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Salinity-induced modulations in growth and biochemical traits in callus cultures of *Allium cepa* L.

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ABSTRACT

Callus cultures of *Allium cepa* L. were exposed to salt stress by inoculating on MS medium containing different levels of salt, including 0, 100, 150 and 200 mM NaCl, in order to evaluate growth and biochemical responses. A significant decline in callus relative growth rate, tissue water content and fresh weight /dry weight ratio was observed under salt stress. Osmotic adjustment was revealed by the accumulation of proline and total soluble sugars in calli stressed with all the concentrations of NaCl, as compared to control. This osmotic adjustment however, did not support growth at high (150 and 200 mM) NaCl levels but might be implied in survival and maintenance of callus under such stressful conditions. Salinity induced significant oxidative damage as indicated by higher values of MDA content in stressed calli as compared to control. Activity of peroxidase elevated with increasing NaCl levels whereas for catalase it increased only till 100mM and thereafter declined, indicating the greater role of peroxidase in detoxifying H_2O_2 at high salt levels thereby preventing oxidative damage and thus contributing to survival of calli.

Keywords: *in vitro*, growth, osmotic balance, oxidative stress, salinity tolerance

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INTRODUCTION

Salinity is one of the most important environmental constraints affecting more than 800 million ha of arable land [1] and therefore, the agricultural productivity. Salinity causes osmotic and oxidative stress and plants respond to that by changing physiological, biochemical and molecular processes. The best characterized biochemical response of plant cell to osmotic stress is synthesis of compatible solutes like proline, glycine betaine and soluble sugars [2]. Oxidative stress is characterized by generation of reactive oxygen species and one of the main tolerance mechanisms against it in plants is activation of antioxidative defense system. The antioxidative enzymes like superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and others are efficiently involved in scavenging reactive oxygen species (ROS) produced by salt stress.

Studies aimed at understanding the mechanism of tolerance under salt stress has generated considerable interest. The response of plants to salt stress has involved a set of adaptations at cellular, tissue and whole plant level [3] and therefore, plants can be evaluated for biochemical and physiological changes under salinity at all these levels. Many studies have demonstrated the similarity in salt tolerance mechanism operating at the whole-plant and cellular level in plants [4]. Cell and tissue culture offers a good system for monitoring salinity-induced biochemical and physiological responses at cellular level [5,6] and may thus prove useful in identifying the effect of salt and the processes associated with tolerance at higher level of plant organization. Tissue culture studies for testing salt tolerance offers advantage in terms of faster responses, under controlled environment as compared to field conditions [7].

Studies on salinity tolerance in *A. cepa* L., a crop classified as salt sensitive species [8] have been carried out by many workers [9,10, 11] but none of them except [12] were focussed on evaluation of cellular responses to salt stress. The present investigation was undertaken to evaluate the influence of salinity stress on growth and biochemical characteristics in callus cultures of *Allium cepa* L. with the aim to gain information on changes taking place at the cellular level.

MATERIALS AND METHODS

Seeds of *A. cepa* L. Variety NHRDF-red were obtained from the National Research Centre for Onion and Garlic, Rajgurunagar, Pune, India. They were surface sterilized for one minute with 0.1% $HgCl_2$, rinsed, and soaked in distilled water for one hour for imbibition. Seeds were subsequently inoculated aseptically on water agar (0.8%agar) and were placed for germination in a growth chamber at 28±2^{o}C, with a photoperiod of 16 h providing 45 µmoles.m^{-2}.s^{-1} illumination provided by cool white fluorescent tubes.

Callus induction and multiplication

Root segments (ca. 1.5 cm) were excised from four week old aseptic seedlings and inoculated for callus induction on Murashige and Skoog (1962) (MS) medium containing 3 %

sucrose, 0.8 % agar , 1.0 mg/l 2,4-Dichlorophenoxyacetic acid and 0.5 mg/l casein hydrolysate. The pH of the medium was adjusted to 5.8 prior to autoclaving at 121˚C for 15min. The cultures were incubated as described earlier. After 3 weeks of culture, callus induction was observed at the cut ends of root segments. The callus was then separated from the initial explants and subcultured regularly on freshly prepared callus multiplication medium (MS medium supplemented with 0.5 mg/l 2,4-D and 0.5 mg/l casein hydrolysate) at an interval of 25 days and after maintenance for 3 months under control conditions, was used for salt stress experiments. The callus obtained was white and friable.

