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SALICYLIC ACID ALTERS ANTIOXIDANT AND PHENOLICS METABOLISM IN *CATHARANTHUS ROSEUS* GROWN UNDER SALINITY STRESS

Neelam Misra[#], Rahul Misra, Ajiboye Mariam, Kafayat Yusuf, Lateefat Yusuf

College of Natural and Applied Sciences (CONAS), Crescent University. Abeokuta (Ogun State), Km 5, Ayetoro Road, Lafenwa, P.M.B. 2104, Sapon, Abeokuta, Ogun State, Nigeria.
***E-mail:** neelam_misra@rediffmail.com

Abstract

Background: Salicylic acid (SA) acts as a potential non-enzymatic antioxidant and a plant growth regulator, which plays a major role in regulating various plant physiological mechanisms. The effects of salicylic acid (SA; 0.05 mM) on physiological parameters, antioxidative capacity and phenolic metabolism, lignin, alkaloid accumulation in salt stressed *Catharanthus roseus* were investigated.
Materials and Methods: *Catharanthus roseus* seeds were grown for two months in a glass house at 27–30^{0}C in sunlight, and then divided into four different groups and transplanted with each group with the following solutions for one month: group I (non-saline control), group II, 100 mM NaCl, group III, 0.05 mM SA, group IV, 100 mM NaCl+0.05 mM SA and to determine the physiological parameters (DW, FW, WC), chlorophyll contents, carotenoid contents, lipid peroxidation, phenolics, lignin, alkaloid and enzymatic assays in each leaf pairs and roots.
Results: SA exhibited growth-promoting property, which correlated with the increase of dry weight, water content, photosynthetic pigments and soluble proteins. SA has additive effect on the significant increase in phenylalanine ammonia-lyase (PAL) activity, which is followed by an increase in total soluble phenolics and lignin contents in all leaf pairs and root of *C. roseus*. SA enhances malondialdehyde content in all leaf pairs and root. The antioxidant enzymes (catalase, glutathione reductase, glutathione-S-tranferase, superoxide dismutase, peroxidase) as well as alkaloid accumulation increased in all treatments over that of non-saline control but the magnitude of increase was found more in root. Further, the magnitude of increase of alkaloid accumulation was significantly higher in 100 mM NaCl, but highly significant was found in presence of 0.05 mM SA and intermediate in presence of both 0.05 mM SA+100 mM NaCl.
Conclusion: We concluded that applied SA to salt stress, antioxidant and phenolic metabolism, and alkaloid accumulation were significantly altered and the extent of alteration varied between the SA and salt stress.

Key words: Antioxidant enzymes; *Catharanthus roseus;* indole alkaloids; phenolic metabolism; salicylic acid; salinity stress.

Abbreviations: CAT - catalase; Chl - chlorophyll; Car - carotenoids; DTNB - 5,5-dithiobis-2-nitrobenzoic acid; GR - glutathione reductase; GST - Glutathione-S-transferase; H_2O_2 - hydrogen peroxide; MDA- malondialdehyde; PAL -phenylalanine ammonia lyase; POD - peroxidase; ROS – reactive oxygen species; SA - salicylic acid; SOD - superoxide dismutase; TBA - thiobarbituric acid.

Introduction

Salicylic acid (SA), a natural plant phenolics, acts as an endogenous signal molecule responsible for inducing abiotic stress tolerance in plants and involved in many growth responses (Misra and Saxena 2009, Park and others 2003) as well as also providing defence against pathogen attack both local (hypersensitive response) and systemic acquired resistance (Durner and Klessig 1996). Stimulation of growth after supplementation of SA has been reported in various plants, like wheat (Shakirov and others 2003), soybean (Gutierrez-Coronado and others 1998), lentil (Misra and Saxena 2009) and maize (Gunes and others 2007). SA acts as a potential non-enzymatic antioxidant and a plant growth regulator, which plays a major role in regulating various plant physiological processes/mechanism including phenolic metabolism (Kova´cˇik and others 2009).

Plant phenolics are naturally occurring substances, characterized as aromatic metabolites, many of which play physiological roles such as antibacterial, antiviral, anticancer agents and scavengers of most types of oxidizing agents (Solecka and Kacperska 2003). Under biotic and abiotic stresses such as pathogen attack, physical wounding, and UV-light exposure, plants can induce a defense response and increase secondary metabolite levels (Ali and others 2006). Moreover, phenolices are essential for the continued survival of all types of vascular plants. In relation to this, increased activity of phenylalanine ammonia lyase (PAL) and other related enzymes such as peroxidase (POD) has been reported in plants subjected to various stresses (Dixon and Paiva 1995).

