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In Vitro Screening of *Catharanthus Roseus* L. Cultivars for Salt Tolerance Using Physiological Parameters

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***Abstract*— *In vitro* shoots of the *Catharanthus roseus* L. cvs. Rosea, (pink flowered) and Alba, (white flowered) were subjected to salinity stresses using different NaCl levels (0 to 100 mM) in a shoot proliferating and callus induction Murashige and Skoog (MS) basal medium. The cultivar Rosea was the more tolerant cultivar. As the level of NaCl increased, there were corresponding linear increases in the levels of total phenol, proline and sugar, while the leaf chlorophyll content showed a marked decline. The foliar mineral contents showed distinct patterns between the salinity tolerant and susceptible cultivars. The level of Na^+ increased in the foliar tissue with increases in NaCl, while K^+ showed a slow decrease. The trends of lipid per oxidation and antioxidative enzymes activity are completely disparate. The foliar malandialdehyde (MDA) content was lowered (2-2.5 fold) and antioxidative enzymes activity (superoxide dismutase, catalase and peroxidase) were significantly higher in the tolerant cultivar than that of susceptible cultivar. Leaves of rosea cultivar incubated in callus induction MSB medium having 2,4 D (4.06 µM) and Kn (2.3 µM) responded favorably and produce good friable, high biomass callus. The results of this study suggest that *C. roseus* (L.) cv. Rosea may be considered as stress tolerant, candidate cultivar for future breeding and can be cultivated at commercial level to meet the ever increasing demand of medicine, as well as the pharmaceutical industries of northern India.**

***Index Terms*— Antioxidative enzymes, callus induction, Lipid per oxidation, Phenol, Salt stress.**

I. Introduction

Environmental factors influence the characteristics, composition, growth and development of individual plants and plant communities. When any of these environmental factors exceed the optimum tolerance of a plant, the result is stress to the plant, which in turn influences its developmental, structural, physiological and biochemical processes. Every year, more and more land becomes non productive owing to salt accumulation. Increase in salinization of arable land is expected to have disturbing global effects, resulting in 30% land loss within next 25 years and up to 50% by the middle of 21st century [1]. Soil salinity is one of the major environmental abiotic stresses especially in arid and semi-arid regions and can severely limit plant growth and yield. Salt stress can lead to stomatal closure, which reduces CO_2 availability in the leaves and inhibits carbon fixation, exposing chloroplasts to excessive excitation energy, which in turn could increase the generation of reactive oxygen species (ROS) and induce oxidative stress [2], [3]. ROS has potential to interact with many cellular components, causing significant damage to membranes and other cellular structures. However, an elaborate and highly redundant plant ROS network composed of antioxidative enzymes and antioxidants are responsible for maintaining the levels of ROS under tight control. Cell and tissue culture may serve as a useful tool for the assessment of salt tolerance competence in plants since it allows for relatively fast responses, short generation time and controlled environment. Among the several approaches to solving the problem of saline soils, the biological approach aims to identify and grow salt tolerant plants under these conditions. It is essential to test important medicinal plants for their salinity tolerance as research efforts aim to derive economic benefits under saline soil conditions [4]. *Catharanthus roseus* (L.) is an important medicinal plant of the family Apocynaceae that is used to treat many fatal diseases. In addition, *C. roseus* has a good antioxidant potential. There are two common cultivars of *C. roseus* named on the basis of the observed flower colour, the pink flowered 'Rosea' and the white flowered 'Alba' [5]. *C. roseus* is extensively commercially cultivated in northern India to meet the ever increasing demand for this medicinal plant in the indigenous systems of medicine, as well as for the pharmaceutical industry. However, the cultivation of this plant is severely hampered by many factors, of which soil salinity is a dominant concern. Wide genetic variability exists for salt tolerance in the two cultivars of *C. roseus* [1], [5] and therefore these two cultivars can be screened to future identify tolerant types that can be used for commercial cultivation. The purpose of this study was to screen and evaluate a salinity tolerant cultivar of *C. roseus*. We also compared the physiological responses, growth abilities and callus induction of the two cultivars under different concentrations of NaCl.

