CHAPTER V

DISCUSSION

Effect of salinity treatment on germination

A dwindle in germination percentage and a swell in the time required for germination is irrespective of genus and species of plants under biotic and abiotic stress. Comparable observations were also found in the experiments conducted with Vigna radiata by Abdel Haleem Mohammed (2007) and Arul Balachandran et al. (2009). Salinity treatment not only reduced germination but also the time taken for initiation of germination. The factors that influence the rate and germination percentage, however, may also include the species, nutrient concentration in the soil. Inhibition or delay in germination under saline conditions may be due to osmotic effect limiting the uptake of water during seed germination that deter the membrane and/or cytosolic enzymes involved in the process as described by El-Baz et al. (2003). Still higher concentrations were not tried as the seedlings failed to survive with much truncated growth and bleached leaves.

Effect of salinity treatment on seedling growth

Abridged root and shoot growth in response to salt stress has already been reported in different plant species (Saha et al., 2010;
Ghoghdi et al., 2012). The experiments demonstrated that the root and shoot lengths of NaCl-treated seedlings decreased with the increasing NaCl concentration over water control. The decline in seedling vitality under salinity stress is due to the condensed ability of imbibitions resulting in limited hydrolysis of food reserves from storage tissues (Ghosh et al., 2015).

**Effect of salinity treatment on pigments**

Already it has been reported that green gram plants grown under salinity stress showed excess accumulation of leaf Na\(^+\) and Cl\(^-\) resulting in excess reactive oxygen species (ROS) production, reduced photosynthesis, and plant growth (Roy Choudhury and Ghosh, 2015). In accordance with previously quoted literature, there was a significant decrease in chlorophyll content in treatments with various concentrations of sodium chloride. Salinity caused reduction in chlorophyll and carotenoids contents which in turn resulted in pronounced chlorosis and necrosis in leaves.

Salinity stress caused swelling of membranes in chloroplasts of sensitive plants which affected their chlorophyll content, or it occurred due to excess ions (Na\(^+\) and Cl\(^-\)) in leaves which induced loss of chlorophylls (Wahid et al., 2004; Arul Balachandran et al., 2009). Accretion of toxic ions under salinity stress reduced the water and
osmotic potential that further caused instability in photosynthetic processes (Khan et al., 2010). Loss of chlorophyll content caused chlorosis of leaves.

The results are in agreement with the earlier findings on mung bean (Sehrawat et al., 2013b; 2013c) for the levels of pigment contents in leaves of plants under abiotic stress (Saha et al., 2010) In earlier works on crop plants, carotenoid content was reported to decline significantly from the pre-flowering to the post-flowering stage under natural environmental conditions.

**Effect of salinity treatment on oxidative stress markers**

Membrane lipid peroxidation is a manifestation and a measure of stress induced damage at the cellular level. In the present study, an increase in the MDA content of leaves of mung bean seedlings treated with increasing concentrations of NaCl was observed, an indication of membrane damage due to peroxidation of lipids which in turn results in enhanced reactive oxygen species production leading to oxidative stress.

Similar results have been observed in reports on salinity stress effects in sugarcane, wheat etc. MDA is regarded as a marker for evaluation of lipid peroxidation or damage to plasmalemma and organelle membranes that increases with environmental stresses. Lipid peroxidation is linked to the activity of antioxidant enzymes e.g. with the
increase of SOD, POX, CAT, oxidative stress tolerance is enhanced and MDA is decreased.

In plants, ROS like H$_2$O$_2$ is continuously produced as byproducts of aerobic metabolic processes like photosynthesis and respiration localized in chloroplast, mitochondria and peroxisomes. Production and removal of ROS must be controlled to ensure normal growth of plants. As demonstrated in the present work and also works of other researchers (Saha et al., 2010), when the equilibrium between production and scavenging of ROS is perturbed by any abiotic stress factor like salinity, the plant becomes hypersensitive to stress and H$_2$O$_2$ level increases. Stone and Yang (2006) have shown that during abiotic stress, hydrogen peroxide serves as a signaling molecule and plays a dual role in plant defence. It also effectively increases the activities of ROS scavenging enzymes.

