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Original Research Article

In Vitro Salt Stress Induced Enhancement of Ascorbic Acid in *Emblica Officinalis*

Priyanka[1], Upendra Kumar[2]*, Mohammad Ishaq Rather[1], Deepti[1] and Y Vimala[1]

[1]Stress Physiology and Plant Tissue Culture Laboratory, Department of Botany, Ch. Charan Singh University, Meerut INDIA.

[2]Molecular Cytogenetics Laboratory, Department of Molecular Biology & Genetic Engineering College of Basic Sciences & Humanities, G.B. Pant University of Agriculture & Technology Pantnagar (Udham Singh Nagar) Uttarakhand, INDIA.

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Abstract: *Emblica officinalis* is highly nutritious and important dietary source of vitamin C, minerals and amino acids. It also contains phenolic compounds, tannins, phyllembelic acid, phyllemblin, rutin, curcuminoides and emblicol callus was raised from cotyledonary explant on Murashige and Skoog (M S) medium supplemented with different concentrations of auxin and cytokinin. Callus initiation and Best callus growth was observed on 1.0 mg/l 2, 4-Diphenoxy acetic acid (2, 4-D). Callus was transferred from 1.0 mg/l 2, 4-D to different concentrations of Sodium Chloride (NaCl) i.e. 50, 100, 150, 200 mM NaCl. The effect of NaCl on proline, protein, phenolics and ascorbic acid were observed after four weeks. Addition of salt (50 mM) led to decrease in protein and proline but increase in phenolics and ascorbic acid content. Further increase in NaCl (100,150 and 200 mM) led to increase in protein, proline, phenolics and ascorbic acid content. Interestingly, ascorbic acid content in the salt (200 mM) treated calli was approximately as much as reported in fruits *in vivo*.

Keywords: *Emblica officinalis,* proline, protein, phenolics, ascorbic acid and salt

Introduction

Emblica officinalis (E. officinalis), syn: *Phyllanthus emblica*, also known as amla belongs to the Euphorbiaceae family. *E. officinalis* has been reported to possess potential antioxidant effect (Liu *et al*., 2008). In folk medicine, all parts of the plant, including fruit, seed, leaf, root, bark and flower are used in various Ayurvedic/Unani herbal preparations (Habib-ur- Rehman *et al*., 2007). Amla is highly nutritious and could be important dietary source of vitamin C, minerals and amino acids. It also contains phenolic compounds, tannins, phyllembelic acid, phyllemblin, rutin, curcuminoides and emblicol (Zhang *et al*., 2000; Jeena *et al.,* 2001)

During salinity induced oxidative stress, several cytotoxic reactive oxygen species (ROS) are continuously generated in the mitochondria, peroxisomes and cytoplasm, which can destroy the normal metabolism through oxidative damage of lipids, proteins and nucleic acids (Apel and Hirt, 2004; Foyer and Noctor, 2005; Turkan and Demiral, 2009). ROS mainly comprises of superoxide radicals (O^{2-}) hydrogen Peroxide (H_2O_2), hydroxyl radicals ($OH^{\bullet}$) and singlet oxygen ($^1O_2$).

Under salt-stress, plants restrict the uptake of salt and adjust their osmotic pressure by the synthesis of compatible organic solutes. Compatible solutes are low molecular weight, highly soluble compounds that are usually nontoxic at high cellular concentrations. These solutes include proline, sucrose, polyols, trehalose and quaternary ammonium compounds (QACs) such as glycine betaine, alaninebetaine, prolinebetaine, choline O-sulfate, hydroxyprolinebetaine and pipecolatebetaine (Ashraf and Foolad, 2007).

Since amla cultivation is spreading to ecologically different parts of the country, technology development needs careful attention to suit the widespread requirements. The research and development programme on amla is fairly weak in the country. Lack of scientific knowledge regarding amla, restricts its market. In future, demand of amla will grow manifold, as now its attributes are known to the international community. The main research

***Corresponding Author:**
Dr. Upendra Kumar,
Molecular Cytogenetics Laboratory,
Department of Molecular Biology & Genetic Engineering,
College of Basic Sciences & Humanities, G.B. Pant University of Agriculture & Technology,
Pantnagar (Udham Singh Nagar), Uttarakhand, INDIA.

in the country almost has been production oriented. There is need to have improved products through technology intervention.

