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In vitro selection and characterization of salt tolerant cell lines in cassava plant (*Manihot esculenta* Crantz)

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Abstract: To identify and characterized a tolerant cassava cell lines for salt stress. Cassava suspension culture grow on MS media containing 50, 100, 150, 200 and 250 mM NaCl were established from cassava callus cultures and some traits, including viability percentage, average and concentration of total viability cells and biochemical indicators including sodium, potassium, calcium and chloride (Na^+, K^+, Ca^{2+}, Cl^-), proline content, peroxidase, glutathione reductase and glutathione peroxidase enzymes were all dramatically induced in response to salt treatment. The results indicated that the high concentration of NaCl 200 and 250 mM decrease the viable cell number one fold comparing to lower concentrations of NaCl and control sample. Surprisingly at 50, 100 mM and 150 mM NaCl we found that the number of viable cells was higher than the control sample. However, the cell viability in 12 days under NaCl stress shows high tolerance against salt stress and the cell numbers also higher comparing to other NaCl concentrations. Ionic status suggested that 200 mM NaCl accumulated less Na^+, Cl^- and Ca^{2+} and maintained better K^+ in comparison to other NaCl stress cell samples. The ion homeostasis data of cassava cell culture under NaCl stress showed that the Na^+ and K^+ accumulation increased very much under lower concentrations of NaCl and gradually decrease in higher concentration. There is a positive relationship between salt tolerance and proline content in in cassava cultures up to 200 mM NaCl stress and the highest proline content compared to other treatments. Gel activity assay of superoxide dismutase (SOD), peroxidase (GPX) and Total peroxidase (POX) activity increased in tolerant cell lines as compared to control. Analysis of the above enzymes suggests that selected cassava cell lines possessed more efficient scavenging system of reactive oxygen species under 200 mL NaCl. we can concluded that in cassava suspension culture we can realize that viability of cell under 200 mM NaCl stress after 15 day will be the perfect time to isolate and identify the intercellular and extracellular protein or/and peptides which could be produced abundantly.
Key words: cassava, salt stress, cell viability, ion concentrations, detoxification enzyme, proline.

Introduction

Cassava (*Manihot esculenta* Crantz) is a woody shrub belongs to family of Euphorbiaceae. Cassava is a crop widely grown as a staple food, animal feed, and as an industrial raw product in the tropical and subtropical regions between 30° N and 30° S of Africa, Latin America and Asia. Cassava It is a staple for more than 800

million people in the world and one of the six most important crops around the world and displays a unique ability to grow on low-fertility soils and tolerate drought conditions [1,2,3]. Salinity is one of the major environmental stress factors that have adverse effects on plant growth and limiting plant productivity [4,5]. Salt stress results from a number of detrimental processes including an ion imbalance and toxicity, the impairment of mineral nutrition, a reduction in the water status of the plant tissues thus affecting plant growth, morphology, and survival, however, the metabolic imbalances caused by ionic toxicity, osmotic stress, and nutritional deficiency under salinity may lead to oxidative stress, linked to the production of reactive oxygen species (ROS), which cause damage to lipids, proteins and nucleic acids[6,7,8]. Also, when there is an imbalance (either by abiotic or biotic reasons) in the cellular compartment between ROS production and antioxidant defense, there is dramatic physiological challenges which results in oxidative stress[9]. With oxidative damage [10] and [11] reported that several enzymes including superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) are activated in plant cells. In addition, plants exposed to salt stress display complex molecular responses including the production of stress proteins and compatible osmolytes, and increases in enzymes that modulate reactive oxygen species, such as superoxide dismutase, ascorbate peroxidase and 162 glutathione reductase[12, 13].

During the last decade, transcriptome and microarray technology [14,15,16] has revealed that genes induced by stress could be categorized into two groups according to the functions of their products. The first group consists of functional proteins such as membrane proteins that maintain water movement through membranes (water channel proteins and membrane transporters); key enzymes for osmolyte biosynthesis (proline, betaine and sugars, etc.); the detoxification enzymes enabling cellular, physiological or biochemical metabolism to maintain a normal level (glutathione *S*-transferase, peroxidase, catalase, superoxide dismutase and ascorbate peroxidase, etc.); and other proteins for the protection of macromolecules (LEA protein, osmotin, antifreeze proteins, chaperons and mRNA binding protein, etc.). The antioxidative defense system is a complex network of compounds and reactions that depend on each other. Biochemical defense systems may provide sensitive tracers of environmental impacts on plants which can be used as a diagnostic tool of in vitro studies[17]. Proline accumulates in a diverse taxonomic group of higher plants in response to biotic and abiotic stresses i.e water deficits, salinity stress, and cold stress [18,19]. However, in vitro culture techniques for tissues and cells are the best way to study and characterized the salt tolerance in plant and for obtaining a salt tolerant plant [20]. Also, tissue culture techniques have been used as a useful tool to elucidate the cellular mechanisms involved in salt tolerance by using as study system in vitro selected NaCl-tolerant cell lines[21,22]. Furthermore, no information was given about the mechanisms involved in salt adaptation either in the selected cassava cell line. Moreover, the selection of crop varieties for greater tolerance to saline environment will allow greater productivity from large saline lands[23]. In addition, [24] suggested that in vitro culture can be a promising method for the study of the plant response to salt and for fast selection of tolerant cassava clones. Therefore, we report an efficient procedure to select cassava cell lines tolerant to different concentrations of NaCl and analyzed in order to contribute to better understanding of the mechanisms underlying salt tolerance for the identification and isolation of tolerant cassava cell lines.

Materials and Methods

Plant material

This study was carried out in Plant Biotechnology Department, National Research Centre and Natural Resources Department, Institute of African Research and Studies, Cairo University, during period from 2013 to 2015. Two years old cassava plants (*Manihot esculenta* Crantz), were obtained from Agricultural Research Centre, Giza, Egypt. For initiation of suspension culture, the supreme callus derived from stem explants were transferred to Erlenmeyer flasks containing 20 ml liquid MS medium fortified with 8 mg/l 2, 4-D for 15 days.

Methods

Cell numbers counting: this parameter was used to measure the growth of cassava plant cell suspension culture through a time course. Counting the number of cells per volume of suspension can be done for fine cell suspension cultures with a hemocytometer; however, the cell suspension cultures were containing a larger cell aggregates which we change it into free cells before counting[25].

Enzymes Extraction

The extraction for the enzymes was done as suggested by[26]. A 0.5 g of callus was harvested and ground to a fine powder in liquid nitrogen. Ground powder was homogenized in 1.5 ml of cold phosphate buffer (100 mM, pH 7.0) containing 1% polyvinylpyrrolidone (PVP) and 1 mM EDTA and then centrifuges at 4 ºC for 15 min at 10000 x g. The supernatant was separated and stored on ice till the assay of enzyme activity. This extract was used for the assay for gel native PAGE assay of all three enzymes.

