Isolation and characterization of NaCl-tolerant mutations in two important legumes, *Clitoria ternatea* L. and *Lathyrus sativus* L.: Induced mutagenesis and selection by salt stress

Dibyendu Talukdar

Department of Botany, R. P. M. College (University of Calcutta), Uttarpara, Hooghly, 712258, West Bengal, India.

E-mail: dibyendutalukdar9@gmail.com.

Accepted 30 May, 2011

Six best performing mutants exhibiting NaCl-tolerance were identified in gamma ray induced M2 progeny of three different varieties of *Clitoria ternatea* L. and *Lathyrus sativus* L. each by preliminary assessment of plant growth under salt stress. Salt tolerance of these six mutants, designated as CR1 and CR2 in *C. ternatea* and as LR1, LR2, LR3 and LR4 in *L. sativus* was evaluated by their responses to 22 different morpho-physiological and biochemical parameters under 130 mM NaCl treatment on 15-day-old seedlings. A positive control set (mutant plants-watering only with distilled water) and a negative control (unmutagenised mother variety-treated with 130 mM NaCl) was also maintained. Among the six mutants, LR4 out performed all others showing significantly higher plant biomass production, leaf K+/Na+ ratio and lower level of lipid peroxidation and electrolyte leakage as compared to positive control. It was closely followed by CR2, LR3 and LR2 seedlings. Enhanced salt tolerance of these four mutants was attributed to increased activities of reactive oxygen species (ROS)-scavenging enzymes-superoxide dismutase and ascorbate peroxidase along with non-enzymatic ascorbate and carotenoids in leaves. Both CR1 and LR1 manifested moderate level of salt tolerance, but significantly higher than negative control (NC) plants.

Key words: Induced mutation, NaCl-tolerance, growth, lipid peroxidation, antioxidant enzymes, *Clitoria ternatea* L., *Lathyrus sativus* L.

INTRODUCTION

*Clitoria ternatea* L. or butterfly-pea belonging to family Leguminosae is an important medicinal plant commonly grown as a garden ornamental on fencerow. It is an evergreen, herbaceous perennial legume having slender, twining stems with leaves of 5 to 7 leaflets elliptical to oblong in shape. Flowers are usually solitary, axillary, bright blue or sometimes white with a pale-yellow base. This plant has been used in traditional as well as ‘Ayurvedic’ system of Indian medicine for the treatment of chronic bronchitis, dropsy, goiter, leprosy, mucous disorders, sight weakness, skin diseases, sore throat and tumors (Jain and De Filippis, 1991; Kulkarni et al., 1988). In Eastern Himalayan states of India, its root power mixed with water was taken orally in treatment of ascariasis and fever, while root mixture with a milk product (‘ghee’) or turmeric is generally used immediately after snake bite (Dolui et al., 2004).

*Lathyrus sativus* L. or grass pea is one of the oldest legume crops with cultivation period of more than 8000 years (Smartt, 1984). It has been widely used as ‘poor man’s crop’ in Indian subcontinent, Australia, South America, North Africa and The Mediterranean countries for human consumption and animal feed stock (Biswas, 2007; Campbell, 1997; Jackson and Yunus, 1984; Smartt, 1984; Talukdar, 2009a). This crop has astonishing combinations of many desirable properties like requirement of very low agronomic
input, high N₂ fixing capacity, high seed protein value with number of important amino acids, presence of antioxidant polyphenols, tolerance to various insects and pests and capacity to grow at extreme environmental conditions. A renewed interest in grass pea cultivation and research in Europe and its reintroduction in China have been justified by urgent need to recover marginal and degraded lands over-exploited by cereal cultivation (Granati et al., 2003; Yang and Zhang, 2005). Its potentiality as a possible phytoremediation for lead accumulation has recently been explored (Brunet et al., 2008). Significant accumulation of arsenic in plant parts, particularly in roots, was also reported in Indian genotypes of grass pea (Talukdar, 2011a).

Soil salinity is one of the most severe abiotic stresses affecting production of the legumes worldwide (Bayuelo-Jiménez et al., 2002; Wang et al., 2003). This problem is more severe in arid and semi-arid regions, and legume plants already face or in the near future will face a notable impact of salt stress in these regions (Graham and Vance, 2003). *C. ternatea* L. is commonly considered as low to moderate salt tolerant (Keating et al., 1986; Naidu and Harwood, 1997). In comparison, *L. sativus* L. has been reported to tolerate a wide range of biotic and abiotic stresses (Vaz Patto et al., 2006), and is commonly known as moderate salt-tolerant (Campbell, 1997). Thus, there remains the possibility of developing genotypes with improved salt tolerance if appropriate parents and breeding techniques are adopted. However, low level of genetic variability in cultivated varieties and poor understanding of physiological parameters on its tolerance to environmental stresses are the two prime hindrances for fully exploitation of the remarkable potentiality of these two crops through conventional breeding methods. In grass pea, extensive researches are now going on to augment its high nutritional quality with low seed toxin (ß-N-oxaly L α, ß-diamino propionic acid) content in improved lines through the creation of additional variability by induced mutagenesis and biotechnological approaches, and number of high yielding lines with low seed toxin level have been reported (Asthana, 1995; Talukdar et al., 2001). On the other hand, development of a rapid in vitro micropropagation technique and introduction of *C. ternatea* L. as a new and promising pasture legume crop in Australia are related to enormous potentiality of this medicinal herb in tropical regions (Hackney et al., 2007; Pandeya et al., 2010). Despite the importance for their cultivation in rapidly increasing saline areas, detail investigation has not been undertaken to study the salinity tolerance of these two crops.

