Chapter - FIVE

Discussion
Salinity is a major threat of irrigated agriculture in arid and semi-arid regions, which affects crop productivity. Unlike drought, salinity stress is a complicated phenomenon which includes osmotic stress, specific ion effect, nutrient deficiency etc., thereby affecting various physiological and biochemical mechanisms associated with plant growth and development. Plants have developed various combating mechanisms to cope with the deleterious effects of salinity stress. Among the several approaches to solve the problem of saline soils, identification of crop plants tolerable to salt stress may be helpful in combating salt stress when grown in saline soils. These plants can also be used for reclamation of salt affected soils. In order to work out this approach, we have screened eight chickpea genotypes for evaluating differential response to various levels of salt treatments in terms of growth and physiological processes of chickpea. The level of tolerance against salt stress was evaluated through the level of induction of antioxidant defense system. The salt sensitive and salt tolerant were used for the development of biomolecular marker under salt stress.

Eight genotypes of chickpea, SKUA-01, SKUA02, SKUA-03, SKUA-04, SKUA-05, SKUA-06, SKUA-07 and SKUA-08, were procured from Sher-e-Kaskmir University of Science and Technology and were grown hydroponically. Fifteen-day-old plants of chickpea genotypes were subjected to 0(T0), 25(T1), 50(T2), 75(T3), 100 (T4) and 150 (T5) mM of NaCl and the observations were recorded at 1\textsuperscript{st}, 3\textsuperscript{rd}, 5\textsuperscript{th}, and 7\textsuperscript{th} day of sampling. Salt treatments affected the growth of the chickpea genotypes significantly; however, the level of effect of salt on the growth of the genotypes was variable. Three genotypes of chickpea (SKUA-03, SKUA-04 and SKUA-05) were able to tolerate the salt treatment only up to 25 mM, as observed at all DAT. These genotypes are termed as salt-sensitive genotypes. SKUA-01, SKUA-02 and SKUA-08 genotypes could tolerate the salt treatment upto 50 mM at all DAT. The growth of SKUA-06 and SKUA-07
was not affected by the salt treatments up to the level of 100 mM as observed at all DAT and are termed as salt tolerant genotypes. The results obtained in this study are discussed in this chapter.

5.1 GROWTH PARAMETERS

Plant growth and biomass yield are classically used to evaluate plant tolerance to abiotic stress (Ashraf and Harris, 2004). In the present investigation, the salt treatments resulted in the significant decrease in the growth of chickpea genotypes measured in terms of length, fresh weight and dry weight of root and shoot. However, there were remarkable difference in the response of the genotypes to the dose and duration of salt treatments. The reduction of plant growth under saline conditions may either be due to osmotic reduction in water availability which resulted in increasing stomatal resistance as reported by Gunes et al. (1996), or to excessive ions, Na and Cl accumulation in the plant tissues (Munns et al., 2002; Rogers et al., 2003; Ashraf and Harris, 2004). These results are in good agreement with those reported by (Cuartero et al., 2006; Joseph and Jini, 2011). SKUA-06 and SKUA-07 genotypes were able to grow well up to the level of 100 mM of salt treatment as there was no significant effect of salt treatments on the fresh weight and dry weight of root and shoot of these genotypes at all DAT. These genotypes are tolerant as no significant change was observed in growth. The genotypes, SKUA-03, SKUA-04 and SKAU-05, were not able to cope up with the salt treatments beyond 25 mM as there was dose dependent decrease in the root and shoot fresh and dry weight of these genotypes at all DAT. Generally, salt-tolerant plants differed from salt-sensitive ones mainly in having a low rate of Na⁺. It was suggested that the capacity of ion accumulation of plants is related to their tolerance to salt stress. It was found that tolerant species accumulated lower Na⁺, and the decrease of K⁺ was lower than in the sensitive species (Essa, 2002; Yasar et al., 2006; Kusvuran et al., 2007). Reduction in root and shoot fresh weight and dry weight
as a result of salt stress has also been reported in several other plant species (Ashraf and Mc Neilly, 1990; Turkmen et al., 2008). Growth of SKUA-01, SKUA-02 and SKUA-08 was not affected significantly by salt treatment up to 50 mM, as observed at all DAT. These varieties may arise as a result of breeding between tolerant and sensitive genotypes.