Protein Estimation

Enzymatic protein in the callus extract was determined according to the coomassie G-250 Dye binding method[27]. Dissolved 100 mg Coomassie Brilliant Blue G-250 (CBBG-250) in 50 ml ethanol. To this solution, 100 ml of 85% o-phosphoric acid was added. The concentrated solution was diluted to 1 litre and then filtered through Whatman No. 1 filter paper. Stored in dark bottle at room temperature. A 100 µl of extract was taken and to it 1.5 ml of Bradford's reagent was added. After 5 min incubation at room temperature, absorbance was noted at 595 nm against a reagent blank. Calculations were made using standard curve prepared from the graded concentrations of bovine serum albumin (BSA).

Measurement of Proline Content:

Proline content was estimated colorimetrically according to the method of [28]. Hundred mg of callus was ground in liquid nitrogen and homogenized in 5 ml of 3% w/v sulfosalicylic acid and centrifuged at 5000 x g for 15 min. The supernatant was used for proline estimation using ninhydrin reagent which was prepared by dissolving 0.5 g of ninhydrin in 30 ml acetic acid and 20 ml of distilled water with continuous stirring. For 1 ml aliquot, 1 ml of ninhydrin reagent was added and the mixture was heated on a boiling water bath for 20 min. Then the samples were cooled at room temperature. On attaining the room temperature, the contents of test tube were shaken vigorously with 4 ml of toluene. Upper pink coloured organic phase was separated from the lower aqueous phase and absorbance of organic phase was read at 520 nm using toluene as a blank. Standard curve was prepared using graded concentrations of L-proline and data were expressed as µmoles g^{-1} fresh weight.

In Gel Assay for Analysis of ROS Scavenging Enzymes

Changes in proteins having isozymic activity of the ROS scavenging enzymes were studied using PAGE under non-reduced, non-denatured conditions at 4 ^{o}C according to the method suggested by[29]. Specific conditions were maintained for keeping native protein intact. PAGE was carried out for superoxide dismutase, guaiacol peroxidase, and glutathione peroxidase.

Sample preparation: the enzyme extract was used to load onto to the gels on equal protein quantity basis. A variation whenever applicable is mentioned along with the individual enzyme.

Procedure for PAGE: electrophoresis was carried out using vertical gel electrophoresis apparatus (Bio Rad) with glass plates 9 x 10 cm with 1.5 mm spacer. Glass plates, spacers, combs and buffer reservoirs of the gel apparatus were thoroughly cleaned with detergent, rinsed and dried. A monomer solution for the appropriate resolving gel was prepared by combining all reagents given in table 2, except ammonium per sulfate and TEMED. Then APS and TEMED were gently mixed into monomer solution and the mixture was poured between the gel plates with the help of a pipette up to the mark delimiting the resolving gel. It was immediately over layered with distilled water. Polymerization was achieved in approximately 30 min at room temperature.

After polymerization of resolving gel, water layer was removed with the help of filter paper strips. Stacking gel solution was prepared as given in Table 2 and poured on the top of the resolving gel. A well forming comb was then placed between the gel plates avoiding trapping of bubbles immediately. The stacking gel was allowed to polymerize at room temperature for 30-45 min.

Electrophoresis apparatus was assembled and filled with electrode buffer the comb was removed from the stacking gel and the wells were washed 2-3 times with electrode buffer. Prepared samples were then loaded into the wells in the stacking gel using a micropipette. Electrophoresis was carried out at 4 ^{o}C temperature at a constant current of 20 mA till the samples were in the stacking gel and at 30 mA once the samples entered into the resolving gel. The run was continued till the tracking dye reached 5 mm above the bottom edge of the gel.

Superoxide dismutase: 10% acrylamide-bisacrylamide gel was prepared and 40 µg protein was loaded.

Staining: gel was soaked in 2.45 mM NBT for 20 min followed by immersion for 15 min in a solution containing 28 mm TEMED, 3 µM riboflavin and 50 mM potassium phosphate at pH 7.8. The gel was then placed on dry white tray and illuminated for 5 to 15 min[30]. During illumination gel became uniformly blue except at positions containing SOD. Illumination was discontinued when maximum contrast between the achromatic zones and the general blue colour had been achieved. The gel was then photographed.

Peroxidases: Same extract was used as it was for superoxide dismutase assay. Ten percent acrylamide-bisacrylamide gel was prepared and 50 µg protein was loaded.

Staining: Gel was incubated for 10 min in a solution composed of 10 mM guaiacol, 50 mM potassium phosphate buffer of pH 7.0 and 10 mM H_2O_2 solution.

Glutathione peroxidase:same extract as for superoxide dismutase was used. 10% acrylamide-bisacrylamide gel was prepared and 50 µg protein was loaded.

Staining: Staining was done according to the methods suggested by[31]. The gel was submerged in a 50 mM Tris-HCl buffer (pH 7.9) containing 13 mM GSH and 2 mM H_2O_2 for 15 min, followed by incubation in a solution containing 1.2 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 1.6 mM phenazine methosulfate (PMS). After this, achromatic bands on a purple background showing the activity of glutathione peroxidase were photographed immediately.

Ion Analysis

Sodium, potassium, calcium and chloride content were determined from the oven-dried material. Hundred mg of the dry cells were extracted in 50 ml of deionized water with continuous shaking and estimated on microprocessor based ion analyzer (Elico, India) using ion specific electrode sodium, potassium, calcium and chloride (Na^+, K^+, Ca^{2+}, Cl^-). Na/K ratio was calculated Faithfull.

Statistical Analysis

The resulted data were subjected to statistical analysis, employing F-test for significance at $P \leq 0.05$ using MSTAT C program and computing of "Least Significant Difference (L.S.D.)" values to separate means in different statistical groups according to described method by[32] Gomez and Gomez (1984). All the observations were recorded in three replications and data were analyzed statistically for standard errors of means (SEM).

Results and Discussion

Effect of NaCl on viability percentage, average and concentration of total viability cells:

To study the effect of NaCl on growth of cassava suspension culture, cells were exposed to different concentrations of NaCl (50, 100, 150, 200, and 250 mM). The cell viability was estimated by direct cell counting chamber in different time points 3, 6, 9, 12, and 15 days (Table 1 and 2).

Table (1): Effect of NaCl concentrations on cassava (*Manihot esculenta* Crantz) cell viability number and percentage after 15 days.