Salt stress treatment

Fresh, proliferating calli were weighed (500 mg) and inoculated on callus multiplication medium supplemented without (control) and with various levels of NaCl (100, 150, 200 mM). The cultures were incubated under controlled conditions as described earlier. Calli were evaluated after 30 days of salt treatment for growth parameters, proline and soluble sugar contents, and antioxidative enzyme activities.

Relative growth rate measurement (RGR)

Samples were weighed to calculate relative growth rate. Calli were weighed initially at the time of their transfer (Wi) and finally after 30 days of salt treatment (Wf), and the mean callus RGR calculated by: [(Wf-Wi)/Wi]x100.

Determination of tissue water content (TWC)

The fresh weight (FW) of the calli was determined immediately after removal from the medium and blotted with tissue paper to remove excess water. Dry weight (DW) was recorded after drying the calli at 60˚C in the hot air oven for 48 h. The percent tissue water content (TWC %) of the calli was determined using the following equation:
TWC (%)=[(FW-DW)/FW]x100

Free Proline Content

Proline content was estimated following the procedure of Bates et al. [13]. Calli (ca. 500 mg) were homogenized in 5.0 ml ice cold 3% aqueous sulphosalicylic acid in an ice cold mortar and pestle. The homogenate was centrifuged at 15, 652 g for 15 min. at 4^{o}C. To an aliquot of 2 ml supernatant, 2 ml of acid ninhydrin was added followed by addition of 2 ml of glacial acetic acid, boiled for 1 hour and allowed to cool. The reaction mixture was extracted with toluene (4.0 ml), vortexed and free proline estimated from the organic phase by recoding absorbance at 520 nm using a UV-Visible spectrophotometer. A 1 mg.ml^{-1} solution of proline, as a standard, was used to calculate proline concentration in the samples.

Total soluble sugars (TSS) content

TSS content was estimated as per the anthrone method [14] with some modifications. About 200 mg of calli were homogenized with ice-chilled 80% ethanol in mortar and pestle. The extract was prepared by centrifugation at 5,000g for 10 min at 44˚C, and final volume was adjusted to 10 ml with 89% ethanol. Then 1 ml of supernatant was reacted with 3 ml of freshly prepared anthrone reagent by incubating the reaction mixture for 10 min at 100˚C in a hot water bath. The reaction was terminated by quick cooling in an ice bath and allowed to cool at room temperature. The optical density was measured spectrophotometrically at 620 nm. A standard curve was prepared using D-glucose; the TSS was calculated and expressed as $mg.g^{-1}$ FW.

Measurement of lipid peroxidation

The level of lipid peroxidation was measured in terms of malonaldehyde (MDA) content as described by Lokhande et al. [15]. Fresh calli (200 mg) was ground in liquid nitrogen and homogenised in 5% (w/v) trichloroacetic acid (TCA) and 0.67% (w/v) thiobarbituric acid (TBA).The mixture was incubated at 100˚C in a hot water bath for 30 min; the reaction was terminated in an ice bath and allowed to cool to room temperature. The mixture was centrifuged at 10,000 g for 10 min at room temperature. Absorbance of the supernatant was measured at 532 and 600 nm for the correction of non-specific turbidity. The level of lipid peroxidation was expressed as µmol of MDA formed g^{-1} FW using an extinction coefficient of 155 mm^{-1} cm^{-1}.

Measurement of total proteins and enzyme activities

Extraction

Calli (ca 500 mg) from the control and various treatments were homogenized using a chilled mortar and pestle in (tissue/buffer ratio 1:2, w/v) ice cold extraction buffer, pH 8.0, containing 0.1 M phosphate buffer and 1% polyvinylpoly pyrolidine (w/v). The homogenate was centrifuged at 13,300 g for 15 min at 4°C. The resultant supernatant was used for determination of total protein, and enzyme activities

Total Proteins

Total soluble protein was determined by the method of Lowry et al. [16] using bovine serum albumin (BSA) as a standard. To a suitably diluted 1 ml extract (prepared as described above) was added 5.0 ml alkaline copper sulphate, mixed well and incubated at room temperature for 10 min. To the resultant solution was added 0.5 ml Folin Ciocalteau reagent and incubated for 30 min. and absorbance was read at 660 nm. Concentration of total soluble protein was estimated using BSA standard curve.