Plant organelle, mitochondria and chloroplasts through their respective electron transport mechanism can generate reactive oxygen species (ROS), such as superoxide (O_2▪-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH•), and singlet oxygen ($^1O_2$) (Mittler 2002). However, ROS are scavenged by plant antioxidant defense systems, comprising both enzymatic and non-enzymatic components (Noreen and others 2009). Under natural conditions of growth and development, plants are inevitably exposed to various stresses, which may cause increased production of ROS (Smirnoff 1993). Abiotic stresses, such as salt stress, may lead to an imbalance between antioxidant defenses and ROS levels, resulting in oxidative stress (Apel and Hirt 2004, Smirnoff 2005) and the efficiency of the antioxidative systems is directly correlated with tolerance to salinity stress (Athar and others 2008, Munns and Tester 2008). The H_2O_2 produced is scavenged by catalase (CAT) and a variety of peroxidases (POD). CAT, which is located in peroxisomes, glyoxysomes and mitochondria, apparently absent in the chloroplast, dismutates mostly photorespiratory/respiratory H_2O_2 into water and molecular O_2 (Willekens and others 1997), whereas POD decomposes H_2O_2 by oxidation of co-substrate such as phenolic compounds and/or antioxidants. POD has also been catalyzed the coupling of vindoline and catharanthine into vinblastine (an antitumor agent) in leaves and in the conversion of ajmalicine to serpentine in roots of *Catharanthus roseus* (Misra and others 1999). Exogenous SA can regulate the activities of these antioxidant enzymes and increase plant tolerance to abiotic stress (He and others 2002). Therefore, it is suggested that salt tolerance can be induced by enhancing antioxidant capacity of plants. Moreover, SA cannot induce abiotic stress tolerance in all types of plants or in other words the effectiveness of SA in inducing stress tolerance depends upon type of species or, developmental stage, the mode of application, and the concentration of SA applied (Borsani and others 2001, Waseem and others 2006, Arfan and others 2007, Horva´th and others 2007).

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It can also contribute to stress tolerance by stimulating highly-branched metabolic responses (Horva´th and others 2007). The pivotal step in the biosynthesis of phenolics, yielding trans-cinnamic acid from phenylalanine, is controlled by phenylalanine ammonia-lyase (PAL, EC. 4.3.1.5). ROS have been implicated in the signaling between stress perception and PAL expression (Dixon and others 1994). Phenolic metabolites can also cooperate with POD in H_2O_2 scavenging. In addition, POD is implicated in other processes, such as the formation of monomers for lignin biosynthesis (Lewis and Yamamoto 1990). Phenylalanine is the precursor for the phenylpropanoid pathway that synthesizes phenolics (Randhir and others 2006, Ratledge 1982). The phenylpropanoid pathway is the main metabolic route for the synthesis of phenolics, flavonoids and lignin, etc. PAL is the most extensively studied enzyme in the phenylpropanoid pathway, perhaps in all secondary metabolism, is a crucial enzyme catalyzing the formation of *trans*-cinnamic acid via the L-deamination of phenylalanine. Cinnamyl alcohol dehydrogenases catalyze the reversible conversion of *p*-hydroxycinnamaldehydes to their corresponding alcohols, leading to the biosynthesis of lignin in plants (Blanco and others 2002). PODs are ionically bound to the cell walls and are involved in the polymerization of phenylpropanoid lignin precursors (Cvikrová and others 2006).

Catharanthus roseus, a medicinal plant which contain most important antitumor agents (vinblastine and vincristine) in leaves and ajmalicine in roots (Misra *et al*. 1996). Many physiological reactions to exogenous applied SA are known, but extensive studies focusing on the effects of SA on antioxidative and phenolic metabolism are still not well understood. Therefore, the main aim of this paper was to investigate the effects of exogenous applied SA on plant growth parameters, phenolics metabolism, lignin, alkaloid accumulation and antioxidant system of salt stressed *Catharanthus roseus*.

Materials and methods

Seeds of *Catharanthus roseus* (L.) G. Don (Pink flower, Family *Apocyanacea*) and wild type (control) were screened in lab for their saline tolerance (100 mM NaCl). After surface sterilization with 1% (v/v) sodium hypochlorite solution for 10 min, followed by rinsing with distilled water, seeds were imbibed for 2 h in distilled water and sown in pots with sterilized sand (Misra and Gupta 2006). After germination, the plants were grown for two months in a glass house at 27–30^{0}C in sunlight, and then divided into four different groups and transplanted with each group was watered every other day with one of the following solutions for one month: group I, distilled water, (non-saline control), group II, 100 mM NaCl, group III, 0.05 mM SA, group IV, 100 mM NaCl+0.05 mM SA.

