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Studies on germination, seedling vigour, lipid peroxidation and proline metabolism in *Catharanthus roseus* seedlings under salt stress

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Abstract

In the present investigation, 'rosea' and 'alba' varieties of *Catharanthus roseus* (L.) G. Don. seeds were grown with different concentrations (15, 30, 45 and 60 mM) of sodium chloride (NaCl), in order to study the effects of salinity on germination behaviour, seedling vigour (root and shoot length), lipid peroxidation (LPO) and proline metabolism. It was found that germination was delayed at lower salinity levels and inhibited at higher salinity regimes. NaCl treatment caused a serious decrease in the early seedling growth by means of reduced seedling vigour at higher salinity levels. The LPO was estimated as thiobarbituric acid reactive substances (TBARS) and found increased under salt stress. Glycine betaine (GB) and proline (PRO) contents significantly accumulated in both the varieties of seedlings under salt stress. Under NaCl stress, the activity of proline oxidase (PROX) decreased and the γ-glutamyl kinase (γ-GK) activity increased.

Keywords: *Catharanthus roseus*; Glycine betaine; Proline; Proline oxidase; Salinity; γ-Glutamyl kinase

1. Introduction

Salinity is one of the major abiotic stresses which adversely affect the crop growth and yield. High concentrations of salt resulting from natural processes or disarrangement in irrigated agriculture result in inhibition of plant growth and yield (Demiral and Turkan, 2006). Salinity also induces water deficit even in well watered soils by decreasing the osmotic potential of soil solutes, thus making it difficult for roots to extract water from their surrounding media (Sairam and Srivastava, 2002). Germination of seeds, one of the most critical phases of plant life, is greatly influenced by salinity (Misra and Dwivedi, 2004). Salinity either completely inhibits germination at higher levels or induces a state of dormancy at low levels (Iqbal et al., 2006). Pahlavani et al. (2006) proved that genetic information regarding seed germination and related traits could help improve seedling emergence in saline soils through breeding programs.

The reduction in plant growth under salinity is a consequence of several physiological responses including modification of water status, photosynthetic efficiency, carbon allocation and utilization (Nabil and Coudret, 1995). Under saline environments, the plant lipid metabolism is interrupted as a result of oxidative damage to membrane lipids by active oxygen species and lipid peroxidation (LPO) (Hernandez and Almansa, 2002; Misra and Gupta, 2006). LPO can also be initiated enzymatically by lipoxygenases (Axelord et al., 1981) and this enzyme incorporates molecular oxygen in to linoleic and linolenic acids, to form lipid hydroperoxides (Elkahoui et al., 2005). Compatible solutes accumulation in the cytoplasm is considered as a mechanism to contribute salt tolerance (Hare et al., 1998). Compatible solutes such as proline (PRO) and glycine betaine (GB) are thought to function as osmoprotectants for proteins (Bohnert and Jenson, 1996). Accumulation of PRO (Misra and Gupta, 2005) and GB (Khan et al., 1998) provides an environment compatible with macromolecular structure and function and helps to adapt the salinity injury (Girija et al., 2002). Protein hydrolysis under salt stressed plants is associated with increased PRO content (Irigoyen et al., 1992). Proline oxidase

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(PROX) and γ-glutamyl kinase (γ-GK) play an important role in controlling the level of PRO, PROX catalyzes the conversion of PRO to glutamate and γ-GK plays an important role in the synthesis of PRO (Girija et al., 2002). Though the previous works demonstrated the effects of salinity on agricultural crops (Muthukumarasamy and Panneerselvam, 1997; Panneerselvam et al., 1997, 1998; Muthukumarasamy et al., 2000), it is not so with respect to medicinal plants.