Catalase (CAT)activity

Catalase (EC 1.11.1.6) activity was determined by the method of Braber [17]. To 300 µL of solution containing 0.1 M phosphate buffer (pH 7.0) and 200 µL 0.005M hydrogen peroxide, 100 µL of protein extract was added and left at 25°C for 5 min. Then 1 ml of sulphuric acid (0.7 N) was added and the reaction mixture titrated with potassium permanganate (0.01 N) until a pink color was achieved and persisted for at least 15 sec. The Cat activity was expressed as µmoles of H_2O_2 used $min^{-1}.g^{-1}$ FW.

Peroxidase (POD)activity

Peroxidase (EC 1.11.1.7) activity was carried out according to Miranda et al. [18] The reaction mixture contained in 1ml: 8mM H_2O_2, 40mM guaiacol, 50mM sodium acetate buffer, pH 5.5 and least amount of enzyme preparation. The change in absorbance at 470 nm due to guaiacol oxidation was followed for 1min using a spectrophotometer. One unit of peroxidase activity was defined as the amount of enzyme producing 1 µmol.min^{-1} of oxidized guaiacol.

Statistical Analysis

The experiment was arranged in a completely randomized design with six replications for each treatment. Data were subjected to analysis of variance (ANOVA) using SPSS (ver. 14, SPSS Inc., Irvine, Calif.) and the treatment means were separated with LSD at 1% level of probability.

RESULTS

Calli growth and water status

Observations recorded after 30 days of salt treatment showed a significant decline in RGR of stressed calli with increase in salt levels, and was around 7-fold less than control at high salt level (150 and 200 mM NaCl) (Table 1). At low NaCl level, RGR was around three-fold less than control. The TWC of salt stressed calli decreased significantly with increase in NaCl level as compared to control calli (Table 1). It was comparable to control at 100mM NaCl whereas around 34% less than control at highest (200mM) NaCl level. The FW/DW ratio also appreciably declined with increasing salt levels. It was 52.3% less than control in calli subjected to 200mM NaCl stress (Table 1).

Proline, TSS and Total Protein contents

Significantly higher levels of proline and TSS were observed in calli grown under salt stress (Table 2). As compared to control calli an approximately 4.0-fold and 3.5-fold increase in proline and TSS levels respectively, was observed in calli exposed to 200mM NaCl. TSS contents at 100 and 150 mM NaCl were comparable. Salinity stress induced considerable elevation in

total protein content of calli as compared to control. This increase however, was most significant (34% of control) only at highest NaCl concentration (Table 2).

Table 1: Growth characteristics of Onion calli grown under NaCl stress

NaCl Concentration (mM)	Relative Growth Rate (%)	Tissue water content (%)	FW/DW ratio
0 (control)	145.4[a]	93.66[a]	16.76[a]
100	42.61[b]	90.0[a]	10.56[b]
150	20.8[c]	84.66[b]	11.96[c]
200	20.4[c]	59.66[c]	7.83[d]

Means within column followed by different letters differ significantly at P<0.01, LSD.
Observations were recorded after 30 days of culture. The values represent the averages of six independent replicates (n = 6).

Lipid Peroxidation

The lipid peroxidation of membrane measured in terms of MDA content was appreciably higher in calli stressed at higher NaCl levels as compared to control, and was around 5-fold higher in calli stressed at 200 mM NaCl (Table 2).

Antioxidative enzyme activity

Activities of CAT and POD were significantly affected by salinity in calli of *A. cepa* L. POD activity increased progressively with increasing level of NaCl (Table 2). It was almost 2.0 fold higher than control calli at 200mM NaCl. CAT activity was significantly the highest (3.0-fold) in calli stressed at 100 mM NaCl compared to control and further decreased with increasing salt concentration thereafter (Table 2).

Table 2: Biochemical characteristics of Onion calli grown under NaCl stress

NaCl Concentration (mM)	Protein Content (mg g^{-1} FW)	Proline Content (µM g^{-1} FW)	Total Soluble Sugars (mg g^{-1}FW)	MDA content (µM g^{-1}FW)	CAT Activity (µM of H_2O_2 used min^{-1}. g^{-1} FW)	POD Activity (U.mg^{-1}protein)
0 (control)	57.66[c]	4.04[d]	11.03[c]	0.08[c]	16.02[d]	100.77[d]
100	60.23[c]	5.73[c]	25.31[a]	0.12[c]	41.30[a]	116.93[c]
150	78.61[b]	8.78[b]	24.61[a]	0.25[b]	25.14[b]	152.22[b]
200	87.89[a]	16.46[a]	38.54[b]	0.54[a]	22.41[c]	198.82[a]

Means within column followed by different letters differ significantly at P<0.01, LSD.
Observations were recorded after 30 days of culture. The values represent the averages of six independent replicates (n = 6

DISCUSSION

Many studies have demonstrated that tissue cultures are adequate systems to evaluate salinity tolerance and to research adaptive mechanisms of plants living in saline conditions [19].