The shoot and root length are the most important parameters for salt stress because roots are in direct contact with soil and absorb water from soil and the shoot supply it to the rest of the plant. For this reason, root and shoot length provides an important clue to the response of plants to salt stress (Jamil et al., 2004). Our results showed that there was large variability in the response of chickpea genotypes to salt treatments in terms of the root and shoot lengths. Again, SKUA-06 and SKAU-07 genotypes of chickpea showed no significant effect of doses and duration of salt treatments except at 150 mM on the length of root and shoot. This provides added tolerance capacity to these genotypes against salt treatments. Whereas SKUA-03, SKUA-04 and SKUA-05 appeared to be the salt sensitive genotypes as inferred from significant reduction in length of root and shoot in response to the all level and duration of NaCl treatments except at 25 mM. The reason for reduced shoot and root development may be due to toxic effects of the NaCl used as well as unbalanced nutrient uptake by the seedlings. High salinity may inhibit root and shoot elongation due to slowing down of the water uptake by the plant (Werner et al., 1995) may be another reason for this decrease. Neumann (1995) indicated that salinity can rapidly inhibit root growth and hence capacity of water uptake and essential mineral nutrition from soil. SKUA-01, SKUA-02 and SKUA-08 genotypes of chickpea were considered as medium between tolerant and sensitive genotypes because of their tolerance behaviour upto the 50 mM of NaCl. Reduction in plant growth by means of salt stress has also been reported for a number of plant species in scientific studies (Essa, 2002; Kusvuran et al., 2007).
Leaf area measurement indicates the ability of plant to assimilate carbon as leaves are the site for sunlight trapping. Leaf area of chickpea genotypes was also affected by salt treatments, however, there was large variability in the chickpea genotypes. Our results are in agreement with the reports of the immediate response of salt stress on the reduction in the rate of leaf surface expansion that leads to cessation of expansion as salt concentration increases (Wang and Nil, 2000). Salinity reduced leaf area; this is compatible with reports by Chartzoulakis and Klapaki (2000). Ashraf and Bashir (2003) have reported significant reduction in fresh and dry weights of shoots and roots, and shoot length and leaf area of *P. vulgaris* and *Sesbania aculeata* plants under salt stress. Meena et al. (2003) also suggested that leaf area and relative water content decreased significantly with variable magnitude by increasing salinity.

### 5.2 BIOCHEMICAL PARAMETERS

#### 5.2.1 Total chlorophyll content

A decrease in photosynthetic pigment content of chickpea plants under salt stress was observed. Total Chlorophyll content decreased significantly at 50 mM onwards of NaCl in SKUA-01, SKUA-02 and SKUA-08. The decrease in chlorophyll concentration might possibly be due to changes in the lipid protein ratio of pigment-protein complexes or increased activity of the chlorophyll-degrading enzyme chlorophyllases (Parida et al., 2004). SKUA-06 and SKUA-07 genotypes showed the tolerance level up to 100 mM of NaCl level at all the days of treatment. While as genotypes SKUA-03, SKUA-04 and SKUA-05 are the most salt sensitive genotypes showing the reduction in total chlorophyll content at all the levels of NaCl treatment at all DAT. The results obtained in this study are in concurrence with those of Azooz et al. (2004) for sorghum and Dager et al. (2004) for *Salvadora persica*. The reduction in leaf chlorophyll content under NaCl stress has been attributed to the destruction of chlorophyll pigments and the instability of the pigment protein complex (Levit, 1980). It is also attributed to the intrusion.
of salt ions with the \textit{de novo} synthesis of proteins, the structural component of chlorophyll, rather than the breakdown of chlorophyll (Jaleel et al., 2007). It has therefore, proven that soil salinity had negative effects on the growth and photosynthetic metabolism of chickpea genotypes.

