

## Changes in non Enzymatic Antioxidants and Ajmalicine Production in *Catharanthus roseus* with Different Soil Salinity Regimes

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**Abstract:** Here the experiment was carried out with different concentrations of sodium chloride (NaCl) in *alba* and *rosea* varieties of *Catharanthus roseus* (L.) G. Don. to determine the changes that occurring in antioxidant potentials and to evaluate the possibility of increasing the anti-hypertension alkaloid, ajmalicine, present in the roots. The two varieties were evaluated by using four different concentrations of NaCl (25, 50, 100 and 200 mM) treatment in pot culture, which were repeated in four different stages of growth like 30, 45, 60 and 75 days after sowing (DAS). The plants were uprooted randomly on 90 DAS and the non-enzymatic [ascorbic acid (AA), reduced glutathione (GSH),  $\alpha$ -tocopherol ( $\alpha$ -toc)] and ajmalicine content were analysed. High salinity caused an enhancement in total AA contents. GSH content decreased insignificantly only at NaCl concentration of 25 mM and increased at higher concentration treatments. The  $\alpha$ -toc content had no significant change under 25 mM NaCl but increased under high salinity regimes. NaCl treatment significantly increased the content of root alkaloid ajmalicine in both the varieties. The increase in ajmalicine content was resulted from the increased secondary metabolite production in the plant under salinity stress. Therefore it can be concluded that, *C. roseus* is an ideal plant for cultivation in salt affected areas in order to obtain maximum amount of secondary metabolites.

**Key words:** Abiotic stress • Antioxidants • Ajmalicine • *Catharanthus roseus* • Medicinal plant

### INTRODUCTION

Salinity is the major environmental stress limiting plant growth and productivity around the globe. An important consequence of salinity stress in plants is the excessive generation of reactive oxygen species (ROS) such as superoxide anion,  $H_2O_2$  and the hydroxyl radicals particularly in chloroplast and mitochondria [1-3]. Generation of ROS such as superoxide,  $H_2O_2$  and hydroxyl molecules cause rapid cell damage by triggering off a chain reaction. Cellular damage or oxidative injury arising from free radicals or ROS now appears the fundamental mechanism underlying a number of human diseases [2-5]. Antioxidants are radical scavengers, which protect the human body against free radicals that may cause pathological conditions such as anaemia, asthma, arthritis, inflammation, neuro-degeneration, Parkinson's diseases, mongolism, ageing process and perhaps dementias [3-6]. Free radicals can be scavenged through chemoprevention utilizing natural antioxidant compounds present in foods

and medicinal plants. Some medicinal plants have been shown to have both chemopreventive and/or therapeutic effects on human diseases [5-8].

*Catharanthus roseus* (L.) G. Don. (Family: Apocynaceae) is one of the important medicinal plants, due to the presence of indispensable anti-cancer drugs, vincristine and vinblastine. Roots of this plant are the main source of an anti-hypertension alkaloid ajmalicine [9]. It is also a popular ornamental plant. There are commonly two varieties in this plant based on the flower colour viz., pink flowered *rosea* and white flowered *alba*. Efforts are on to develop superior pharmaceutical varieties of *C. roseus* plants by using a number of approaches. In industrial crops such as medicinal plants the content of the economically important metabolite is more important than the yield of the plant part containing the metabolite, as it determines the cost of its extraction. The *Catharanthus* alkaloids have attracted considerable attention of researchers due to their high price and low contents in the plant [10-12]. The cell and tissue culture

and biotechnological aspects of this plant are being extensively investigated to increase the yield of alkaloids [13]. Hence in this context, it is interesting to note that, the content of secondary metabolites in plants is greatly affected by abiotic stresses like salinity, wounding stress and water stress [14].

Though the previous works demonstrated the effects of salinity on agricultural crops [15], it is not so with respect to medicinal plants. To the best of our knowledge, no information on the physiological response interms of ajmalicine metabolism of *C. roseus* to NaCl application is available. The purpose of this study was to provide additional information on the non-enzymatic (AA, GSH,  $\alpha$ -toc) and indole alkaloid (ajmalicine) accumulation in *C. roseus* under different concentrations of NaCl treatments.