In order to meet the ever increasing demand of medicinal plants, for the indigenous systems of medicine as well as for the pharmaceutical industry, some medicinal plants need to be cultivated commercially, but the soil salinity and other forms of pollutions pose serious threats to plant production (Qureshi et al., 2005). So it seems valuable, to test the important medicinal plants for their salt tolerance capacity. *Catharanthus roseus* (L.) G. Don. (Family: Apocynaceae) is one of the highly exploited and studied medicinal plants. This plant contains alkaloids which are valuable source of antitumour agents like vinblastine and vincristine used in chemotherapy of leukemia and in the treatment of Hodgkin's disease, and also a popular ornamental plant (Filippini et al., 2003). *C. roseus* is classed as a glycophyte. Two distinct varieties of this plant, the pink flowered 'rosea' and white flowered 'alba' were taken for the present study. Many investigations have been already carried out in this plant on its medicinal importance (Jaleel et al., 2006), but the salinity effects on this medicinal plant attracted a little attention. This investigation was aimed to find out the extent of changes in germination behaviour, seedling vigour, LPO, GB and PRO contents, PRO synthesizing (γ-GK) and PRO degrading (PROX) enzyme activities in 'rosea' and 'alba' varieties of *C. roseus* under NaCl treatment.

2. Materials and methods

2.1. NaCl treatments and germination behaviour

The seeds of both the varieties of *C. roseus* were collected from the Department of Horticulture, Annamalai University, Tamil Nadu, India. Germination trials were conducted in 9 cm sterile petri dishes lined with Whatman No.1 filter papers and moistened with distilled water to ensure adequate moisture for the seeds. Seed treatments included 15, 30, 45 and 60 mM NaCl concentrations. In an attempt to remove germination inhibitors, the seeds were leached with distilled water for 5 days before the experiment. Seeds were then surface sterilized in aqueous solution of 0.1% $HgCl_2$ for 60 s to prevent fungal attack and rinsed in several changes of sterile water. The seeds were sowed in petri dishes and placed in seed germinator at 34±1 °C. The seeds were examined daily and considered germinated when the radicle was visible. The germination percentage was calculated from 8 days after sowing (DAS) to 12 DAS. The morphological parameters like shoot length and root length were measured on 20 DAS.

2.2. Lipid peroxidation (TBARS content)

LPO was estimated as TBARS (Heath and Packer, 1968). Fresh sample (0.5 g) was homogenized in 10 ml of 0.1% trichloroacetic acid (TCA), and the homogenate was centrifuged at 15000 rpm for 15 min. To 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 °C for 30 min in the laboratory electric oven and then cooled in an ice bath. After centrifugation at 10,000 rpm for 10 min the absorbance of the supernatant was recorded at 532 nm in spectrophotometer (U-2001-Hitachi). The TBARS content was calculated according to its extinction coefficient of 155 $mM^{-1}cm^{-1}$ and expressed in units (U). One 'U' is defined as μmol of MDA formed min^{-1} mg^{-1} protein.

2.3. Glycine betaine content

The amount of GB was estimated according to the method of Grieve and Grattan (1983). The plant tissue was finely ground, mechanically shaken with 20 ml deionised water for 24 h at 25 °C. The samples were then filtered and filtrates were diluted to 1:1 with 2 N H_2SO_4. Aliquots were kept in centrifuge tubes and cooled in ice water for 1 h. Cold KI-I_2 reagent was added and the reactants were gently stirred with a vortex mixture. The tubes were stored at 4 °C for 16 h and then centrifuged at 10,000 rpm for 15 min at 0 °C. The supernatant was carefully aspirated with a fine glass tube. The periodide crystals were dissolved in 9 ml of 1,2-dichloroethane. After 2 h, the absorbance was measured at 365 nm using GB as standard and expressed in mg g^{-1} DW.

2.4. Proline content

The PRO content was estimated by the method of Bates et al. (1973). The plant material was homogenized in 3% aqueous sulfosalicylic acid and the homogenate was centrifuged at 10,000 rpm. Supernatant was used for the estimation of PRO content. The reaction mixture consisted of 2 ml acid ninhydrin and 2 ml of glacial acetic acid, which was boiled at 100 °C for 1 h. After termination of reaction in ice bath, the reaction mixture was extracted with 4 ml of toluene and absorbance was read at 520 nm.