Treatments (A)	Timing (B)	Viable	Non-viable	Total	Viability (%)
Control	Initial	2.5 ±0.29	1.75 ±0.48	4.25 ±0.63	60.42 ±6.25
	3rd Day	14.50 ±3.97	7.25 ±2.93	21.75 ±6.73	74.75 ±8.99
	6th Day	7.25 ±1.70	7.75 ±4.03	15.00 ±3.67	54.46 ±16.23
	9th Day	6.25 ±1.84	2.00 ±1.22	8.25 ±2.59	77.19 ±14.42
	12th Day	19.25 ±4.55	3.50 ±2.02	22.75 ±4.52	85.52 ±8.56
	15th Day	5.50 ±0.29	1.00 ±0.00	6.50 ±0.29	84.51 ±0.78
50 mM	Initial	24.25 ±6.55	4.00 ±1.68	28.25 ±8.18	88.68 ±4.04
	3rd Day	14.50 ±3.97	7.25 ±2.93	21.75 ±6.73	74.75 ±8.99
	6th Day	18.50 ±0.87	3.00 ±0.41	21.50 ±1.19	86.18 ±1.32
	9th Day	17.25 ±2.69	8.00 ±1.96	25.25 ±2.53	68.16 ±7.98
	12th Day	23.00 ±3.14	11.25 ±1.70	34.25 ±1.97	66.43 ± 6.36
	15th Day	2.50 ±0.29	2.00 ±0.00	4.50 ±0.29	55.00 ±2.89
100 mM	Initial	27.00 ±4.38	4.75 ±2.17	31.75 ±4.09	85.05 ±6.82
	3rd Day	17.75 ±1.44	11.75 ±1.80	29.50 ±2.47	60.51 ±3.55
	6th Day	21.00 ±1.68	6.75 ±1.03	27.75 ±1.03	75.40 ±4.33
	9th Day	9.75 ±2.50	8.00 ±1.87	17.75 ±4.17	54.29 ±4.57
	12th Day	12.50 ±2.53	17.75 ±2.32	30.25 ±3.79	40.88 ±5.58
	15th Day	9.50 ±3.28	6.00 ±1.58	15.50 ±4.25	60.13 ±9.54
150 mM	Initial	21.75 ±4.91	6.75 ±0.75	28.50 ±5.12	74.40 ±4.49
	3rd Day	9.75 ±2.50	7.25 ±1.60	17.00 ±3.03	54.66 ±9.02
	6th Day	11.00 ±3.34	4.75 ±2.50	15.75 ±5.72	74.35 ±4.69
	9th Day	15.50 ±3.66	8.25 ±1.70	23.75 ±4.46	62.62 ±8.66
	12th Day	11.00 ±4.42	13.50 ±2.60	24.50 ±5.33	42.20 ±9.01
	15th Day	8.25 ±3.35	8.00 ±3.34	16.25 ±6.52	50.63 ±4.87
200 mM	Initial	13.00 ±1.08	3.00 ±0.58	16.00 ±0.91	81.10 ±3.69
	3rd Day	7.25 ± 1.89	2.50 ±0.87	9.75 ±2.39	71.88 ±8.57
	6th Day	7.50 ±1.44	6.00 ±0.71	13.50 ±1.32	54.34 ±6.78
	9th Day	9.50 ±1.85	7.75 ±3.15	17.25 ±4.42	58.31 ±6.45
	12th Day	11.50 ±0.96	9.75 ±4.29	21.25 ±5.19	60.26 ±9.09
	15th Day	8.50 ±1.89	8.25 ±2.69	16.75 ±3.38	53.54 ±9.20
250 mM	Initial	13.00 ±6.01	0.75 ±0.48	13.75 ±6.83	94.27 ±3.93
	3rd Day	11.00 ±2.74	5.75 ±2.39	16.75 ±5.12	73.51 ±9.15
	6th Day	5.00 ±1.68	8.50 ±2.18	13.50 ±3.59	35.00 ±7.97
	9th Day	9.50 ±2.25	7.50 ±1.44	17.00 ±2.16	53.63 ±10.58
	12th Day	11.25 ±0.95	7.25 ±2.10	18.50 ±2.99	63.08 ±5.31
	15th Day	3.25 ±0.63	6.75 ±3.66	10.00 ±4.26	44.85 ±10.86
LSD $_{0.05}$	**A**	3.56	2.56	5.22	9.34
	B	3.39	2.40	4.64	8.62

Error bars are ±SD

Table (2): Effect of NaCl concentrations on cassava (*Manihot esculenta* Crantz) average and concentration of total viability cells after 15 days

Treatments (A)	Timing (B)	Average Number of Cells/Square	Concentration of Viable Cells /ml
Control	Initial	0.69 ±0.06	13000 ±1224.74
	3rd Day	3.63 ±0.99	72500 ±19843.13
	6th Day	1.81 ±0.43	36250 ±8508.57
	9th Day	1.56 ±0.46	31250 ±9213.89
	12th Day	4.81 ±1.14	96250 ±22764.65
	15th Day	1.38 ±0.07	27500 ±1443.38
50 mM	Initial	6.06 ±1.61	121250 ±32747.46
	3rd Day	3.63 ±0.99	72500 ±19843.13
	6th Day	4.63 ±0.22	92500 ±4330.13
	9th Day	4.31 ±0.67	86250 ±13443.55
	12th Day	5.75 ±0.78	115000 ±15679.07
	15th Day	0.63 ±0.07	12500 ±1443.38
100 mM	Initial	6.75 ±1.09	135000 ±21889.88
	3rd Day	4.44 ±0.36	88750 ±7180.70
	6th Day	5.25 ±0.42	105000 ±8416.25
	9th Day	2.44 ±0.62	48750 ±12479.15
	12th Day	3.13 ±0.63	62500 ±12665.57
	15th Day	2.38 ±0.82	47500 ±16393.60
150 mM	Initial	5.44 ±1.23	108750 ±24526.77
	3rd Day	2.44 ±0.62	48750 ±12479.15
	6th Day	2.75 ±0.84	55000 ±16708.28
	9th Day	3.88 ±0.92	77500 ±18314.38
	12th Day	2.75 ±1.10	55000 ± 22079.40
	15th Day	2.06 ±0.84	41250 ±16754.97
200 mM	Initial	3.25 ±0.27	65000 ±5400.62
	3rd Day	1.81 ±0.47	36250 ±9437.29
	6th Day	1.88 ±0.36	37500 ±7216.88
	9th Day	2.38 ±0.46	47500 ±9242.11
	12th Day	2.88 ±0.24	57500 ±4787.14
	15th Day	2.13 ±0.47	42500 ±9464.85
250 mM	Initial	3.25 ±1.50	65000 ±30069.36
	3rd Day	2.75 ±0.68	55000 ±13693.06
	6th Day	1.25 ±0.42	25000 ±8416.25
	9th Day	2.38 ±0.56	47500 ±11273.12
	12th Day	2.81 ±0.24	56250 ±4732.42
	15th Day	0.81 ±0.16	16250 ±3145.76
LSD 0.05	**A**	0.97	19317
	B	0.85	16941