## MATERIALS AND METHODS

The seeds of two varieties of *Catharanthus roseus* (L.) G. Don. were collected from Botanical garden, Department of Botany, Annamalai University, TamilNadu. Seeds were sown in plastic pots (300 mm diameter) filled with soil mixture containing red soil, sand and Farm Yard Manure (FYM) at 1:1:1 ratio. Before sowing the seeds, the pots were irrigated with the respective treatment solutions (25, 50, 100 and 200 mM NaCl) and the Electrical Conductivity (EC) was measured and found as 0.10 dSm<sup>-1</sup> (Control), 3.9 dSm<sup>-1</sup> (25 mM NaCl), 7.8 dSm<sup>-1</sup> (50 mM NaCl), 12.2 dSm<sup>-1</sup> (100 mM NaCl) and 13.9 dSm<sup>-1</sup> (mM NaCl). 5-8 seeds were sown per pot and the pots were watered to the field capacity with deionized water up to 30 DAS and every care was taken to avoid leaching. The initial EC level of the soil was maintained by flushing each pot with required volume of corresponding treatment solution at 30, 45, 60 and 75 DAS.

The pot culture experiment was carried out in a completely randomized design (CRBD) with six replicates for each treatment. The position of each pot was randomized at four days intervals to minimize spatial effects in the green house, where the temperature was  $\pm 28^{\circ}\text{C}$  at night and the relative humidity (RH) varied between 60-70%. The seedlings were thinned to one per pot on the 10 DAS. The plants were uprooted carefully on 90 DAS, keeping the root system intact and washed thoroughly with tap water and surface dried. The roots were separated from the plants; shade dried and kept separately according to treatments. The roots of different concentration NaCl treated plants for both *rosea* and *alba* varieties were then finely powdered in an electronic

blender and the powder was kept in separate containers for ajmalicine extraction.

Ascorbic acid content was assayed as described by Omaye *et al.* [16]. The extract was prepared by grinding 1 g of fresh material with 5 ml of 10% trichloro acetic acid (TCA), centrifuged at 3500 rpm for 20 min, reextracted twice and supernatant made upto 10 ml and used for assay. To 0.5 ml of extract, 1 ml of DTC reagent (2,4-dinitrophenyl hydrazine-thiourea-CuSO<sub>4</sub> reagent) was added and incubated at 37 °C for 3 hrs and 0.75 ml of ice-cold 65% H<sub>2</sub>SO<sub>4</sub> was added, allowed to stand at 30°C for 30 min, resulting colour was read at 520 nm in spectrophotometer (U-2001-Hitachi). The AA content was determined using a standard curve prepared with AA.

The reduced glutathione content was assayed as described by Griffith and Meister [17]. 200 mg fresh material was ground with 2 ml of 2% metaphosphoric acid and centrifuged at 17000 rpm for 10 min. Adding 0.6 ml 10% sodium citrate neutralized the supernatant. 1 ml of assay mixture was prepared by adding 100  $\mu\text{l}$  extract, 100  $\mu\text{l}$  distilled water, 100  $\mu\text{l}$  5, 5-dithio-bis-(2-nitrobenzoic acid) and 700  $\mu\text{l}$  NADPH. The mixture was stabilized at 25 °C for 3-4 min. Then 10  $\mu\text{l}$  of glutathione reductase was added, read the absorbance at 412 nm in spectrophotometer.

$\alpha$ -Toc content was assayed as described by Backer *et al.* [18]. 500 mg of fresh tissue was homogenized with 10 ml of a mixture of petroleum ether and ethanol (2:1.6 v/v) and the extract was centrifuged at 10,000 rpm for 20 min and the supernatant was used for estimation of  $\alpha$ -toc. To one ml of extract, 0.2 ml of 2% 2,2-dipyridyl in ethanol was added and mixed thoroughly and kept in dark for 5 min. The resulting red colour was diluted with 4 ml of distilled water and mixed well. The resulting colour in the aqueous layer was measured at 520 nm. The  $\alpha$ -toc content was calculated using a standard graph made with known amount of  $\alpha$ -toc.

