ISSN: 0976-3031

Available Online at http://www.recentscientific.com

International Journal of Recent Scientific Research
Vol. 3, Issue, 10, pp.812 - 821, October, 2012

International Journal of Recent Scientific Research

RESEARCH ARTICLE

DEVELOPMENT OF NACL-TOLERANT LINE IN AN ENDANGERED ORNAMENTAL, *ADENIUM MULTIFLORUM* KLOTZSCH THROUGH *IN VITRO* SELECTION

Tulika Talukdar[1]

Department of Botany, Krishnagar Govt. College, University of Kalyani, Krishnagar, West Bengal, India

ARTICLE INFO

Article History:

Received 10th September, 2012
Received in revised form 20th September, 2012
Accepted 29th September, 2012
Published online 6th October, 2012

Key words:

Adenium multiflorum Klotzsch, Antioxidant enzymes, *In Vitro*, NaCl-tolerant callus line, Organogenesis, Reactive oxygen species

ABSTRACT

Plants were developed through *in vitro* organogenesis of a NaCl-selected callus line of an endangered ornamental, *Adenium multiflorum* Klotzsch. Stepwise selection by gradual increase of NaCl concentration (0-100 mM) in the MS medium was found to be more effective for survival and growth of callus than direct transfer to salinity. Callus was developed aseptically from leaf explants in MS medium supplemented with NAA and kinetin. Comparing control, the selected (100 mM NaCl, stepwise) callus line exhibited significant increase in activities of antioxidant enzymes. Stability of salt tolerance of the selected callus was checked by growing the calli in NaCl free medium for 3 successive months, followed by re-exposure to higher salinity stress (120 mM). Under elevated salt stress (200 mM NaCl), the regenerants (S1) obtained from selected calli showed significantly higher antioxidant enzyme activities over both positive control (control callus derived plants in NaCl free medium) and negative control (control callus derived plants in 200mM NaCl). This ensured low level of lipid peroxidation and consequent good growth of S1 plants under high NaCl stress. Based on growth performance and antioxidant capacity, the S1 plants could be selected as NaCl-tolerant line.

© Copy Right, IJRSR, 2012, Academic Journals. All rights reserved.

INTRODUCTION

Adenium (*A. multiflorum* Klotzsch), also known as Impala Lily, is a beautiful ornamental plant in the family Apocynaceae. Due to its 'bon-sai' like appearance and flowers having brilliant combinations of red, white, pink and crimson coloration *A. multiflorum* has gained tremendous popularity as an ornamental cash crop in domestic (India) as well as in international market. It is native to tropical Africa and Arabia, but introduced and naturalized in different parts of the world including South-east Asia (Oyen, 2008). The plant, recently, has been enlisted in Red Data Lists of several tropical African countries where it is regarded as threatened. Habitat destruction, over-exploitation for medicinal and illegal horticultural trade, and poor propagation in its natural habitat are the main threats to Adeniums. In India, the interest for growing it as a pot plant has been increasing markedly due to its rising demand for landscape and indoor decoration (Bhattacharjee, 2006). *A. multiflorum* can be propagated by seeds, cuttings or transplants. However, both are inefficient propagation methods for the pot plant, since the germinability of seed is very poor and the planting material not only has a very low multiplication rate but it requires a large area of stock plants also (Kanchanapoom *et al*., 2010). *In Vitro* regeneration technique has emerged as a feasible and cost-effective alternative tool for rapid production of ornamentals in recent years. In a developing economy like India, the market for ornamentals has steadily increased over the last few years (Hossain *et al*., 2007) and Adeniums have good opportunity to occupy a significant share of this nascent market.

Soil salinity, mostly due to the presence of NaCl, is an ever increasing threat to crop yield, particularly in arid and semi-arid areas (Yamaguchi and Blumwald, 2005). Millions of hectares of land in India is suffering with problems of salinity and lying either un-utilized or semi-utilized. Massive efforts are going on to develop techniques for the identification of successful plant genotypes for proper utilization of such degraded soil. Development of NaCl induced stress tolerant plant through *in vitro* selection has been reported in many crops including ornamentals (Hossain *et al*., 2007; Patnaik and Debata, 1997; Rai *et al*., 2011). However, no systematic effort has been made for screening the beautiful *A. multiflorum* suitable for cultivation in salt affected soil to meet the increasing demand of floriculture trade.

Generation of reactive oxygen species (ROS) is an inevitable event in aerobic plant life, which usually put ROS metabolism under tight regulation. However, in response to environmental stress, such as salinity, the critical balance between the formation of ROS and its scavenging by antioxidant defense molecules is disturbed. Like other stress conditions, NaCl can generate excess ROS which has the potentiality to unbalance the cellular redox system heavily in favor of oxidized state, resulting in oxidative damage to lipids, proteins and nucleic acids (Zhu, 2002). Plants have evolved a diverse but well integrated antioxidant defense system comprising of both enzymatic [superoxide dismutase (SOD), ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), glutathione reductase (GR), catalases (CAT) etc.] and non-enzymatic [glutathione, ascorbate, carotenoids and phenolic compounds]

* Corresponding author: +9109231624902
E-mail address: talukdartulip12@gmail.com

compounds to protect themselves against biotic and abiotic stress conditions (Noctor and Foyer, 1998; Talukdar, 2011a, 2012a,b). Selection of salt-tolerant genotypes through induction of antioxidant enzymes has become a major strategy in many crops including ornamentals, vegetables, cereals and legumes (He *et al*., 2009; Hossain *et al*., 2007; Olmos *et al*., 1994; Rai *et al*., 2011; Talukdar, 2011a,c).

ROS mainly comprises of superoxide radical ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$) and singlet oxygen ($^1O_2$).Within plant cells, SODs, a group of metalloenzymes, constitute the first level of defense against superoxide radicals. However, as has frequently been pointed out that dismutation of superoxide radical results in simultaneous production of hydrogen peroxide (H_2O_2), another predominant ROS (Noctor and Foyer, 1998).Within ascorbate (AsA)-glutathione (GSH) cycle, APX is the principal H_2O_2-scavenging enzyme (Asada, 2006). APX uses reduced ascorbate as its co-factor. The resultant dehydroascorbate (DHA), the oxidized form of ascorbate, is reduced back to ascorbate by the activity of DHAR. GR plays a crucial role by maintaining the GSH (reduced glutathione)/GSSG (oxidized glutathione) ratio favorable to ascorbate reduction (Gossett *et al.*, 1996). For this reason GR is considered as a rate limiting enzyme for defense against active O_2 toxicity (Tanaka, 1994). Outside this cycle, H_2O_2 is removed by the catalase, which does not consume any type of reducing power to decompose H_2O_2 (Talukdar, 2011a; Willikens *et al*., 1997). Increased generation of ROS including H_2O_2 often results in peroxidation of membrane lipids with consequent production of malondealdehyde (MDA), a cytotoxic aldehyde, the level of which is often used as an indicator of ROS induced oxidative damage (Demiral and Turkan, 2005; Talukdar, 2011a-c).

Accumulation of compatible solute is one of biochemical strategies plants adopt to restrict the uptake of salt and adjust their osmotic pressure (Rai *et al.*, 2011). Proline is one of most studied compatible solute protecting plants from osmotic stress (Rai *et al*., 2011; Sumithra *et al*., 2006; Talukdar, 2011a). However, the significance of proline accumulation in osmotic adjustment is still debatable and varies according to the species (Talukdar, 2011a).

