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EFFECT OF SALINITY STRESS ON SEED GERMINATION AND ANTIOXIDATIVE DEFENSE SYSTEM OF *Catharanthus roseus*

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ABSTRACT

In this study the effect of responses of *Catharanthus roseus* to NaCl stress has been explored. Periwinkle plants were exposed to different concentrations of salt and the effect of treatment on germination, growth parameter and antioxidative defense system investigated. Seeds were presoaked in solutions with different salinity after sterilization. Experimental samples irrigated once every two days with solutions containing given NaCl concentration. Increasing the NaCl concentration to 25, 50, 75 and 100 µM salt reduced germination percentage by 8, 29, 41 and 81 percent respectively. Plant fresh and dry weights of treated plants showed a decrease, compare to the control. Ascorbic acid content increased to 42, 56, 129 and 218 percent when treated by 25, 50, 75 and 100 µM salt, respectively, compared to the control. Glutathione concentration showed a significant ($P<0.05$) increase at all treatments. NaCl caused to significant decrease of SOD activity. Salinity enhanced the activities of catalase, peroxidase and glutathione reductase. The MDA content increased with the increasing concentrations of NaCl. MDA content of samples treated by 100 µM NaCl increased up to 45 percent more than the control.

Keywords: *Catharanthus roseus*, antioxidative defense system, germination, lipid peroxidation, periwinkle, salinity

1. INTRODUCTION

Plants are widely recognized as manufacturer of complex, medically related molecules (Ryan and Moore, 2009). Terrestrial plants have been used as medicines in Egypt, China, India and Greece. Herbal cancer therapy includes some different treatments in the attack on cancer. All monoterpene indole alkaloids, including more than130 created by Madagascar periwinkle, are considered to be generated from the central intermediate strictosidine, which is produced via a condensation of tryptamine, derived from l-tryptophan, and secologanin (Facchini & De Luca, 2008), likely from a nonmevalonate biosynthetic pathway. Strictosidine synthase, the enzyme responsible for this Pictet-Spengler condensation, is located at a juncture point, controlling the flux of metabolites to downstream products. As a result, the substrates accepted by this enzyme limit the types of modified monoterpene alkaloids that can be created (McCoy, Galan, and O'Connor, 2006).

Catharanthus roseus (L.), Madagascar periwinkle, is a temperate season, permanent, cold-sensitive, ornamental, therapeutic plant belonging to the family Apocynaceae. This family has 114 genera and 4650 species that most of them are both ornamental with therapeutic value (Simpson, 2006). It has extended, ellipsoid, glossy, dark-green colored leaves and its veins are light colored (Rezaee, Chehrazi, and Moalemi, 2012). The flowers have five petals with pink, white, red, and purple colors. Madagascar periwinkle is a good source of non-enzymatic and enzymatic antioxidants and anti-hypertension. Long before modern researcher learned about plant's worthy and varied properties, people in faraway places were using the Madagascar periwinkle, for a host of medicinal purposes. In India, they treated wasp sting with the juice from the leaves. In Hawai'i they prescribed an extract of the boiled plant to arrest bleeding. In Central America and parts of South America, they made a gargle to ease sore throats and chest ailments and laryngitis. In Cuba, Puerto Rico, Jamaica and other islands, an extract of the flower was commonly administered as eyewash for the eyes of infants. In Africa, leaves are used for menorrhagia and rheumatism (Dobelis, 1997; Walts, 2004). It is discovered that Catharanthus extracts are antineoplastic, in vitro, leading ultimately to the licensing of the alkaloids vinblastine and vincristine (El-Sayed and Cordell, 1981).

Biosphere's continued exposure to abiotic stress, e.g. drought and salinity, cause imbalance in the natural status of the environment. Thus growth and development of plants, as immobile organisms, are harmfully affected by a variety of abiotic and biotic stress factors (Mohammad Reza Amirjani, 2010, 2011a, 2011b, 2012b; Mahajan and Tuteja, 2005).