Ajmalicine extraction was carried out by following standard extraction method [19]. Identification and quantification of ajmalicine was done by preparative thin layer chromatography using silica gel G (Merck) in CHCl<sub>3</sub> by comparison of R<sub>f</sub> values with authentic ajmalicine standard (Himedia, Mumbai). Ajmalicine was spotted by Dragendorff's reagent. The spot corresponding to the R<sub>f</sub> value of standard ajmalicine was scraped and eluted with 2 ml methanol. The silica gel was removed from sample by centrifugation at 1000 rpm and the final volume of the supernatant was made to 5.0 ml. The concentration of ajmalicine separated from the crude extract was determined at 254 nm using a spectrophotometer. A

standard graph was prepared using authentic ajmalicine standard at various dilutions and the amount of ajmalicine in each sample was determined. UV spectrum of standard and ajmalicine sample was also recorded (Fig. 3). Three experiments were performed independently for each sample and ajmalicine content was expressed as mean of three replicates in  $\text{mg g}^{-1}$  dry weight (DW).

Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). The values are mean  $\pm$  standard deviation (SD) for six samples in each group.  $P$  values  $\leq 0.05$  were considered as significant.

## RESULTS

There was a significant alteration in the non-enzymatic antioxidant potentials under salinity stress when compared to control plants. AA showed an increase in all the parts of the plant, under salinity conditions when compared to untreated plants. A significant increase ( $P \leq 0.05$ ) was recorded under 50 mM NaCl concentration in both *rosea* and *alba* varieties. The higher concentrations have the similar effect as 50 mM. Among the samples, the root samples recorded the highest AA content (Fig. 1a).