Induced mutagenesis is a powerful tool to create additional variability in crop plants. Recently, a number of valuable mutant lines and different cytogenetic stocks including aneuploids, tetraploids and translocation lines have been developed in grass pea through induced mutagenesis which can be used as effective tools to explore the underlying genetic mechanisms of various biotic and abiotic stress responses of this crop (Vaz Patto et al., 2006; Talukdar and Biswas, 2007; Talukdar, 2008, 2009b, 2010a, b, 2011b). But, no such reports are available in *Clitoria*. Recently, mutagenesis has been successfully induced through gamma ray treatment in seeds of *Clitoria* in present author’s laboratory, and preliminary investigation revealed variation in response to salinity between *Clitoria* and grass pea seedlings. The objective of the present study is to assess the salt stress responses based on different morpho-physiological and biochemical parameters at seedling stage of *C. ternatea* L. and *L. sativus* L and to identify salt tolerant mutants in these two crops.

### MATERIALS AND METHODS

#### Plant materials and induction of mutation

Fresh and healthy seeds of *C. ternatea* L. var. CAZR I466, 752, and IGFR 12-1 and *L. sativus* var. RLS-188, Pusa-90-2 and WBK-CB-14 were irradiated each with 100, 150, 200, 250, 300 and 350 Gy doses of gamma rays to induce mutation. Treated seeds were sown treatment-wise during March 2006 for *Clitoria* and October 2006 for grass pea to grow M₁ generation in separate plots with 30 and 50 cm uniform distances between plants and rows, respectively. Untreated plants were used as control. Seeds of individual M₁ plants in all the treatments along with control were separately harvested and sown in different rows in a randomised block design to raise M₂ progenies. M₂ seeds were used to test the effect of salinity at seedling stage of the two crops.

#### Experiment set-up

Dry, healthy and uniform-sized 20 M₂ seeds genotype¹ treatment¹ were surface sterilized in 70% ethanol for 2 min, rinsed twice in deionized water and then placed on water-moistened filter papers in 9 cm diameter Petri dishes in an incubator at 25°C with 12-h light (ISTA, 2008). Germinated seeds were immediately transferred to twelve inches earthen pots containing a mixture of fine soil, vermiculite and farm yard manure (1:1:1). Each pot was labeled with accession number, treatment dose and planting date. Seedlings were thinned to two per pot after emergence and watered evenly for their uniform growth until 7 days after first emergence. Initially, pots were kept away from direct sunlight but with good light intensity, and watered very carefully to keep the soil moist but not wet. Once the seedlings reached 10 cm in height, the pots were kept under normal field conditions with mean day/night temperature 32°C/27°C, 12-h photoperiod and relative humidity of 75±2% for *Clitoria* and 27°C/20°C, 10-h photoperiod and relative humidity of 77±5% for grass pea at climatic condition of lower Indo-Gangetic plain during March and October, respectively. The salt treatment commenced on 15-day-old seedlings. The control plants from each of the six varieties were irrigated with distilled
water, while others were subjected to salinity stress by watering them with 130 mM NaCl supplemented distilled water (300 ml water in each pot), respectively, thrice in a week. Five pots (two plants per pot) of genotype 1 were arranged in a randomized complete block design with three replications of each treatment (radiation dose). Salt concentration was regularly checked by measuring electrical conductivity with a conductimeter (Systronics M-308, Kolkata, India), and evaporranspirational losses were compensated with deionized water. Based on overall plant growth and yield, six best-performing mutants- two in C. ternatea L. (300 Gy, CAZR-1466 and 250 Gy, IGFR 12-1) and four in L. sativus L. (one each from variety RLS-188, 200 Gy, and PUSA-90-2, 300 Gy and rest two from WBK-CB-14, 350 Gy) was selected for further morphological and biochemical study in the next growing season (M₂ generation). The mutants selected in Clitoria were tentatively designated as CR1 and CR2, while those from Lathyrus varieties were named as LR1, LR2, LR3 and LR4, respectively.