Soil salinity is one among the several environmental stresses causing drastic changes in the growth, physiology and metabolism of plants. Saline environment can induce a wide number of responses in plants ranging from readjustment of transport and metabolic processes to growth inhibition (Azooz *et al*. 2004). Among responses is delay in primary germination process by this stress (Almansouri, Kinet, and Lutts, 2001). Seeds have similar responses to salinity stress for halophyte and non-halophyte plants (Ungar, 1996).

The effect of sodium chloride on seed germination is due to osmotic effects and/or ions toxicity (Tobe, Li, and Omasa, 2004). In halophytes, the decrease in germination usually is due to osmotic effects while this happens due to ion toxicity in non-halophytes (Bajji, Kinet, and Lutts, 2002).

Most plants sensitive to saline environment due to a combination of adverse osmotic gradients and inhibitory effects of salts and ions on cell metabolism and of nutrient imbalance and secondary stresses such as an oxidative stress linked to the production of toxic reactive

oxygen intermediates (Hasegawa, Bressan, Zhu, and Bohnert, 2000).

Salinity effects on a broad range of metabolic processes in plants and induces changes in contents and activities of many enzymes (Dubey, 1994, 1997; Khan and Panda, 2008; Richharia, Shah, and Dubey, 1997). As a result of ion imbalance and hyperosmotic stresses, which are primary effects of salt stress, secondary stresses such as oxidative damage may occur. Stress environment decreases carbon reduction by the Calvin cycle and decrease in oxidized $NADP^+$ to serve as an electron acceptor in photosynthesis. When ferrodoxin is over reduced during photosynthetic electron transfer, electrons may be transferred from PSI to oxygen to form superoxide radicals ($O_2^{\cdot}$) by the process called Mehler reaction, which triggers chain reactions that generate more aggressive reactive oxygen species (ROS). Any imbalance in the cellular redox homeostasis can be called as an oxidative stress and results in the production of ROS because of the univalent reduction of oxygen. Salt stress increases the rate of production of ROS such as superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$), alkoxyl radical ($RO^{\cdot}$) and singlet oxygen ($^1O_2$) formation via enhanced leakage of electron to oxygen. It is already known that these cytotoxic ROS, which are also generated during metabolic processes in the mitochondria and peroxisomes, can destroy normal metabolism through oxidative damage of lipids, proteins, and nucleic acids (Grant and Loake, 2000; Gueta-Dahan, Yaniv, Zilinskas, and Ben-Hayyim, 1997). Lipid peroxidation, induced by free radicals, is also important in membrane deterioration (Demiral and Turkan, 2005; Mandhania, Madan, and Sawhney, 2006).

Plant cells, however, have evolved a highly efficient antioxidant defense system to ROS detoxification, mitigate and repair the damage initiated by ROS. This specific protective mechanisms include either the nonenzymatic constituents, like ascorbic acid (AsA), tocopherols, anthocyanin, flavonoids, carotenoids and glutathione (GSH) (Lee and Lee, 2000) or the enzymatic components, such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX) and glutathione reductase (GR).

SOD (EC 1.15.1.1) is the enzyme that catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide, H2O2. Thus, they are an important antioxidant defense in nearly all cells exposed to oxygen.

CAT (EC 1.11.1.6) is a common enzyme found in nearly all living organisms exposed to oxygen. It catalyzes the decomposition of hydrogen peroxide to water and oxygen. It is a very important enzyme in reproductive reactions. Likewise, catalase has one of the highest turnover numbers of all enzymes; one catalase molecule can convert millions of molecules of hydrogen peroxide to water and oxygen each second (Chelikani, Fita, and Loewen, 2004).

POD (EC 1.11.1.7) can be coupled to other proteins via its amino groups, as well as its carbohydrate moiety. POD also protects the cells against the destructive influence of H2O2 by catalyzing its decomposition through oxidation of phenolic and endiolic cosubstrates (Lin and Kao, 2002).

GR (EC 1.8.1.7) is an enzyme that reduces glutathione disulfide (GSSG) to the sulfhydryl form GSH, which is an important cellular antioxidant (Meister, 1988).

The peroxidases then scavenged the produced H_2O_2 by and by oxidation of co-substrates such as phenolics or other antioxidants (Almeselmani, Deshmukh, Sairam, Kushwaha, and Singh, 2006)

In the present study the effect of salinity on seed germination, growth parameters and antioxidative defense system of *C. roseus* was investigated.

