions, where drought stress in both the seedling and terminal growth stages results in low grain and fodder yields. Field bean is especially adapted to drought (Maundu et al., 1999) and has been reported to have better drought tolerance than common beans (*Phaseolus vulgaris*) or cowpea [*Vigna unguiculata*] (Piper and Morse, 1915). There is overwhelming evidence for existence of both strong drought tolerance and considerable diversity in drought tolerance within the species (Ewansiha and Singh, 2006). Genetic studies and plant improvement for the same were conducted at Tamil Nadu Agricultural University in Coimbatore, India since the 1930s (Rangaswami and Krishnan, 1935) resulting in development of a number of cultivars. A high-yielding, photo-insensitive cultivar, HA-4 has been recently bred (Mahadevu and Byre Gowda, 2005) and was used for our studies.

Despite the proven ability to adapt to wider ecological conditions, and economic and agronomical values, which have been constantly improved by classical breeding studies; (Adebisi and Bosch, 2004), there are no reports of biochemical and molecular basis of adaptation of the plant to various abiotic stresses. In the light of these lacunae, the present investigation was imperative to evaluate the response of Field bean to various abiotic factors.
such as salt, drought, heat and heavy metal stress in terms of antioxidant and antioxidant enzymes and other stress markers. As the plant is a proven performer under drought, it would be a mine of drought resistance genes. Therefore, during the investigation, an attempt was also made to gain an insight into the nature of genes expressed/suppressed during drought stress. The results of this investigation are presented in the following chapters of the thesis.

**Fig 1.17:** Field bean (*Dolichos lablab*) growing under field conditions; (Panel 1); fresh seeds (A), fresh pods (B), daal/ split seeds (C) and full seeds (D) (Panel 2).