5.2.2 Soluble protein content

One of the mechanisms affected by salt stress in plants was protein synthesis. It is known that soluble protein content is an important indicator of physiological status of plants. After 25 mM of NaCl treatment, the total soluble protein content decreased in SKUA-03, SKUA-04 and SKUA-05. This situation demonstrated that these genotypes were first affected by salt stress. Similar to our findings, Sibole et al. (1998) and Yurekli et al. (2004) reported that short term NaCl stress severely reduced leaf protein contents in \textit{Phaseolus vulgaris} plants. Chickpea genotypes SKUA-01, SKUA-02 and SKUA-08 showed non-significant change in soluble protein content up to 50 mM of NaCl treatment but significant change was observed with the increase in the levels of NaCl treatment. Inhibitory effect of salinity on leaf protein was also reported by Ashraf and Waheed (1993) in wheat and Ashraf and Fatima (1995) in safflower. Helal and Mengel (1981) have suggested that there is marked decomposition of protein under saline conditions. This may suggest an alternative explanation of chlorophyll destruction under saline conditions. In our experimental findings genotype SKUA-06 and SKUA-07 showed an increase in soluble protein content with the increase in NaCl treatment. Similar results were reported in salt tolerant cultivars of barley, sunflower, finger millet and rice plants (Parvaiz and Satyavati, 2008). Yurekli et al. (2004) reported that total soluble protein content significantly decreased in salt sensitive \textit{Phaseolus vulgaris} but increased in salt tolerant \textit{P. acutifolius} plants. Similarly, Porgali and Yurekli (2005) reported that compared with control plants, protein amount in salt sensitive \textit{L. esculentum} plants decreased with the salt application. In salt tolerant \textit{L. pennellii} plants, total soluble protein
content was more than control plants. Demiral and Turkan (2006) detected that while total soluble protein content of salt tolerant *O. sativa* cv. Pokkali plants increased with salinity, sensitive (*O. sativa* cv. IR-28) rice cultivars showed a decrease under salt stress. These different results on salt stress showed that the responses to salt stress depends on plant species even in varieties of same plant species, plant developmental stage, duration and severity of the salt application (Parvaiz and Satyavati, 2008). It is likely that NaCl induced lipid peroxidation and fragmentation of proteins due to the toxic effects of reactive oxygen species that leads to the reduced protein content. Rai et al. (2004) reported the same results.

5.2.3 Proline content

Proline is a compatible solute that accumulates in great quantities under osmotic stress and participates in osmo-regulation and osmo-protection (López-Carrión et al., 2008). Proline is also considered to be involved in the protection of enzymes (Solomon et al., 1994), cellular structures (Van Rensburg et al., 1993) and to act as a free radical scavenger (Alia et al., 1995). Its biosynthesis could be associated with the regulation of cytosolic pH (Venekamp, 1989) or with the production of NADP for the stimulation of the pentose phosphate pathway. Proline accumulation in salt stressed plants is a primary defense response to maintain the osmotic pressure in a cell, which is reported in salt tolerant and salt sensitive cultivars of many crops (Demiral and Türtkan, 2005; Koca et al., 2007). In the present study, proline accumulation in the salt tolerant genotypes SKUA-06 and SKUA-07 was significantly higher than that in the salt sensitive genotypes SKUA-03, SKUA-04 and SKUA-05. Similar results have been reported in rice [IR28 (salt susceptible) < Pokkari (salt tolerant)] and sorghum [CSF18 (salt susceptible) < CSF20 (salt tolerant)] grown under salt stress (Demiral and Türtkan, 2005). Salt tolerant plant species may possibly survive in salt stress condition using other defense mechanisms such as ion homeostasis, antioxidation and
hormonal systems (Zhang et al., 2006). Due to this, evaluation of a number of parameter in salt stressed plant would result in the identification of some effective criteria to classify plants for salt tolerance. Many plants, both halophytes and glycophytes, accumulate proline as a non-toxic and protective osmolyte under salinity, including mangrove (Parida et al., 2002), maize (Cicek and Cakirlar, 2002), sorghum (de Lacerda et al., 2005) and mulberry (Harinasut et al., 2003). Some authors have, however, argued that excessively high levels of proline accumulation may be a response to leaf damage when exposed to high NaCl concentration and a higher level of proline accumulation is associated with salt sensitive traits in tomato (Bolarin et al., 1995) and sorghum (de Lacerda et al., 2005). Proline accumulation in response to lower salt concentration may contribute positively to salt tolerance, whereas the high concentration in leaf tissues under high salinity treatment may be partly due to leaf damage.

5.2.4 Lipid peroxidation

Lipid peroxidation is a well-known parameter for determination of oxidative damage to membranes (Da Costa et al., 2007). The adverse effects of the salt on membranes are results of the accumulating toxic ions and ROS. The ion balance of membranes changed by Na\(^+\) and Cl\(^-\) accumulation. These ions replace K\(^+\) and Ca\(^{2+}\) ions, which have important roles in the functioning of the membrane proteins, under salt stress. On the other hand, ROS, especially the hydrogen peroxide and hydroxyl radicals, damage the membrane lipids and result in lipid peroxidation, damaging the membrane structure and integrity.