2. MATERIALS AND METHODS

2.1 Plant cultivation and salt stress induction

Healthy seeds of periwinkle were collected from the Pakan bazr Insttute, Esfahan, Iran. Seeds were surface sterilized using sodium hypochlorite for 10 min with frequent shaking and then washed with deionized water (Idrees, Naeem, Aftab, Khan, & Moinuddin, 2011; Rezaee, *et al*., 2012). The seeds were pre-soaked in deionized water, as control, 25, 50, 75 and 100 µM NaCl solutions for 12 h. Prior to seed sowing, perlite was saturated with water or solutions with given concentration of NaCl. Saturated perlite was filled in the 20 cm wide pots.

2.2 Determination of germination percentage

Healthy and uniform surface-sterilized seeds were transferred in Petri dishes on two sheets of sterile filter paper moistened with deionized water, as control, and four stress levels at 25, 50, 75 and 100 µM NaCl to test germination The Petri dishes were kept at 24 ± 1°C and the number of seeds germinated in each Petri dish recorded daily for 8 days. Seeds that presented approximately 2 mm of root length were considered as germinated. The germination percentage was calculated.

2.3 Measurement of growth parameters

Morphological parameters such as root length, shoot height and fresh weight were measured in fresh samples. Samples were oven-dried at 80 °C for three days and the dry weight was calculated.

2.4 Estimation of ascorbic acid content

AsA content was assayed as described by *Guo et al.* (2006). AsA was extracted by grinding 1 g of fresh leaves using 5 ml of 10% trichloroacetic acid (TCA). The content was centrifuged at 3,500 rpm for 20 min, re-extracted twice and the supernatant was collected in a test tube. The content was made up to 10 ml using the TCA. To 0.5 ml of the extract, 1 ml of 6 mM 2, 4-dinitrophenyl hydrazine ethiourea-CuSO4 (DTC) reagent was added. The content was incubated at 37°C for 3 h and 0.75 ml of ice-cold 65% H2SO4 was added, followed by allowing the content to stand at 30°C for 30 min. The OD of the content was recorded at 520 nm using a spectrophotometer. The AsA content was determined using a standard curve prepared using AsA.

2.5 Determination of glutathione content

Glutathione (GSH) were determined as described by Guo *et al*. (2006). Fresh leaves (1 g) were sliced and ground to a fine powder in liquid nitrogen using a pestle and mortar. The powder were then extracted with 5 ml of 5% trichloroacetic acid (TCA) and centrifuged at 13,000g for 15 min. 0.2 ml of extract was neutralized with 2.6 ml of 150 mM NaH_2PO_4 (pH 7.4) and incubated with 0.2 ml of 5,5′-dithio-bis(2-nitrobenzoic acid) (75.3 mg of 5,5′-dithio-bis(2-nitrobenzoic acid was dissolved in 30 ml of 100 mM phosphate buffer, pH 6.8) at 30 °C for 10 min. Absorbance was determined at 412 nm and the GSH concentration was calculated by comparison with standard curve (Lu, Wang, Niu, Guo, and Huang, 2008).

2.6 Determination of antioxidant enzymes

Catalase (CAT) activity was measured by the method of Tan *et al* (2008). Enzyme extract and 4 ml of 50 mM phosphate buffer (pH 7.0) were mixed and incubated at 30°C for 10 min. The reaction was started by adding 1 ml of 50 µM H_2O_2, and terminated after 1min by adding 2 ml of 10% H_2SO_4. CAT activity was then determined by estimating the residual H_2O_2 in the reaction solution using 10 mM $KMnO_4$ titration to pink. CAT activity was expressed as U mg-1 FW.

Superoxide dismutase (SOD) was assayed on the basis of its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT), according to the methods of Beauchamp and Fridovich (1971) and Beyer and Fridovich (1987). The reaction mixture contained 50mM phosphate buffer (pH= 7.8), 13 mM methionine, 75 mM NBT, 100 µM EDTA, 200 mL of enzyme extract and 2 mM riboflavin. The reaction mixture was read at 560 nm. The increase in absorbance in the absence of enzyme was taken as 100 and 50% initial was taken an equivalent to 1 unit of SOD activity.