Error bars are ±SD

The NaCl tolerant cassava cell lines obtained in this study were selected from callus cultures initiated from stem explants. The direct recurrent selection procedures were successful in selection of cassava cell lines exhibiting tolerance to 200 mM NaCl. Data showed that cassava cell viability have been affected by sodium chloride treatment comparing to the previous growth curve in normal condition. The high concentration of NaCl 200 and 250 mM decrease the viable cell number one fold comparing to lower concentrations of NaCl and control sample (Fig. 1). Surprisingly at 50, 100 and 150 mM NaCl we found that the number of viable cells was higher than the control sample (zero NaCl) (Fig. 1). However, during 15 days under NaCl stress conditions data showed that viable cell number of 250 mM NaCl was very low comparing to remaining concentrations in whole experiment. While, after 3, 6, and 9 days the growth cell value was almost same and lower than the initial

inculcation of cassava cells (Fig 2), (Table 1, 2). The cell viability in 12 days under NaCl stress shows high tolerance against salt stress and the cell numbers also higher comparing to other NaCl concentrations (Fig 2), (Table 1, 2). The suspension cell growth data revealed that the biomass accumulation of cassava plant *Manihot esculenta* Crantz was less than the normal callus on solidified media, while under salt stress condition cassava growth curve showed a good improvement especially under 50, 150 mM NaCl. However, the previous in vitro study reported that this plant can tolerate severe drought stress based on its strong and deep root system, also cassava plant have a robust detoxification enzyme system to break the toxicity of the reactive oxygen species ROS which produced from stress condition[33]. So, may be those cells responded to salt stress by the same way to tolerate drought stress. However, the higher concentration like 200, 250 mM NaCl cassava cell viability and number has decreased after 12 and 15 days (Fig. 1, 2 and 3) (Table 1, 2). That may indicate to the best time for us to harvest the cells before starting their cell death program for further analysis. Similar trends were found by [34] reported that the growth profile of control and 85.5mM cells were similar its ability to resist to 85.5mM NaCl can be on evidence that culture might have salt tolerance features in citrus cell suspension culture. Moreover, [35] reported that increasing NaCl resulted in reduction of growth: 50 mM delayed maximum biomass; 100 mM imposed a severe decline in biomass; 150 mM resulted in biomass loss and cell viability loss in *Pinus pinaster* suspension cells. Also, [36] indicated that NaCl significantly reduced cell viability of all genotypes of safflower compared to the control. While [37] suggested that a treatment with the higher concentration of NaCl caused rapid cell death during this time in tomato cell suspension culture. However [38] found that the treatment of 100 mM NaCl reduced the number of viable cells over a 24 h period in wheat whereas the barley cells did not exhibit any marked change.

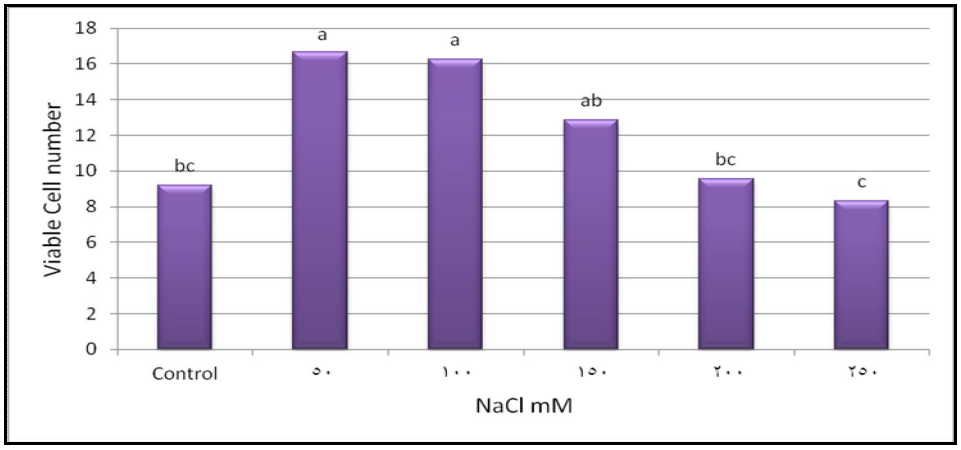

Fig (1): Effect of different NaCl concentrations on of cassava (*Manihot esculenta* Crantz) cells suspension viability numbers, derived from stem explants on MS medium plus vitamins supplemented with 8mg/l 2,4-D after 15 days.

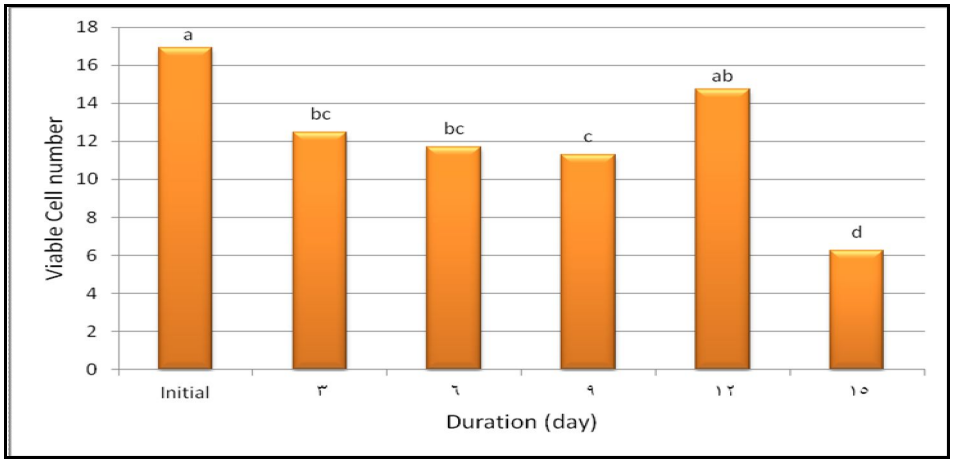

Fig (2): Effect of NaCl treatments on cassava (*Manihot esculenta* Crantz) cells suspension viability during 15 days, derived from stem explants on MS medium plus vitamins supplemented with 8mg/l 2,4-D after 15 days.