Lipid molecules, in general, and unsaturated lipids, in particular, are sensitive to oxidation by ROS. Consequently, the presence of elevated levels of TBARS, a product of lipid peroxidation, is generally an indicator of free radical damage to cell membranes causing severe oxidative stress (Metwally et al., 2005). Lipid peroxidation, measured as the amount of MDA, is produced when polyunsaturated fatty acids in the membrane undergo oxidation by the
accumulation of free oxygen radicals. However in the present study, the maximum and minimum sensitivity being associated with SKUA-03, SKUA-04, SKUA-05 and SKUA-06, SKUA-07 respectively. The SKUA-06, SKUA-07 genotypes showed the maximum tolerance indicating that they had the greater ability to survive with the oxidative stress while as the latter is prone to salt stress induced production of super-oxide radicals resulted in the increase in lipid peroxidation. These results are in agreement with the reports of higher increases in the amount of MDA with the increase in salt stress in the salt-sensitive cultivar as compared to tolerant cultivar of rice and in roots of *Lemna minor* (Demiral and Turkan, 2005; Mandhania et al., 2006). MDA has been widely used as selection to assess salt injury as criterion in various plants (Jaleel et al., 2007; Ahmad et al., 2011). MDA concentration was found the maximum in plants exposed to stress (Alia et al., 1993; Giannakoula et al., 2008). Lipid peroxidation can occur in both chloroplasts and mitochondria (Elstner, 1982; Bowler et al., 1992). Our study clearly demonstrates that NaCl treatment induced an oxidative stress as assessed by measurement of extra-cellular TBARS levels. However, lipid peroxidation is not the only oxidative stress damage, because reactive oxygen species (ROS) may also damage macromolecules such as DNA and proteins (Alscher et al., 1997; Pastori and Foyer, 2002).

5.3 ANTIOXIDANT DEFENCE SYSTEM

In order to scavenge ROS and to avoid oxidative damage, plant possess an antioxidative system comprising of antioxidative enzymes like, SOD, APX, CAT, GR, and non-enzymes like ascorbate, glutathione (Hernandez et al., 2000; Khan et al., 2002; Bor et al., 2003). Tolerance to NaCl-stress in higher plants correlates to the levels of antioxidant systems and substrates (Koca et al., 2007; Athar et al., 2008).To overcome the effects of salinity-induced oxidative stress, plants make use of these complex antioxidant system. Relatively higher activities of ROS scavenging enzymes have been reported in tolerant genotypes when compared
to susceptible ones, suggesting that the antioxidant system plays an important role in plant tolerance against environmental stresses.

5.3.1 Superoxide dismutase (SOD)
Superoxide dismutase (SOD) is the first enzyme in detoxification process and converts superoxide radicals to H$_2$O$_2$ at a very fast rate. SOD is a critical enzyme responsible for the elimination of superoxide radicals and is considered to be a key anti-oxidant in aerobic cells. Accumulation of ROS in cellular oxidative stress can lead to the damage of important biomolecules such as membrane lipids, proteins and DNA. The combined action of SOD and APX is critical in mitigating the effects of oxidative stress, since the former merely acts on the superoxide intermediate (H$_2$O$_2$) and the latter acts on H$_2$O$_2$ converting anion into water and oxygen. In the present study, a significant increase in SOD activity was observed in chickpea genotypes under saline conditions, this was most prominent in SKUA-06, and SKUA-07 genotypes suggesting that SOD may function as a ROS scavenger, by converting O$_2^-$ to H$_2$O$_2$ (Alscher et al., 2002). The increase in SOD activity could be due to a \textit{de novo} synthesis of enzymatic proteins. The low enzyme activity at higher NaCl treatments in many genotypes could be attributed to inactivation of H$_2$O$_2$ produced in different cellular compartments where SOD catalyses dismutation of superoxide radicals (Yamaguchi et al., 1995). The SOD activity in genotypes SKUA-01, SKUA-02 and SKUA-08 increased with increasing the levels of NaCl up to 50 mM when compared to the control at all the DAT. These results showed that these genotypes are moderately tolerant to NaCl stress. But with the increase in the levels of treatment the SOD activity in these genotypes were slightly low. This may be attributed to the inactivation of SOD enzyme by H$_2$O$_2$ that is produced in different cellular compartments where the disproportion of superoxide radicals is catalysed by SOD (Yamaguchi et al., 1995). Whereas in SKUA-03, SKUA-04 and SKUA-05 genotypes, there was no significant change in the SOD activity in response to NaCl stress, means that
SOD activity was not strong enough to detoxify the superoxide radicals completely showing the less tolerance towards NaCl stress. Similar results has been found in many plant species, it has been shown that salinity increases SOD activity in leaves of salt-tolerant cultivars, whereas in salt-sensitive cultivars SOD activity is decreased (Dionisio-Sese et al., 1998; Hernandez et al., 2000; Hernandez et al., 2001; de Azevedo Neto et al., 2006; Singh et al., 2007). Thus, induction of SOD activity may enable the plants to overcome the salt-induced oxidative stress.