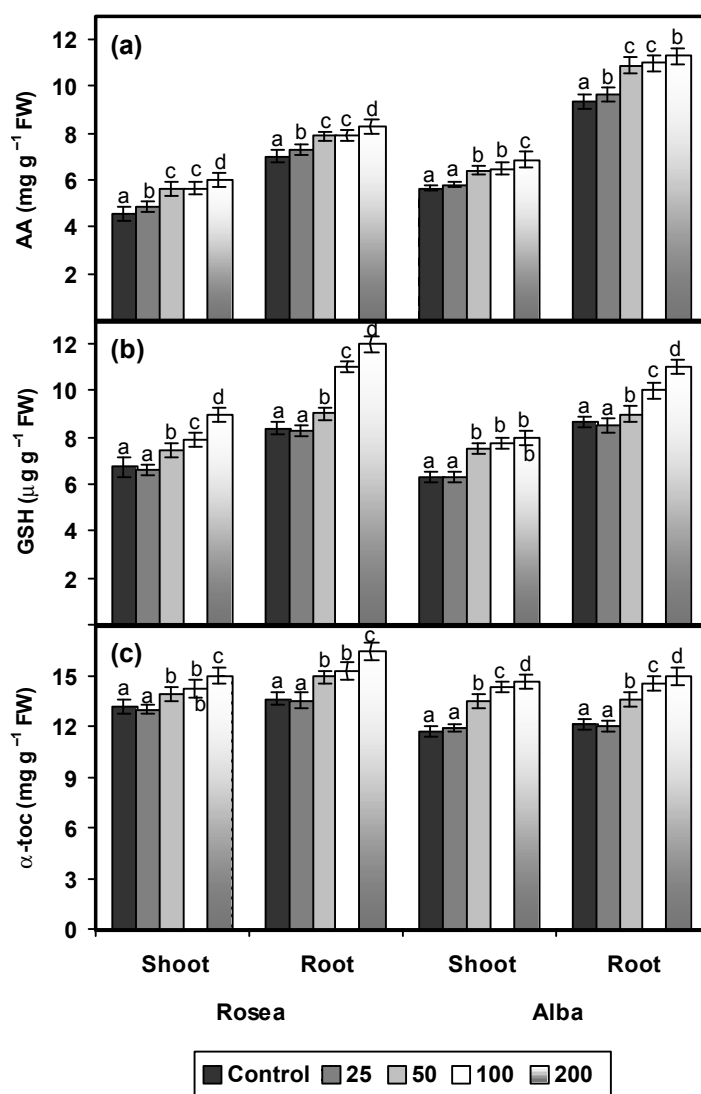


Fig. 1: Effect of different concentrations of NaCl (25, 50, 100 and 200 mM), on (a) ascorbic acid (AA), (b) reduced glutathione (GSH) and (c)  $\alpha$ -tocopherol ( $\alpha$ -toc) contents of *rosea* and *alba* varieties of *Catharanthus roseus* on 90 DAS. Values are given as mean  $\pm$  SD of six experiments in each group. Bar values are not sharing a common superscript (a,b,c,d,e) differ significantly at  $p \leq 0.05$  (DMRT)

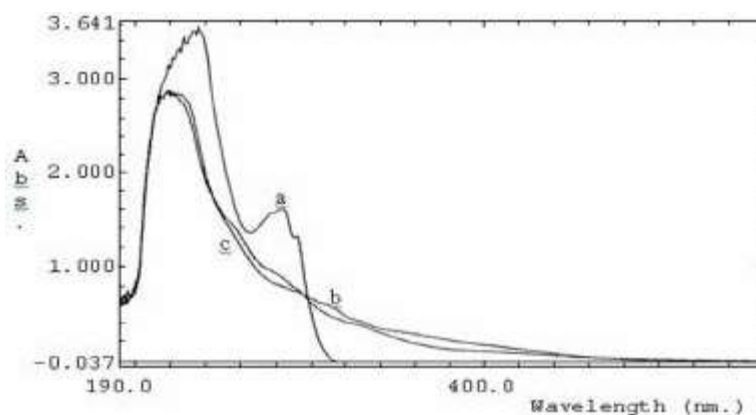


Fig. 2: UV spectrum of ajmalicine standard and ajmalicine samples from *rosea* and *alba* varieties of *Catharanthus roseus*.

- a. Ajmalicine standard
- b. Ajmalicine sample from roots of *rosea* variety
- c. Ajmalicine sample from roots of *alba* variety

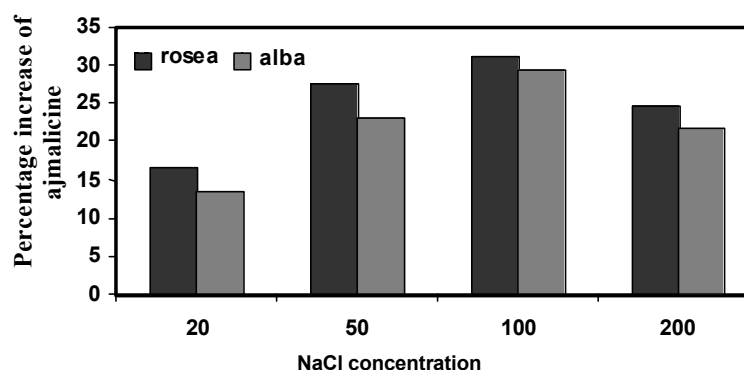


Fig. 3: Effect of different concentrations of NaCl (25, 50, 100 and 200 mM), on ajmalicine content (% increase from control) of *rosea* and *alba* varieties of *Catharanthus roseus* on 90 DAS

The variations found in the GSH content under NaCl treatment in *rosea* and *alba* varieties of *C. roseus* are shown in Fig. 1b. On 90 DAP, root sample from NaCl treated *alba* variety showed a highest content of GSH.

The  $\alpha$ -toc content in the NaCl treated plants was higher than that of control on 90 DAP. The highest  $\alpha$ -toc content was recorded on 90 DAP root sampling of NaCl treated *rosea* variety (Fig. 1c).