POD activity was measured as the change of absorbance of 470 nm due to guaiacol oxidation according the method described by Polle and Seifert (1994). The reaction mixture (3 ml final volume) was composed of 50 mM potassium phosphate buffer (pH 7.0), 2.7 mM guaiacol, 2 mM H_2O_2 and 150 µl of enzyme extract.

2.7 Calculation of the APX and GR activities

Specific activities of APX and GR were calculated as: $(\Delta A/(\varepsilon * d)) * (V_{assay}/V_{sample})/gFW$), where ΔA = (absorption change min^{-1} in mixture with extract) – (absorption change min^{-1} in mixture with blank) = net absorption change min^{-1}; ε = molecular absorption coefficient of either ascorbate (APX) or NADPH (GR), in μM^{-1} cm^{-1}; d = cuvette light path (cm); V_{assay} = total assay volume (ml); V_{sample} = volume of extract in the assays (ml); gFW = fresh weight of used tissue to obtain protein extract, in g. Activities of APX [µmol ascorbate g^{-1} min^{-1}] and GR [µmol NADPH $g^{-1}min^{-1}$] are by definition equal to enzyme units; the latter were graphically presented as: U $(g\ FW)^{-1}$.

2.8 Determinations of lipid peroxidation

MDA, a by-product of the lipid peroxidation caused by active oxygen species (hydroxyl radicals) in living cells, is widely used as a biomarker of oxidative damage. MDA reacts with 2-thiobarbituric acid (TBA) to form the condensation product MDA–TBA. The level of lipid peroxidation was measured by estimation a decomposition product of peroxidized polyunsaturated fatty acid component of membrane lipid, malondialdehyde (MDA).

MDA was measured by a colorimetric method (Heath and Packer, 1968) using thiobarbituric acid (TBA) as the reactive material. The tissues (0.5 g) were homogenized with 10 ml of 1% (w/v) trichloroacrtic acid (TCA). Then the mixture was centrifuged at 10000 g for 15 minutes. One ml of e supernatant was vortexed with 4 ml of 20% (w/v) TCA containing 0.5% (w/v) 2-thiobarbituric acid (TBA). The reaction mixture were heated at 95ºC for 30 min in water bath and then quickly cooled in an ice bath for 5 min and centrifuged at 10000 g for 10 min. The absorbance of coloured supernatant was measured at 532 nm and was corrected for non-specific absorbance at 600 nm. The non-specific absorbance at 600 nm was subtracted from the absorbance at 532 nm. The following formula was applied to calculate MDA content using its absorption coefficient (ε) and expressed as nmol MDA g^{-1} fresh weight: MDA (nmol g^{-1} FW) = $[(A_{532}-A_{600}) \times V \times 1000/\varepsilon] \times W$. Where, ε is the specific extinction coefficient (=155 mM^{-1} cm^{-1}), V is the volume of homogenizing medium, W is the fresh weight of leaf, A_{600} and A_{532} are the absorbance at 600 nm and 532 nm wavelength respectively.

2.9 Statistical data analysis

Statistical analysis was performed using SPSS 16. The data represent means calculated from three replicates. The analysis of variance procedure (ANOVA) followed by Duncan's multiple range Test (DMRT) used to compare the effect of salinity. The values are mean ± S.D. for three samples in each group and statistical significance was set at $P \leq 0.05$.

3. RESULTS

3.1 Effect of salinity stress on the germination percentage

As Table-1 shows increase of NaCl concentration resulted to decrease of the germination percentage. The maximum seed germinations occurred at the control. Increasing the NaCl concentration to 25, 50, 75 and 100 µM reduced germination percentage by 8, 29, 41 and 81 percent respectively. These levels had significant differences ($P \leq 0.05$). The minimum germination was related to the 100 µM salt.