II. Materials and Methods

Catharanthus roseus (L.) cvs. Rosea and Alba ranging in age from 7 to 8 months were taken from the green house of the Horticulture and Floriculture Division, Indian Agriculture Research Institute, India. Lateral shoots that were between 10 and 15 cm long were cut off and washed with running tap water for about 1 hour. The shoot pieces obtained in this manner were then disinfected in a 1% sodium hypochlorite solution for 25 minutes with constant shaking. The disinfected material was placed in distilled water, sterilized in an autoclave and kept there for 10 minutes. All

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treatments were carried out in sterile inoculating boxes under aseptic conditions. The shoots were removed from the sterile water and cut in a sterilized Petri dish with a scalpel into 2-4 leaf pieces, so that the shoot below the lowest leaf was about 1cm long. These cuttings were established in 200 ml glass flasks containing 100 ml of modified [6] medium supplemented with 2 µg glycine, 100 µg myoinositiol, 0.5 µg nicotinic acid, 0.5 g pyridoxine hydrochloride, 0.1 µg thiamine hydrochloride, 1 mg/l kinetin (Kn), 30 g/l sucrose and 10 g/l agar-agar (Qualigens, Mumbai). The pH of the medium was adjusted to 5.8 before autoclaving for 15 min. at 1.06 kg cm^{-2}. The cultures were kept at 25°C under alternating light and dark conditions, with illumination periods using an artificial light at an intensity of 10,000 lux, and dark periods alternating in a sequence of 16 hours and 8 hours. The top end cuttings grew further but did not ramify. After one week, buds appeared in the axis of the cuttings, which sprouted to 10-12 leaf stage shoots (about 7 cm in length) within 20-25 days. These lateral shoots were separated from the cuttings under sterile conditions and cut into 2-4 leaf pieces. The cuttings prepared in this manner were sub cultured on the same medium to obtain sufficient numbers of explants for *in vitro* screening. For screening, the 2-4 leaf piece micro cuttings were aseptically excised and cultured onto NaCl containing a shoot proliferating medium. This salt stress media contained half the amounts of glycine, myo inositol, nicotinic acid, pyridoxine hydrochloride and thiamine hydrochloride that were used in the MS medium. Analytical reagent sodium chloride (Sigma, St. Louis, USA) was added to the above mentioned culture medium in concentrations ranging from 15
mM to 100 mM. The pH of the medium was adjusted to 5.8 before autoclaving, and the medium was then distributed equally in 100 ml conical flasks. The cultures were incubated at a temperature of 25 ± 2°C under a 16/8 h photo-period (65 µmol $m^{-2}s^{-1}$). The non stress control medium contained no salt. The surviving cultures were further sub cultured with similar NaCl levels. The explants were maintained under the same conditions for 55 days, after this time the foliar tissue mineral contents were estimated. The Na and K contents were measured using flame photometer (Systronics 121, Ahmadabad, India). Total chlorophyll (a+b) was estimated according to [7]. The total phenols in the leaf samples were determined using the method of [8]. The phenolic concentration was estimated by comparing the sample with a calibration curve of gallic acid (prepared with 0-50 µg). The total sugar and the proline contents of the shoot samples were measured according to the methods suggested by [9] and [10], respectively. The activity of antioxidative enzyme (superoxide dismutase, catalase and peroxidase) in the fresh tissue was determined using the method of [11]. The leaf samples of each treatment were homogenized in ice cold 0.1M phosphate buffer (pH 7.5) containing 0.5 mM EDTA. The supernatant was used for the enzymatic assay. The enzymes activity of superoxide dismutase (EC 1.15.1.1), catalase (EC 1.11.1.6) and peroxidase (EC 1.11.1.7) were determined using the methods suggested by [12, 13, 14] respectively. Superoxide dismutase (SOD) activity was estimated by recording the decrease in absorption of superoxide-nitroblue tetrazolium complex by the enzyme at 560nm. The catalase (CAT) and peroxidase (POD) were estimated as consequences of H_2O_2 consumption at 240 nm and purpurogallin formation at 420 nm respectively. Enzyme activity was expressed in U (U= 1mM of H_2O_2 reduction min^{-1} mg^{-1} protein). Enzyme protein was determined [15] for all three enzymes to express specific enzyme activity. The malandialdehyde (MDA) content in the leaves samples was measured by the colorimetric method [16]. Consecutively to study the effect of different concentrations of NaCl (0 to 100 mM) on callus induction and callus growth disinfected leaves of 7-8 months old *C. roseus* cvs. Rosea and Alba were lacerate into small pieces (7-9 mm) and aseptically transferred onto MS basal medium [6] supplemented with combination of growth hormones (Table I). Cultures were kept on cultured racks at 26 ± 2°C, 16 h photoperiod with white fluorescent light for four weeks. The percent induction of callus was calculated using the following formula: Frequency = (Number of explants showing response/ Total number of explants) x100. The tissue dry weight was determined after the samples were dried in a hot air oven at 65°C. Experiment was conducted with three replications (25-30 units per treatment). The significance of differences between means (for stressed and control) for different parameters was measured using Student's t-Test, and difference were considered significant if $P<0.05$.