2.5. γ-Glutamyl kinase [ATP: L-glutamate 5-phosphotransferases (EC 2.7.2.11)] activity

γ-GK activity was assayed by the method of Hayzer and Leisinger (1980). Plant samples (1 g) were extracted with 50 mM Tris–HCl buffer and centrifuged at 40,000 *g* for 30 min at 4 °C. 0.1 ml reaction buffer was prepared by adding 0.1 ml 10 × ATP and 1.8 ml of extract and incubated at 37 °C for 30 min, 2 ml of stop buffer was added. γ-GK activity was measured at 535 nm and expressed in units (U mg^{-1} protein). One unit (U) of enzyme activity is defined as μg of γ-glutamylhydroxamate formed min^{-1} mg^{-1} protein.

2.6. Proline oxidase [L. proline: O_2 oxidoreductase (EC 1.4.3.1)] activity

PROX activity was determined according to the method outlined by Huang and Cavelieri (1979). Plant samples (1 g)

were extracted with 5 ml of Tris–HCl buffer (pH 8.5) grinding medium and centrifuged at 10,000 *g* for 10 min at 4 °C. The supernatant was again centrifuged at 25,000 *g* at 20 min at 4 °C. 3 ml assay mixture was prepared by taking 0.1 ml of extract, 1.2 ml of 50 mM Tris–HCl buffer (pH 8.5), 1.2 ml of 5 mM

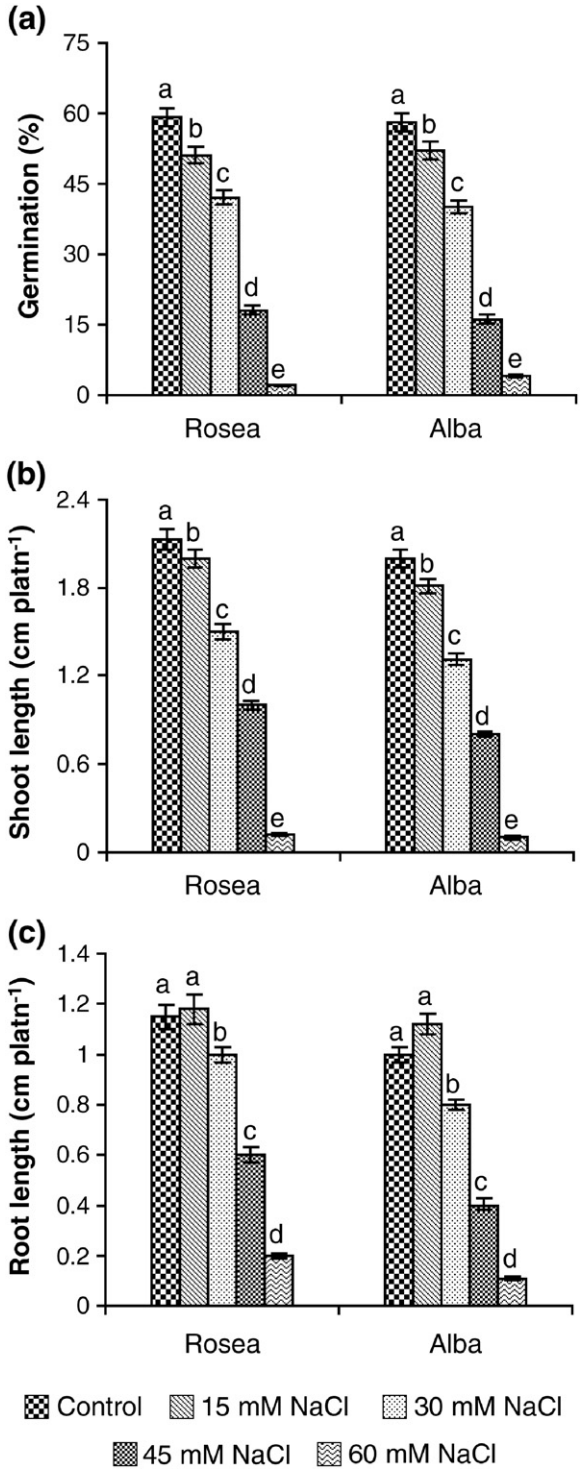

Fig. 1. Effect of increasing concentrations of NaCl on germination percentage (a), shoot length (b) and root length (c) of *C. roseus* seedlings. Values are given as mean±SD of six experiments in each group. Bar values are not sharing a common superscript (a,b,c,d,e) differ significantly at $P \leq 0.05$ (DMRT).