Assessment of growth parameters of selected mutants (M₂)

To assess the effect of salt treatment, ten seedlings mutant 1 with un-mutagenised mother variety were subjected to 130 mM NaCl treatment exactly in the same procedure followed in previous M₁ generation. The treated variety was designated as negative control (NC), whereas mutant plants given only distilled water were served as positive control (PC). Growth performance of the selected mutant plants along with PC and NC was assessed by measuring the following parameters, plant height (cm), number of primary branches/leaf, leaves/branch, leaflets/leaf, leaf injury level, root and shoot dry weight (g)/plant after 25 days of commencement of salt treatment. To determine dry weights, after harvesting, plants were separated into roots and shoots. Roots were washed in tap water and rinsed in de-ionised water. Plant materials were dried at 65°C for 48 h and weighed. For ascertaining level of leaf injury, individual seedling was rated on a scale of 0 to 4, based on the following salt injury gradation of Chen et al. (2007) with some modifications for grass pea and Clitoria, 0: No salt injury (100% survival with no damage); 1: Mild salt injury, indicated by small area (approximately 1/5) of leaflet apex and margin turning brownish yellow; 2: Moderate salt injury, indicated by ½ of the leaflet turning whitish-yellow; 3: Severe salt injury, when over 80% of total leaflet area turned whitish-yellow and very thin, and 4: Extreme injury, when leaflets became necrotic, crinkled, finally fell off and the plant ultimately died. Leaflets, borne on first formed primary branches, were considered for visual scorings of the trait.

Estimation of proline

Leaf proline content was estimated according to the method of Bates et al., (1973) from fully expanded leaf samples collected from first formed primary branches on 17th stress day of treatment.

Estimation of Na⁺ and K⁺ contents

Fully expanded leaves of control and salt-treated plants were analysed for total Na⁺ and K⁺ contents following the method of Kumak and Sharma (1989). The oven-dried leaf (0.2 g) was ground to fine powder and transferred to a digestion flask (50 ml) containing acid mixture (3 ml) of concentrated H₂SO₄ and HClO₄ in the ratio of 9:1 (v/v). The flask was heated gently over a hot plate for 10 to 12 min until the solution became colorless. The cooled digest was then diluted by adding double distilled water and volume was made up as required. The estimation of Na⁺ and K⁺ contents in acid extracts was carried out using an atomic absorption spectrophotometer (Perkin Elmer, Analyst-100).

Estimation of relative water content (RWC)

Leaf RWC was estimated according to the method of Whetherley (1950). The turgid weight of 1 g of fresh leaf sample was determined by keeping it in water for 4 h. Dry weight (DW) was measured by drying the same sample in a hot air oven (80°C) until constant weight was achieved. RWC was expressed in percentage following the formula, RWC (%) = [(FW-DW) / (Turgid weight-DW)] × 100.

Estimation of chlorophyll and carotenoids contents

Leaf chlorophyll and carotenoid contents were determined by the method of Lichtenthaler (1987). Leaf tissue (50 mg) was homogenized in 10 ml chilled acetone (80%). The homogenate was centrifuged at 4000 g for 12 min. Absorbance of the supernatant was recorded at 663, 647 and 470 nm for chlorophyll a, chlorophyll b and carotenoids, respectively. The contents were expressed as mg chlorophyll or carotenoids g⁻¹ FW.

Estimation of ascorbic acid (AA)

Ascorbic acid content will be estimated following the methods of Mukherjee and Choudhari (1983). Five hundred milligrams of leaf tissue was homogenized in 10 ml of 6% trichloroacetic acid. Four ml of extract was mixed with 2 ml of 2% dinitrophenylhydrazine in acidic medium followed by the addition of 1 drop of 10% thiourea in 70% ethanol. The mixture was boiled for 15 min in water bath and after cooling to room temperature, 5 ml of 80% v/v H₂SO₄ was added in an ice bath (0°C). The absorbance will be recorded at 530 nm. Concentration of AA will be determined from a standard curve plotted with known concentration of ascorbic acid.

Lipid peroxidation

Lipid peroxidation rates were determined by measuring the malondialdehyde equivalents following the method of Hodges et al. (1999). About 0.5 g of fresh leaf tissue was homogenized in a mortar with 80% ethanol. The homogenate was centrifuged at 3000 × g for 12 min at 4°C. The pellet was extracted twice with the same solvent. The supernatants were pooled and 1 ml of this sample was added to a test tube with an equal volume of either the solution comprised of 20% TCA and 0.01% butylated hydroxy toluene (BHT) or solution of 20% TCA, 0.01% BHT and 0.65% TBA. Samples were heated at 95°C for 25 min and cooled to room temperature. Absorbance was measured at 440, 532 and 600 nm. Lipid peroxidation rate equivalents (nmol malondialdehyde ml⁻¹) were calculated by using the formulae of Hodges et al. (1999).