3.1 Measurement of growth parameters

Morphologic and growth properties of seedling were estimated by means of shoot height and root length of seedlings as well as their fresh and dry weight. The shoot and root growth were inhibited by salinity stress

(Table-1). The effect of salinity on decrease of root and shoot growth under different salinity levels were significant (P≤0.05) compare to the control except the effect of 25 µM NaCl on shoot height. Salt stress significantly decreased the fresh weights and dry weights of plants (Table-1). Plant fresh weights showed a decrease of 32, 60, 73 and 93 percent at concentrations of 25, 50, 75 and 100 µM, respectively, compare to the control. All differences were significant (P≤0.05). Reductions of dry weight were 24, 42, 60 and 80 percent compare to the control.

3.2 Estimation of ascorbic acid content

As Figure-1 shows ascorbic acid (AsA) concentration under salt stress was changed. AsA concentration was significantly (P≤0.05) affected when treated with all treatment compared to control. AsA increased to 42, 56, 129 and 218 percent compared to the control. Difference between results of treatment 25 and 50 µM, however, was not significant.

3.3 Glutathione concentrations

Changes in GSH concentrations under heat stress are shown in Figure-2. GSH concentration showed a significant (P≤0.05) alternation at all treatments. GSH concentrations were 4%, 16%, 59% and 98% higher when treated by concentrations of 25, 50, 75 and 100 µM NaCl, respectively, as compared to the control.

3.4 Antioxidative enzymes

To manage the oxidative stress, the cells activate several enzymatic antioxidants, such as SOD, CAT, POD and GR. In order to evaluate the oxidative stress generated by salinity stress, activities of antioxidative enzymes in *C. ruseos* plants subjected to different treatments were determined (Figure-2). The antioxidant enzymes showed deviation in their activities under salinity stress. The concentration of 25 µM had no significant changes on level of SOD but all other treatments caused to significant decrease of the enzyme activity. Salinity enhanced the activities of catalase and peroxidase maximally (Figure-2). Relative to control, the activity of CAT showed increase at all treatments. CAT activities at all treatments were significantly different. Change between samples treated by 25 and 50 µM, however was not significant. Ascorbate peroxidase activity showed no significant increase when treated by 25 µM but increased in seedling under other treatments. The activity of POD was increased with all treatments. Treated by different treatments plants showed an increase in GR activity, which was about 138 % of GR activity of control. Seedlings treated with 25 and 50 µM NaCl, however, had a no significant increase.

3.5 Lipid peroxidation

Oxidative damage to tissue lipid was estimated by the content of MDA. The MDA content increased with the increasing concentrations of NaCl (Figure-3). MDA content of samples treated by 100 µM NaCl increased up to 45 percent more than the control.

Table-1. Effect of different concentration of NaCl on morphologic and growth properties of Periwinkle.

NaCl (µM)	**Germination** (%)	**Root length** (cm)	**Shoot height** (cm)	**Fresh weight** (g)	**Dry weight** (g)
0	96 ± 2	1,23 ± 0,09	5,79 ± 0,13	0,72 ± 0,03	0,11 ± 0,02
25	88 ± 4	1,31 ± 0,07	3,02 ± 0,21	0,49 ± 0,03	0,08 ± 0,01
50	68 ± 2	0,96 ± 0,02	2,10 ± 0,19	0,28 ± 0,02	0,06 ± 0,01
75	56 ± 2	0,61 ± 0,06	0,82 ± 0,1	0,19 ± 0,02	0,04 ± 0,01
100	18 ± 2	0,22 ± 0,04	0,53 ± 0,03	0,04 ± 0,01	0,02 ± 0,01

Data are the mean value ± SD of three individual experiments.