5.3.2 Catalase (CAT)
Catalase is the main scavenger of H$_2$O$_2$ in peroxisomes, converting it to water and molecular oxygen (Willekens et al., 1995). The CAT destroys the H$_2$O$_2$ produced by SOD and other reactions (Foyer et al., 1994). In this study, CAT activities increased markedly in the genotypes (SKUA-06 and SKUA-07), while they reduced in the SKUA-03, SKUA-04, and SKUA-05 genotypes. This showed that genotypes SKUA-06 and SKUA-07 were more efficient scavenger of H$_2$O$_2$, which may result in better protection against H$_2$O$_2$. Moreover, increasing body of evidence suggests that high salinity levels induce oxidative stress (Savoure et al., 1999). In genotypes SKUA-03, SKUA-04, SKUA-05 the CAT activity was inhibited, which might have resulted in H$_2$O$_2$ accumulation that reacts with (O$_2$) to produce hydroxyl-free radicals (OH') via the Herbert-Weiss reactions (Elstner, 1982; Bowler et al., 1992). CAT activity of SKUA-01, SKUA-02 and SKUA-08 was significantly increased up to 50 mM of NaCl level of treatment. The decrease in catalase activity might be because Na$^+$ probably binds or replaces some components such as Fe$^+$ in the enzyme indicating that it interacts with iron somewhere in the metabolic pool. On the other hand, increase in the H$_2$O$_2$ might inactivate the enzyme (Luna et al., 1994). The tolerance of some genotypes to environmental stresses has been associated with higher activities of antioxidant enzymes. For example, the wild NaCl-tolerant species Lycopersicon pennellii had
higher activities of SOD, POD and CAT than the cultivated species *L. esculentum* (Shalata and Tal, 1998). Costa et al. (2005) suggested that a strong correlation between salt tolerance and POD activity in sorghum genotypes. Agarwal and Shaheen (2007) reported that a higher CAT activity in *Momordica charantia* was associated with tolerance of plant to NaCl. Ahmad et al. (2010b) also demonstrated the positive correlation between increase in salt concentration and catalase activity in mulberry.

5.4 ASCORBATE GLUTATHIONE CYCLE

Ascorbate-glutathione cycle in chloroplasts is the major defence system for scavenging H$_2$O$_2$, which finally converts H$_2$O$_2$ to H$_2$O and O$_2$. The cycle involves mainly ascorbate peroxidase and glutathione reductase enzymes, ascorbate and glutathione as oxireductants, H$_2$O$_2$ as an electron acceptor, and NADPH as an H$^+$donor, which are strictly compartmentalized and act in a highly coordinated manner (Asada, 1992; Foyer et al., 1994).

5.4.1 Ascorbate peroxidase (APX)

Ascorbate peroxidases (APX) are antioxidants performing the same general function as catalases. However, unlike catalase, they catalyse removal of H$_2$O$_2$ by using ascorbate as a reductant. APX play an important role in regulation of intracellular level of H$_2$O$_2$ in higher plants (Shigeoka et al., 2002). It has been reported that APX activity may have an important role in the mechanism of salt tolerance in plants. Genotypes SKUA-06 and SKUA-07 showed significant APX activity with the increase in the salt treatment up to 100 mM, but at 150 mM there was no change in the activity of APX at all DAT. Several studies have reported a large increase in APX activity after salinity stress in rice (Lee et al., 2001), pea (Hernández et al., 2000) and cotton (Gossett et al., 1994). Gueta-Dahan et al. (1997) indicated that acquisition of salt tolerance might also be a consequence of improving resistance to salt stress, via increased APX activity. Increased activity
of APX in salt-adapted cells seems to be more important for their salt tolerance. Bor et al. (2003) found induced APX activity in salt-tolerant wild beet. Mittova et al. (2000) also reported same relation between APX and salt tolerance in tomato. While as in SKUA-03, SKUA-04 and SKUA-05 genotypes, there was no significant change in the APX activity with increase in the level of treatment at all the DAT. It has been reported that the activity of H$_2$O$_2$ scavenging enzymes APX and the level of antioxidants like ascorbate in legumes decreases with salinity (Swaraj and Bishnoi, 1999). APX activity of SKUA-01, SKUA-02 and SKUA-08 genotypes increased only up to 50 mM of NaCl treatment at all the DAT. Similar results were reported by Comba et al. (1998) that the antioxidant enzyme activity increased at 50 mM of NaCl treatment. It was hypothesized that the increase in activity of APX could be due to activation of pre-existing APX or due to synthesis of new APX upon salt exposure (Parida et al., 2004). Although an increase in APX activity in the presence of salinity stress has been reported for other plants as well (Sairam and Srivastava, 2002).