Hydrogen peroxide estimation

The H₂O₂ content of fully matured leaves was colorimetrically
measured as described by Mukherjee and Choudhur (1983) by extracting leaf samples in cold acetone and mixing of an aliquot (3 ml) of the extracted solution with 1 ml of 0.1% titanium dioxide in 20% (v/v) H$_2$SO$_4$. After centrifugation at 6,000 g for 15 min, the intensity of yellow colour of the supernatant was measured at 415 nm. The concentration of H$_2$O$_2$ was calculated from a standard curve plotted within the range of 100 to 1000 nM H$_2$O$_2$.

**Electrolyte leakage**

Electrolyte leakage (EL) was assayed by measuring the ions leaching from leaf tissue into deionised water (Dionisio-Sese and Tobita, 1998). Fresh leaf samples (100 mg) were cut into small pieces (about 5 mm segments) and placed in test tubes containing 10 ml deionised water. Tubes were kept in a water bath at 32°C for 2 h. After incubation, electrical conductivity (EC) of the bathing solution was recorded with an electrical conductivity meter (Systronics M-308, Kolkata, India). The samples were then autoclaved at 121°C for 20 min to completely kill the tissues and release all electrolytes. Samples were then cooled to 25°C and final electrical conductivity (EC) was determined. The EL was expressed as a percentage by the formula, EL (%) = (EC$_1$)/(EC$_2$) x 100

**Antioxidant enzyme assays**

**Superoxide dismutase**

Plant material was homogenized in 50 ml phosphate buffer, pH 7.0, containing 1% PVP. The homogenate was filtered and then centrifuged in a refrigerated centrifuge at 15,000 x g for 20 min. The resultant supernatant was used as source of enzyme. The extraction was performed at 4°C. The activity of superoxide dismutase (SOD, EC 1.15.1.1) was determined by the nitro-blue tetrazolium (NBT) photochemical assay method as described by Beyer and Fridovich (1987). The reaction mixture (3 ml) contains 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 μM NBT, 0.1 mM EDTA, 2 μM riboflavin and 0.1 ml of enzyme extract.

The test tubes with reaction mixture were shaken carefully and kept for 25 min under 30 cm below a light source of 30 W fluorescent lamps. A parallel control was run where buffer was used instead of sample. The reaction was then stopped by switching off the lights, and the tubes were covered with black cloth. The absorbance of the solution was measured at 560 nm in a UV-Vis spectrophotometer. A non-irradiated complete reaction mixture served as a blank. SOD activity was expressed as Enzyme unit mg$^{-1}$ protein. One unit of SOD was defined as the amount of protein causing a 50% NBT photoreduction.

**Ascorbate peroxidase**

100 mg of plant samples were homogenized in 1 ml of 50 mM phosphate buffer (pH 7.8) containing 5 mM ascorbate, 5 mM DTT, 5 mM EDTA, 100 mM NaCl and 2% (w/v) PVP. The homogenized material was centrifuged at 15,000 x g for 15 min at 4°C. Ascorbate peroxidase (APX, EC 1.11.1.11) activity was assayed following the method of Nakano and Asada (1981). The hydrogen peroxide-dependent oxidation of ascorbate was followed by a decrease in the absorbance at 290 nm with extinction constant 2.8 mM$^{-1}$ cm$^{-1}$. The enzyme activity was expressed as change in absorbance units, mg$^{-1}$ protein.

**Statistical analysis**

The results are presented as mean values ± standard errors (SE). Statistical significance between mean values was measured by simple t-test. A probability of $p<0.05, 0.01$ was considered significant.

**RESULTS AND DISCUSSION**

**Effects of salinity on plant growth**

In comparison to PC plants, significant reduction in plant height, primary branches/plant, leaves/branch, leaflets/leaf, respectively was exhibited by LR1 mutant under salinity stress condition. Mutant CR1 also manifested decrease in mean values of plant height and number of primary branches, but the reduction was not significant for leaf and leaflet number under salt stress (Table 1). The CR2 mutant showed marginal decrease in these four traits. Among the Lathyrus mutants, increase in mean value of above four parameters was observed in both LR3 and LR4 mutants in different magnitudes, whereas LR2 showed non-significant variation for the traits as compared to PC plants (Table 2). Both shoot and root dry weight (g) decreased significantly in CR1, while marginal reduction for both the traits was estimated for CR2 plants. Mean value of shoot dry weight varied marginally in LR1, LR2 and LR3 mutants, but it increased significantly in LR4 mutant. Root dry weight decreased in all the four mutants, but significantly only in LR1 under salinity stress. Drastic reduction in mean of all the six growth parameters was observed in NC plants, showing much higher rate of reduction than the mutant lines under NaCl treatment (Tables 1 and 2).