Strategies for the development of NaCl-tolerant plants have been reported in cereals (Vajrabhaya *et al*., 1989), legumes (Talukdar, 2011a) and other economically important crops including ornamentals (Hossain *et al*., 2007; Suprasanna, 2010). *In vitro* selection procedure, induced mutagenesis and *Agrobacterium*-mediated transformation offer powerful tools for the development of such tolerant lines. Handsome reports are available regarding development and isolation of NaCl-tolerant cell/callus lines using *in vitro* technique (Kumar and Sharma, 1989; Olmos *et al*., 1994; Patnaik and Debata, 1997; Tal, 1994; Vajrabhaya *et al*., 1989). However, information is scanty about successful regeneration of plants from such tolerant lines showing stability in salt tolerance traits (Binh *et al*., 1992; Patnaik and Debata, 1997; Winicov, 1996; Zhang *et al*., 2001). The main constrain behind the development of NaCl-tolerant plants from such callus/cell lines is the loss of regeneration potentiality or genetic instability of salt tolerance at the whole plant level (Hossain *et al*., 1997).

In the present investigation, an attempt has been made to develop stable NaCl-tolerant *A. multiflorum* plants by selection of NaCl-tolerant callus line and their subsequent differentiation under NaCl stress condition. Development of stable micro propagated plants would help to meet growing demand of *A. multiflorum* in floriculrure trade through direct utilization of saline soil.

MATERIALS AND METHODS

Plant material

The plant of *A. multiflorum* grown in earthen pot was used as an experimental donor plant. Juvenile leaves from 3 months old donor plant were used as explants. Leaf explants were washed thoroughly under running tap water for 30 min and for another 10 min with 5% (v/v) aqueous solution of Teepol, followed by washing with single distilled water three times. Teepol, a liquid detergent was used to remove the dust and dirt particles from the leaf surface. Leaves were then treated with bavistin (a systemic fungicide) solution for 4-5 min, followed by rinsing thoroughly with sterile distilled water. The leaf explants were then quickly dipped (for 30 sec) in 70% ethanol and surface sterilized with 0.2% (w/v) aqueous $HgCl_2$ solution for 2 min, followed by repeated washing with sterile distilled water. All leaves were dissected into small pieces (approximately 0.5-0.75 cm length) and trimmed, so that maximum part can be exposed to media.

Culture media and growth condition

Murashige and Skoog (1962) medium (MS) were used as basal medium fortified with 3% (w/v) sucrose and 0.8% Difco-bactoagar. The plant growth regulators used were α – naphthalene acetic acid (NAA) and kinetin. The $_pH$ of the medium was adjusted to 5.6-6.0 prior to autoclaving at 121 $^{\circ}$C for 15 min. All the experiments were carried out in culture tubes (150x25mm) containing 25-30ml of culture medium. Temperature of the culture room was maintained at 25 ± 1 $^{\circ}$C under a 16 h photoperiod with a light intensity of 3000lux and relative humidity of 65-70%.

Callus induction and establishment

For callus induction juvenile leaf section (approximately 0.5cm long) with cut end surface was placed in contact with culture medium supplemented with growth regulators- α-naphthaleneacetic acid (NAA), 1-2.5mg l^{-1} and kinetin, 0.5mg l^{-1}. The inoculated calli were subcultured after every 10-15 days for successive three times in the same medium composition.

In vitro NaCl treatment of callus and selection protocol (for callus)

After three successive subcultures, comparatively green fast growing calli were selected for NaCl treatment. The lethal concentration of NaCl in the medium for callus survival was first determined and it was found to be 150 mM for *A. multiflorum.* Two selection protocols viz. direct selection (DS) and stepwise selection (SS) were utilized to develop NaCl-tolerant callus line. In DS protocol, the callus pieces, approximate 500 mg fresh mass (FM) were transferred to the same callus induction medium but supplemented with different concentrations of NaCl (0, 50, 75 and 100 mM) and maintained for 9 consecutive months with regular subculture at 30 days interval. Calli cultured on callus induction medium

(MS medium supplemented with growth regulators) devoid of NaCl were considered as control. In stepwise selection protocol, approximately 500 mg of callus (fresh mass) was first transferred to 50mM NaCl supplemented medium and maintained for 3 consecutive months with regular subculture at 30 days interval. After 3 months, only surviving calli were transferred to next higher NaCl concentration i.e.75mM NaCl supplemented medium and maintained for another 3 months. Finally, surviving calli were transferred to 100mM NaCl medium and maintained again for 3 months. All total 60 replicates were maintained for each treatment. Out of this, 36 replicates were used (four replicates during each subculture) up to 9 months for measuring growth and biochemical parameters. During subcultures soft, necrotic callus portions were rejected and healthy growing callus portions (Fig.1A and B; arrow marks) were transferred to fresh medium.

After 9 months of long NaCl treatment, based on growth performance and antioxidant capacity, in stepwise protocol, well growing green calli were selected as 100mM NaCl-tolerant line that showed all positive responses towards the stress (Fig.1C). In DS method, same procedure of growth performance and antioxidant capacity were used in screening tolerant line at each NaCl concentration. The selected lines were then allowed to grow in NaCl free medium for 3 months with regular subculture followed by retransfer to slightly higher saline medium (120 mM) to check the stability of NaCl-tolerance character.

Plant regeneration

For shoot organogenesis, calli of NaCl-selected line (100 mM NaCl-tolerant callus line) were inoculated on 100mM NaCl supplemented MS medium containing 3% sucrose, 0.8% difcobactoagar and growth regulators (Table 1). The pH adjustment of medium and autoclaving were done as earlier. Separate control calli were cultured on MS medium having the same composition except for NaCl and considered as control. All the cultures were incubated at 25 ± 1 °C under a 16h photoperiod with a light intensity of 3000lux and relative humidity of 65-70%.

In-vitro NaCl treatment of regenerated plants

Regenerated shoots (1–1.5 cm in length) of both control and selected calli were transferred separately to MS medium (containing 3% sucrose and 0.8% bactoagar) supplemented with 200 mM NaCl and considered as negative control (NC) and S1 line, respectively. Separate control line (shoots developed from control calli) was maintained in MS medium having the same compositions except for the NaCl and treated as positive control (PC).

Assessment of callus growth

Callus fresh mass (FM) was measured at the time of every subculture. Callus growth was expressed in terms of percentage of fresh mass increased.

Antioxidant enzyme assays

Fresh callus/leaf tissue (250 mg) was homogenized in 1mL of 50mM potassium phosphate buffer (pH 7.8) containing 1mM EDTA, 1mM dithiotreitol and 2% (w/v) polyvinyl pyrrolidone (PVP) using chilled mortar and pestle kept in ice bath. The homogenate was centrifuged at 15,000×*g* at 4 °C for 30 min. Clear supernatant was used for enzyme assays. For measuring APX (ascorbate peroxidase) activity, the tissue was separately ground in homogenizing medium containing 2.0mM ascorbate in addition to the other ingredients. All assays were done at 25 °C. Soluble protein content was determined according to Bradford (1976) using BSA as a standard. All spectrophotometric analyses were conducted using an UV/visible Spectrophotometer.

Superoxide dismutase (SOD)

SOD (EC 1.15.1.1) activity was determined by nitro blue tetrazolium (NBT) photochemical assay according to Beyer and Fridovich (1987). In this method 1ml of solution containing 50mM potassium phosphate buffer (pH 7.8), 9.9mM L-methionine, 57 µM NBT, 0.025% triton-X-100 was added into small glass tubes, followed by 20 µl of enzyme extract. Reaction was started by adding 10 µl of riboflavin solution (0.044 mg ml^{-1}) and placing the tubes in an aluminium foil-lined box having two 20-W fluorescent lamps for 7 min. A parallel control was run where buffer was used instead of sample. After illumination, the absorbance of solution was measured at 560 nm. A non-irradiated complete reaction mixture was served as a blank. SOD activity was expressed as U (unit) mg^{-1} protein. One unit of SOD was equal to that amount which causes a 50% decrease of SOD-inhibited NBT reduction.