The plants from each group were uprooted; leaf pairs (apical, middle, basal) were removed node by node and used for physiological parameters (DW, FW, WC), chlorophyll contents, carotenoid contents, lipid peroxidation (in terms of MDA content), phenolics, lignin content, alkaloid content and enzymatic assays. The plant growth (DW and FW) was evaluated using twenty plants from each group in triplicate. At specified periods of growth, all tissue parts (leaf pairs and root) were separated node by node and FW of these tissue parts was measured. For the determination of DW, these tissue parts were dried for three days in an oven at 70^{o}C (or till there is no decrease in weight). Water content as percentage of fresh weight (FW) was calculated using formula-WC (%) = [(FW–DW)/FW] x 100.

Salicylic acid obtained from Sigma Chemical Co.UK, was initially dissolved in 100µl dimethyl sulfoxide and concentration of 0.05 mM SA (pH 6.5) were made up with distilled water containing 0.02 % Tween 20 (Polyoxyethylenesorbitan monolaurate, Sigma Chemicals, UK) **(**Khan and others 2003).

Photosynthetic pigments (chlorophyll a+b, carotenoid) were measured in each leaf pair. Leaf samples (500 mg) were homogenized with acetone (90% v/v), filtered and make up to a final volume of 50 ml. Pigment concentrations were calculated from the absorbance of extract at 663, 648 and 470 nm using the formula of the method of Lichtenthaler (1987) with some modification, given as below:

Chlorophyll a (mg/g FW) = (11.75 x A663-2.35 x A645) x 50/500,
Chlorophyll b (mg/g FW) = (18.61 x A645-3.96 x A663) x 50/500,
Carotenoid (mg/g FW) = (1000 x A470) – (2.27 Chl a) - (81.4 x Chl b)/227 x 50/500

Lipid peroxidation was measured in terms of malondiadehyde (MDA) content. Leaf pairs and root (500 mg) were homogenized in 10 ml 0.1% TCA. The homogenate was centrifuged at 15,000g for 30 min. Took 1.0 ml aliquot of the supernatant 4.0 ml of 0.5% thiobarbituric acid (TBA) in 20% TCA was added. The mixture was heated at 95^{0}C for 30 min and then quickly cooled in an ice bath. After centrifugation at 10,000g for 10 min, the absorbance of the samples was recorded at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The MDA equivalent was calculated as follows- MDA (nmol /ml FW) = ([A532 –A600] / 155,000) x 1000000 (Heath and Packer 1968).

A crude enzyme extract was prepared by homogenizing 500 mg of tissue (each leaf pair and root) in 0.1 M Tris HCl buffer (pH 7.5), 0.5 mM EDTA and 1% PVP (MW 360,000), at 4^{o}C. The homogenates were centrifuged at 18,000-x g for 30 min. The supernatant was used as the crude enzyme preparation (Misra and Gupta 2006).

Glutathione reductase (GR, EC 1.6.4.2) activity was assayed according to method of Smith and others (1988) by following the increase in absorbance at 412 nm due to 5, 5'-dithiobis- 2-nitro benzoic acid (DTNB) reduction by glutathione reduced form (GSH) generated from glutathione oxidized form (GSSG). The reaction mixture consisted of 0.1 M sodium phosphate buffer (pH 7.5), 1 mM EDTA, 0.75 mM DTNB in 0.01 M sodium phosphate buffer (pH 7.5), 0.1 mM NADPH, 1mM GSSG. The reaction was started by the addition of enzyme extract.

Glutathione-S-transferase (GST, EC 1.8.1.7) activity was measured by the method of Mannervik and Gutherberg (1981) following the decreased in the absorbance at 340 nm due to glutathione (GSH) oxidation. The final assay volume of 1 ml contained 100 mM sodium phosphate buffer, pH 6.5, 1 mM GSH, 1 mM 1-chloro-2, 4-dinitrobenzene (CDNB) in ethanol. Reaction was started by the addition of enzyme extract.

Catalase (CAT, EC 1.11.1.6) activity was determined after the slight modification of Upadhyaya and others (1985). The assay mixture contained 20 mM sodium phosphate buffer, pH 7.5, 0.025 % H_2O_2 and enzyme extract. The decomposition of H_2O_2 was measured at 240 nm.