5.4.2 Glutatione reductase (GR)
The last enzyme of Halliwell-Asada cycle, glutathione reductase (GR), catalyses the NADPH-dependent reduction of oxidized glutathione. GR is the rate-limiting enzyme in H$_2$O$_2$ scavenging pathway and it is involved in the maintenance of high ratio of GSH/GSSG, which is required for the regeneration of ascorbate (Sudhakar et al., 2001). In our study the GR activity seemed to be enhanced in all the genotypes. The highest activity of GR was found in salt tolerant genotypes SKUA-06 and SKUA-07. Glutathione reductase is activated under low levels of salt treatment and could increase the ratio of NADP$^+$/NADPH, thereby ensuring the availability of NADP$^+$ to accept electrons resulting into less flow of electrons to O$_2$ for generation of ROS (Reddy et al., 2004). Under such situation, there is flow of electrons to O$_2$ and therefore, the formation of ROS can be minimized. In genotypes SKUA-01,
SKUA-02 and SKUA-08 the GR activity increased up to 50 mM of NaCl treatment while as SKUA-03, SKUA-04 and SKUA-05 genotypes showed the minimum activity in a dose dependent manner. This decrease in GR activity in the stressed leaves in these genotypes might be due to the tendency of the plant to acclimate or inactivation of the enzyme losing its ability to maintain the higher ratio of GSH/GSSG (Mittova et al., 2000). Several authors investigating salt-tolerant and salt-sensitive cultivars have suggested that the salt tolerance character is related to increased GR activity in salt-tolerant cultivars (Hernandez et al., 2000; Sudhakar et al., 2001; Meloni et al., 2003).

5.4.3 Ascorbate content
Ascorbate is a primary as well as secondary antioxidant found in plants and has diverse physiological roles. Ascorbate has been shown to have an essential role in several physiological processes in plants, including growth, differentiation, and metabolism. It functions as a reductant for many free radicals, thereby minimizing the damage caused by oxidative stress. Ascorbate is the terminal electron donor in the processes, which scavenge the free radicals in the hydrophilic environments of plant cells. It scavenges hydroxyl radicals at diffusion-controlled rates (Mc Kersie, 1996). Ascorbate also reacts non-enzymatically with \( \text{H}_2\text{O}_2 \) at a significant rate, producing water and monodehydroascorbate. In addition to its role in antioxidant defence system, ascorbate has a broad spectrum of functions in plants. It accumulates at high concentrations in photosynthetic tissues, where it has been known to be intimately involved with the regulation of photosynthesis, protecting the chloroplast against damage by ROS. In our study the highest increase in ascorbate content was observed in the SKUA-06 and SKUA-07 which increased with dose and duration of treatment. This observation also was reported by Yu et al. (2003) in wild soybean. The increased ASC content is a stress-protecting mechanism of plants under salinity conditions (Shalata et al., 2001). ASC is an
important antioxidant, which reacts not only with $\text{H}_2\text{O}_2$ but also with $\text{O}_2^-$ (Reddy et al., 2004). ASC can also be used as the terminal antioxidant because the redox potential of the ASC/monodehydroascorbate pair is lower than that of most other bioradicals (Jaleel et al., 2007). A high level of endogenous ASC is essential for maintaining the non-enzymatic scavenging system that protects plants from oxidative damage due to salinity stress (Shigeoka et al., 2002). Genotypes SKUA-03, SKUA-04 and SKUA-05 genotypes showed decrease in ascorbate content with the increase in NaCl treatment at all the DAT, it can refer that these genotypes have low ability to detoxify $\text{H}_2\text{O}_2$. Our results are in agreement with reports that salt-tolerant species have higher ascorbate and glutathione contents and higher redox states in comparison with salt-sensitive species (Shalata et al., 2001; Khan and Panda, 2008).