In the present investigation we have evaluated the response to salinity in *A. cepa* L. using callus culture system. All the growth and biochemical parameters measured for onion calli were significantly affected by salinity. Salinity impose both ionic and osmotic stresses [20] and thus, disrupts the plants' ability to uptake nutrient at various levels of tissue organization. Whole plants, exhibiting higher level of organization, may be able to cope with ion toxicity by intracellular compartmentalization more efficiently whereas for callus cultures this may not be possible owing to its dedifferentiated and unorganised nature. This might be the reason for reduction in relative growth rate of salinity –stressed calli in the present study and other species like *Saccharum officinarum* [21] and *Arabidopsis thaliana* [22], onion [12] and *Salvadora* [23]. In our study, although the RGR of calli declined significantly at high salt levels but they survived and remained fresh. According to Lokhande et al. [15] slow growth is one of the strategies developed by plants for survival under salinity stress.

The availability of water to the cell is reduced in hyper-osmotic medium [24] and this leads to loss of cell turgor and hence slows down of cell division and elongation [25]. Dose dependent reduction in FW, DW, and relative water content of salinity stressed calli was observed in the present study. Patade et al. [21] correlated reduced callus growth (FW/DW ratio) with nutritional imbalance as a result of ion interference. Cicek and Cakirlar [26] associated salinity-induced reduction in water content, FW, DW of maize leaves with restricted uptake of water and essential nutrients from the culture medium.

One of the important adaptive mechanisms of salinity tolerance in plants is maintenance of osmotic balance by accumulation of compatible solutes such as carbohydrates and proline [27]. Significant accumulation of proline in stressed calli was recorded in the present study. Survival and maintenance of calli on high NaCl levels could be attributed to the osmotic adjustment due to proline. In addition, enhanced total soluble sugars of salt stressed calli observed in our study also seemed to have contributed in osmotic adjustment. Many investigators have demonstrated proline and soluble sugar accumulation as an adaptive trait in response to hyperosmotic stresses [14,28] correlated it with osmotic regulation. Our earlier report [21] has demonstrated the role of proline in osmotic adjustment under salinity stress in onion at seedling establishment stage.

Salt concentration in plants is known to influence the regulation of protein expression [6]. Proteins that accumulate in plants under salinity are cytoplasmic, which can cause alterations in cytoplasmic viscosity of the cells [23]. In addition, proteins also provide a storage form of nitrogen that is re-utilized later and therefore, may play a significant role in osmotic adjustment [29]. Enhancement of protein content of stressed calli was observed in the present study, which is in consistent with observations reported by Bekheet et al. [12], who have demonstrated a positive correlation between protein content and salt levels. Our results suggested the contribution of proteins also in osmotic homeostasis.

The lipid peroxidation of membrane measured in terms of MDA content has frequently been used as an indicator of oxidative stress. In the present study salinity induced significant oxidative damage in calli of *A. cepa* L. as indicated by high MDA content compared to control

calli. Similar to our observations Yang et al. [6] also observed an increased lipid peroxidation of membrane in calli of *Nitraria tangutorum* Bobr. exposed to salt stress.

Activation of antioxidative enzyme system is frequently reported in plants exposed to salinity and has been correlated with a plant's capacity to tolerate and adapt to salinity conditions [30]. Catalase is one of the main H_2O_2 scavenging enzymes that dismutase H_2O_2 into water and O_2 [31,32]. Lokhande et al. [15] observed a decline in catalase activity in *Sesuvium portulacastrum shoot cultures at 400mM NaCl*, while increased CAT activity was reported in *Nitraria tangutorum* callus culture at 50 or 100 mM NaCl stress [6]. Thus, catalase enzyme activity varied with different plant species and with salt concentration. In the present study, CAT activity of salt stressed calli increased till 100mM NaCl and thereafter decreased with further increase in NaCl level, indicating the declining ability of callus tissues in CAT-mediated detoxification of H_2O_2 with increasing salt level. However, the activity of POD, an another H_2O_2 scavenger, in stressed calli showed a dose dependent rise, suggesting that the neutralization of H_2O_2 at higher salt level was mainly due to POD and this might have contributed in survival of calli at high NaCl level.