Peroxidase (POD, EC 1.11.1.7) activity was assayed in aliquots of crude enzyme preparation as described by Putter (1974) with some modifications. The assay mixture consisted of 25 mM guaiacol and 0.020% H_2O_2 in 0.1M sodium phosphate buffer, pH 6.5 at 30^{o}C. The product of the reaction was measured at 470 nm.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured by photochemical method as described by Giannopoliti and Ries (1977) with slight modification. The reaction mixture consisted 20 mM sodium phosphate buffer pH 7.5, 0.1 mM EDTA and 10 mM methionine, 0.1 mM p-nitro blue tetrazolium chloride (NBT) in ethanol, 0.005 mM riboflavin and enzyme extract. Blanks were kept in the dark and others were illuminated for 30 min. Total SOD activity was defined as the amount of enzyme required to cause 50 % inhibition of the rate of NBT reduction at 560 nm.

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) activity was determined by HPLC method as the production of trans-cinnamic acid from phenylalanine (measured at 275 nm). Samples were homogenized in sodium borate buffer (pH 8.8) and supernatants were used for preparation of reaction mixture comprising 0.5 ml sodium borate buffer, 0.25 ml supernatant and 0.3 ml 50 mM phenylalanine (Kova´c˘ik and others 2007). After

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one h incubation at 40^0C, the reaction was stopped by adding 0.05 ml 5N HCl. The endogenous content of t-cinnamate in samples not incubated with L-phenylalanine was between 5 and 11%; this was subtracted from values recorded in the L-phenylalanine-incubated samples prior to calculating PAL activity.

All the enzyme activities were expressed in katal.

Total alkaloid extraction and determination was carried out as described earlier (Misra and Gupta 2006). Samples of leaf pairs and root were homogenized in 90% ethyl alcohol. The ethanol extract was evaporated to dryness. The residue was dissolved in distilled water and mixed with conc. HCl (final concentration of HCl was 3%). An equal volume of ethyl acetate was added. The aqueous phase was collected, adjusted to pH 9.0 with ammonia and extracted with chloroform. The chloroform phase containing the alkaloids was collected and evaporated to dryness to get total alkaloid. The residue was dissolved in methanol and then analyzed by HPLC (Water, USA). The samples of alkaloids were loaded separately on a C_{18} reverse phase analytical cartridge and eluted at a flow rate of 1.0 ml/min with the elution buffer (methanol: acetonitrile: 0.01 M ammonium acetate + 0.1% triethyl amine in 1:1:2.25 ratio) at pH 8.2. The alkaloids were detected at 254 nm.

Soluble phenolics were extracted by 80% methanol and calculated from calibration curve prepared using gallic acid by the Folin-Ciocalteu method (Kova´cˇik *et al*. 2007). Root lignin content was determined from the cell wall fraction (removing of other compounds by phosphate buffer, 1% Triton X-100, 1 M NaCl and acetone) by thioglycolic acid reaction (Kova´cˇik and Klejdus 2008).

Soluble protein was measured by the Bio-Red micro assay modification of the Bradford procedure (1976) using bovine serum albumin as standard.

Statistical analysis

Each treatment was analyzed with at least three replicates and standard deviation (S.D.) was calculated. Statistical analysis was performed using the students t-test; $p<0.05$ and $p<0.001$ were considered statistically significant and highly significant, respectively.

Results

The growth parameters were analyzed in all leaf pairs and root of *Catharanthus roseus* grown under four different treatments, non-saline control, 0.05 mM SA, 100 mM NaCl, and in combination of 0.05 mM SA+100 mM NaCl. The *C. roseus* accumulated less biomass over the same growth period in presence of salinity (Table-1). SA significantly stimulated growth of the leaf pairs and roots. Moreover, SA increased tissue water content (Table-1), soluble proteins (data not shown) and MDA content in all leaf pairs and roots of *C. roseus* (Fig.1). Chlorophylls and carotenoids contents were increased in all leaf pairs in presence of SA. The water content of salt stressed plants was lower as compared to non-saline control. Salinity affected the growth of leaf pairs more than roots. The FW and DW decreased significantly with exposure to salinity stress. Exogenous treatment of 0.05 mM SA increased dry yield significantly both in saline and non-saline control (Table-1). The FW and DW of apical, middle and basal leaf pairs and roots increased considerably in presence of SA and intermediate in presence of both treatments as compared to non-saline control. The plants grew much better in the presence of SA ($p<0.05$) than that of other treatments (Table-1).