Munns (2005) described dry weight of plants as one of the realistic criteria in determining salt responses in plants. An increase in height, primary branches and leaf number might be attributed to higher shoot dry weight in LR3 and LR4 plants. Therefore, high tolerance was found associated with high total plant dry weight, which was increased by positive contribution from other components, least affected by salt induced injury in tolerant lines. In grass pea, superior performance of dwf1 and dwf2 dwarf mutant lines at 170 mM NaCl treatment was indicated by their normal plant dry weight, while low plant biomass accumulation in the third dwarf line, dwf3, was associated with symptoms of increased salt sensitivity (Talukdar, 2011b). Reduction in plant dry weight under salt stress was reported in different varieties of grass pea (Mahdavi and Sanavy, 2007) and Phaseolus (Bayuelo-Jime’nez et al., 2002). Interestingly, root development was more sensitive to salt treatment than shoot development in the present mutant lines, and the result was in accordance with earlier reports in grass
Table 1. Assessment of 12 growth parameters in positive control (PC), negative control (NC) and two selected mutant lines (CR1 and CR2) of *C. ternatea* L. at harvest (25 days after commencement of 130 mM NaCl treatment on 15-days-old seedlings).

<table>
<thead>
<tr>
<th>Traits</th>
<th>PC</th>
<th>CR1</th>
<th>CR2</th>
<th>NC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant height (cm)</td>
<td>49.9±0.11</td>
<td>33.6±0.09*</td>
<td>41.9±0.10</td>
<td>22.4±0.15**</td>
</tr>
<tr>
<td>Primary branches plant⁻¹</td>
<td>8.8±0.21</td>
<td>6.0±0.13*</td>
<td>8.2±0.09</td>
<td>2.9±0.10**</td>
</tr>
<tr>
<td>Leaves branch⁻¹</td>
<td>5.7±0.19</td>
<td>4.9±0.11</td>
<td>5.5±0.10</td>
<td>3.0±0.16*</td>
</tr>
<tr>
<td>Leaflets leaf⁻¹</td>
<td>6.3±0.20</td>
<td>5.9±0.04</td>
<td>6.0±0.05</td>
<td>2.3±0.02**</td>
</tr>
<tr>
<td>Shoot dry weight (g)</td>
<td>0.097±0.31</td>
<td>0.077±0.22*</td>
<td>0.089±0.18</td>
<td>0.041±0.24**</td>
</tr>
<tr>
<td>Root dry weight (g)</td>
<td>0.10±0.21</td>
<td>0.072±0.15*</td>
<td>0.098±0.18</td>
<td>0.031±0.17**</td>
</tr>
<tr>
<td>Leaf injury level</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Chlorophyll a (mg g⁻¹ fr. wt.)</td>
<td>4.34±0.56</td>
<td>3.89±0.34*</td>
<td>4.29±0.29</td>
<td>1.79±0.31**</td>
</tr>
<tr>
<td>Chlorophyll b (mg g⁻¹ fr. wt.)</td>
<td>1.70±0.49</td>
<td>0.94±0.25*</td>
<td>1.73±0.28</td>
<td>0.75±0.30*</td>
</tr>
<tr>
<td>Carotenoids (mg g⁻¹ fr. wt.)</td>
<td>1.22±0.18</td>
<td>1.09±0.25</td>
<td>1.30±0.17</td>
<td>0.79±0.23*</td>
</tr>
<tr>
<td>Na⁺ content (mg g⁻¹ fr. wt.)</td>
<td>2.39±0.30</td>
<td>2.89±0.12</td>
<td>2.41±0.17</td>
<td>5.01±0.27*</td>
</tr>
<tr>
<td>K⁺ content (mg g⁻¹ fr. wt.)</td>
<td>3.43±0.24</td>
<td>3.51±0.20</td>
<td>4.67±0.14*</td>
<td>2.22±0.17**</td>
</tr>
</tbody>
</table>

+ Values are means ± standard error (n = 10); * and ** significant from PC at 5 and 1% levels, respectively.

Table 2. Assessment of growth performance of positive control (PC), negative control (NC) and four selected mutant lines (LR1, LR2, LR3 and LR4) of *L. sativus* L. at harvest (25 days after commencement of 130 mM NaCl treatment on 15-day-old seedlings).