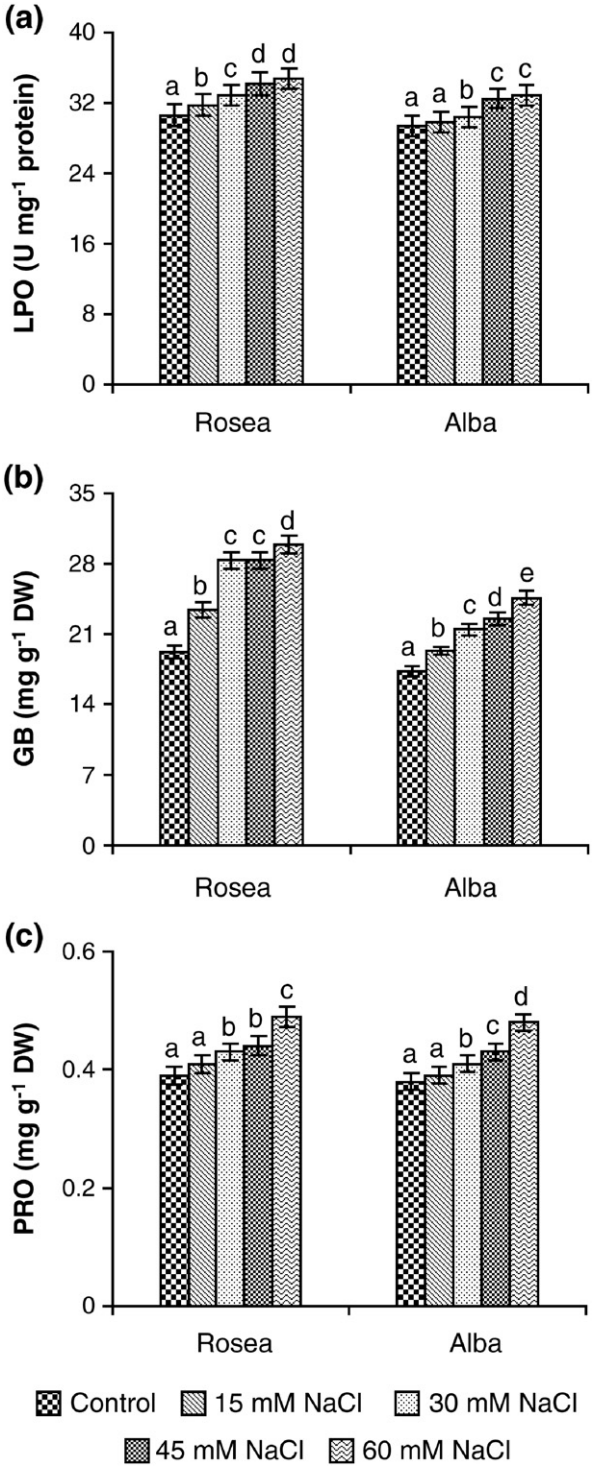

Fig. 2. Effect of increasing concentrations of NaCl on LPO (a), GB (b) and PRO (c) contents of *C. roseus* seedlings. Values are given as mean±SD of six experiments in each group. Bar values are not sharing a common superscript (a, b,c,d,e) differ significantly at $P \leq 0.05$ (DMRT).