Ascorbate peroxidase (APX)

APX (EC 1.11.1.11) activity was assayed according to the method of Nakano and Asada (1981). Three milliliter of the reaction mixture contained 50mM potassium phosphate buffer (pH 7.0), 0.1mM EDTA, 0.5mM ascorbate, 0.1mM H_2O_2 and 0.1ml enzyme extract. The hydrogen peroxide-dependent oxidation of ascorbate was followed by a decrease in the absorbance at 290 nm (extinction coefficient 2.8mM^{-1} cm^{-1}).APX activity was expressed as µmol ascorbate oxidized min^{-1} mg^{-1} protein.

Dehydroascorbate reductase (DHAR)

DHAR (EC 1.8.5.1) enzyme activity was measured following the protocol of Nakano and Asada (1981). The complete reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 2.5 mM GSH, 0.2 mM DHA and 0.1 mM EDTA in a final volume of 1 ml. Reaction was started by addition of suitable aliquots of enzyme extract and the increase in absorbance was recorded at 30 s intervals for 3 min at 265 nm. Enzyme activity was expressed as µmol ascorbate formed min^{-1} mg^{-1} protein.

Glutathione reductase (GR)

GR (EC 1.6.4.2) activity was determined by monitoring the glutathione dependant oxidation of NADPH, as described by Carlberg and Mannervik (1985). In a cuvette, 0.75 ml 0.2M potassium phosphate buffer (pH 7.0) containing 2mM EDTA, 75 µl NADPH (2 mM), and 75 µl oxidized glutathione (20 mM) were mixed. Reaction was initiated by adding 0.1 ml enzyme extract to the cuvette and the decrease in absorbance at 340 nm was monitored for 2 min. GR specific activity was expressed as µmol NADPH oxidized min^{-1} mg^{-1} protein.

Catalase (CAT)

CAT (EC 1.11.1.6) was measured according the method given by Chandlee and Scandalios (1984) with a small modification. The assay mixture contained 2.6 ml of 50 mM potassium phosphate buffer (pH 7.0), 0.4 ml of 15 mM H_2O_2 and 0.04 ml

of enzyme extract. The decomposition of H_2O_2 was followed by the decline in absorbance at 240 nm. The enzyme activity was expressed in U mg-1 protein (U = 1 mM of H_2O_2 reduction min^{-1} mg^{-1} protein).

Estimation of free proline content

Proline content was measured spectrophotometrically using the method of Bates *et al*. (1973). Two hundred and fifty milligrams of callus/leaf tissue was homogenized with 5mL 3% (w/v) sulphosalicylic acid and centrifuged at 2000 rpm for 10 min. Supernatant was treated with acid-ninhydrin and acetic acid, boiled for 1 h at 100 °C. The reaction was then terminated in an ice bath. Reaction mixture was extracted with 2mL toluene. Absorbance of chromophore containing toluene was determined at 520 nm. Proline content was expressed as µmol g^{-1} DW.

Lipid peroxidation

Lipid peroxidation rates were determined by measuring the malondialdehyde (MDA) 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 450, 532 and 600 nm. Level of lipid peroxides was calculated following Hodges et al. (1999) and expressed as nmol MDA g^{-1} fresh weight.

Statistical analysis

The results presented are the mean values ± standard errors obtained from at least four replicates. Statistical significance between mean values was assessed by conventional Duncan's multiple range test (DMRT) using SPSS-10 statistical software. A probability of $p < 0.05$ was considered significant.

RESULTS

NaCl-tolerant callus line selection
Changes in fresh mass

Increase percentage of callus fresh mass over time (recorded in month) were used for preparing the growth curve of calli subjected to two different selection procedures (DS and SS), along with control calli (Fig. 2). Control calli showed a more or less stable growth rate throughout the entire experimental period. In DS protocol, Calli subjected to different salt concentrations exhibited considerable variations in growth rate (Fig. 2). In comparison to control, calli of 75 and 100mM NaCl treatments showed much lower growth rate. The growth rate slightly increased after 2nd month in both 75 and 100 mM NaCl treatments. In 100 mM treatment highest growth rate was observed on 3rd month (34.5% increase in FM), then in successive months a sudden lowering in growth rate was noticed and in 9th month it became very insignificant causing 83% death of callus. While after 3rd month, growth rate of calli subjected to 75 mM NaCl treatment was slightly lower than control but was comparatively stable than calli of 100mM treatment. Among the different treatments, 50 mM NaCl treatment showed highest and more or less uniform growth rate though slightly lower than that of control callus.

In stepwise selection method, the apparent growth rate of calli were much higher than that of direct method , although a slight decline in growth rate was observed after calli were transferred from 50 to 75 mM NaCl medium (at 4th month) and from 75 to 100 mM NaCl medium (at 7th month). During these decline stages calli showed better growth rate (56.4% and 55.3% increase in FM at 4th and 7th month respectively) as compared to 75mM and 100mM direct calli (Fig. 2). After 9 months these calli exhibited slightly higher growth rate (70.2% increases in FM) than the control calli (68.5% increase in FM).

Changes in callus SOD activity with treatment period

The effect of NaCl on callus SOD activity is represented in Fig. 3A. More or less uniform SOD activity was exhibited by control calli throughout the treatment period. In DS protocol, calli at 50mM NaCl treatment showed gradual increase in SOD activity up to fourth month after which the activity marginally decreased and ultimately reached a plateau. However, upon transfer to 75 and 100 mM NaCl supplemented media calli showed a sharp increase in SOD activity on first month. In 75mM NaCl treated callus, the SOD activity was remained high on second month, decreased slightly at third month and then became stable. In 100 mM NaCl treatment, a steep decline in SOD activity following the first month's sudden increase was noticed in the successive months.

In comparison with the direct selection procedure a reverse trend in SOD activity was observed in stepwise method. Abrupt increase in SOD activity was observed on fourth month when calli were transferred from 50 to 75mM NaCl supplemented medium. This level was maintained in subsequent months, resulting in much higher SOD activity over control and other treatments.

Alteration in the activity of APX

Modifications in APX activity of callus under NaCl treatments are presented in Fig. 3B. In control, the APX activity gradually increased up to fourth month and finally reached a plateau. In DS method, salinity had positive impact on callus APX activity. Upon 50mM NaCl treatment, calli showed APX activity nearly similar to control level throughout the treatment period. APX activity, however, registered a sharp increase on first month and remained more or less uniform on successive months in 75 mM NaCl treated callus. Calli, in 100mM NaCl treatment, exhibited an initial increase in APX activity. After second month activity started decreasing gradually and fifth month onwards APX activity was recorded much lower, compared to control callus.

In comparison to DS method, an initial increase in APX activity over control was recorded in stepwise route while growing on 50mM NaCl supplemented medium. Drastic increase in activity was noticed when calli were subjected to 75 and 100 mM NaCl concentration at fourth and seventh months, respectively. On eight month these calli showed much higher APX activity than calli of rest of the treatments and it was maintained on ninth month also.