<table>
<thead>
<tr>
<th>Traits</th>
<th>PC</th>
<th>LR1</th>
<th>LR2</th>
<th>LR3</th>
<th>LR4</th>
<th>NC</th>
</tr>
</thead>
<tbody>
<tr>
<td>X1</td>
<td>37.3±0.22</td>
<td>27.8±0.19*</td>
<td>33.9±0.22</td>
<td>38.2±0.11</td>
<td>40.2±0.09</td>
<td>21.2±0.19</td>
</tr>
<tr>
<td>X2</td>
<td>7.5±0.22</td>
<td>5.3±0.17*</td>
<td>7.0±0.09</td>
<td>7.8±0.03</td>
<td>8.8±0.11*</td>
<td>3.9±0.19**</td>
</tr>
<tr>
<td>X3</td>
<td>5.5±0.15</td>
<td>4.2±0.11*</td>
<td>5.2±0.17</td>
<td>6.3±0.12*</td>
<td>7.0±0.03*</td>
<td>2.5±0.11**</td>
</tr>
<tr>
<td>X4</td>
<td>6.0±0.12</td>
<td>4.7±0.08*</td>
<td>5.7±0.09</td>
<td>6.2±0.02</td>
<td>5.8±0.10</td>
<td>3.1±0.20**</td>
</tr>
<tr>
<td>X5</td>
<td>0.082±0.23</td>
<td>0.077±0.15</td>
<td>0.080±0.19</td>
<td>0.085±0.13</td>
<td>0.094±0.25*</td>
<td>0.037±0.19**</td>
</tr>
<tr>
<td>X6</td>
<td>0.094±0.31</td>
<td>0.076±0.22*</td>
<td>0.086±0.32</td>
<td>0.083±0.18</td>
<td>0.090±0.20</td>
<td>0.026±0.22**</td>
</tr>
<tr>
<td>X7</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>X8</td>
<td>4.98±0.69</td>
<td>3.34±0.22*</td>
<td>4.74±0.31</td>
<td>5.21±0.18*</td>
<td>5.18±0.19*</td>
<td>1.89±0.30**</td>
</tr>
<tr>
<td>X9</td>
<td>1.67±0.54</td>
<td>0.91±0.14*</td>
<td>1.58±0.21</td>
<td>1.89±0.69</td>
<td>2.02±0.25*</td>
<td>0.79±0.33**</td>
</tr>
<tr>
<td>X10</td>
<td>1.17±0.29</td>
<td>0.67±0.20*</td>
<td>1.10±0.22</td>
<td>1.29±0.21</td>
<td>1.45±0.09*</td>
<td>0.70±0.31*</td>
</tr>
<tr>
<td>X11</td>
<td>1.89±0.40</td>
<td>2.71±0.32*</td>
<td>2.19±0.27</td>
<td>2.10±0.16</td>
<td>2.05±0.17</td>
<td>4.35±0.29**</td>
</tr>
<tr>
<td>X12</td>
<td>4.30±0.34</td>
<td>4.39±0.25</td>
<td>4.81±0.17*</td>
<td>4.92±0.22*</td>
<td>5.15±0.09*</td>
<td>2.09±0.27**</td>
</tr>
</tbody>
</table>

Effect of salinity on leaf injury

Among the mutants, CR1 and LR1 manifested level 1 injury level on leaflets, while CR2, LR2, LR3 and LR4 showed complete absence (level 0) of leaf injury under salt treatment (Tables 1 and 2). Marking of injury was also absent (level 0) in leaflets of PC seedlings, but severe injury was exhibited on leaflets of NC plants as level 3. Initially, the injury was marked by small chlorotic spots on leaflet tips, but as treatment progressed further, large areas in lamina became yellow. The lower leaflets in NC plants also showed marking of necrosis at later stages of growth. Leaf injury has been considered as one of the important salt response traits in plants (Munns, 2005). Among the five levels (0 to 4) standardized on the basis of visual scoring of leaf injury under salt stress, level 3 injury was manifested by NC plants, indicating highest level of susceptibility of these plants to salt treatment. The level 1 injury in the leaves of CR1 and LR1 mutants suggested lower level of salt sensitivity, while absence of injury (level 0) on leaves of CR2, LR2, LR3 and LR4 genotypes as per PC level indicated their better tolerance to salt stress. The cause of leaf injury was attributed to salt load exceeding the capability of leaf
cells to compartmentalize salts in the vacuole. Salts would then build up rapidly either in the cytoplasm inhibiting enzyme activity or in the cell walls resulting in dehydration of the cell (Munns, 2005).

Salt stress effect on leaf proline content

Among the parameters responding to salt treatment, rapid accumulation of free proline content is one of the significant events in plants. In the present material, compared with PC plants, endogenous free proline content increased at highest level in LR4, and it was followed by CR2, LR3 and LR3 plants, whereas its accumulation decreased significantly in NC plants (Figure 1A). However, the exact role of proline in abiotic stress tolerance is being debated. A positive correlation between proline over accumulation and increasing salinity/drought tolerance has also been found in different crop plants including transgenics that were engineered for overproduction of proline (Kavi, 1989; Anoop and Gupta, 2003). Increase in proline content under NaCl stress was also reported in Pisum sativum (Ahmad et al., 2008) and Phaseolus aureus (Misra and Gupta, 2005). In contrast, proline accumulation in plants was regarded by some workers as a symptom of stress in less-salinity-tolerance species, and its contribution to osmotic adjustment was found negligible as compared with K⁺ (Wang et al., 2004). In the present study, higher accumulation of proline was estimated in those genotypes which manifested tolerance phenotypes for other traits also. Further study, however, is needed to ascertain its exact role in salt-induced Lathyrus and Clitoria genotypes.