The content of ajmalicine in the roots of both *rosea* and *alba* varieties of *C. roseus* increased with increasing concentrations of NaCl and it was found maximum in 100 mM concentration (Fig. 2). The two varieties, *rosea* and *alba* responded differently to NaCl treatment. In *rosea* the increase in ajmalicine content was 16.44, 27.64, 31.26, 24.48 % and in *alba* it was 13.40, 23.11, 29.21 and 21.67% over control in 25, 50, 100 and 200 mM

concentrations of NaCl treatment respectively. The percentage of increase was higher in *rosea* variety when compared to *alba* variety.

## DISCUSSION

The non-enzymatic antioxidative mechanisms like GSH and AA responded differently to NaCl treatment. In this investigation, we observed a significant increase in AA content in all the parts of *Catharanthus* plants under NaCl treatment. The increased AA content is correlated with the stress protecting mechanism of the plant under salinity conditions [20-22]. AA is an important antioxidant, which reacts not only with  $H_2O_2$  but also with  $O_2^-$ , OH and lipid hydroperoxidases [21,22]. AA can function as the "terminal antioxidant" because the redox potential of AA/monodehydro ascorbate (MDA) pair

(+ 280 nm) is lower than that of most of the bioradicals. However, very little is known about the regulation of AA biosynthesis in higher plants. It is conceivable that adaptations of intracellular AA to oxidative stress might clearly depend on the balance between the rates and capacity of AA biosynthesis and turnover related to antioxidant demand [4-8]. A high level of endogenous ascorbate is essential effectively to maintain the antioxidant system that protects plants from oxidative damage due to the biotic and abiotic stress.

We noticed a reduction in GSH content in different parts of *Catharanthus* only at NaCl concentration of 25 mM and increased at higher concentration treatments. The main functions of GSH in the protection against oxidative stress are its involvement in the ascorbate-glutathione cycle and in the regulation of protein thiol-disulphide redox status and also play a protective role in salinity tolerance by the maintenance of the redox status [23-25]. The decrease in GSH found in this study might be due to the predominant oxidation under salinity conditions and it is necessary for increasing the stress protectant antioxidant enzyme glutathione reductase.

The  $\alpha$ -toc content in the NaCl treated plants was higher than that of control on 90 DAP.  $\alpha$ -toc (Vitamin E) is lipophilic antioxidant synthesized by all plants.  $\alpha$ -toc are major lipid soluble antioxidant present in the polyunsaturated fatty acid (PUFA) enriched membranes of chloroplasts and are proposed to be an essential component of plastid antioxidant network.  $\alpha$ -toc interacts with the polyunsaturated acyl groups of lipids, stabilize membranes and scavenge species and lipid soluble by products of oxidative stress [9-14].

NaCl treatment increased the ajmalicine content in both varieties when compared to control. Therefore the variations among the varieties in production of ajmalicine in response to NaCl treatment may partly be due to the genotypic differences and salinity tolerance capacities, which however were not determined in the present study. However, *rosea* variety gives higher yield of foliage and roots and total alkaloids [8-11]. Abiotic stresses can alter the secondary metabolite content in plants [12-14]. The leaf alkaloid content increased by manipulating the frequency of irrigation and the root and leaf alkaloid contents in *Catharanthus* plants were found increased by nitrogen fertilization. The alkaloid content in *C. roseus* plants have been found influenced by individual factors such as stages of plant growth.

## CONCLUSION

Soil salinity is one among the several environmental stresses causing drastic changes in the growth, physiology and metabolism of plants, which can leads to the increased accumulation of secondary metabolites like alkaloids. Here, it can be concluded that, at high concentrations, NaCl leads to oxidative stress and inturn cause a significant increase in antioxidative responses. So it is clear that, the NaCl salinity has its own role in the increasing of root alkaloid ajmalicine in *rosea* and *alba* varieties of *C. roseus* thus can be recommended for commercial application to maximize the production of valuable anti-hypertension drug, ajmalicine from this medicinal plant.

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