Total chlorophyll content was measured in all four treatments in leaf pairs and results depicted in Fig.1a. Total chlorophyll content decreased in presence of 100 mM NaCl and found to be NaCl stress dependent. However, better chlorophyll stability was found in SA and SA+NaCl treated leaves as compared to saline and non-saline control. The concentration of carotenoids increased with SA treatments (Fig.1b). Lipid peroxidation (MDA concentration) of salinity stressed plants was higher than that of non-saline control. Exogenous application of SA increased lipid peroxidation significantly (Fig.1c).

Phenylalanine ammonia-lyase activity increased in presence of NaCl and significantly higher in SA-treated plants in all leaf pairs and roots (Fig.1e). The increase of PAL activity was found highly significant in roots than the leaf pairs. Moreover the increased accumulation of total soluble phenolics was observed in all leaf pairs and root (Fig.1f), but the apical leaf pair had more significant phenolics accumulation than the others tissues. Same pattern was observed for lignin accumulation (Fig.1d). But root lignin content was highly significant affected by the SA (Fig. 1d).

Fig. 2a depicted the developmental profile of GR activity in *C. roseus* under four different treatments. The presence of NaCl caused an increase in GR activity throughout the plant growth (≈2.8 fold). While SA increased the GR activity ≈3.2 fold and ≈2.9 fold in presence of both treatments in *C. roseus* roots over that of non-saline control. The increased activity of GR in presence of SA was found more significantly in root than that of leaf pairs.

Plants grown under SA showed higher GST activity in leaf pairs and roots (Fig. 2b) than the other treatments. Apical leaf pair had higher activity than the other leaf pairs, while the roots of SA treated plants expressed the highest activity ($p<0.001$).

The presence of NaCl increased the CAT activity in all leaf pairs and root. SA treatment resulted in the increase in the CAT activity (Fig.2c) while with SA+NaCl-treatment *C. roseus* showed an intermediate increase throughout over that of non-saline control. SA increased the activity ≈2.0 fold and ≈1.5 fold in combination of NaCl+SA. The increase was more pronounced in roots than the leaf pairs.

Fig.2d depicted the developmental profile of SOD activity in leaf pairs and root of *C. roseus* grown under four different treatments. The presence of NaCl caused an increase in SOD activity (≈1.65 fold). SA increased the SOD activity ≈2.05 fold, while ≈1.28 fold increased in the combination of both over that of non-saline control. The increase was more pronounced in roots than the leaf pairs. The developmental profile of POD in *C. roseus* under four different treatments is shown in Fig.2e. The presence of NaCl caused an increase in POD activity. SA increased the activity ≈1.18 fold over that of non-saline control. The combination of NaCl+SA resulted is ≈ 1.0 fold increase.

The total alkaloid accumulation increased in presence of salinity in all leaf pairs and root over that of non-saline control (Fig.2f). The alkaloid content was found to be maximal ($p<0.05$) in SA treated plants in apical leaf pair as well as highly significantly found in roots of *C. roseus* while there was no significant increase in basal leaf pair. Apical leaf pair maintained higher indole alkaloid accumulation than the other leaf pairs. A highly significant ($p<0.001$) indole alkaloid accumulation was found in the roots of all treatments than the leaf pairs.

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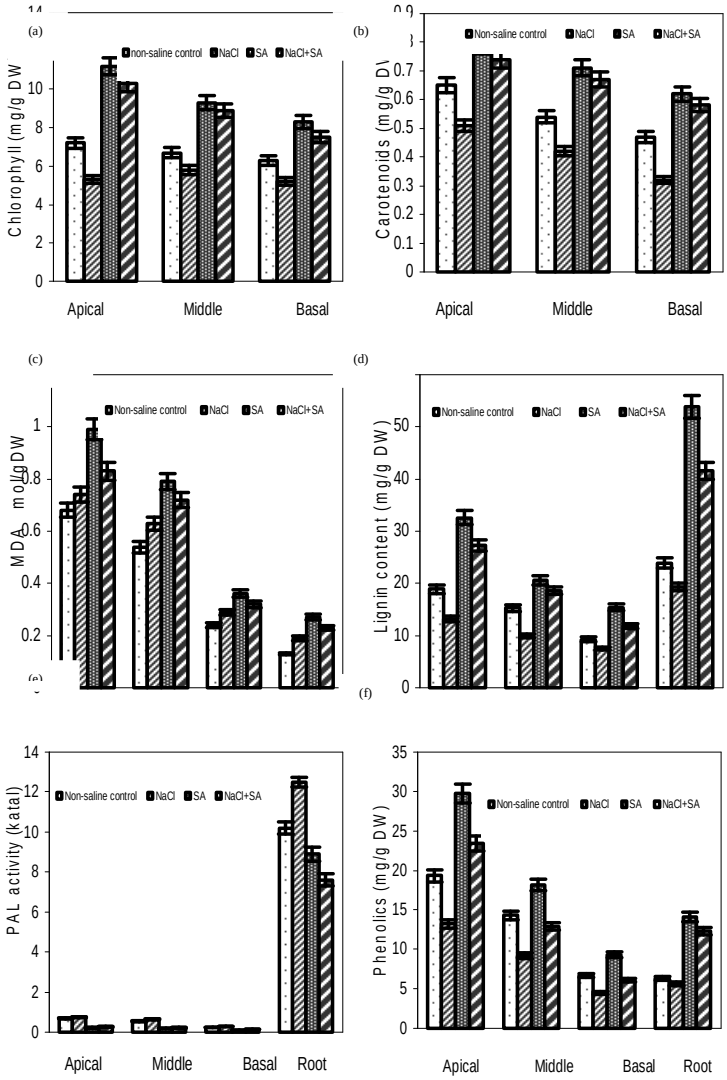