**Effect of salinity treatment on Osmoprotectants**

It is understood that under stress conditions proline and glycine betaine serve as attuned cytoplasmic solutes that balance osmotically for external osmolarity or for ions sequestered in the vacuole (Gorham, 1995). Compatible solute accumulation in the cytoplasm is considered a means to impart salt tolerance (Jaleel et al., 2007).
Proline is a major organic molecule that acts as a mediator of osmotic adjustment under salinity stress, a stabilizer of sub-cellular structures, a sink for energy and even a stress related signal. It is also involved in cell osmoregulation, protection of proteins during dehydration and can act as an enzymatic regulator during stress conditions (Rontein et al., 2002).

In this study, NaCl treatment caused an increase in pro content in leaf tissues of NaCl treated plants and this is in accord with the outcomes of the experiments of Nakamura et al., (2002), Demiral and Turkan (2006), Jaleel et al. (2007, 2008), Abbas et al. (2010). Proline accumulated in response to excessive salts in growth medium (Munns and Tester, 2008). Superfluous NaCl in growth medium restrict the availability of water to plant. This restriction results in dehydration of cytoplasm. This dehydration affects the metabolism of the cells and the functions of macromolecules and eventually retards the growth of plant (Le Rudulier, 2005; Taffouo et al., 2009). Proline has role in defense of plant cell against undesirable effects of salt by maintaining osmotic balance, stabilizing subcellular structures and scavenging ROS (Ashraf and Foolad, 2007).

Glycine betaine is a small water soluble, organic metabolite and nontoxic at high concentrations, that can potentially take part in a crucial role in effectual protection against abiotic stress (Ashraf and
Harris, 2004; Ashraf and Foolad, 2007; Chen and Murata, 2008). The major role of glycine betaine in plants open to the elements to saline soil is probably defending plant cells from salt stress by osmotic adjustment (Gadallah, 1999), protein stabilization (Makela et al., 2000), photosynthetic apparatus protection (Allakhverdiev et al., 2003) and reduction of oxygen radical scavengers (Heuer, 2003). Glycine betaine has strong osmoprotective properties and is known to confer tolerance to salinity stress (McNell et al., 2001). Glycine betaine increased with increase in NaCl concentration up to 200mM. NaCl stress which induced increase in Glycine betaine has also been studied by other researchers (Abbas et al., 2010; Syeed and Fatma 2011).

**Effect of salinity treatment on enzymic antioxidant potentials**

Salinity manipulates complex biochemical responses and quite a lot of defensive mechanisms including production of enzymatic as well as nonenzymatic antioxidants, which detoxify ROS that swiftly occurs in plants due to rising salt concentration. Increased activities of many of the antioxidant enzymes in plants combat oxidative stress induced by salinity stress and various environmental stresses. Upholding of a high antioxidant capacity to forage the toxic ROS has been associated to increased tolerance of plants to these environmental stresses (Zaefyzadeh et al., 2009; Chen et al., 2011 and Sehrawat et al., 2015). SOD is a major superoxide forager and provides a first line of resistance
against cellular damage due to abiotic stress. The highly reactive superoxide is then transformed to \( \text{H}_2\text{O}_2 \) by SOD. The surfeit \( \text{H}_2\text{O}_2 \), which itself is toxic for the plant, is then scavenged by CAT activity. In this investigation, with increase in salinity stress, a significant enhancement in CAT activity (2 times) with respect to water control in the seedlings was observed. However with steep increase in hydrogen peroxide and counteracting SOD, a small drop off in catalase activity was observed.

SOD and CAT activity has been reported to be negatively correlated with the degree of damage to plasma lemma, chloroplast and mitochondrial membrane systems, and positively correlated with stress resistance indices (Elkahoui et al., 2005).

The experiments performed made known that the activity of enzymes polyphenol oxidase and peroxidase activities in the leaves of mung bean plant under salinity stress (NaCl) was found to be appreciably and very significantly increased with increasing NaCl concentration. However, catalase activity notably decreased in highly stressed mungbean plants. The magnitudes of increase or decreases in enzyme activities of mungbean plants due to salinity were found to be higher at high NaCl concentration. The decrease in catalase activity in mungbean plant under salt stress might lead to accumulation of toxic amount of \( \text{H}_2\text{O}_2 \).
In this respect, Kocsy et al. (1991) observed that catalase activity decreased in salinity stressed resistant wheat variety. Moreover, Lee et al., 2001 found that salt stress enhances the content of H$_2$O$_2$ and the activity of superoxide dismutase, peroxidase while it decreases catalase activity in Oryza sativa plant. Nandini et al. (2002) reported that application of NaCl on mungbean caused increase in polyphenol oxidase activity in leaves. Chakrabarti and Mukherji (2003) found that peroxidase activity increased under salt stress.