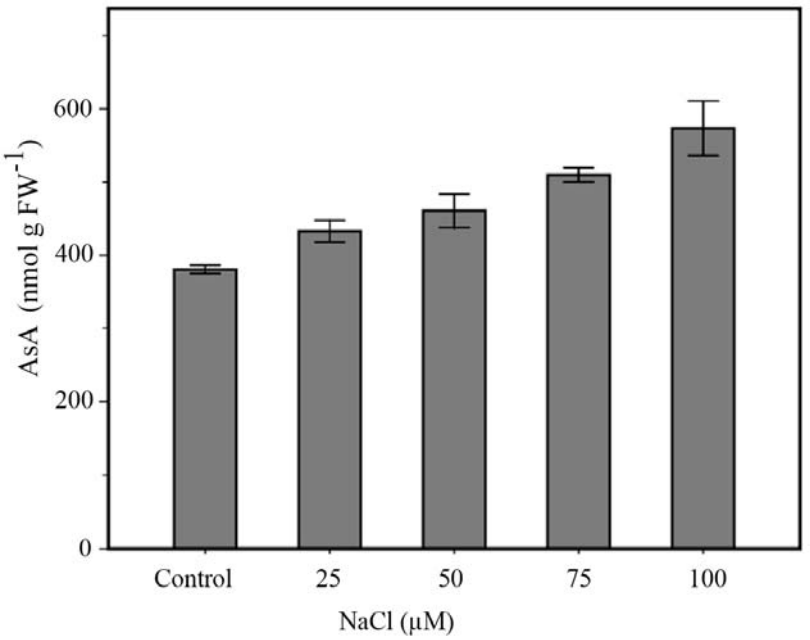

Figure-1. Effect of salt stress on amount of ascorbic acid in *C. roseus* seedlings. Data are the mean value ± SD of three individual experiments.

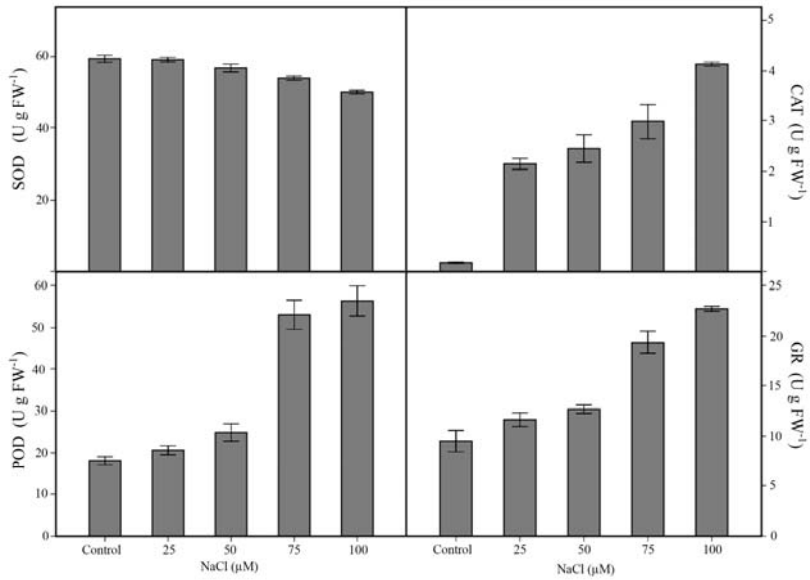

Figure-2. Effect of salt stress on antioxidative enzymes in *C. roseus* seedlings. Data are the mean value ± SD of three individual experiments.

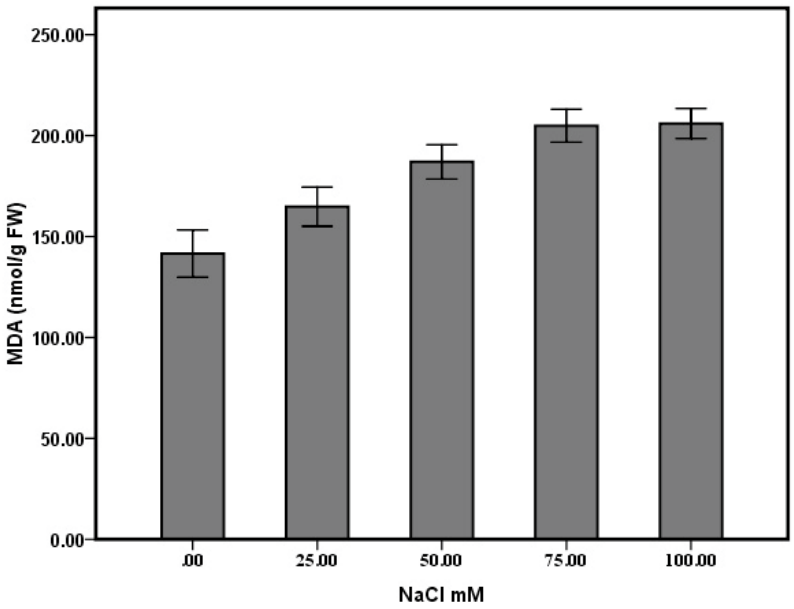

Figure-3. Effect of salt stress on amount of MDA in *C. roseus* seedlings
Data are the mean value ± SD of three individual experiments.