5.4.4 Glutathione content

Glutathione is a strong cellular reductant with a broad redox potential. It acts as a scavenger of peroxides and serves as a storage and transport form of reduced sulphur (May et al., 1998). It has been shown that glutathione acts as a regulator of gene expression (Baier and Dietz, 1997), and is a precursor of phytochelatins (Grill et al., 1989). Due to the redox active thiol group GSH may be involved in the regulation of the cell cycle and can act as a defence compound against oxidative stress. The main functions of GSH is the protection against oxidative stress and its involvement in the ascorbate-glutathione cycle and in the regulation of protein thiol-disulphide redox status (Alscher et al., 1997). GSH has been shown to participate in the regeneration of the reduced form of ascorbate through non-enzymatic reduction of DHA at an alkaline pH (Noctor et al., 1998). GSH plays an important role in the response of plants to oxidative stress due to the generation of active oxygen species (Huckelhoven and Kogel, 2003). In our study salt tolerant genotypes SKUA-06 and SKUA-07 showed a maximum increase in glutathione content with the increase in salt treatment at all the days.
of treatment. The increase in glutathione (GSH) content may be due to the role of the enzymes APX, GR and SOD which are involved in the regeneration of glutathione and ascorbate that are important in detoxification of ROS (Foyer et al., 1994). While as salt sensitive genotypes SKUA03, SKUA-04 and SKUA-05 showed decrease in the GSH content with the increase in the levels of treatment at all the DAT. The decrease in GSH content found in the study might be due to its oxidation under salinity conditions (Jaleel et al., 2007). Active participation of GSH content in detoxification of oxygen species and free radicals has been found in several studies including those by Cao et al. (2004) and Israr et al. (2006).

5.5 ISOZYME ANALYSIS

Isozymes are protein markers and have been used as useful markers in genetic studies of many plant species. This technique is based on the principal that allelic variation exists from many different proteins. For example, alleles of malic dehydrogenase would both perform the correct enzymatic function, but the electrophoretic mobility of the two may differ. Therefore, alleles would not migrate to the same location in the gel. The utilization of multiple isoforms of enzymes is one of the primary control mechanisms of cellular metabolism in plants.

Superoxide dismutase (SOD) is one of the enzyme of the antioxidant system and generally exists in all kinds of plants. SODs are ubiquitous metalloenzymes that catalyze dismutation of superoxide radicals and prevents organisms from oxidative damage of too much oxygen free radicals, especially the damage to cytoplasm membrane. To some extent, the activity of SODs in adversity may be used as an index to evaluate the resisting capacity of plants to adversity. As already mentioned in results, three isoforms of SOD were found i.e. copper/zinc (Cu/Zn) SOD, manganese (Mn) SOD, and iron (Fe) SOD and they can be differentiated on the basis of the metal co factors present. Plants generally contain Cu/ZnSOD in the cytosol, FeSOD and/or Cu/ZnSOD in the chloroplasts.
and MnSOD in the mitochondria (Tertivanidis et al., 2004). Different forms of SODs are inhibited by either potassium cyanide (Carter and Thornburg, 2000; Alscher et al., 2002) or by hydrogen peroxide (Carter and Thornburg, 2000; Alscher et al., 2002). For this study, two genotypes one salt tolerant i.e. SKUA-06 and another salt sensitive SKUA-03 were selected. It is clear that in SKUA-06 all the isoforms of SOD were found in the control but with increase in the level of treatment only MnSOD and Cu/ZnSOD were found. Where as in SKUA-03 only the MnSOD was found with the increase in the level of treatment. A significant decrease in FeSOD after the stress in leaf tissues of chickpea seems to diminish the ability to scavenge oxygen radicals, favoring an accumulation of oxygen radical species, which may be one of the reason for the increase in lipid peroxidation in leaves. Our results coincide with the reports of Eyidogan and Oz (2005) where they have noted three SOD activity bands (MnSOD, FeSOD and Cu/ZnSOD) in C. arietinum under salt stress. Furthermore, significant increase in the activities of Cu/ZnSOD and MnSOD isozymes was observed under salt stress. The results are in contrast to the previous reports (Gomez et al., 1999; Hernandez et al., 2000) that SOD isozymes were only found in salt-tolerant pea genotypes, but not in salt-sensitive ones. It may be suggested that the synthesis of the new SOD isozyme might contribute to increased SOD activity under salt stress. Similar results have been reported using transgenic plants (Tanaka et al., 1999; Badawi et al., 2004; Wang et al., 2004).