Peroxidases (EC 1.11.1.7) forage H$_2$O$_2$ by catalyzing the divalent reduction of H$_2$O$_2$ to water using a variety of reductants. Peroxidase is a stress marker enzyme known to be very active under stressful conditions. Under natural conditions, the enzyme activity declines sharply from 6 to 25 DAS, followed by a slight increase at 45 DAS. Peroxidase activity is possibly high at 6 DAS to maintain an optimum metabolic status by efficient scavenging of free radicals.

**Ribosomal Proteins**

Ribosomes are the particles that catalyse mRNA-directed protein synthesis in all organisms. The codons of the mRNA are exposed on the ribosome to allow tRNA binding. This leads to the incorporation of amino acids into the growing polypeptide chain in accordance with the genetic information. Incoming amino acid monomers enter the ribosomal A site
in the form of aminoacyl-tRNAs complexed with elongation factor Tu (EF-Tu) and GTP. The growing polypeptide chain, situated in the P site as peptidyl-tRNA, is then transferred to aminoacyl-tRNA and the new peptidyl-tRNA, extended by one residue, is translocated to the P site with the aid of the elongation factor G (EF-G) and GTP as the deacylated tRNA is released from the ribosome through one or more exit sites [PMID: 11297922, PMID: 11290319]. About 2/3 of the mass of the ribosome consists of RNA and 1/3 of protein. The proteins are named in accordance with the subunit of the ribosome which they belong to - the small (S1 to S31) and the large (L1 to L44). Usually they decorate the rRNA cores of the subunits.

Many ribosomal proteins, particularly those of the large subunit, are composed of a globular, surfaced-exposed domain with long finger-like projections that extend into the rRNA core to stabilise its structure. Most of the proteins interact with multiple RNA elements, often from different domains. In the large subunit, about 1/3 of the 23S rRNA nucleotides are at least in van der Waal's contact with protein, and L22 interacts with all six domains of the 23S rRNA. Proteins S4 and S7, which initiate assembly of the 16S rRNA, are located at junctions of five and four RNA helices, respectively. In this way proteins serve to organise and stabilise the rRNA tertiary structure. While the crucial activities of decoding and peptide transfer are RNA based, proteins play an active role
in functions that may have evolved to streamline the process of protein synthesis. In addition to their function in the ribosome, many ribosomal proteins have some function 'outside' the ribosome [PMID: 11290319, PMID: 11114498].

Ribosomal protein L22 is one of the proteins from the large ribosomal subunit. In Escherichia coli, L22 is known to bind 23S rRNA. It belongs to a family of ribosomal proteins which includes: bacterial L22; algal and plant chloroplast L22 (in legumes L22 is encoded in the nucleus instead of the chloroplast); cyanelle L22; archaeabacterial L22; mammalian L17; plant L17 and yeast YL17.

Ribosomal proteins make up the protein portion of the ribosome and with rRNA are essential for protein synthesis (9, 10). However, recent studies have shown that some ribosomal proteins may also participate in cellular events apart from protein biosynthesis, and this has inspired research interest in the genes that encode them. Such extraribosomal functions include DNA repairation, RNA chaperone activity, cell development, regulation of differentiation, pathogen resistance mechanisms. Ribosomal protein genes are regulated in response to environmental stresses (16, 23,–25). However, the mechanism of their resistance to abiotic stresses has not been described (Xiao et al.2014).
Defensins

Plant defensins are cationic peptides that are ubiquitous within the plant kingdom and belong to a large superfamily of antimicrobial peptides found in several organisms collectively called defensins. The primary structure of these peptides includes 45 to 54 amino acid residues with considerable sequence variation. At the level of three-dimensional structure, they are small and globular, composed of three anti-parallel β-sheets and one α-helix, which is highly conserved among these peptides.