Figure 1: Total chlorophyll content (a), Carotenoids (b), MDA (c), Lignin (d), PAL activity (e) and Phenolics (f) in leaf pairs (apical, middle, basal) and root during plant growth of *C. roseus* under four different treatments; non-saline control, NaCl, SA, NaCl+SA. Each value represents mean of three independent observations and SD determined. Data are statistically significant and highly significant at $p<0.05$ and $p<0.001$, respectively.

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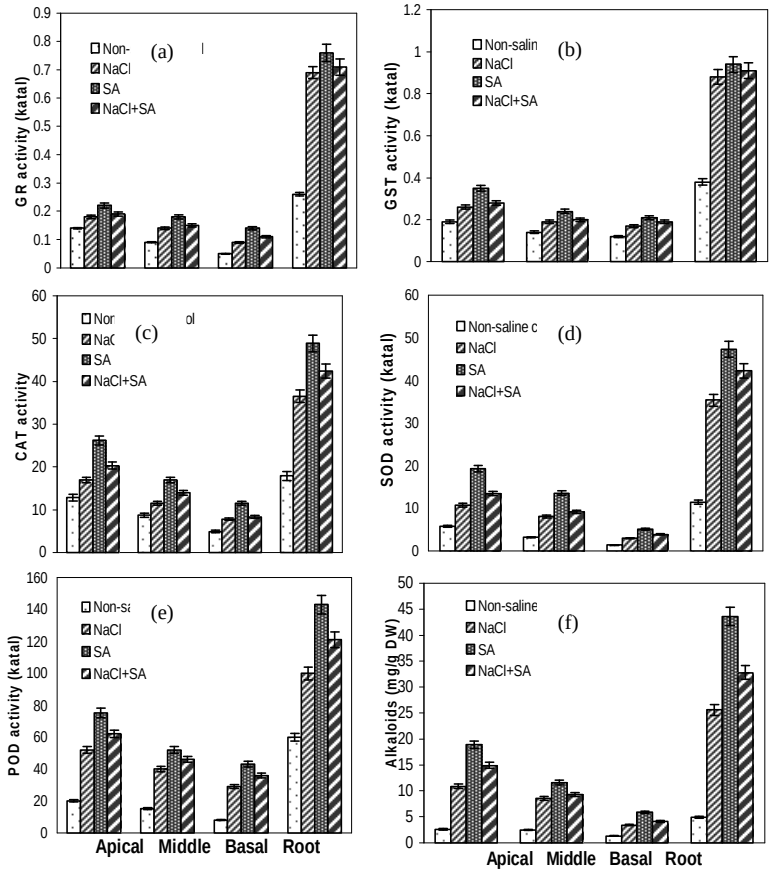

Figure 2: Activities of GR (a), GST (b), CAT (c), SOD (d), POD (e) and alkaloid content (f) in leaf pairs (apical, middle, basal) and in root during *C. roseus* plant growth under four different treatments; non-saline control, NaCl, SA, NaCl+SA. Each value represents mean of three independent observations and SD determined. Data are statistically significant and highly significant at $p<0.05$ and $p<0.001$, respectively.

Table 1: Growth parameters: Fresh weight, dry weight and water content in *Catharanthus roseus* seedlings grown under four different treatments, non-saline control, 100 mM NaCl, 0.05 mM SA, and combination of 100 mM NaCl+0.05 mM SA. Each value represents mean of three replicates and SD determined.