Effect of salinity on Na⁺ and K⁺ relative water content (%)

NaCl-induced salinity exhibited highest accumulation of Na⁺ and reduction of K⁺ concentration in leaves of NC plants for both Clitoria and Lathyrus mutants. Among the six mutants, Na⁺ accumulation was as per with PC plants in five mutants except LR1 where its content increased significantly under salt treatment. All the mutant showed an increase in K⁺ content but the change was significant in CR2, LR2, LR3 and LR4 mutant plants (Tables 1 and 2). The K⁺/Na⁺ ratio increased significantly over PC plants in CR2 and LR4 mutants, but decreased significantly in NC plants for both crops (Figure 1B).

As compared to control, significant decrease in RWC% was recorded in NC plants (1.3-fold) and in CR1 (1.2-fold) mutants under salt stress (Figure 1C). Marginal decline was recorded in rest of the genotypes.

Equilibrium of cellular Na⁺ and K⁺ content is absolutely essential in imparting salinity tolerance in plants. Excessive accumulation of Na⁺ and Cl⁻ in the leaves has been considered highly harmful for normal metabolism of plants, and tolerant genotypes has the capacity of successful salt exclusion (Greenway and Munns, 1980; Yeo and Flowers, 1986; Munns, 2005). The K⁺:Na⁺ ratio has been used as a discriminating factor between tolerant and sensitive genotypes with greater capacity of former to block or reduce the uptake or exclude the excess amount of Na⁺ and associated increase in K⁺ content (Munns, 2005). In the present material, significant increase in K⁺ content and marginal variation in Na⁺ level, in comparison to PC, might be one of the reasons for better plant growth in CR2, LR2, LR3 and LR4 plants. High accumulation of Na⁺ and decrease in K⁺ content has led to lowest K⁺:Na⁺ ratio, rendering NC plants highly susceptible to salt treatment. Leaf RWC content followed the same trend.

Chlorophyll, carotenoids contents and ascorbate pool

Salinity has significant effect on the chlorophyll a and b content. As compared to PC plants, mean value decreased significantly in CR1 and LR1 seedlings but increased in LR3 and LR4 under salinity stress (Tables 1 and 2). The change, however, was not significant in case of CR2 and LR2 mutants. Among the 10 genotypes, the ratio of chlorophyll a/b was highest (4.13) in CR1 and lowest (2.38) in NC-Clitoria (Figure 1D). The change in carotenoid content was not significant in the mutant plants except LR1 where it reduced and in LR4 where its content enhanced significantly under salt stress. The NC plants exhibited lowest mean value for both chlorophyll and carotenopid contents.

Ascorbate content was increased by nearly 2-folds in the leaves of LR4 plants. Significant increase was also estimated in LR3, LR2 and CR2 plants (Figure 1). Its content, however, decreased in other genotypes, but the value was significant in NC plants under salinity stress (Figure 1E).

Photosynthetic pigment contents-chlorophyll a, b and carotenoids were also affected by NaCl treatment in different magnitudes. Among the ten genotypes, LR4 and LR3 exhibited higher level of these three pigment components, while CR2 and LR2 maintained level of PC plants. In contrast, reduction of chlorophyll contents in NC, CR1 and LR1 plants was, presumably, due to the increased activity of chlorophyllase enzyme or due to the disruption of ultrastructure of pigment-protein complex by ion toxicity (Saha et al., 2010).
However, relatively higher reduction in chl b content as compared to chl a led to an increase in chl a/b ratio in CR1 plants, and this can be explained by the fact that degradation of chl b under salt stress begins with its conversion to chl a, as reported in mung bean (Saha et al., 2010). In plants, carotenoids and ascorbate play a primary role as a non-enzymatic antioxidant defense in combating stress by quenching reactive oxygen species (ROS). Elevated levels of these two components in leaves of NaCl-treated CR2, LR2, LR3 and LR4 seedlings indicated their better free radical scavenging capacity. A positive correlation between ascorbate contents and anti-oxidant defense enzymes, particularly peroxidase has recently been established (Diaz-Vivancos et al., 2010).