The three-dimensional structure is stabilized by four disulfide bridges formed by eight strictly conserved Cys residues. Two of these bridges compose the Cys-stabilized α-helix β-strand motif, which is found in other peptides with biological activities.

Plant defensins present numerous biological activities, such as inhibiting protein synthesis, ion channel function and α-amylase and trypsin activity; impairing microbial, root hair and parasitic plant growth; mediating abiotic stress and Zn tolerance; altering ascorbic acid redox state; stimulating sweet taste sensation; serving as epigenetic factors; affecting self-incompatibility; and promoting male reproductive development.
Some of these biological activities, such as microbial growth inhibition and sweet taste induction, coupled with a scaffold that provides these peptides with incredible physicochemical resistance to harsh environments and the potential for simple amino acid substitution, raise the opportunity to improve the function of defensins or introduce new activities, endowing these peptides with great biotechnological and medical significance.

**Kunitz type trypsin inhibitors**

The Kunitz-type soybean trypsin inhibitor (STI) family consists mainly of proteinase inhibitors from Leguminosae seeds [PMID: 14705960]. They belong to MEROPS inhibitor family I3, clan IC. They exhibit proteinase inhibitory activity against serine proteinases; trypsin (MEROPS peptidase family S1, IPR001254) and subtilisin (MEROPS peptidase family S8, IPR000209), thiol proteinases (MEROPS peptidase family C1, IPR000668) and aspartic proteinases (MEROPS peptidase family A1, IPR001461) [PMID: 14705960].

Inhibitors from cereals are active against subtilisin and endogenous alpha-amylases, while some also inhibit tissue plasminogen activator. The inhibitors are usually specific for either trypsin or chymotrypsin, and some are effective against both. They are thought to protect the seeds against consumption by animal predators, while at the
same time existing as seed storage proteins themselves - all the actively inhibitory members contain 2 disulphide bridges. The existence of a member with no inhibitory activity, winged bean albumin 1, suggests that the inhibitors may have evolved from seed storage proteins.

Proteins from the Kunitz family contain from 170 to 200 amino acid residues and one or two intra-chain disulphide bonds. The best conserved region is found in their N-terminal section. The crystal structures of soybean trypsin inhibitor (STI), trypsin inhibitor DE-3 from the Kaffir tree Erythrina caffra (ETI) [PMID: 1988676] and the bifunctional proteinase K/alpha-amylase inhibitor from wheat (PK13) have been solved, showing them to share the same 12-stranded beta-sheet structure as those of interleukin-1 and heparin-binding growth factors [PMID: 1738162]. The beta-sheets are arranged in 3 similar lobes around a central axis, 6 strands forming an anti-parallel beta-barrel. Despite the structural similarity, STI shows no interleukin-1 bioactivity, presumably as a result of their primary sequence disparities. The active inhibitory site containing the scissile bond is located in the loop between beta-strands 4 and 5 in STI and ETI.

The STIs belong to a superfamily that also contains the interleukin-1 proteins, heparin binding growth factors (HBGF) and histactophilin, all of which have very similar structures, but share no sequence similarity with the STI family.
In general, families of serine protease inhibitors predominate, followed by a few families of inhibitors of cysteine and metallo-proteases, while aspartic protease inhibitors are rare and dispersed in different families. The soybean trypsin inhibitor was the first plant protease inhibitor to be isolated (Kunitz, 1945) and similar proteins, subsequently characterized, have been named Kunitz trypsin inhibitors (family I3). They are widespread in plants and encoded by families of genes that are expressed in all plant tissues, but mostly in the seeds of some leguminous plants. They mainly inhibit serine proteases (SPs), while some inhibit aspartic or CPs (Rawlings, 2010).

Studies aimed at increasing our understanding of proteases and their inhibitors in plants were first focused on the occurrence of proteolysis and on the proteases active during different stages of plant development, such as germination, differentiation, morphogenesis, senescence and programmed cell death.