$MgCl_2$, 0.1 ml of 0.5 mM NADP, 0.1 ml of 1 mM KCN, 0.1 ml of 1 mM phenazine methosulphate (PMS), 0.1 ml of 0.06 mM 2,6-dichlorophenol indophenol (DCPIP) and 0.1 ml distilled water instead of PRO. The reaction was monitored at 600 nm at 25 °C using PRO to initiate reaction, the OD value increased

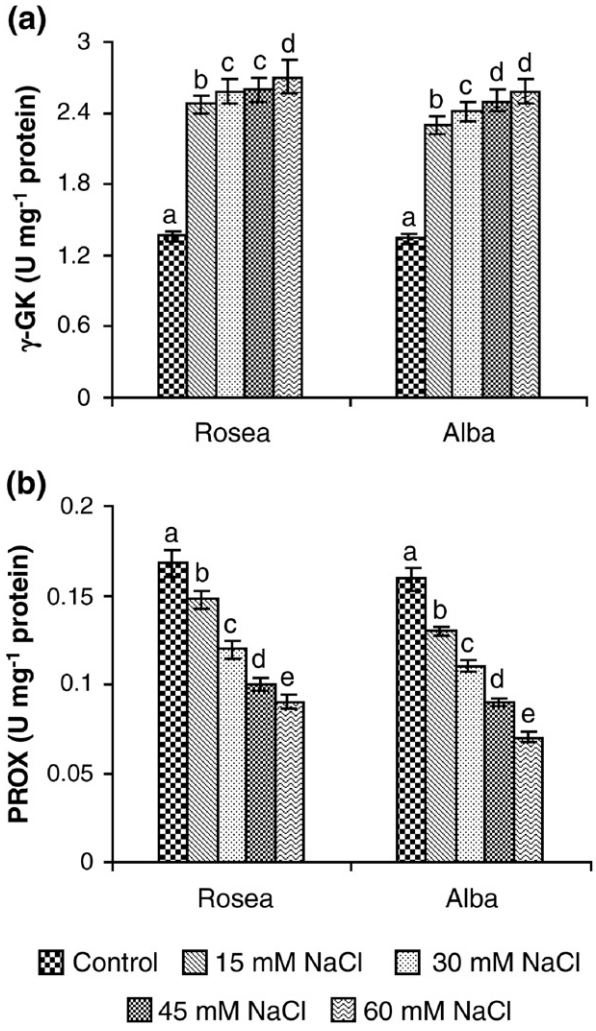

Fig. 3. Effect of increasing concentrations of NaCl on γ-GK (a) and PROX (b) activities of *C. roseus* seedlings. Values are given as mean±SD of six experiments in each group. Bar values are not sharing a common superscript (a, b,c,d,e) differ significantly at $P \leq 0.05$ (DMRT).

was noted at 0, 1, 2, 3, 4 and 5 min. PROX activity was expressed in U mg^{-1} protein (one U = mM DCPIP reduced min^{-1} mg^{-1} protein).

2.7. Statistical analysis

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

3. Results and discussion

3.1. Germination percentage

The effect of increasing levels of NaCl salinity on the germination percentage of *C. roseus* is presented in Fig. 1a. A trend of decreasing germination percentage with increasing NaCl concentrations was found. At 60 mM NaCl, the germination was highly inhibited. The inhibition was high in the case of rosea variety when compared to alba. Inhibition of germination due to salinity has been reported earlier in greegram cultivars (Misra and Dwivedi, 2004). The decreasing germination due to increasing salinity can be correlated to the nature of salinity to reduce imbibition of water due to lowered osmotic potentials of the medium and causes changes in metabolic activity (Yupsanis et al., 1994). Moreover, salinity perturbs plant hormone balance (Khan and Rizvi, 1994) and reduces the utilization of seed reserves (Ahmad and Bano, 1992).

3.2. Seedling vigour

The seedling vigour was estimated by means of shoot and root length of seedlings. The shoot and root growth was inhibited by salinity stress (Fig. 1b and c). The extent of decrease under higher salinity levels is more or less equal in both rosea and alba varieties. 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 (Kumar et al., 2005).

3.3. Lipid peroxidation (TBARS content)

Oxidative damage to tissue lipid was estimated by the content of total TBARS. The TBARS content increased with the increasing concentrations of NaCl (Fig. 2a). The extent of increase was more significant in rosea when compared to alba. In NaCl-treated plants, oxidative stress might be induced due to the decreased stomatal conductance in response to the osmotic imbalance and reduced leaf water potential. LPO has been associated with damages provoked by a variety of environmental stresses (Hernandez et al., 2003). Poly unsaturated fatty acids (PUFA) are the main membrane lipid components susceptible to peroxidation and degradation (Elkahoui et al., 2005). The increase in LPO can be correlated with the accumulation of ions and active oxygen species (AOS) production under salt stress (Hernandez et al., 2001; Misra and Gupta, 2006). The level of LPO, indicates the extent of salt tolerance as reported by Bor et al. (2003) in sugarbeet and wild beet under NaCl treatment.