Modulation in the activity of DHAR

The activities of DHAR in NaCl treated and control callus lines are presented in Fig. 3C. Control calli showed no marked

Table 1 Effects of plant growth regulators on shoot induction in control and NaCl-selected callus (100mM NaCl-tolerant callus line, developed through SS method) of *Adenium multiflorum* Klotzsch

Plant growth Regulators (mgl^{-1})	Control callus line (MS medium)		NaCl-selected callus line (MS medium + 100 mM NaCl)	
	% of callus producing Shoot buds	No. of shoot buds/ responded callus	% of callus producing shoot buds	No. of shoot buds/ responded callus
NAA (2.0) +				
Kinetin 0.5	0	0	0	0
Kinetin 1.0	38.2 ± 0.88	5.3 ± 0.54	0	0
Kinetin 1.5	41.3 ± 1.11	5.6 ± 0.39	0	0
Kinetin 2.0	48.1 ± 1.87	7.0 ± 0.44	4.2 ± 0.16	1.6 ± 0.32
Kinetin 2.5	53.3 ± 1.00	9.2 ± 0.54	13.2 ± 0.21	3.5 ± 0.19
Kinetin 3.0	59.6 ± 2.21	11.4 ±0.91	20.3 ± 0.16	5.2 ± 0.22

Data were recorded after 30 days of culture initiation and presented as mean ± SE (n = 4).

Table 2 Comparison between control callus line and 100 mM NaCl-tolerant callus line developed through SS (stepwise selection) of *A. multiflorim* Klotzsch

	Control callus line	Selected callus line NaCl withdrawn from the medium[a]	Retransfer to 120 mM NaCl medium[b]
Callus growth (% of FW increased)	68.3a ± 3.33	71.3a ± 5.18	69.6a ± 4.52
SOD activity (U mg^{-1} protein)	91.15a ± 3.91	131.57a ± 5.18	297.58b ± 7.78
APX activity (µmol AsA oxi. min^{-1}, mg^{-1} pro)	0.37a ± 0.06	0.41a ± 0.06	0.71b ± 0.04
DHAR activity (µmol AsA form. min^{-1}, mg^{-1} pro)	0.61a ± 0.04	0.63a ± 0.07	0.98b ± 0.04
GR activity (nmol NADPH oxi. min^{-1}, mg^{-1} pro)	20a ± 0.8	30a ± 1.2	25b ± 1.8
CAT activity (mM H_2O_2 red.min^{-1} mg^{-1} pro)	3.11a ± 0.09	2.98a ± 0.04	17.46b ± 0.15
Proline content (µmol g^{-1} dry weight)	27.89a ± 4.76	33.31a ± 3.99	166.12b ± 18.32

Data are means ± SE of four independent experiments. Mean values in rows with different letters are significantly different at $P < 0.05$ following Duncan's Multiple Range Test.
[a] Data were taken at 90 days after withdraw of NaCl from the medium.
[b] Data were taken at 30 days after re-transfer to saline medium.

Table 3 Assessment of growth of callus-derived plants grown in MS medium supplemented with 200 mM NaCl in *A. multiflorum* Klotzsch

Plant types [a]	Plant height (cm)	Days to root initiation	Root length (cm)	Number of roots/plant
PC	4.88a ± 0.18	11.2a ± 0.22	5.67a ± 0.35	10.27a± 0.29
S1	3.51b ± 0.11	13.5b ± 0.16	5.12a ± 0.32	9.82a ± 0.20
NC	1.78c ± 0.16	17.3c ± 0.37	2.26b ± 0.17	4.67b ± 0.18

[a] PC: positive control (control plants grown under unstressed condition); NC: negative control (control plants subjected to *in vitro* NaCl-stress); S1: regenerated plants of selected callus (100mM NaCl-tolerant callus line developed through SS) subjected to 200mM NaCl-stress. Data were taken after 30 days of culture initiation and presented as mean ± S.E. (n = 10). Mean values in columns with different letters are significantly different at $P < 0.05$ following Duncan's multiple range test (DMRT).

changes in DHAR activity on different periods of measurement. DS procedure, calli upon 50mM NaCl treatment showed an increase in DHAR activity on first month and maintained the trend up to three months. Thereafter, it started to decrease gradually and got stabilized at control level on six month. In 75 mM NaCl treated callus, DHAR activity enhanced markedly on first month and only slight changes were noticed on successive months. Calli of 100mMNaCl treatment exhibited an initial increase in DHAR activity which started decreasing gradually and from fifth month onwards the activity was found much lower, compared to control callus.
During growing on 50mM NaCl supplemented medium, calli exhibited an initial increase of DHAR activity over control in stepwise method. Marked increase in activity was noticed when calli were transferred to 75 and 100 mM NaCl concentration at fourth and seventh months, respectively. On nine month these calli showed much higher DHAR activity than calli of rest of the treatments.

Changes in the activity of GR

The GR activities of NaCl treated and control callus lines are given in Fig. 3D. Control calli did not show any remarkable changes in GR activity on different dates of measurement.

Calli of 50mMNaCl treatment in DS protocol showed a gradual increase in GR activity up to third month. Thereafter, it slightly decreased and ultimately became stabilized from five month onwards. GR activity increased remarkably in 75 and 100mMNaCl treated calli on first month. In 75 mM NaCl treated callus, activity decreased significantly on second month and was followed by a gradual increase in successive months. In 100mM NaCl treatment, the GR activity started decreasing sharply after very first month.

The GR activity gradually increased with treatment period in calli of stepwise selection method. The increase was more marked at the next higher treatment concentration. At ninth month, these calli exhibited much elevated GR activity than calli of rest of the treatments.

Changes in CAT activity

The CAT activities of NaCl treated and control callus lines are given in Fig. 3E. Control calli showed nearly uniform level throughout the experiment. Comparing control, calli of 50 mM in DS method did not show any remarkable change in CAT activity throughout the treatment periods. In 75 mM NaCl, calli exhibited marked enhancement in CAT activity on first month, maintained this level up to fifth month, and then began

declining. In 100mM, calli showed sudden increase in activity on first month, after which it fell sharply.

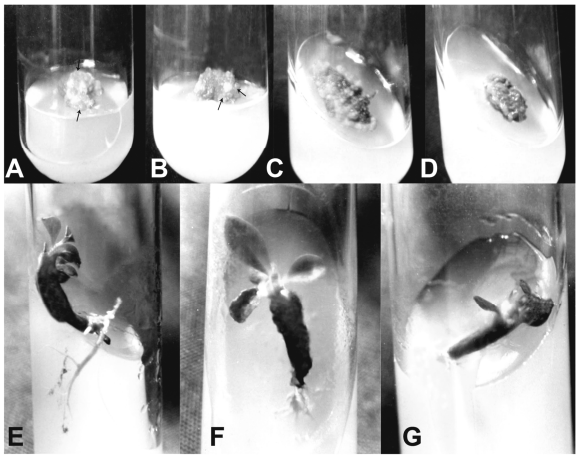

Fig. 1 Growth of callus and subsequent plant regeneration, and performance of regenerants under *in vitro* NaCl stressed condition in *Adenium multiflorum* Klotzsch, (A) Green sectors (arrow marks) of callus developed on 50mM NaCl supplemented medium in stepwise selection (SS); (B) Green sectors (arrow marks) of callus developed on 75mM NaCl supplemented medium (SS); (C) Green, well growing callus of selected 100mM NaCl-tolerant line developed through SS; (D) Dead callus (brown and soft tissue) of 100mM NaCl medium (direct selection) after 9 months of treatment. Plant regeneration from selected callus line, showing comparative growth performance of (E) PC, (F) S1 and (G) NC plants under *in vitro* salinity stressed condition

An initial increase in CAT activity over control was recorded in stepwise method in 50mM NaCl supplemented medium. Sudden increase in activity was noticed when calli were subjected to 75 and 100 mM NaCl concentration at fourth and seventh months, respectively. On ninth month these calli showed much higher CAT activity than calli of rest of the treatments.