TABLE I: COMBINATIONS OF GROWTH HORMONES FOR INITIATION OF CALLUS IN TWO CULTIVARS OF *CATHARANTHUS ROSEUS* (L.)

S. No.	Medium	Growth Hormone	Successful callusing	
			White Flower 'alba'	Pink Flower 'rosea'
1.	MSA	2,4 D (4.09 µm)+ BAP (11.53 µm)	+++	++++
2.	MSB	2,4 D (4.09 µm) + Kn (2.30 µm)	++++	+++++
3.	MSC	2,4 D (8.13 µm) + Kn (2.30 µm)	++	+++
4.	MSD	2,4 D (8.13 µm) + BAP (11.53 µm)	+	++

[2, 4 D (2, 4-Dichlorophenoxy acetic acid); BAP (6-Benzyl amino purine); Kn (Kinetin)]
(+++++ = 85%, ++++ = 70%, +++ = 55%, ++ = 40%, + = 25%)

TABLE II: SALT TOLERANCE IN TWO CULTIVARS OF *CATHARANTHUS ROSEUS* (L.)

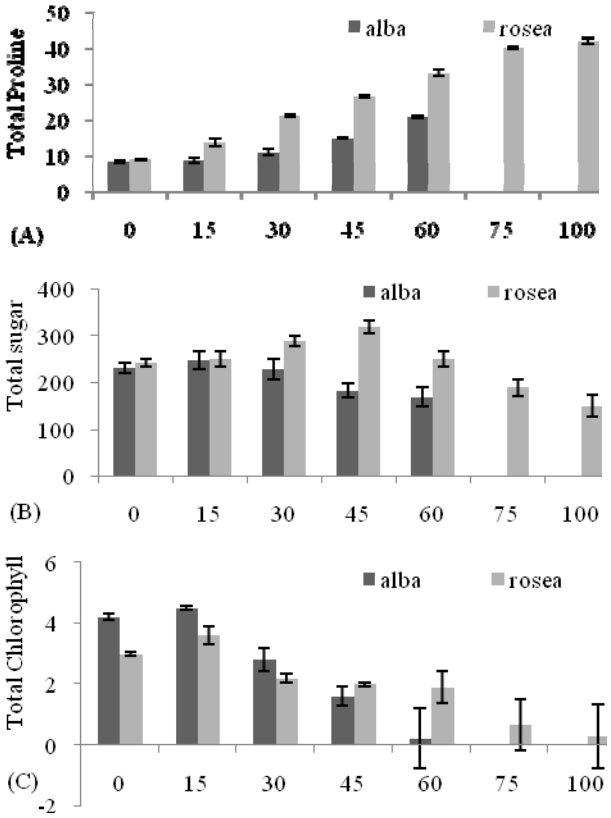
Cultivars		Explant Survival						
		NaCl (mM)						
		0	15	30	45	60	75	100
White Flower 'Alba'	mean	97.8	80.0	69.2	67.2	26.1	--	--
	SD (±)	0.002	0.011	0.007	0.09	0.008	--	--
Pink Flower 'Rosea'	mean	99.1	97.0	93.4	87.2	55.4	30.7	21.3
	SD (±)	0.008	0.003	0.01	0.021	0.006	0.007	0.004

ANOVA significance at $p<0.05$; the values are mean of three replicates ± Standard Deviation (SD)