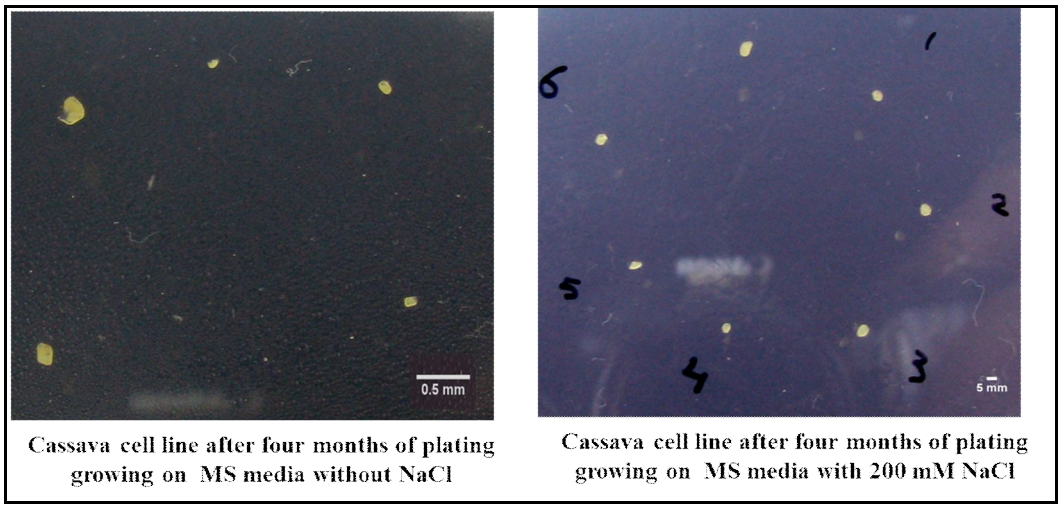

Fig (3): Cassava cell lines colonies growing under control and 200 mM NaCl

Influence of NaCl on intracellular ion concentrations (Sodium, potassium, calcium and chloride (Na^+, K^+, Ca^{2+} and Cl^-)

To compare the ionic status in NaCl stressed cassava cell culture, Na^+, K^+ Ca^{2+} and Cl^- content were determined in oven-dried cells after exposing the cell culture to different concentration of sodium chloride (50, 100, 150, 200 and 250 mM) for 15 days. Data showed that Na^+ concentration in stressed cells were about 5 to 10 times higher than the control either in suspension culture. Accumulation of Na^+ was observed to be higher in 100 and 250 mM NaCl concentrations as compared to 50, 150, 200 mM NaCl concentrations in cell culture throughout the experiment. In 15 days of NaCl exposure, cassava cells decreased the accumulation of Na^+ under 200 mM NaCl which was similar 50 mM NaCl concentration (Table 3). K^+ content was marginally decreased in most of stressed samples comparing to controls when subjected to NaCl exposure. However, at 50, 100 and 200 stress cells of cassava exhibited higher K^+ content as compared to 150 and 250 mM NaCl as well as control (40%). Accordingly, Na^+/K^+ ratio under 200 and 50 mM NaCl remained lower than that in 100, 150 and 250 mM NaCl concentrations. In 250 mM Na^+/K^+ ratio was 2.7% and in 150 mM was 2.2% and 1.9% in 100 mM NaCl stressed cells after 15 days, however, 50 mM and 200 mM NaCl exhibited 0.8 at the same time period (Table 3). Chloride content was again found to be higher in 50 mM and 250 mM NaCl stressed cassava cells as compared to 100, 150, 200 mM NaCl, also cassava callus which was grown in solidified medium showed higher accumulation of chloride similar to 50 mM NaCl concentration, however differences were obvious. After 15 days of NaCl stress, cassava accumulated 60% less Cl^- in 200 mM NaCl, which was little pet similar to zero NaCl (control) (Table 3). Ca^{2+} content date showed high accumulation in 100, 250 mM NaCl and it decreased gradually tell became lesser under 200 mM NaCl after 15 days of stress excuser. The zero NaCl (control) accumulation of Ca^{2+} was very much similar to 200 mM (Table 3). Data presented above on ionic status suggested that 200 mM NaCl accumulated less Na^+, Cl^- and Ca^{2+} and maintained better K^+ in comparison to other NaCl stress cell samples. The ion homeostasis data of cassava (*Manihot esculenta* Crantz) cell culture under NaCl stress showed that the sodium and potassium accumulation increased very much under lower concentrations of NaCl and gradually decrease in higher concentration. This data may be reveal the behavior of cassava cells to eliminate accumulation of Na^+ and K^+ in the in the cells through its detoxification enzymatic system until the NaCl reach 250 mM the cell could not tolerate more Na and k even the viability cell number has been decreased under 250 mM NaCl. This view gathers support from the studies of [39] who reported that NaCl tolerant cell lines accumulate higher amount of Na^+ and Cl^- ions than NaCl sensitive cell lines in sour orange cultures. However, [40](Ghoulam et al. 2002) found that sugar beet cultivars accumulated high concentrations of Na^+, K^+ and Cl^- in the leaves under salt stress. Also, [41](Summart et al. 2010) found that in rice cells accumulated high level of Na^+ during stress, whereas the accumulation of K^+ and Ca^{2+} was decreased. Exclusion or inclusion of Na^+, Cl^-, K^+, and Ca^{2+}, antioxidant enzymes and malondialdehyde concentration play a key protective role against salt stress in tomato callus-cultured [38]. Contrary, increased levels of NaCl increased the intracellular concentrations of Na^+ and K^+ increased with decreased Na^+ while, Cl^- did not show any important change in Medicago cultures[42]. Also, [36] Soheilikhah et al. (2013) indicated that the accumulation of Na^+ ions and osmolytes could play an important role in osmotic adjustment in safflower cells under saline

stress. Furthermore, increasing K/Na ratios in the growing media of date plam significantly reduced the absorption of Na^+ less than 200 mM and also balance ions compartmentalization [43]. This could indicate to triggering the programmed cell death in cassava cells after 15 days of stress. The synthesis of secondary metabolites in plant suspension culture is part of the defense responses of plants to all kind of stresses. Salt stress may act as an elicitor of plant defense response to stimulate the secondary metabolite synthesis [44]. [45] have hypothesized that when plants are stressed, an exchange occurs between carbon to biomass production or formation of defensive secondary compounds. A stress response is induced when plants recognizes stress at the cellular level. Secondary metabolites are involved in protective functions in response to both biotic and abiotic stress conditions.

Table (3): Effect of different concentrations of NaCl on ions uptake (Na^+, K^+, Ca^+, Cl^-) on Cassava cell suspension culture after 15 days.