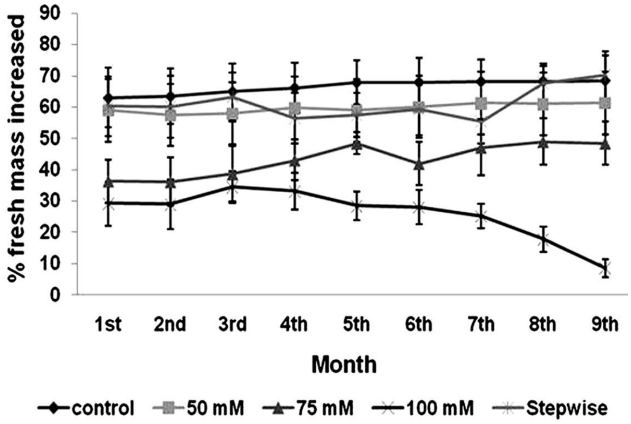

Fig. 2 Growth (percent increase in fresh mass) of *Adenium multiflorum* Klotzsch callus grown on MS medium containing NAA 1mg l^{-1}, BAP 0.5 mg l^{-1} and 0, 50, 75 or 100mM NaCl. Calli were grown for 9 months treatment durations either directly on different NaCl supplemented medium (DS) or NaCl concentration was increased in a stepwise route (SS). Vertical bars indicate mean ± standard errors of four replicates (n = 4).

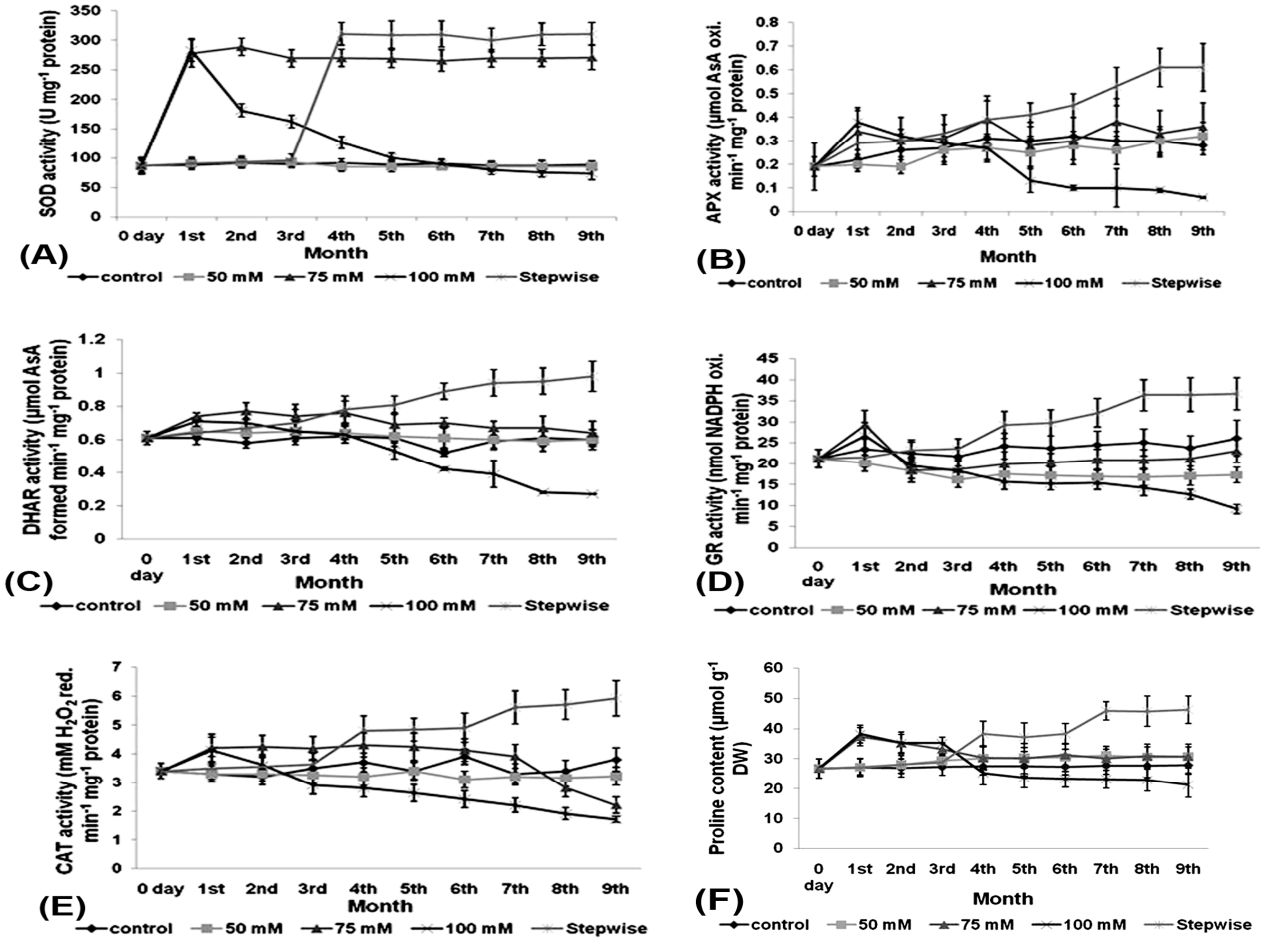

Fig. 3 Changes in callus (A) SOD, (B) APX, (C) DHAR, (D) GR, (E) CAT and (F) endogenous free proline content in *A. multiflorum* Klotzsch. Calli were subcultured either on the MS medium having the same NaCl concentration (0, 50, 75 or 100mM NaCl) (DS) or NaCl concentration of the medium was increased in a stepwise way (SS). Results are presented as means ± S.E. of four replicates (n = 4).

The effects of salt treatment, on proline contents of treated and control calli, are presented in Fig. 3F. Control callus maintained more or less uniform proline level throughout the experimental regime. In DS protocol, NaCl treated calli compared to control accumulated higher proline. In 50mM NaCl treatment, proline level gradually increased and finally from fifth month it reached a plateau. In the 75mM treated calli proline content increased significantly on the first month, which then slightly decreased and ultimately reached a stable level. Highest proline content was observed in 100mM NaCl treated callus on first month and was sharply declined from forth month onwards.

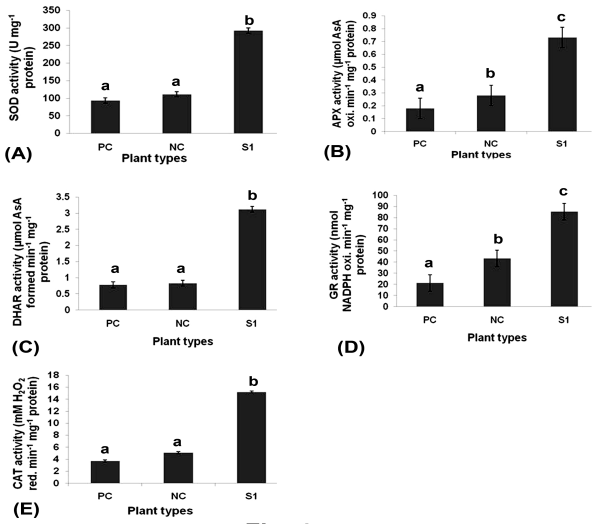

Fig. 4 Activities of (A) SOD, (B) APX, (C) DHAR, (D) GR, and (E) CAT in leaves of control (PC) and NaCl stressed (S1 and NC) plants of *Adenium multiflorum* Klotzsch. Vertical bars indicate mean ± standard errors of four independent extracts (n = 4). Mean values in columns with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test (DMRT).