III. RESULTS

The *in vitro* NaCl tolerances of the two cultivars of *C. roseus* (L.) varied markedly when subjected to different salt concentrations (Table II). The data suggested that the Rosea cultivar, could tolerate a NaCl concentration of up to 100 mM, while Alba showed tolerance only up to 60 mM (Plate 1A, B). When the cultures were subjected to higher levels of NaCl, symptoms of toxicity appeared as leaf damage, i.e., reduced size, marginal necrosis and ultimately explant necrosis. Enhanced proline accumulation with increasing

salinity levels was also observed in both cultivars (Fig. 1A). The total sugar content in the shoots declined after a slight initial increase (1.5 fold) in the tolerant cultivar, while in the susceptible cultivar there was a linear decline under NaCl stress (Fig. 1B). As the concentration of NaCl increased, there was a sharp reduction in the chlorophyll content of the leaf tissues (Fig. 1C). In the tolerant cultivar (Rosea), the phenol content increased linearly, while in the susceptible cultivar (Alba) there was no observed change, and the levels remained almost static (Fig. 1D). Tissue ionic concentrations were similarly affected by the *in vitro* induced salinity in both cultivars. The level of Na^+ increased in the foliar tissue with increasing levels of NaCl (Fig. 2A), while the K^+ level decreased ($P<0.05$) significantly (Fig. 2B). The K/Na ratio ranged between 1.5 and 3.0 in the tolerant cultivar, while in susceptible cultivar it was < 1.5 (Fig. 2C). The superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) enzymes activity in leaves declined after a initial increase (50-60 %) in the tolerant cultivar, while in the susceptible cultivar there were a linear decline under NaCl stress (Fig. 3A, B, C). The malandialdehyde (MDA) content of both *C. roseus* cultivars were lowest in the control plants. The level of malandialdehyde increased linearly in foliar tissue with the increase in concentration of NaCl in susceptible cultivar, while it was 2-2.5 folds lowered in tolerant cultivar (Fig. 3D). The maximum induction of callus in both the *C. roseus* cultivars were observed on MSB medium (Fig. 4A, B) supplemented with 2, 4 D (4.06 µM) and Kn (2.3 µM) [Plate 2A,B]. The dry weight of callus of both *C. roseus* cultivars illustrated in Fig. 5A. The maximum dry weight of callus were observed in both *C. roseus* cultivars on MSB medium having 45 mM NaCl concentration after 60 days of incubation [Plate 2C]. The dry weight biomass of callus declined linearly in both *C. roseus* cultivars incubated on MSC and MSD media.



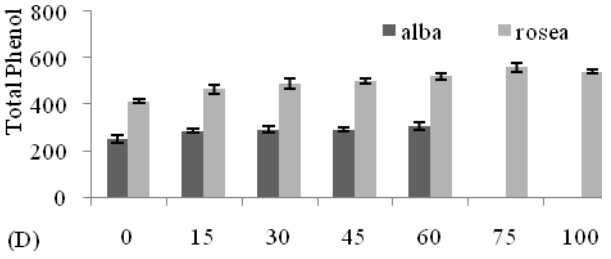


Fig. 1 (A) Total Proline (mg g^{-1} dry wt.); (B) Total Sugars (mg g^{-1} dry wt.); (C) Total Chlorophyll (mg g^{-1} fresh wt.); (D) Total Phenol (mg g^{-1} fresh wt.) contents of two cultivars of *C. roseus* at 55 days. (Vertical bars represent mean ± SE; *significant at $p<0.05$)