Treatments	Growth parameters Leaf pairs									Root		
	Basal			Middle			Apical					
	FW (mg)	DW (mg)	WC (%)	FW (mg)	DW (mg)	WC (%)	FW (mg)	DW (mg)	WC (%)	FW (mg)	DW (mg)	WC (%)
Control	21.4± 1.2	13.1± 0.6	38.7± 0.89	82.9± 3.1	41.1± 1.7	50.4± 1.1	32.4±2 .5	14.3± 0.82	55.9± 1.19	31± 2.6	17.1± 0.52	44.8± 1.10
NaCl	12.2±3 .12†	8.16± 1.01$^{\delta}$	33.1± 1.04$^{\delta}$	64.12± 2.11†	35.2± 1.11†	45.1± 0.19$^{\delta}$	20.1±2 .1$^{\delta}$	11.2± 0.88†	44.2± 1.02†	22± 2.3*	14.1± 0.66†	35.9± 1.34†
SA	38.8± 4.20*	18.15 ±1.24†	53.2± 2.17†	108.4± 5.34†	51.1± 1.21$^{\delta}$	52.8± 3.09†	68.3±2 .4†	28.5± 1.26$^{\delta}$	58.2± 2.83$^{\delta}$	59.2± 3.12†	28.2± 2.01†	52.3± 2.19†
NaCl +SA	29.2± 3.48$^{\delta}$	16.19± 0.72*	44.5± 3.11$^{\delta}$	80.21± 4.44†	39.5± 0.65†	50.8± 1.98†	42.2±4 .9†	18.5± 0.12†	56.1± 2.17†	49.4± 4.20†	23.8± 1.98†	51.8± 1.92†

Data are 'mean ± SD' and are the averages of 10 seedlings.
*$P \geq 0.05$ (Insignificant); $P < 0.05$ (Probably significant); $^{\dagger}P < 0.01$ (Definitely significant)

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Discussion

Since salt stress limits plant growth by adversely affecting various physiological, biochemical and metabolic processes, such as, photosynthesis, antioxidant phenomena, nitrogen metabolism, ion homeostasis, proline metabolism and osmolytes accumulation (Misra and others 2006, Misra and Gupta 2006, Misra and Dwivedi 1990, Ashraf 2004, Misra and Gupta 2005). Germination of seeds, plant growth and metabolism are absolutory influenced by salinity and alter under salt stress (Misra and Dwivedi 1990, Misra and Gupta 2005). Salinity decreased *C. roseus* growth significantly. SA treatments alleviated the deleterious effects of salinity on plant growth (Shakirova and others 2003). Increases in dry matter of salt stressed plants in response to SA may be related to the induction of protective role of membranes that increase the tolerance to plant damage. Coronado and others (1998) reported that SA used as a spray to shoots of soybean significantly increased the growth of shoots and roots in either greenhouse or field conditions. It has also been reported that SA treated maize plants showed higher dry mass as compared to untreated seedlings grown under salinity stress (Gunes and others 2007).

Salinity treatment caused a depletion of protein in all leaf pairs and root, whereas, SA enhanced the total protein content in all leaf pairs and root of *C. roseus*. Combination of both treatments also showed an intermediate increase in protein content over that of non-saline control (data not shown). The increased protein content induced by SA might be helping in maintaining osmolarity in the cells during saline stress (data not shown). Lipid peroxidation (MDA concentration) increased significantly with salinity stress, and increasing levels of exogenously applied SA alleviated this oxidative damage. Reactive oxygen species (ROS) play a major role in the defense mechanism of plants against abiotic stress conditions. ROS are produced by plant cells via the enhanced enzymatic activity of plasma membrane-bound NADPH oxidases, cell wall-bound PODs and amine oxidases in the apoplast. Hydrogen peroxide (H_2O_2) produced during this response is considered to diffuse into cells through aquaporins and along with SA, and nitric oxide (NO) through signal pathway activate many plant defense systems.

The activity of CAT was found significantly higher in NaCl-treated plants and intermediate in NaCl+SA-treatment throughout plant growth over that of non-saline control. The inhibitory effect of SA on CAT has also been reported in tobacco leaves (Durner and Klessig 1996). The inhibitory effect of SA on CAT increases H_2O_2 concentration, which might be activating genes responsible for ameliorating salt stress. POD showed significantly higher activity in NaCl-treated plants, and decreased in NaCl+SA- treated plants. Further the POD activity increased with the increase of plant growth and overall activity was higher. The higher activity of POD increases lipid peroxidation, which intern causes damage to cell membranes. SA might be protecting the cell membranes by reducing the POD activity. SOD activity showed the significant higher activity in *C. roseus* grown under SA, lower in NaCl-treated plants and an intermediate activity in NaCl+SA-treated plants than the non-saline control. The SOD activity also increased with increase in plant growth from day 4 to 10 and overall activity was higher in all leaf pairs and root. GR activity was significantly higher in SA-treated plants, lower in NaCl-treated seedlings and an intermediate activity was observed in NaCl+SA- treated plants over that of non-saline control. Furthermore, the GR activity increased with increase in plant growth and overall activity was higher. Same pattern was found for GST enzyme activity.