A steady increase in proline content was observed in stepwise increase of NaCl concentration. This increase was more marked at fourth and seventh month where the NaCl concentration was changed to the next higher dose.

Comparison between control and selected callus line

Callus growth of selected line (100mM NaCl-tolerant callus line developed through SS) was as good as control, both under unstressed and stressed conditions (Table 2). Antioxidant enzyme activities (SOD, APX, GR, DHAR and CAT) of selected calli were decreased on withdrawal of NaCl from the medium (for 90 days), while activities were increased significantly in comparison to the control calli on re-exposure to saline medium. Accumulation of proline followed the similar trend. Callus proline content increased up to 5-fold on retransfer of callus to the 120mM NaCl medium (Table 2).

Plant regeneration

Effect of NAA (2 mgl^{-1}) in combination with kinetin on shoot bud induction are presented in Table 1. In control callus, shoot bud differentiation was observed in all the treatments except for 0.5 mgl^{-1} kinetin, whereas 2.0, 2.5 and 3.0 mgl^{-1} kinetin were found to be effective for shoot organogenesis in NaCl-selected callus line. No shoot bud formation was observed in rest of the combinations. In NaCl-selected callus, highest shoot bud organogenesis frequency (20.3%) was recorded in 3.0 mgl^{-1} kinetin along with 2 mgl^{-1} NAA.

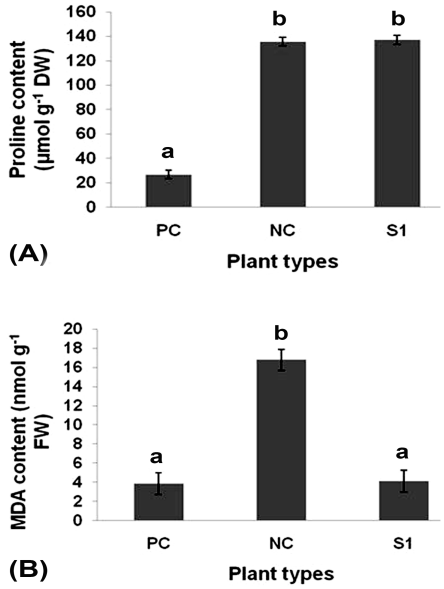

Fig. 5 Changes in (A) proline and (B) molondealdehyde (B) content in leaves of control (PC) and NaCl stressed (S1 and NC) plants of *Adenium multiflorum* Klotzsch. Vertical bars indicate mean ± standard errors of four replicates (n = 4). Mean values in columns with different letters are significantly different at $P < 0.05$ following Duncan's multiple range test (DMRT).

Growth and antioxidant enzymes capacity of regenerated plants under NaCl stress

In the medium supplemented with 200mM NaCl, S1 plants exhibited superior performance to NC both in terms of growth and antioxidant enzymes capacity (Table 3 and Fig. 1E-G, 4). Although plant height of S1 line decreased in comparison with PC, the decrease was even more drastic in case of NC, where plants exhibited stunted growth (Fig. 1E-G). Root initiation got delayed as well as number of roots/ plant and root length were decreased significantly in NC plants in comparison with S1 and PC lines. Stress induced increase in SOD, APX, DHAR and CAT activities were recorded in both NC and S1 lines. The magnitude of the increase, however, was more in case of S1 plants (Fig. 4), whereas GR activity increased nearly 2-fold in NC and 4-fold in S1 plants over PC (Fig. 4). Both NC and S1 plants accumulated significantly high levels of proline (5-fold over PC) under NaCl stress (Fig. 5A).MDA content was nearly normal in S1 plants, while it was 4-fold higher in NC than PC (Fig. 5B).

DISCUSSION

In vitro culture of plant cells, tissues or organs on a medium containing selective agents offers the opportunity to select and regenerate plants with desirable characteristics including tolerance to stress (Purohit *et al*., 1998). However, selection protocol of callus line seems to play a pivotal role in recovery of stable variant plants showing improved salt tolerance (Hossain *et al*., 1997; Rai *et al*., 2011; Winicov, 1996). In the present investigation, stepwise increase of NaCl concentration

from relatively low level (50 mM) to cytotoxic level (100 mM) is found to be a far better way to develop stable NaCl-tolerant variant than direct transfer of calli to highest selection pressure (100 mM NaCl). The suitability of stepwise selection method over direct transfer is evidenced by better fresh mass of calli which is, obviously, orchestrated by stepwise increase in antioxidant capacity towards development of stable tolerants, as has been reported earlier in other crops, also (Kumar and Sharma, 1989; Patnaik and Debata, 1997).

Modulation of growth is one of the important adaptive strategies plants adopt in response to salinity stress (Munns, 2005). The genetic basis of this modulation observed in a hardy legume, grass pea, in different ploidy levels (Talukdar, 2009, 2010, 2011c) can bring all cellular responses to salt stress together. Growing evidences suggest role of ROS as signaling molecule in these adaptive processes (Apel and Hirt, 2004). On the first month, sudden increase in the activities of SOD, APX, DHAR, GR and CAT in calli (direct selection) of 75 and 100 mM NaCl supplemented medium indicates increased production of ROS. Presumably, this is the reason that callus on first month exhibited slower growth rate than others, despite there was a marked enhancement of its antioxidant enzyme activities. The situation got worse once the callus was directly subjected to 100 mM. The callus growth was poor at initial months in spite of a sudden surge in activities of antioxidant enzymes and it got poorer at successive months with declining levels of enzyme activities in longer treatment regime. The results obtained from the present experiment strongly favors the idea that an increase of NaCl concentration from 0 to 100 mM in just one step severely jeopardized the ROS-scavenging machinery, impeding survival and growth potential of callus under long term salt stress. However, the capacity to withstand same level of salinity can be acquired by stepwise increase of NaCl concentration. This was substantiated by the fact that stepwise elevation of calli to 75mM supplemented medium showed better fresh mass than its direct selection in the same concentration. Calli growth was accelerated further even after 9 months of long-term treatment in the stepwise route to 100 mM. Obviously, increased antioxidant enzyme activities coupled with high endogenous proline accumulation is instrumental for ensuring good growth of callus in step-by-step exposure to elevated salt stress. Higher SOD activity coupled with considerable increase in APX, CAT, GR and DHAR suggests better ROS scavenging capacity of salt-tolerant cell lines, which is in agreement with earlier observations on different crops (Garratt *et al.*, 2002; Hernández *et al*., 2000). Based on the present performance, calli of stepwise method were selected as 100 mM NaCl-tolerant line that exhibited all positive responses towards the salinity stress. Comparing with control, calli at low concentration (50 mM) showed no considerable change in growth performance and biochemical parameters as also reported earlier in different crops (Hossain *et al.*, 1997; Kumar and Sharma, 1989; Patnaik and Debata, 1997).

The growth and free radical scavenging capacity of 100 mM NaCl-tolerant line developed through *in vitro* stepwise selection was checked as stable even after the withdrawal of NaCl from the medium for 3 months and the retransfer to the saline condition. Similar performance was noticed in chrysanthemum (Hossain *et al*., 1997) and citrus (Ben-Hayyim and Kochba, 1983).