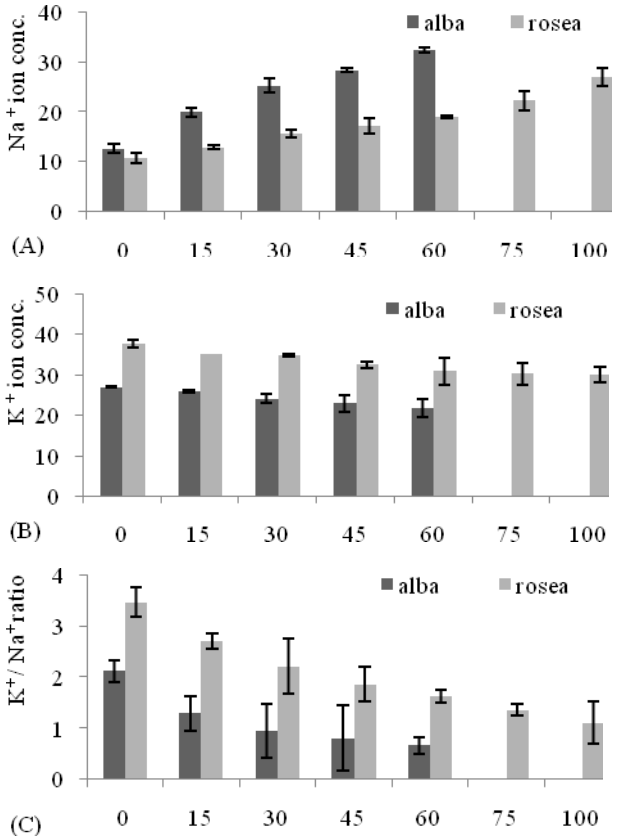


Fig. 2 (A) Tissue Sodium ion (Na^+) (mg g^{-1} dry wt.); (B) Tissue Potassium ion (K^+) (mg g^{-1} dry wt.) contents; (C) Na^+/K^+ ratio as affected by *in vitro* induced salinity in two cultivars of *C. roseus* at 55 days. (Vertical bars represent mean ± SE; *significant at $p<0.05$)

Plate.1 A) Explant sub culture for *in vitro* screening, onto NaCl containing proliferating medium (MS+0.05 µg Thiamine HCl); B) Sub culturing of surviving cultures of two cultivars of *C. roseus* at 55 days.

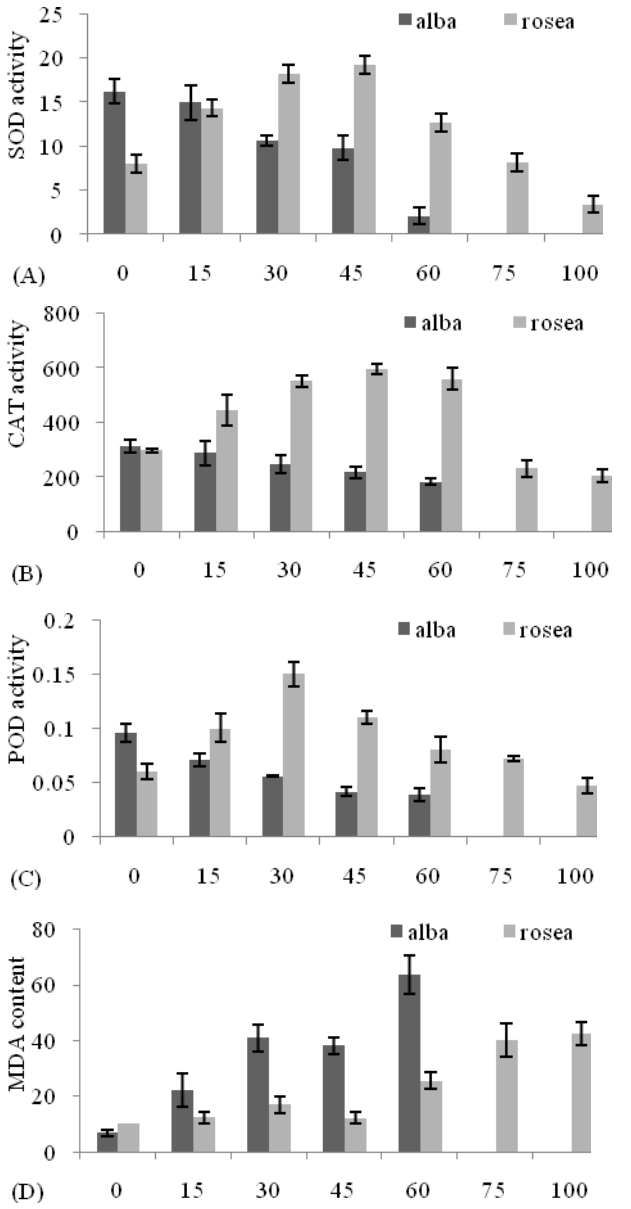


Fig. 3 (A) Superoxide dismutase (SOD) (Units mg^{-1} Pro.); (B) Catalase (µ mol H_2O_2 red. mg^{-1} $Pro.min^{-1}$); (C) Peroxidase (Δ A_{420} mg^{-1} $Pro.min^{-1}$) activity; (D) Malandialdehyde (MDA) (n mol g^{-1} FW) contents of two cultivars of *C. roseus* at 55 days. (Vertical bars represent mean ± SE; *significant at $p<0.05$)