Gradually research has become focused on the involvement of proteases and PIs in response to environmental stress. For a long time the main interest was directed towards biotic stress, such as attack by herbivores and pathogens, as well as the often associated physical wounding. In addition to basic reasons, research in this field has been, and still is, inspired by potential applications in biotechnology (Brzin and Kidrič, 1995; Sabotič and Kos, 2012).
Two decades ago abiotic stress attracted little attention and the relatively small number of studies were focused on the involvement of proteases and their inhibitors in plants under drought, high salinity and at high or low temperatures (Brzin & Kidrič, 1995). Recent developments have started to change this picture of the field. The suggestion that proteolytic enzymes are specifically involved in the response of plants to abiotic stress originated in observations that stress conditions often bring about senescence of plant tissue and that the senescence is closely connected with enhanced proteolysis that involves several proteases in the same tissue (Huffaker, 1990). This complicates differentiation between the different possible causes of change in protease activity. Further, in the natural environment, factors that induce a state of stress seldom act individually. However, at the same time, different stresses can have the same effect at the cell level and responses to them may share common molecular mechanisms. Such a relationship between drought, salt stress and cold is well known (Bartels and Nelson, 1994).

Studies also suggest that functional Kunitz trypsin inhibitors with antifungal activity act against several important phytopathogens in the tobacco defense response. (Huang, 2010)
Prolyl isomerase (also known as peptidylprolyl isomerase or PPIase) is an enzyme (EC 5.2.1.8) found in both prokaryotes and eukaryotes that interconverts the cis and trans isomers of peptide bonds with the amino acid proline. Proline has an unusually conformationally restrained peptide bond due to its cyclic structure with its side chain bonded to its secondary amine nitrogen. Most amino acids have a strong energetic preference for the trans peptide bond conformation due to steric hindrance, but proline's unusual structure stabilizes the cis form so that both isomers are populated under biologically relevant conditions. Proteins with prolyl isomerase activity include cyclophilin, FKBPs, and parvulin, although larger proteins can also contain prolyl isomerase domains.

Protein folding in vivo is mediated by an array of proteins that act as molecular chaperones, foldases or both. In folded proteins, the peptide bonds occur only in two confirmations, cis or trans. The peptide bonds not preceding proline are almost always trans in folded proteins, but 5.7% of all Xaa–Pro peptide bonds show cis confirmation in proteins with known three dimensional structure.

The slow isomerization about proline imidic bonds are frequently the rate-determining events in folding. In a cell, folding should not be too
slow and partially folded intermediates should not be present for an extended time in order to minimize the risk of their aggregation. Peptidyl prolyl cis–trans isomerasers (PPIases), also called rotamases or immunophilins, are the only enzymes evolved to stabilize a transition state that is separated from a ground state only by a difference in torsional angle.

Significant increase in leaf-, root- and seed PPIase activity in response to water stress was observed only in the drought-tolerant cultivar ICSV-272, which was due to specific induction of PPIases. However, a direct relationship between stress tolerance and expression of total PPIase activity has not been reported as yet.

Cyclophilins apart from playing an important role in protein folding may perform specific functions via interacting partner proteins in larger multi-component complexes. The search for their interacting partners under high stress plant response and thereby gene interference will provide in-depth understanding of their physiological roles and potential function in stress alleviation. Multiple studies have been focused to identify the interacting proteins and to elucidate their effects on peptidyl prolyl cis-trans isomerase activity (Dipesh et al. 2013).
**Pentatricopeptides**

Pentatricopeptide repeat (PPR) proteins are RNA binding proteins with functions in organelle RNA metabolism. They are found in all eukaryotes but have been most extensively studied in plants.

Given that these post-transcriptional processes are highly diverse, one would expect such functions to be catalysed by many different proteins. Indeed, each post-transcriptional event often involves several proteins, amongst which a large family of helical repeat proteins have been found to play important roles in organelle gene expression. These complex proteins are known as pentatricopeptide repeat (PPR) proteins and were originally identified during the sequencing of the genome of the model plant *Arabidopsis thaliana*. The PPR family is now known as one of the largest protein families to exist in angiosperms with over 450 PPR-encoding genes identified in *A. thaliana*.