Treatments	Mineral Contents (ppm)					Proline (ug/g)
	Na	K	Ca	Na/K	Cl	
Control	142 ±8^{f}	2611 ±14^{d}	1428 ± 16^{d}	0.05 ±0.003^{e}	194 ±6.16^{d}	1.03±0.0^{d}
50 mM	3575 ±197^{e}	4159 ±125^{b}	1917 ±74^{c}	0.86 ±0.022^{d}	630 ±8.46^{a}	2.65±0.9^{d}
100 mM	7851 ± 97^{b}	4187 ±60^{b}	2266 ±86^{b}	1.88 ±0.004^{c}	268 ±5.00^{c}	7.30±0.4^{c}
150 mM	5948 ±181^{c}	2684 ±105^{d}	2060 ±160^{c}	2.22 ±0.020^{b}	265 ±10.66^{c}	9.50±0.98^{c}
200 mM	4196 ±115^{d}	5033 ±177^{a}	1379 ±16^{d}	0.83 ±0.007^{d}	201 ±1.51^{d}	27±1.79^{a}
250 mM	9982 ±326^{a}	3648 ±105^{c}	2624 ±116^{a}	2.74 ±0.011^{a}	367 ±10.56^{b}	16.55±1.16^{b}

Treatments that are not significantly different at the 5% level are indicated by the same letters in the same column. Error bars are ±SE

Proline Content:

Proline content in the selected cell line is presented in Table (3) and proline contents were significantly ($p \leq 0.05$) affected with an increase in the concentration of NaCl. However, there is a positive relationship between salt tolerance and proline content in in cassava cultures up to 200 mM NaCl stress and the highest proline content (27 ug/g) was observed in cell line under 200 mM NaCl compared with control (1.03 ug/g). supporting results were found by [46] who reported that proline content has been widely use as parameter of cell lines selection for salt tolerance. The positive relationship between salt tolerance cell and proline accumulation has been provide in different plants, potato[47], tomato[48,49], Canola[50], Sunflower[51]. Furthermore,[52] in tobacco, indicated that proline accumulation increased gradually with increasing concentrations of NaCl. Also,[53] found that in Jerusalem artichoke plantlets were observed to accumulate proline in roots, stems and leaves during salt stress. On other hand, [40] indicated that the proline contents could not be used as a reliable criterion for screening sugar beet varieties for salt tolerance.

Isoenzymes activity:

Data illustrated in Fig (4) shows that the activities of all of these enzymes were increased on 200 mM NaCl treatment compared to control. However, the peroxidase, glutathione reductase and glutathione peroxidase activities of different cassava cell line under salt stress (control and 200 mM NaCl) were presented in Figure (4). It showed that there was an important change in peroxidase and glutathione peroxidase activities cassava cell line under 200 mM NaCl stress compared to control while less change was observed in glutathione reductase activity and 200 mM NaCl and control treatments. Basically , drought, salt, and cold stress all induce the accumulation of ROS such as superoxide, hydrogen peroxide, and hydroxyl radicals also, stimulate the accumulation of compatible osmolytes and antioxidants[54]. However, the primary effects of salt stress are the enhanced accumulation of reactive oxygen species (ROS) that are harmful to plant cells at high concentrations [55]. When there is an imbalance (either by abiotic or biotic reasons) in the cellular compartment between ROS production and antioxidant defense, there is dramatic physiological challenges which results in oxidative stress[9]. A defensive system called the antioxidant enzyme system is also activated under stress conditions including several ROS-scavenging enzymes such as superoxide dismutase, peroxidase, glutathione reductase, monohydroascorbate reductase, ascorbate peroxidase and catalase[56]. Moreover, failure of the antioxidant defense system may result in oxidative damage to several cell constituents such as proteins, enzymes, DNA and membrane lipids[57,58]. Our results are supported by the findings of [59] indicated that NaCl alters the peroxidase

activity of cassava cuttings cultivated *in vitro*. Also,[52] hypothesis that glutathione reductase is a key element in the evaluation of salinity tolerance of tobacco varieties. Furthermore, [60] found that salt stress led to significant increases, much more pronounced in ascorbate peroxidase (APX), glutathione reductase (GR) and guaiacol peroxidase (GPX) in cowpea. On other hand,[61] cleared that guaiacol specific peroxidase was significantly elevated glutathione reductase and catalase less increased under salt stress in French bean. Also, [62] reported that the enzymatic defense mechanism against salt stress might be a vital selection marker for some species but not for others.

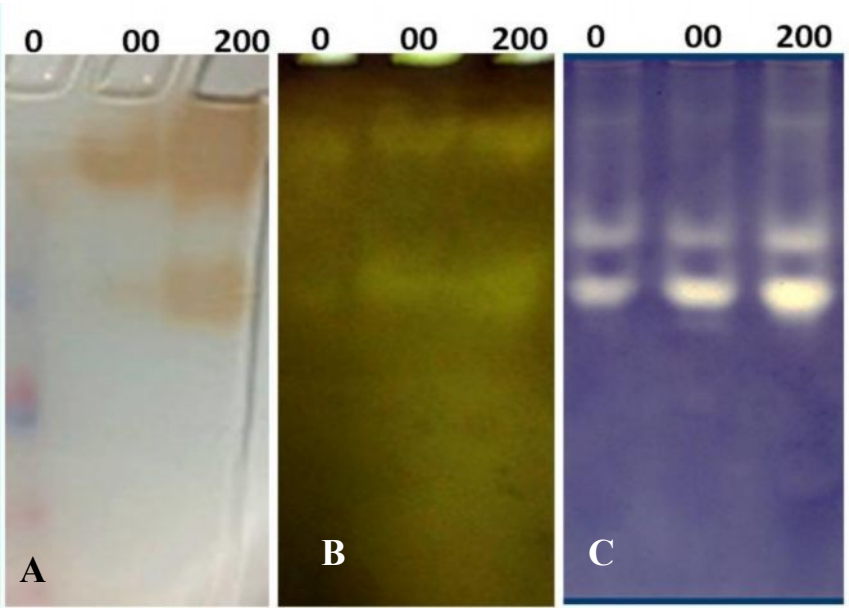

Fig (4): Detoxification enzyme activities in control cells growing on normal conditions & selected cassava cell line grown under 200mL NaCl stress treatment. Twelve days old suspension cells were either grown on 200 mM NaCl or in normal complete MS medium. (A) POX, (B) GPX and (C) SOD. (0) is cassava callus grown under solid MS media without salt. (00) is cassava suspension cells grown under lequid MS media without salt. (200) is the selected cassava cell line grown under MS media plus 200 mL NaCl.

Conclusions

We can concluded that in Cassava suspension culture we can realize that viability of cell under 200 mM NaCl stress after 15 day will be the perfect time to isolate and identify the intercellular and extracellular protein or/and peptides which could be produced abundantly.

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References

1. El-Sharkawy, M. A. Cassava biology and physiology. Plant Molecular Biology, 2004, 56(4): 481–501.
2. Sakurai, T., Plata, G., F. Rodrıguez-Zapata, F., Seki, M., Salcedo, A., Toyoda, A. Ishiwata, A., Tohme, J., Sakaki, Y., Shinozaki, K. and Ishitani, M. Sequencing analysis of 20,000 full-length cDNA clones fromcassava reveals lineage specific expansions in gene families related to stress response. BMC Plant Biology, 2007, 7(66):1-17.
3. Lebot, V. Tropical root and tuber crops. CABI . CAB International. Oxford-shire. UK. 2009.
4. Munns, R. Comparative physiology of salt and water stress. Plant Cell Environ., 2002, 25: 239-250.