**Effect of salinity on lipid peroxidation, tissue H$_2$O$_2$ content and EL%**

Cell membrane stability is often related to salt tolerance in plants, and the conductance measurement of electrolyte leakage from leaf cells is usually used as an indicator of membrane damage in salt-treated plants. Change in cell membrane integrity is closely linked with extensive membrane lipid peroxidation in plants including legumes (Hernández et al., 2000). Reactive oxygen species, namely superoxide radicals induces the degradation of phospholipids and the resulting polyunsaturated fatty acids released by such breakdown are then peroxidised (Kellogg and Fridovich, 1975). In the present study, enhanced rate of lipid peroxidation was recorded as indicated by gradually increasing malondialdehyde (MDA) contents in seedlings of both *Clitoria* and *Lathyrus* mutant and NC plants exposed to salinity. The MDA content increased significantly in leaves of NC, CR1 and LR1 plants, and the increase was the highest in NC seedlings (Figure 1F). Non-significant variation in MDA content was, however, estimated for the rest of the genotypes. Similar trend was recorded in tissue hydrogen peroxide content in the 10 genotypes under salt treatment (Figure 1G). This result was supported by the significant increase in percentage of electrolyte leakage (EL%) in leaves of NC plants under salt treatment. It was followed by CR1, LR1 and LR2 plants. Electrolyte leakage was increased by 3.1 to 7.8 folds in NC plants, 1.6-fold in CR1, 5.3-fold in LR1 and 2.7-fold in LR2 mutants (Figure 1H). The result strongly indicated enhanced rate of membrane lipid peroxidation in salt-sensitive NC and CR1 as well as LR1 mutants resulting in much higher level of H$_2$O$_2$ content and electrolyte leakage in leaves in comparison to relatively tolerant genotypes of CR2 in *Clitoria* and LR4 as well as LR3 in *L. sativus*. The result is in accordance with earlier findings in legumes like pea, cowpea, mungbean (Hernández et al., 2000; Ahmad et al., 2008; Maia et al., 2010; Saha et al., 2010;) and other crops (Dionisio-Sese and Tobita, 1998; Sairam and Srivastava, 2002; Hossain et al., 2006).

**Salinity-induced response of two antioxidant enzymes activities**

Under salt treatment, leaf SOD activity increased significantly in CR1 (1.7-fold), CR2 (1.8-fold), LR1 (1.7-fold), LR2 (2.5-fold), LR3 (2.7-fold) and LR4 (2.9-fold) plants (Figure 1I), but reduced significantly in NC plants (Figure 1I). The activity of the H$_2$O$_2$- decomposing enzyme APX was significantly higher in CR2 (1.9-fold) mutants of *C. ternatea*, while among four mutants of *L. sativus* L., highest increase in enzyme activity was estimated in leaves of LR4 (3.2-fold) under salt stress. It was closely followed by LR3 (2.9-fold) and LR2 (2.0-fold) plants (Figure 1J). Significant decrease in APX activity, however, was recorded in salt-treated NC, CR1 and LR1 plants (Figure 1J). The regulation of cellular redox state is a crucial factor when a plant experiences environmental stresses like salinity (Apel and Hirt, 2004). The production of different classes of reactive oxygen species (ROS) due to enhanced leakage of electron to oxygen in mitochondria and an increase in the H$_2$O$_2$ content in chloroplasts and their export to cytosol under salt stress conditions trigger the increase in anti-oxidant enzyme activities in plants. Superoxide dismutase (SOD) and ascorbate peroxidase constitute the first line of defense along with other non-enzymatic primary components (Ahmad et al., 2008; Hernández et al., 2000). SOD catalyzes the chemical conversion of superoxide radicals into hydrogen peroxide, which is in turn metabolized by the action of peroxidases. In the present study, SOD activity increased significantly in all the mutant lines, but decreased APX activity in CR1 and LR1 plants might have rendered these two mutant lines less effective in scavenging the H$_2$O$_2$ radicals, leading to the significant accumulation of H$_2$O$_2$ in tissues. In contrast, high SOD level coupled with relatively higher APX activity in CR2, LR3 and LR4 seedlings helped them to remove H$_2$O$_2$ properly. A slight decrease in APX activity as compared with SOD level in LR2 plants might be responsible for accumulation of H$_2$O$_2$ level, close to significant level and significant EL% under salt stress. The decrease of both SOD and APX activity led to NC plants highly susceptible to salinity treatment, as supported by other parameters in the present study. Increase in SOD and APX activity in relatively salt tolerant genotypes was also reported in different crop plants including legumes (Ahmad et al., 2008; Hernández et al., 2000;
Figure 1. Assessment of different leaf biochemical components and antioxidant activities in six selected induced mutant lines of *C. ternatea* L. (CR1 and CR2) and *L. sativus* L. (LR1, LR2, LR3 and LR4) under 130 mM NaCl treatment; PC-positive control (untreated), NC-negative control (treated). (A) Proline content; (B) K'/Na' ratio; (C) Relative water content (RWC) %; (D) Chlorophyll a/b ratio; (E) Ascorbate content; (F) MDA content; (G) Hydrogen peroxide (H$_2$O$_2$) content; (H) Electrolyte leakage (EL%); (I) Superoxide dismutase, and (J) Ascorbate peroxidase activity. Unit of measurement was given within bracket in each case. Data were expressed as the mean of 10 independent values (n = 10); * and ** mean values are significantly different from PC value at 5 and 1% level, respectively.
Maia et al., 2010; Sairam and Srivastava, 2002; Hossain et al., 2006).

Based on the performance of six mutants in their responses to 22 parameters under salt stress in the present study, it was concluded that LR4 exhibited highest tolerance to 130 mM NaCl treatment, and it was closely followed by CR2, LR3 and LR2 mutants. The CR1 and LR1 showed moderate tolerance, while NC plants exhibited highest susceptibility to NaCl-induced salinity stress at seedling stage.

REFERENCES