PPR proteins are characterised by a 35 amino acid motif, often repeated in tandem a variable number of times. Each PPR motif consists of two antiparallel α-helices, which interact with each other. The series of α-helices form a superhelix containing a groove, which can bind its RNA ligand in a sequence-specific manner. Most PPR proteins function as molecular adaptors in the recruitment of catalytic enzymes or effector proteins to target transcripts.
Two classes of PPR proteins exist. The P class is characterised by the canonical 35 amino acid motif and typically lacks additional domains. The second class, the PLS class, consists of slightly longer and shorter PPR motifs, as well as C-terminal domains such as the E, E+, and DYW domains, which often have prominent roles in RNA editing.

Plants display diverse survival mechanisms against microbial infection and other environmental stresses. While specialized host responses do occur, many components of the molecular events underlying plant responses to abiotic and biotic stresses are common.

Passive defenses, such as the cuticle, aid in drought tolerance and protection from UV damage while also acting as a deterrent of herbivory and barrier against pathogen infection (Reina-Pinto and Yephremov, 2009). Similarly, the cellular and biochemical processes associated with active responses to different abiotic and biotic stimuli also share functional overlaps (Fujita et al., 2006). These induced responses are largely mediated by plant hormones and their interactions, which range from simple synergism or antagonism to intricate networks of cross-regulation (Grant and Jones, 2009).

Responses to pathogen infection are modulated by salicylate (SA), jasmonate (JA), and ethylene (ET) with a growing role for abscisic acid (ABA), auxin, and GAs. ABA, a major regulator of environmental stress
responses, is generally regarded as a negative regulator of plant defense, with exogenous application or increased endogenous levels typically correlating with plant susceptibility to pathogens (Mauch-Mani and Mauch, 2005; Fujita et al., 2006).

However, there are instances of ABA positively contributing to disease resistance through modulation of callose deposition, stomatal closure, defense gene expression, and accumulation of reactive oxygen species (Mauch-Mani and Mauch, 2005).

Overall, plant responses to different stresses share significant overlap and points of convergence defined by regulatory factors that integrate signaling from various pathways (Fujita et al., 2006; Robert-Seilaniantz et al., 2010). Among these, the Arabidopsis (Arabidopsis thaliana) R2R3MYB transcription factor BOS1 is a mediator of abiotic and biotic stress responses, its loss of function resulting in susceptibility to necrotrophic infection as well as hypersensitivity to salt, osmotic, and oxidative stress (Mengiste et al., 2003).

Similarly, overexpression of the ATAF1 transcription factor results in susceptibility to Botrytis cinerea and Blumeria graminis f. sp. hordei as well as decreased tolerance to ABA, salt, and oxidative stress (Wu et al., 2009). Phytochrome and flowering time1 (PFT1) regulates plant resistance to Alternaria brassicicola, B. cinerea, and Fusarium oxysporum
through its function in the biosynthesis of anthocyanin, a flavonoid linked to numerous abiotic and biotic stress responses.

PFT1 encodes a subunit of the evolutionarily conserved Mediator complex, which was recently shown to promote transcription of microRNA (miRNA) genes by recruiting RNA polymerase II to their promoters (Kim et al., 2011). miRNAs and small interfering RNAs have emerged as important regulators of plant defense and stress tolerance known to affect gene expression, ROS accumulation, and plant cell death (Borsani et al., 2005; Sunkar et al., 2007).

Natural cis-antisense small interfering RNAs have been associated with Arabidopsis salt tolerance as well as resistance to pathogens (Borsani et al., 2005) Natural small interfering RNA ATGB2 contributes to RPS2-mediated resistance to Pseudomonas syringae by repressing PENTATRICOPEPTIDE REPEAT PROTEIN-LIKE (PPRL) gene expression. (Katiyar-Agarwal et al., 2007).

The functional link of many PPRPs in chloroplast and mitochondrial development and/or regulation suggests they may play a role in managing perturbations in cellular redox elicited by different types of stress (Lurin et al., 2004; Andres et al., 2007; Saha et al., 2007). They described the function of the Arabidopsis pentatricopeptide repeat
protein for germination on NaCl (PGN) in plant resistance to necrotrophic fungi and tolerance to abiotic stress.

The sequence analysis and structure prediction made with the amino acid sequence of PPR protein and gene ontology predictions for molecular function ontology and biological process ontology shows that the proteins plays an important role in combating oxidative stress that may be due to heavy metal ions, salinity, pathogenic infections.