It has been demonstrated that the degree of salt stress adaptation observed in whole plant is also exhibited in callus tissues [4]. However, the results of present investigation revealed that onion calli responded fairly different to salt stress in comparison to responses observed at seedling establishment stage under salinity [11]. Callus growth was more inhibited in comparison to seedling growth [11] under salinity. However, the callus showed a capacity to survive on high (200mM) salinity in our study whereas under same salinity the seedlings could not germinate at all (data unpublished). These observations indicate a possibility of culture-induced adaptation to salt tolerance in onion callus.

CONCLUSION

The callus cultures of *A. cepa L.* showed survival on high salinity levels at the cost of reduced growth and this could be attributed to i) osmotic adjustment by enhanced accumulation of proline and soluble sugars; ii) reduction of oxidative damage by increased activity of POD. Further, the results of our study demonstrates that *in vitro* cell cultures could serve as good model system for the study of stress mechanism, as they are faster and independent from environmental factors.

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REFERENCES

[1] Munns R, Tester M. Annu Rev Plant Biol 2008; 59: 651-681.
[2] Flowers TJ, Colmer TD. New Phytol 2008; 179: 945-963.
[3] Borsani O, Valpuesta V, Bitella MA. Plant Cell Tiss Organ Cult 2003; 73: 101-115.
[4] Arzani A. In vitro Cell Dev Biol Plant 2008; 44: 373-383.
[5] Matkowaski A. Biotech Adv 2008; 26: 548–560.
[6] Yang Y, Wei X, Shi R, Fan Q, An L. J Plant Growth Regul. 2010; 29: 465–476.
[7] Perez-Tornero O, Tallon CI, Porras I, Navarro JM. J Plant Physiol 2009; 166: 1923-1933.
[8] Mangal JL, Lala S, Hooda PS. J Hort Sci 1989; 64: 475-477.
[9] Sidari M, Santonoceto C, Anastasi U, Preiti G, Muscolo A. Amer J Agr Biol Sci 2008; 3: 410-416.
[10] Goudarzi M, Pakymat H. J Appl Sci 2009; 9: 348-353.
[11] Joshi N, Sawant P. Int J Veg Sci 2012; 18: 3-19.
[12] Bekheet SA , Taha HS, Solliman ME. Arab J Biotech 2006; 9: 467-476.
[13] Bates CJ, Waldren RP, Teare ID. Plant Soil 1973; 39: 205–207.
[14] Watanabe S, Kojima K, Ide Y, Sasaki S. Plant Cell Tiss Org Cult 2000; 63: 199–206
[15] Lokhande VH, Nikam TD, Penna S. Plant Cell Tiss Organ Cult 2010; 102: 17-25.
[16] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. J Biol Chem 1951; 193: 265-275.
[17] Braber JM. Z Pflazenphysiol 1980; 97:135-144.
[18] Miranda MV, Fernandez Lahor HM, Cascone O. Appl Biochem Biotechnol 1995; 53: 147-154.
[19] Gondonou CB, Errabii T, Abrini J, Idaomar M, Senhaji NS. Plant Cell Tiss Organ Cult 2006; 87:9-16.
[20] Tester M. Ann Bot 2003; 91: 503–527
[21] Patade VY, Suprassana P, Bapat VA. Plant Growth Regul 2008; 55: 169-173
[22] Zhao X, Tan HJ, Liu YB, Li XR, Chen GX. Plant Cell Tiss Organ Cult 2009; 98:97-103
[23] Sharma V, Ramawat KG. Biotech 2013; 3:11-17.
[24] Chamandoosti F. Pak J Biol Sci 2007;10:3880-3884
[25] Rhodes D, Samaras Y. Genetic control of osmoregulation in plants. In cellular and molecular physiology of cell volume regulation. Strange K. Boca Raton: CRC Press. 1994 pp: 347-361.
[26] Cicek N, Cakirlar H. Bulg J Plant Physiol 2002; 28: 66-74.
[27] Rosa M, Hilal M, Gonza'lez JA, Prado FE, et al. Plant Physiol Biochem 2009; 47:300–307.
[28] Suriyan C, Chalermpol K. Agric Sci China 2009; 8:51–58
[29] Hasegawa PM, Bressan RA, Zhu JK, Bohnert HJ. Annu Rev Plant Physiol Plant Mol Biol 2000; 51: 463–499.
[30] Yazici I, Turkan I, Sekmen AH, Demiral T. Environ Exp Bot 2007; 61:49-57
[31] Moradi F, Ismail AM. Ann Bot 2007; 99: 1161–1173.
[32] Li Y. Plant Soil Environ 2008; 54: 493–497.