3.4. Glycine betaine content

One of the most important mechanisms exerted by higher plants under salt-stress conditions is the accumulation of compatible solutes such as GB. In the present study, the amount of GB content increased with the increasing concentration of NaCl in *C. roseus* plants (Fig. 2b). The GB content was less in alba variety when compared to rosea. GB accumulation resulted from the NaCl-induced oxidative stress, and is helpful in the stimulation of salt tolerance mechanisms (Girija et al., 2002; Demiral and Turkan, 2006).

3.5. Proline content

Another compatible solute which accumulates under salt stress in plants is PRO. In the present study, an increase in PRO accumulation in both the varieties of *C. roseus* seedlings under salinity (Fig. 2c) with a concomitant increase in γ-GK (PRO synthesizing enzyme) and a decrease in PROX (PRO degrading enzyme) activities (Fig. 3a and b). The content was more or less equal in both rosea and alba varieties. Although the precise role of proline accumulation is still debated, PRO is often considered as a compatible solute involved in osmotic adjustment (Azooz et al., 2004). The accumulation of PRO may be through an increase in its synthesis constantly with inhibition of its catabolism (Yoshiba et al., 1997) and may be a mechanism for stress tolerance. However, its role in imparting stress resistance under saline conditions is controversial. Anyway, understanding the biosynthesis, degradation, transport and role of PRO during stress and the signalling events that regulate stress-induced accumulation is vital in developing plants for stress tolerance (Kavikishore et al., 2005).

3.6. γ-Glutamyl kinase activity

The PRO metabolising enzyme, γ-GK increased under the NaCl salinity in both the varieties of *C. roseus* seedlings (Fig. 3a). This enzyme plays an important role in the synthesis of PRO. The γ-GK activity can be inversely correlated with proline oxidase activity and protein content in salt treated plants (Girija et al., 2002). PRO accumulation in NaCl stressed seedlings can be attributed in part to the increased level of γ-GK activity (Sakamoto et al., 1998).

3.7. Proline oxidase activity

PROX activity decreased under NaCl stress in *C. roseus* seedlings when compared to control (Fig. 3b). The activity was more or less equal in both rosea and alba varieties. This enzyme converts free PRO into glutamate. Reduction in PROX activity and simultaneous increase in PRO level were reported in low temperature stressed wheat (Charest and Phan, 1990). PRO may act as a non-toxic osmotic solute preferentially located in the cytoplasm or as an enzyme protectant, stabilizing the structure of macromolecules and organelles. Accumulated proline may supply energy to increase salinity tolerance (Misra and Gupta, 2005). PRO as an osmoprotectant compound, plays a major role in osomoregulation and osmotolerance (Demir, 2000). However its definite role in exerting salinity resistance continues to be a debate (Demiral and Turkan, 2006).

From the results of this investigation, it can be concluded that increasing NaCl concentration inhibits germination and seedling vigour both in rosea and alba varieties of *C. roseus*. The LPO in terms of TBARS content showed an increasing trend under salt stress. The increased TBARS content showed the membrane peroxidation and degradation under salinity. The compatible solutes like GB accumulated under salt stress, with a concomitant increase in PRO content. The present investigation indicates that the responses occurring in the PRO metabolism enable both rosea and alba varieties of *C. roseus* seedlings to withstand the saline conditions to a certain extent, eventhough both varieties are sensitive to salinity. These facts should be taken into consideration in the economic cultivation of this valuable medicinal plant. However, the present study addressed only the range of salinity that was relatively mild in soil. The impact of highly adverse conditions resulting from increased soil salinity on this medicinal plant requires additional investigation, which is underway in our lab.

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