5. Gupta, B. and Huang, B. Mechanism of Salinity Tolerance in Plants: Physiological, Biochemical, and Molecular Characterization, International Journal of Genomics, 2014 (1):701596.
6. Hernández, J.A., Jiménez, A., Mullineaux, P., Sevilla, F. Tolerance of pea (*Pisum sativum* L.) to long-term salt stress is associated with induction of antioxidant defences. Plant Cell Environ., 2000, 23: 853-862.
7. Zhu, J. K. Salt and drought stress signal transduction in plants. Ann Rev Plant Biol., 2002, 53:247-273.
8. Khan, M. N., Siddiqui, M. H., Mohammad, F., Naeem, M. and Khan, M. A. Calcium chloride and gibberelic acid protect Linseed *(Linum usitatissimum*) from NaCl stress by inducing antioxidative defence system and osmoprotectant accumulation. Acta Physiol. Plan., 2010, 32:121-132.
9. Amoako, S., Yahaya, A. and Sarfo, J. K. Catalase activity of cassava (*Manihot esculenta*) plant under African cassava mosaic virus infection in Cape coast, Ghana. African J Biotech., 2015,14 (14): 1201-1206.
10. Li, D., Li, C., Sun, H., Wang, W., Liu, L., Zhang, Y. Effects of drought on soluble protein content and protective enzyme system in cotton leaves. Front. Agric. China, 2010, 4: 56-62.
11. Ge, T. D., Sui, F. G., Bai, L.-P., Lu, Y.-Y., Zhou, G. S. Effects of water stress on the protective enzyme activities and lipid peroxidation in roots and leaves of summer maize. Agric. Sci. China, 2006, 5: 291-298.
12. Davenport, S.B., Gallego, S.M., Benavides, M.P., Tomaro, M.L. Behaviour of antioxidant defense system in the adaptive response to salt stress in *Helianthus annuus* L. cells. - Plant Growth Regul., 2003, 40: 81-88.
13. Smith, C. A., Melino, V. J., Sweetman, C. and Soole, K. L. Manipulation of alternative oxidase can influence salt tolerance in Arabidopsis thaliana. Physiologia Plantarum, 2009, 137: 459–472.
14. Sheveleva, E., Chmara, W., Bohnert, H. J. and Jensen, R. G. Increased salt and drought tolerance by D-ononitol production in transgenic *Nicotiana tabacum* L. Plant Physiol., 1997, 115: 1211-1219.
15. Seki, M., Narusaka, M., Abe, H., Kasuga, M., Yamaguchi-Shinozaki, K., Carninci, P., Hayashizaki, Y. and Shinozaki K. Monitoring the expression pattern of 1300 Arabidopsis genes under drought and cold stresses by using a full-length cDNA microarray. Plant Cell, 2001, 13: 61-72.
16. Zhu, J., Jiang, H. L., Du, X. Y., Wang, J., Xu, W. X. and Liu, S. F. Preparation and characterization of hCG-loaded polylactide or poly(lactide-co-glycolide) microspheres using a modified water-in-oil-in-water (w/o/w) emulsion solvent evaporation technique. J. Microencapsul. 2001, 18: 247-260.
17. Tausz, M., Wonisch, A., Grill, D., Morales, D. and Jimenez, MS. Measuring antioxidants in tree species in the natural environment: from sampling to data evaluation. J Exp Bot., 2003, 54: 1505-1510.
18. Szabados, L. and Savouré, A. Proline: a multifunctional amino acid. Trends in Plant Science, 2010, 15, (2): 89-97.
19. Delauney, A. J. and Verma, D. P. S. Proline biosynthesis and osmoregulation in plants. Plant J., 1993, 4: 215-223.
20. Ochatt, S. J., Marconi, P. L., Radice, S., Arnozis, P. A., Caso, O. H. In vitro recurrent selection of potato: production and characterization of salt tolerant cell lines and plants. Plant Cell, Tissue and Organ Culture, 1999, 55: 1-8.
21. Zhu, J. Plant salt tolerance. Trends in Plant Science, 2001, 6(2):66-71.
22. Gu, R., Liu, Q., Pei, D., Jiang, X. Understanding saline and osmotic tolerance of *Populus euphratica* suspended cells. Plant Cell Tissue Organ Cult., 2004, 78: 261-265.
23. Rao, S. and Patil, P. In vitro selection of salt tolerant calli lines and regeneration of salt tolerant plantlets in mung bean (*Vigna radiata* L. Wilczek) in Biotechnology - Molecular Studies and Novel Applications for Improved Quality of Human Life (Sammour, R. H. Ed.). InTech, Croatia. 2012.
24. Carretero, C. L., Cantos, M., García, J. L. and Troncoso A. In vitro–ex vitro salt (NaCl) tolerance of cassava (Manihot esculenta Crantz) plants. In Vitro Cell. Dev. Biol. Plant, 2007, 43:364–369.
25. Absher, M. Hemocytometer counting. In:P. F. Kruse, Jr., and M. K. Patterson, Jr.(Eds.), Tissue Culture Methods and Applications. Academic Press, New York, 1973, pp.395-397.
26. Bradford, MA rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 1976, 72: 248-254.
27. Larkindale, J. and Huang, B. Thermotolerance and antioxidant systems in *Agrostis stolonifera*: involvement of salicylic acid, abscisic acid, calcium, hydrogen peroxide, and ethylene. J. Plant Physiol., 2004, 161: 405-413.
28. Trotel, P., Bouchereau, A., Niogret, M. F. and Larher, F. The fate of osmo-accumulated proline in leaf disc of rape (*Brassica napus* L) incubated in a medium of low osmolarity. Plant Sci., 1996, 118: 31-45.

29. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophageT4. Nature, 1970, 227: 680-685.
30. Rucinska, R., Waplak, S. and Gwozdz, E. Free radical formation and activity of antioxidant enzymes in lupin roots exposed to lead. Plant Physiol. Biochem. 1999, 37: 187-194.
31. Kho, C. W., Park, S. G., Lee, D. H., Cho, S., Oh, G. T., Kang, S. and Park, B. C. Activity staining of glutathione peroxidase after two-dimensional gel electrophoresis. Mol. Cell., 2004, 18: 369-373.
32. Gomez, K. A. AND Gomez A. A. Statistical Procedures for Agricultural Research, Wiley and sons, New York, 1984.
33. Reilly, K., Gómez-Vásquez, R., Buschmann, H., Tohme, J., Beeching, J. R. Oxidative stress responses during cassava post-harvest physiological deterioration. Plant Mol Biol, 2004, 56: 625-641
34. Lima-Costa, M.E., Ferreira, A.L. Duarte, A., Beltrão J. Saline stress and cell toxicity evaluation using suspended plant cell cultures of horticultural crops grown in a bioreactor. Acta Horticulturae, 2002, 573(573):219-225.
35. Azevedo, H., Amorim-Silva, V. and Tavares, R. M. Effect of salt on ROS homeostasis, lipid peroxidation and antioxidant mechanisms in Pinus pinaster suspension cells. Annals of Forest Science, Springer Verlag/EDP Sciences, 2009, 66 (2), 1-9.
36. Soheilikhah, Z., Karimi, N., Ghasmpour, H. R. and Zebarjadi, A. R. Effects of saline and mannitol induced stress on some biochemical and physiological parameters of *Carthamus tinctorius* L. varieties callus cultures. AJCS, 2013, 7(12):1866-1874.
37. Poór, P. and Tari, I. Ethylene-regulated reactive oxygen species and nitric oxide under salt stress in tomato cell suspension culture. Acta Biologica Szegediensis, 2011, 55(1):143-146.
38. Dong, J., Bowra, S. and Vincze, E. The development and evaluation of single cell suspension from wheat and barley as a model system; a first step towards functional genomics application. BMC Plant Biology, 2010, 10:239, 1-12.
39. Storey, R. and Walker, R. R. Citrus and salinity. Sci. Hort., 1999, 78: 39-81.
40. Ghoulam, C. Ahmed Foursy, A. and Khalid Fares, K. Effects of salt stress on growth, inorganic ions and proline accumulation in relation to osmotic adjustment in five sugar beet cultivars. Environ Exper Bot., 2002, 47 (1):39-50.
41. Summart, J., Thanonkeo, P., Panichajakul, S., Prathepha, P. and McManus, M. T. Effect of salt stress on growth, inorganic ion and proline accumulation in Thai aromatic rice, Khao Dawk Mali 105, callus culture. African J Biotech., 2010, 9 (2): 145-152.
42. Al-Rawahy, S. H. and Farooq, S. A. Influence of intracellular Na^+, K^+ and Cl^- on the salt tolerance in suspension cell cultures of Medicago media. African J Biotech., 2012, 11(20): 4499-4512.
43. Alkhateeb, S. A., Alkhateeb, A. A. and Solliman, M. E. In vitro response of date palm (*Phoenix dactylifera* L.) to K/Na ratio under saline conditions. Biol. Res., 2015, 48 (63):2-9.
44. Thakur M, Sohal BS. Role of elicitors in inducing resistance in plants against pathogen infection, 2013(1): 762412, 10 pages.
45. Bryant JP, Chapin FSI, Klein DR. Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. Oikos. 1983, 40:357- 68.
46. Kavi Kishor, P. B., Hong, Z., Miao, G. H., Hu, C. A. A., Verma, D. P. S. Overexpression of Δ^1-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. Plant Physiol., 1995, 108: 1387-1394.
47. Martinez, C.A., Maestri, M. and Lani, E.G. In vitro salt tolerance and proline accumulation in Andean potato (*Solanum* spp.) differing in frost resistance. Plant Sci., 1996, 116: 177-184.
48. Hernandez S, Deleu C and Larher F. Proline accumulation by leaf tissues of tomato plants in response to salinity. Life Sciences, 2000, 323: 551-557.
49. Mohamed, A.N., Rahman, M.H., Alsadon, A.A. and Islam, R. Accumulation of Proline in NaCl-treated Callus of Six Tomato (Lycopersicon esculentum Mill.) Cultivars. Plant Tissue Cult. & Biotech., 2007, 17(2): 217-220.
50. Xue, X., Liu, A., Hua, X. Proline accumulation and transcriptional regulation of proline biothesynthesis and degradation in Brassica napus. BMB Reports, 2009, 42: 28–34.
51. Shahbaz, M., Ashraf, M., Akram, N. A., Hanif, A., Hameed, S., Joham, S. and Rehman, R. Salt induced modulation in growth, photosynthetic capacity, proline content and ion accumulation in sunflower (*Helianthus annuus* L.). Acta Physiologiae Plantarum, 2011, 33: 1113–1122.
52. Çelik Ö.and Atak Ç. The effect of salt stress on antioxidative enzymes and proline content of two Turkish tobacco varieties. Turk J Biol. 2012, 36: 339-356.

53. Huang, Z., Zhao, L., Chen, D., Liang, M., Liu, Z., Shao, H., Long, X. Salt Stress Encourages Proline Accumulation by Regulating Proline Biosynthesis and Degradation in Jerusalem Artichoke Plantlets. PLoS ONE, 2013, 8(4): 1-11.
54. Hasegawa, P.M., Bressan, R.A., Zhu, J.K., and Bohnert, H.J. Plant cellular and molecular responses to high salinity. Annu. Rev. Plant Mol. Plant Physiol., 2000,51: 463- 499.
55. Pang, C.-H. and Wang, B.-S. Oxidative Stress and Salt Tolerance in Plants. In: Lüttge, U., Beyschlag, W. & Murata, J. (eds.) Progress in Botany. Berlin, Heidelberg: Springer Berlin Heidelberg, 2008.
56. Habib, S. H., Kausar, H. and Saud, H. M. Plant Growth-Promoting Rhizobacteria Enhance Salinity Stress Tolerance in Okra through ROS-Scavenging Enzymes. BioMed Research International, 2016, 6284547, 10 pages.
57. Asada, K. The water–water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons. Annual Review of Plant Physiology and Plant Mol., Biol., 1990, 50, 601– 639.
58. Mittler, R. Oxidative stress, antioxidants and stress tolerance. Trends in Plant Science, 2002, 7: 405-410.
59. Lima, G.P.P., Fernandes, A.A.H., Catâneo, A.C., Cereda, M.P., & Brasil, O.G. Alterações na atividade da peroxidase e do conteúdo de carboidratos em mandioca cultivada in vitro sob estresse salino. Scientia Agricola, 1998, 55(3): 413-417.
60. Praxedes, S. C., Damatta, F. M., De-Lacerda, C. F., Prisco, J. T., Gomes-Filho, E. Salt stress tolerance in cowpea is poorly related to the ability to cope with oxidative stress. Acta Bot. Croat., 2014, 73 (1), 51- 62.
61. Babu, N. R. and Devaraj, V. R. High temperature and salt stress response in French bean (*Phaseolus vulgaris*). Australian Journal of Crop Science, 2008 2, 40-48.
62. Abogadallah, G. M. Antioxidative defense under salt stress. Plant Signaling and Behavior, 2010, 5, 369–374.

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