High frequency of multiple shoot formation was obtained in low salt concentration (up to 45 mM NaCl) in tolerant cultivar (Fig. 5B) in all the medium containing growth regulators, while in the susceptible cultivar there were a linear decline under NaCl stress in all the medium (Plate 2D).

IV. Discussion

Reduced tissue growth in stressful medium is a typical phenomenon that has been interpreted as a change in metabolism initiated to resist stress. The salt tolerances of the two cultivars were different. The relatively high variability in survival among the cultivars of *C. roseus* in response to salt stress reflects the genetic heterogeneity in the species (Table I and II). The results revealed that leaf damage (i.e., reduced size, marginal necrosis, and ultimately explant necrosis) was due to the accumulation of excessive toxic ions in the leaf cell vacuoles. The accumulation of excessive amounts of toxic ions in leaf cell vacuoles reduces carbon fixation [17].

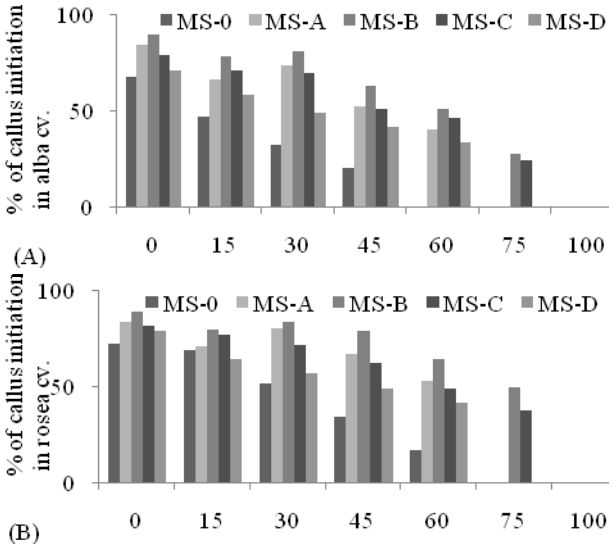
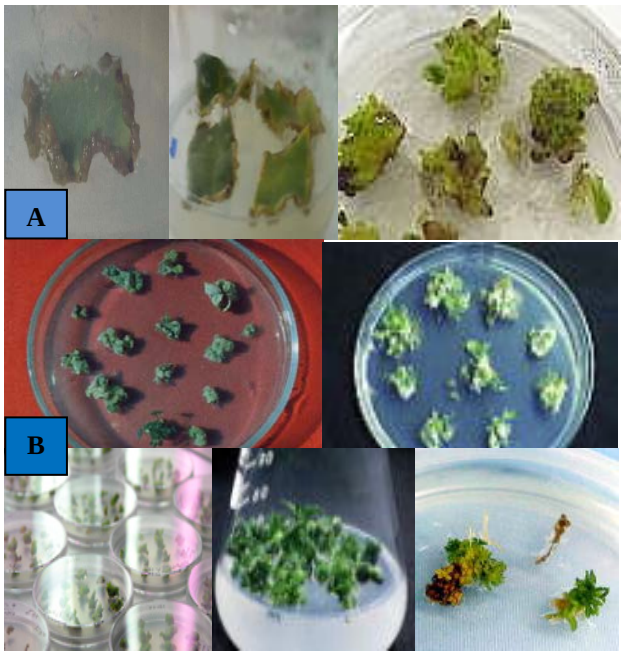


Fig. 4 Frequency of callus initiation (%) from the leaves of 'Alba' (A) and 'Rosea' (B) cultivars of *C. roseus* in different medium after four weeks of incubation.