Plants release phenolic metabolites in response to treatment with signaling and others compounds and these compounds also play a major role in the defence against microbes or as allelochemicals (Badri and others 2008, Bais and others 2004). The toxic effects of some phenolics are mediated through the overproduction of ROS (Badri and others 2008, Weir 2004). Plant POD catalyzes a number of biochemical reactions such as the formation of phenolic monomers for lignin synthesis and the oxidation of phenolic substrates; the latter is often regarded as an antioxidant pathway that protects plant cells/organelle from the destructive influence of ROS to support ascorbate/ascorbate POD system (Ali and others 2006, Kova´cˇik and others 2008) and glutathione metabolic system (Srivastava and Dwivedi 1998). SA induced extracellular secreted PODs, which may contribute to generation of ROS coupled to oxidation of plant hormones and defense-related compounds (Kawano 2003). However, if the plant antioxidant system capacity is up regulated, oxidation of phenolics may proceed to the production of quinones, which can donate electrons to molecular oxygen, forming superoxide anions (Weir and others 2004).

Changes in SOD, POD, GR, GST and CAT enzyme activity during plant growth of *C. roseus* in presence of four different treatments suggested that oxidative stress may be an influent component of environmental stresses on *C. roseus*. Higher enzyme activity was observed in the plants under saline conditions, while just opposite trend was observed in wheat (Meneguzzo and others 1999). The mechanisms of antioxidative enzymes are not yet clear under salinity stress. The role of SA in regulating the salinity stress in maize, acts as potential growth regulator to improve plant growth and nutrient utilization under salt stress (Gunes and others 2007). An overview of the data presented here suggested that during salt stress an enormous amount of ROS are produced which adversely affects the germination and growth parameters of *C. roseus*. ROS also inhibit the activity of antioxidant enzymes which scavenge them resulting in the impairment of growth of *C. roseus*. SA treatment increases the activities of SOD, GR, GST, and POD considering the protective enzymes and thus ameliorates the salt stress in *C. roseus*. From the results of above discussions, we concluded that the role of SA signify in regulating the salt tolerance response in *C. roseus*, and suggested that SA could be used as a potential growth regulator to improve plant growth under salt stress.

POD also plays an important role in the coupling of monomeric indole alkaloids, vindoline and catharanthine into vinblastine (antitumor agent) in leaf pairs and ajmalicine to serpentine conversion in roots of *C. roseus* (Misra and others 2006). Higher POD and SOD activity concomitant with the increased accumulation of alkaloid were found to be in all leaf pairs as well as in roots of *C. roseus* grown under salinity stress and more significantly found in SA treated plants as compared to non-saline control. Hence, there was a close correlation between the POD activity and indole alkaloid accumulation.

In conclusion, exogenously applied SA stimulated growth parameters, photosynthetic pigments, lipid peroxidation, antioxidant capacity, phenolics metabolism, and indole alkaloid accumulation in all leaf pairs and roots of *C. roseus* grown under salinity stress. *C. roseus* plants could be ascribed to the difference in mechanisms underlying oxidation stress injury and subsequent tolerance to salinity with the exogenously applied SA. The results may also suggest that reactions, which produce free radicals, are more pronounced in apical leaf pair among the others leaf pairs. Roots are the first to sense salinity, drought or anoxia and constitute the first line adaptation reactions. In terms of phenolic metabolism, it seems that the SA had a cumulative effect, as indicated by the sharp increase in PAL activity, followed by increases in soluble phenolics and lignin accumulation. These responses may be explained by mechanisms for oxidative stress tolerance induced by SA, which is indicated by the increase of MDA, antioxidant enzymes and alkaloid accumulation. Moreover, the toxic effect generated by 100 mM NaCl was completely overcome with the application of 0.05 mM SA. Further, it is concluded that exogenously SA treatment can ameliorate the negative effect of salinity on the growth of medicinally important *C. roseus* plants and SA could be used as a potential growth regulator to improve plant salinity tolerance. Furthermore, SA ameliorated the stress generated by NaCl through the alleviated antioxidant system.

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