5.6 RAPD Analysis and development of SCAR marker

RAPD (Random Amplified Polymorphic DNA) markers represent an efficient and inexpensive way to generate molecular data and have been used successfully in various taxonomic and phylogenetic studies (Abo-elwafa et al., 1995; Sharma et al., 1995). RAPD analysis can be used to characterize DNA variation patterns within species and among closely related taxa. Within grain legume crops alone, RAPD markers have been widely used for the identification
of genetic relationships among cultivars ([Williams et al., 1990; Brown-Guedira et al., 2000; Subramanian et al., 2000; Amadou et al., 2001; Dwivedi et al., 2001; Galvan et al., 2001; Li and Nelson, 2001; Maciel et al., 2001; Sonnante and Pignone, 2001; Tosti and Negri, 2002], among wild forms (Cattan-Tou-pance et al., 1998), or between cultivars and wild forms (Mimura et al., 2000; Raina et al., 2001). Genetic diversity is normally measured as the average sequence divergence between any two individuals for a given loci. Some of this variation in the extent of polymorphism reflects the choice of genotype, but major differences are also observed for random genes within a single genome. The high degree of polymorphism in this study compared to other reports appears to be due to more diverse material, which belonged to different chickpea genotypes. The polymorphism in RAPD is due to a single-base change. In this study, RAPD produced a higher number of bands because RAPDs are random in nature and can anneal anywhere in the genome.

In the present study, the variation among the chickpea genotypes was also assessed with random amplified polymorphic DNA (RAPD) markers. Fifteen RAPD primers amplified a total of 915 DNA fragments with an average of 61 fragments per primer. Out of the total amplified fragments, only 16 were monomorphic and the remaining 899 (98.1%) were polymorphic. This polymorphism was an indication of prevalence of moderate diversity among these eight chickpea genotypes (Punitha and Raveendran, 2004). In lettuce 110 (65.9%) of the total 167 fragments produced by twenty ISSR (inter-simple sequence repeat) primers showed polymorphism with an average of 8.5 polymorphic bands per primer (Vicente et al., 2008), however, in eggplant nine RAPD primers showed 95.3% (Sadder et al., 2007). RAPD markers showed a high level of polymorphism and a high number of clearly amplified bands. Extensive DNA polymorphism has also been reported using RAPD markers in several other crops plants (Hou et al., 2005). The RAPD-based dendrogram of chickpea genotypes displayed the genetic relationships between these accessions,
(Table 26 and Fig. 38) which accorded with previous studies of chickpea (Ahmad et al., 1992; Iruela et al., 2002). This analysis has proved to be successful in revealing the diversity among the genotypes of chickpea as also reported in Curcuma spp. (Syamkumar and Sasikumar, 2007), Crocus spp. (Grilli Caiola et al., 2004) and in Vigna spp. (Betal et al., 2004). Salt tolerant genotypes SKUA-06 and SKUA-07 showed the similarity co-efficient of 26% and the salt sensitive genotypes SKUA-03 and SKUA-04 with similarity of 16.5%, SKUA-05 showed 14.5% of similarity co-efficient respectively. Thus molecularly identified salt tolerant and salt sensitive genotypes have high potential in chickpea improvement for development of cultivar with wide adaptability. The salt tolerant and salt sensitive genotypes would be ideal parents for development of mapping population. Such information may be useful for selecting the diverse parents and monitoring the genetic diversity periodically in the breeder's working collection chickpea.

For consistent and repeatable amplification, the RAPD markers need to be converted to SCAR markers as suggested by Horejsi et al. (1999) and Gupta (2004). A SCAR marker amplifies only the critical RAPD marker fragment, thus eliminating the problem frequently encountered with RAPD markers that amplify a large number of non-specific fragments as well as overlapping fragments of similar sizes. An RAPD amplicon of 380 bp as obtained by BG-28 RAPD primer was specific to salt-tolerant genotypes of chickpea. This amplicon was eluted, cloned and sequenced to design the SCAR primer pairs that amplified this specific DNA fragment only. The amplification of genomic DNA of chickpea genotypes generate single DNA fragment of 380 bp in the SKUA-06 and SKUA-07 genotypes only. PCR protocols were optimized to facilitate repeatable and consistent amplification of this fragment as a single band amplification, confirming the specificity of SCAR primer for salt-tolerant cultivar of chickpea.
Symbiotic rhizobia of wild legumes are more tolerant to some ecological conditions (salt, severe drought, elevated temperatures, etc.) than rhizobia from cultivated legumes (Zahran 2001., Ogutci et al., 2008). Some rhizobia isolated from wild legumes also successfully establish effective symbiosis under stress conditions (Zahran 2001., Ogutci et al., 2008). However, the ability of chickpea to grow and survive in saline conditions improved when it was inoculated with *Rhizobium leguminosarum* by *ciceri* strains, especially DN7 and TN4, isolated from wild chickpeas. In fact, the best results for symbiotic N2 fixation under salt stress are obtained if both symbiotic partners resist such stress. Therefore, further work is required to select chickpea genotypes that are tolerant to salt stress.