Synthesis of compatible organic solutes has been widely used as the basis of *in vitro* selection of stress tolerant plants (Rai *et al*., 2011). Among the different solutes, rapid accumulation of free proline within the cell is the most significant event under salinity stress. In the present experiment, rise of free proline level was parallel to increase in NaCl concentrations, which was more prominent in case of stepwise method. The transfer of calli from lower to next higher saline medium was associated with steep hike in proline level. Reversely, the withdrawal of NaCl from the medium results in sharp fall in proline content. The results indicate role of proline to balance the osmoregulation as an adaptive feature of tolerant line in saline environment. A positive correlation between proline over accumulation and increasing salinity/drought tolerance has also been found in different crops including cultured lines and transgenics that were engineered for overproduction of proline (Anoop and Gupta, 2003; Talukdar, 2011a). By contrast, accumulation of proline does not play a major role in combating salinity stress at the whole plant level. Both NC and S1 lines in the present study accumulated significantly high proline under salinity stress, but still NC plants showed retarded growth whereas S1 plants attained much better height. Further study, however, is needed.

So far, somatic embryogenesis has been used in most cases as potential tool to regenerate plant from selected callus/cell lines (Binh *et al*., 1992; Patnaik and Debata, 1997; Zhang *et al*., 2001). In the present study, plants were regenerated from callus through shoot and root organogenesis. Growth performance and antioxidant enzyme capacity in these regenerated plantlets was checked in medium supplemented with high (200 mM) NaCl. Compared with PC, plant height, number of roots plant^{-1} and root length decreased in both S1 and NC plants, but the effect of high salinity was more severe on NC plants. The delay in root initiation coupled with poor root growth badly impeded the mineral absorption of NC plants, leading to reduced plant height. By contrast, nearly equal days to root initiation and normal length of root in nascent S1 plants ensured efficient absorption of nutrients to grow faster than NC. This indicated better performance of S1 plants over NC in high selective pressure.

The superior growth performance of NaCl-selected callus regenerants (S1 line) was ensured by efficient scavenging of ROS through significantly high level of antioxidant enzyme activity. Increase in SOD activity about 5-fold in S1 line over PC strongly suggests generation of superoxide radicals in response to high salinity treatment, and SI has the better capacity than NC to quench it. Elevated SOD activity in salt tolerant genotypes, in comparison to salt sensitive ones, has been observed in different ornamentals (Hossain *et al*., 1997), cereals, legumes and other crops (Dionisio-Sese and Tobita, 1998; Hossain *et al*., 2006; Talukdar, 2011a, 2012a,b). However, it is noteworthy that rise of SOD activity under stress should be fine tuned with cellular H_2O_2-scavenging machinery to nullify onset of stress, as dismutation of superoxide radicals by SOD often results in generation of H_2O_2 which as a stable ROS can react with various cellular targets particularly in those locations where thiol-containing molecules and enzymes are functioning (Hossain *et al*., 1997; Talukdar, 2012a,c). Activities of two prominent H_2O_2 -

scavenging enzymes, APX and CAT, increased in both NC and S1 plants, but the magnitude of increase was significantly higher in S1 line than NC, giving higher protection to the S1 plants from the oxidative damage. Likewise, increase in DHAR and GR activity in S1 line higher than NC line indicates more efficient recycling of antioxidant molecules in the regenerants under high salt stress. Together, this helps S1 plants to prevent high salt induced oxidative damage to its membrane by minimizing the ROS-mediated lipid peroxidation. This was strongly evidenced by quite normal level of malondealdehyde (MDA) in leaves of S1 plants. Growing evidences indicate an intimate relationship between excess ROS particularly H_2O_2 generation and consequent damage of membrane, marking the onset of oxidative stress under salinity (Hernández *et al*., 2000; Talukdar, 2011a). Cooperative increase in antioxidant enzyme capacity particularly peroxidase-catalase system in fine regulation of salt-generated ROS has also been reported in rice (Vaidyanathan *et al*., 2003), mulberry (Sudhakar *et al*., 2001), legumes and other crops (Martinez *et al*., 2003; Talukdar, 2012a,b). Failure of antioxidant defense in the present NC plants to cope with high salinity stress ultimately led to membrane damage and poor growth performance.

In conclusion, we propose that antioxidant enzyme activities are more reliable markers for salt-tolerance in regenerated *A. multiflorum* plants than proline content. Significant increase in antioxidant enzyme capacity, low level of lipid peroxidation and better growth of S1 plants under high NaCl-stress suggest that efficient scavenging of ROS is essential to combat the salinity-induced oxidative damage in A. *multiflorum* plants. Considering overall growth performance and antioxidant capability, S1 plants could be selected as NaCl-tolerant line which was exhibiting far better adaptability than NC towards high salinity stress. Future trial, however, are needed for large scale introduction of S1 plants in agronomic purposes.

References

Anoop, N. and Gupta, A.K. 2003. Transgenic indica rice cv IR-50 overexpressing *Vigna aconitifolia* delta(1)-pyrroline-5-carboxylate synthetase cDNA shows tolerance to high salt. J Plant Biochem Biotechnol., 12: 109–116.

Apel, K. and Hirt, H. 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Ann Rev Plant Biol., 55: 373-399.

Asada, K., 2006. Production and scavenging of reactive oxygen species in chloroplasts and their functions. Plant Physiol., 141: 391-396.

Bates, L.S., Waldren, R.P. and Teare, I.D. 1973. Rapid determination of free proline for water stress studies. Plant Soil, 39: 205–207.

Ben-Hayyim, G. and Kochba, J. 1983.Aspects of salt tolerance in a NaCl-selected stable cell line of *Citrus sinensis*. Plant Physiol., 72: 685–690.

Beyer, W.F. and Fridovich, I. 1987. Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. Anal Biochem., 161: 559–566.

Bhattacharjee, S.K., 2006. Advances in Ornamental Horticulture.Vol.1, Eastern Book Corporation, India.

Binh, D.Q.,Heszky, L.E.,Gyulai,G.and Csillag,A. 1992.Plant regeneration of NaCl-pretreatred cells from long-term suspension culture of rice(*Oryza sativa* L.) in high saline conditions. Plant Cell Tissue Organ Cult., 29:75–82.

Bradford, M.M., 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem., 72: 248–254.

Carlberg, I. and Mannervik, B. 1985. Glutathione reductase. In: Meister A (ed) Methods Enzymology. Academic press, California, pp 484–490.

Chandlee, J.M. and Scandalios, J.G. 1984. Analysis of variants affecting the catalase development program in maize scutellum. Theor. Appl. Genet., 69: 71-77.

Demiral, T. and Turkan, I. 2005.Comparative lipid peroxidation, antioxidant defense systems and proline content in roots of two rice cultivars differing in salt tolerance. Environ. Exp. Bot., 53: 247–257.

Dionisio-Sese, M.L. and Tobita, S. 1998. Antioxidant responses of rice seedlings to salinity stress. Plant Sci., 135: 1–9.

Garratt, L.C., Janagoudar, B.S., Lowe, K.C., Anthony, P., Power, J.B. and Davey, M.R. 2002. Salinity tolerance and antioxidant status in cotton cultures. Free Radic. Biol. Med., 15: 502–511.

Gossett, D.R., Banks, S.W., Millhollon, E.P. and Lucas, M.C. 1996. Antioxidant response to NaCl stress in a control and an NaCl-tolerant cotton cell line grown in the presence of paraquat, buthionine sulfoximine and exogenous glutathione. Plant Physiol., 112: 803-809.