4. DISCUSSIONS

During treatment the germination dropped from 98% to 18%. The salinity during germination period imposes damages to cell membranes, especially to the cytoplasm membrane, and consequently increases the cells permeability due to replacing Ca^{++} with Na^{+} which ultimately increases the K^{+} losses (Takel, 2000).

Salt stress inhibits the efficiency of the translocation and assimilation of photosynthetic products (Xiong and Zhu, 2002) and might have caused reduction in shoot growth. Reduction in plant growth has also been attributed to reduced water absorption due to osmotic effect, nutritional deficiency on account of ionic imbalance and decrease in many metabolic activities (Rezaee, *et al.*, 2012). Therefore, the root's cells are unable to absorb water needed for growth from the environment, thus the absorption of some water soluble mineral substances is limited which limits the plant's growth and development due to the difficulties in the metabolism (Kumar, Goyal, and Kuhad, 2005).

Ascorbic acid content of *Catharanthus roseus* plants increased significantly in all treatments. Ascorbic acid is readily oxidised to monodehydro ascorbic acid as part of its antioxidant function (Gomathinayagam, Jaleel, Lakshmanan, and Panneerselvam, 2007; Shao, Chu, Shao, Jaleel, and Mi, 2008). Similar results have been previously reported by Jaleel *et al*. (2009). GSH concentration increased progressively during drought stress, concomitant with high level of GR activity, indicating that GSH plays an important role in scavenging the ROS by ascorbate-glutathione cycle. GSH is an ideal biochemical to protect plants against stress including oxidative stress, and takes part in the control of H_2O_2 levels (Sankar, *et al*., 2007). Ascorbate acid and glutathione are key nonenzymatic antioxidants. There is a close relationship between ascorbate and glutathione dates from soon after the characterization of the chemical formulae of the two molecules. In oxidative stress, they participate in redox regulation in different cell compartments (Foyer and Noctor, 2011).

The presence of stress in the cell leads to the formation of ROS, which cause further severe oxidative damage to different cell organelles and biomolecules. Antioxidant metabolism is one of the common defense processes that plants evolve to counteract the damage generated by the ROS. The ROS scavenging depends on the detoxification mechanism provided by an integrated system of reduced non-enzymatic molecules and enzymatic antioxidants. CAT, POD, GR and SOD (Shi, *et al*., 2006). SOD is a key enzyme that plays an important role in cellular defense and catalyses the dismutation of superoxide radicals to H2O2 and superoxide (Foyer and Noctor, 2011). The expressions of the antioxidant enzyme-related genes such as CAT, GR and POD were all up-regulated by salinity treatment. This suggests that salinity adaptation improved the antioxidant capacity, which may effectively decrease ROS injury during heat stress. These findings are similar to wheat experiencing heat stress where an increase in activity was observed in SOD, CAT, GR and APX at high temperature and other stresses (Almeselmani, Deshmukh, and Sairam, 2009; Mohammad Reza Amirjani, 2012a; Mohammad Reza Amirjani, 2013; Balla K, 2009; Dash and Mohanty, 2002).

Lipid peroxidation has been associated with damages provoked by a variety of environmental stresses (Hernandez, *et al*., 2003). The level of MDA, indicates the extent of salt tolerance as reported by Bor *et al* (2003) in sugar beet and wild beet under NaCl treatment. Poly

unsaturated fatty acids are the main membrane lipid components susceptible to peroxidation and degradation. The increase in MDA can be correlated with the accumulation of ions and active ROS production under salt stress (Hernandez, *et al.*, 2003; Misra and Gupta, 2006).

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