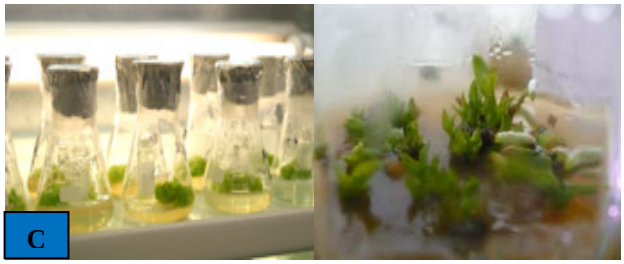


Plate.2 A) Callus initiation from the cut ends of leaf explant after four weeks of incubation; B) Callus growth after 5 weeks of incubation; C) Effect of different concentrations of NaCl on growth and morphogenesis of callus in MSB medium; D) Effect of high salt concentration (45-75mM NaCl) on the growth of callus and shoot multiplication; E) Multiple shoots formation from the callus of *C. roseus* cultivars in MSB medium [2,4 D (4.06µM) + Kn (2.30µM)].

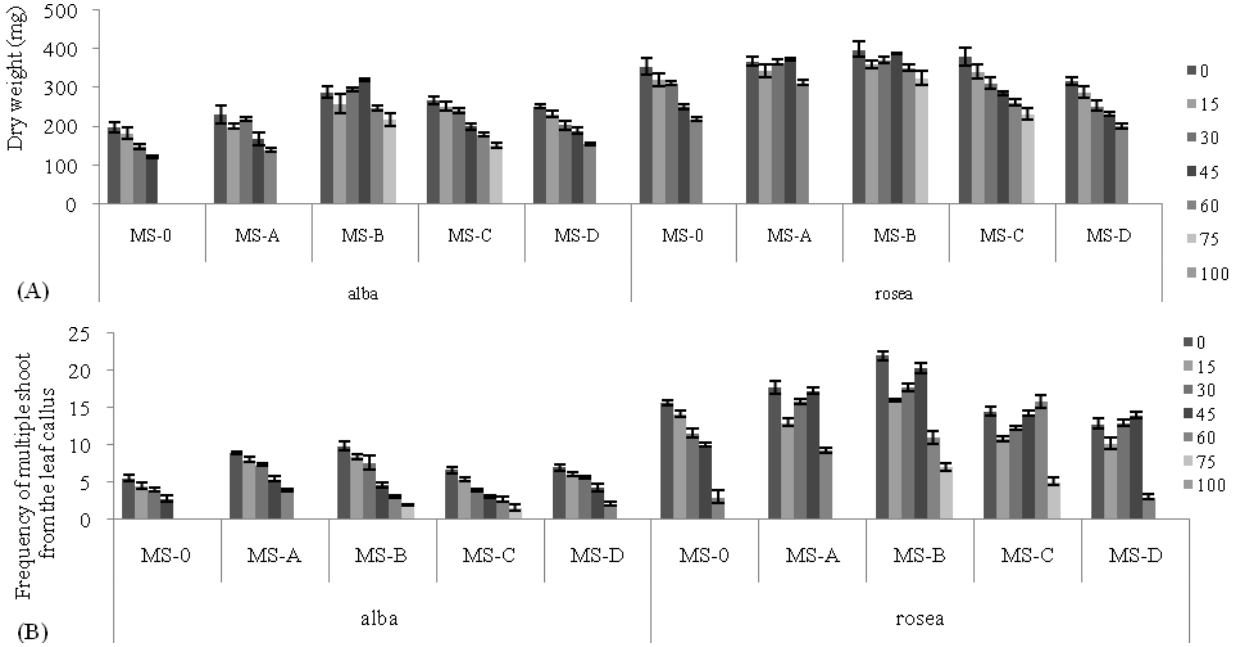


Fig. 5 (A) Dry weight (mg) of callus initiated from the explant (leaves); (B) Frequency of multiple shoot formation from leaf callus of two cultivars of *C. roseus* in different medium after nine weeks of incubation. (Vertical bars represent mean ± SE; *significant at $p<0.05$).