He, S., Han, Y., Wang, Y., Zhai, H. and Liu, Q. 2009. *In vitro* selection and identification of sweet potato (*Ipomoea batatas* (L.) Lam.) plants tolerant to NaCl. Plant Cell Tissue Organ Cult., 9: 69–74.

Hernández, J.A., Jiménez, A., Mullineaux, P. and Sevilla, F. 2000. Tolerance of pea (*Pisum sativum* L.) to long-term salt stress is associated with induction of antioxidant defences. Plant Cell Environ., 23: 853-862 .

Hodges, D.M., Delong, J.M., Forney, C.F. and Prange, R.K. 1999. Improving the thiobarbituric acid- reactive substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. Planta, 207: 604-611.

Hossain, Z., Mandal, A.K.A., Datta, S.K. and Biswas, A.K. 2006. Isolation of a NaCl-tolerant mutant of *Chrysanthemum morifolium* by gamma radiation: *in vitro* mutagenesis and selection by salt stress. Functional Plant Biology, 33: 91-101.

Hossain, Z., Mandal, A.K.A., Datta, S.K. and Biswas, A.K.2007. Development of NaCl-tolerant line in *Chrysanthemum morifolium* Ramat. through shoot organogenesis of selected callus line. J Biotechnol., 129: 658–667.

Kanchanapoom, K., Sunheem, S. and Kanchanapoom, K. 2010. *In vitro* Propagation of *Adenium obesum* (Forssk.) Roem. and Schult. Not. Bot. Hort. Agrobot. Cluj., 38: 209-213.

Kumar, V. and Sharma, D.R. 1989. Isolation and characterization of sodium chloride-resistant callus culture of *Vigna radiata* (L.) Wilczek var *Radiata*. J. Exp. Bot., 210: 143-147.

Martinez, C.A., Maestri, M. and Lani, E.G. 2003. *In vitro* salt tolerance and proline accumulation in Andean potato

(*Solanum* spp.) differing in frost resistance. Plant Sci., 116: 117-184.

Munns, R., 2005. Genes and salt tolerance: bringing them together. New Phytol., 167: 645-663.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol. Plant, 15: 473–497.

Nakano, Y. and Asada, K. 1981. Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplast. Plant Cell Physiol., 22: 867-880.

Noctor, G. and Foyer, C.H. 1998. Ascorbate and glutathione: keeping active oxygen under control. Ann. Rev. Plant Physiol. Plant Mol. Biol., 49: 249-279.

Olmos, E., Hernandez, J.A., Sevilla, F. and Hellin, E. 1994. Induction of several antioxidant enzymes in the selection of a salt tolerant cell line of *Pisum sativum*. J. plant Physiol. 144: 594-598.

Oyen, L.P.A., 2008. *Adenium obesum* (Forssk.) Roem. & Schult. In: Schmelzer GH, Gurib Fakim A (eds) Plant resources of tropical Africa, medicinal plants, Backhuys, Wageningen.

Patnaik, J. and Debata, B.K. 1997. *In vitro* selection of NaCl tolerant callus lines of *Cymbopogon martini* (Roxb.). Wats. Plant Sci., 124: 203-210.

Purohit, M., Srivastava, S. and Srivastava, P.S. 1998. Stress tolerant plants through tissue culture. In: Srivastava PS (ed) Plant Tissue Culture and Molecular Biology: Application and Prospects. Narosa Publishing House, New Delhi, pp 554–578.

Rai, M.K., Kalia, R.K., Singh, R., Gangola, M.P. and Dhawan, A.K. 2011. Developing stress tolerant plants through *in vitro* selection-An overview of the recent progress. Environ. Exp. Bot. 71: 89-98.

Sudhakar, C., Lakshmi, A. and Giridarakumar, S. 2001. Changes in the antioxidant enzyme efficacy in two high yielding genotypes of mulberry (*Morus alba* L.) under NaCl salinity. Plant Sci., 161: 613–619.

Sumithra, K., Jutur, P.P., Carmel, B.D. and Reddy, A.R. 2006. Salinity-induced changes in two cultivars of *Vigna radiata*: responses of antioxidative and proline metabolism. Plant growth Regul., 50: 11–22.

Suprasanna, P., 2010. Biotechnological interventions in sugarcane improvement: Strategies, methods and progress. BARC Newsletter, 316: 47-54.

Tal, M., 1994. *In vitro* selection for salt tolerance in crop plants: theoretical and practical considerations. *In Vitro* Cell Div. Biol., 30: 175-180.

Talukdar, D., 2009. Dwarf mutations in grass pea (*Lathyrus sativus* L.): Origin, morphology, inheritance and linkage studies. Journal of Genetics, 88: 165-175.

Talukdar, D., 2010. Reciprocal translocations in grass pea (*Lathyrus sativus* L.): Pattern of transmission, detection of multiple interchanges and their independence. J. Hered., 101: 169-176.

Talukdar, D., 2011a. 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. J. Med. Plant Res., 5: 3619-3628.

Talukdar,D.,2011b.Flower and pod production, abortion, leaf injury, yield and seed neurotoxin levels in stable dwarf mutant lines of grass pea (*Lathyrus sativus* L.) differing in salt stress responses. Int. J. Curr. Res., 2: 46-54.

Talukdar, D., 2011c. The aneuploid switch: Extra-chromosomal effect on antioxidant defense through trisomic shift in *Lathyrus sativus* L. Indian J. Fund. Appl. Life Sci., 1: 253-273.

Talukdar, D., 2012a. Ascorbate deficient semi-dwarf *asfL1* mutant of *Lathyrus sativus* exhibits alterations in antioxidant defense. Biol. Plant., 56: 675-682.

Talukdar, D., 2012b. A glutathione-overproducing mutant in grass pea (*Lathyrus sativus* L.): Alteration in glutathione content, modification in oxidant defense response to cadmium stress and genetic analysis using primary trisomics. International J. Recent Scientific Research, 3: 234-243.

Tanaka, K., 1994. Tolerance to herbicides and air pollutants. In: Foyer CH, Mullineaux PM (eds) Causes of photooxidative stress and Amelioration of Defence Systems in Plants. CRC, Boca Raton, FL, pp 365-378.

Vaidyanathan, H., Sivakumar, P., Chakrabarty, R. and Thomas, G. 2003. Scavenging of reactive oxygen species in NaCl-stressed rice (*Oryza sativa* L.)—differential response in salt-tolerant and sensitive varieties. Plant Sci., 165: 1411–1418.

Vajrabhaya, M., Thanapaisal, T. and Vajrabhaya, T. 1989. Development of salt tolerant lines of KDML and LPT rice cultivars through tissue culture. Plant Cell Rep., 8: 411-414.

Willikens, H., Charnnongpol, S., Davey, M., Schraudner, M., Langebartels, C., Van Montagu, M., Inzé, D. and Van Camp, W. 1997. Catalase is a sink for H_2O_2 and is indispensable for stress defence in C_3 plants. EMBO J., 16: 4806–4816.

Winicov, I., 1996. Characterization of rice (*Oryza sativa* L.) plants regenerated from salt tolerant cell lines. Plant Sci., 113: 105–111.

Yamaguchi, T. and Blumwald, E. 2005. Developing salt-tolerant crop plants: challenges and opportunities. Trends Plant Sci., 10: 615–620.

Zhang, B.H., Liu, E., Wang, Q.L. and Zhang, W.S. 2001. Selection for salt tolerance in cotton tissue culture and plant regeneration from NaCl-tolerant embryogenic callus. Israel J. Plant Sci., 49: 187-191.

Zhu, J.K., 2002. Salt and drought stress signal transduction in plants. Ann. Rev. Plant Biol., 53: 247–273.

******