Proline continues to be the most studied plant molecule of this decade. Higher plants accumulate free proline in response to external salt and drought stresses. Proline can act as an osmoticum (a protective agent of enzyme and cellular structure) and as a storage compound to reduce nitrogen during the process of rapid re-growth after stresses are relieved. In this study, an increase in proline content was observed in both cultivars with increasing NaCl in the growth medium. The results of the present study are in agreement with earlier reports on proline accumulation under stress conditions in seedlings, as well as in fully grown plants [18] - [20]. The total sugar content in the shoots declined after an initial increase in the tolerant cultivar. This increase in sugar content in the tolerant cultivar may facilitate osmotic adjustment in the plant [18]. This result also suggests that increased carbohydrates in the Rosea cultivar increase the plant's stress tolerance. Photosynthesis, one of the most important metabolic pathways in plants, is a targeted pathway during salt stress. As the concentration of NaCl increased in the medium, there was a corresponding sharp reduction in chlorophyll content in the leaf tissue in both cultivars. Corroboratory results were observed in earlier work by [21], who showed that the net photosynthetic rate declined during periods of salt stress in leaves. Stress induced by salinity, is associated with increased proline synthesis and other metabolic changes, including increased synthesis of phenolic acids and essential oil metabolites that are stimulated in response to stress [19]. Phenolics are synthesized in the leaves and then carried to other tissues and organs. In this study, total phenols increased linearly, as observed by [22]. Metabolized phenols affect tissue culture systems positively, increasing auxin metabolism (rapid cell division and synthesis of the cell wall). Some authors have described phenolics as being positively related to *in vitro* proliferation [23]. The results presented here clearly show different responses with respect to the K^+/Na^+ ratio. Nevertheless, tolerant cell lines accumulate elevated levels of Na^+ and Cl^- [24]. Potassium is an essential macronutrient taken up by the roots. A decrease in the potassium content of *C. roseus* due to NaCl treatment may be the result of the toxic effects of NaCl on plant growth or competition with other ions, which in turn exercises a regulatory control on potassium uptake. Reduced potassium content has also been reported in NaCl stressed grasses [25] and in *Vicia faba* [26]. Decreases in mineral levels due to NaCl stress may be due to competition among Na^+, Cl^- and other mineral ions during uptake [27]. Increased activity of superoxide dismutase, catalase and peroxidase enzymes in tolerant cultivar alleviated the damaging effects of salt concentration on cell membranes. These enzymatic antioxidants removed superoxide radicals, which disturbs vital biomolecules [2]. Malondialdehyde (MDA) is regarded as a marker for assessment of lipid per oxidation or damage to plasmalemma and organelle membranes that increases with environmental stresses. In this study, negative relationship between antioxidative enzyme activity and malandialdehyde contents [28]. These results suggest that

increased antioxidative enzymes activity in Rosea cultivar successfully scavenging reactive oxygen species (ROS), prevent oxidative injury of membrane and enhanced oxidative stress tolerance in plants [29]. Healthy and fast growing friable callus is the prerequisite of different biotechnological investigations. Callus consists of undifferentiated masses of cells developed on a semi-solid medium. The maintenance of such cultures depends on an adequate supply of nutrients, growth hormone and controlled sterile environment [20]. The cells, although undifferentiated, contain all the genetic information present in parent plant. By suitable manipulation of hormone and contents of the medium, it is possible to initiate the development of roots, shoots and complete plants from callus culture [30]. The nutritional requirements of plant cells and tissues vary from species to species and therefore a number of media have been devised for specific tissue by different workers.

V. CONCLUSION

From the results of this investigation, it is clear that the, greater amounts of proline, total sugar and the higher K^+/Na^+ ratio in the shoots of the Rosea cultivar resulted in better ion homeostasis when compared with the Alba cultivar. In addition higher activities of antioxidative enzymes (superoxide dismutase, catalase and peroxidase) in Rosea cultivar alleviated the damaging effect of salt stress. Leaves of rosea cultivars incubated in MSB medium supplemented with 2,4 D (4.06 µM) and Kn (2.3 µM) responded favorably and produce good friable, high biomass callus and multiple shoots formation from the leaf callus, as compared to the Alba cultivar. Consequently, the Rosea cultivar has a higher salinity tolerance than Alba. The results of this study suggest that *C. roseus* (L.) cv. Rosea may be considered as stress tolerant, candidate cultivar for future breeding and can be cultivated at commercial level to meet the ever increasing demand of medicine, as well as the pharmaceutical industries of northern India.

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