Plant cell cultures synthesize and produce several valuable compounds of industrial and pharmaceutical significance (Havkin- Frankel *et al*., 1976). The objective of this work was to investigate the merit of using *in vitro* culture for enhancement of ascorbic acid for commercial purpose.

Materials and Methods

Cotyledons of *E. officinalis* were taken from the seeds of mature fruits. The cotyledons were directly inoculated on nutrient medium under sterilized conditions of laminar flow bench. Standard techniques of media preparation, inoculation and incubation were followed. Throughout, callus was reared on Murashige and Skoog (1962) medium supplemented with various plant growth regulators (PGR). Cultures were maintained at 25 ± 2°C in continuous light of 1400 lux intensity. The observations were taken after fixed time intervals.

Free proline content was determined according to Bates *et al.,* (1973). 200 mg of fresh callus was homogenized in 4 ml of 3% aqueous sulphosalicylic acid and centrifuged at 10 000 g for 10 min to remove debris. 2 mL of the supernatant was mixed with 2 mL of acid ninhydrin (625 mg ninhydrin in 15 ml glacial acetic acid and 10 mL of 6 M orthophosphoric acid) and 2 mL glacial acetic acid in a test tube and boiled at 100 ^{0}C for 1 h. The reaction was stopped by cooling the tubes in an ice bath. The red coloured chromophore formed was extracted with 6 ml of toluene and the absorbance of the resulting organic layer was measured at 520 nm (Shimadzu UV-1800). The concentration of proline was estimated by referring to a standard curve prepared using L-proline.

Ascorbic acid (AsA) was extracted in 5 % (m/v) metaphosphoric acid, at 4 °C. The homogenate was then centrifuged at 3000 *g* for 20 min at 4^{0}C. AsA content was quantified in the supernatant as described by Shukla *et al.* (1979). In brief, an aliquot of 1 ml of the sample was mixed with 2.5 ml of 1 % (v/v) freshly diluted Folin-Ciocalteu reagent. The reaction mixture was allowed to stand at room temperature for 40 min. The absorbance was recorded at 730 nm, using ascorbic acid as a standard.

Bradford method (Bradford, 1976) was used to determine the amount of protein. Bradford reagent was prepared by using 500 mg Coomassie Brillant Blue G-250 in 250 ml 95 % ethanol and 500 ml 85 % phosphoric acid. Finally the solution was diluted to 1 l and filtered. The prepared reagent was 5X. It was diluted to 1X before each use. From each sample 20 ml was taken and put into a test tube. 480 ml of distilled water was added onto the extract. 5 ml of 1X Bradford reagent was put onto the diluted sample. After 10 minutes of incubation at room temperature the absorbances were measured at 595 nm by Schimadzu UV-1800 spectrophotometer against blank of 500 ml water and 5ml Bradford reagent. For the protein standard curve Bovine Serum Albumin (BSA) was used.

Total Phenolic content was determination by (Bray and Thorpe, 1954). For this, 50.0 mg fresh plant material, dried over filter paper, was homogenized in mortar and pestle with small amount (2.0 ml) of 80% ethanol. The supernatant was re-extracted with five volumes of 80% ethanol; the supernatant was evaporated to dryness and the residue was dissolved in DW upto a final volume of 5ml. Varying volumes of aliquots (0.2-1.0 ml) were dispensed and diluted with distilled water upto 1.0 ml. 0.5ml Folin and Ciocalteu's reagent was added. After 3 minutes 20% Na_2CO_3 was added and mixed thoroughly in boiling water for exactly one minute, cooled and measured the absorbance at 650nm against blank (3.0ml DW+ 0.5ml Folin and Ciocalteu's reagent + 2.0ml 20% Na_2CO_3). Total Phenolic content was expressed in mg Cinnamic acid equivalents per g fresh weight of tissue with the help of a previously prepared calibration curve.

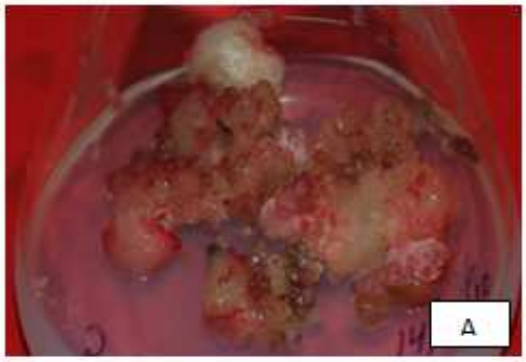
Effect of 2, 4-D and NaCl on four week old callus of *E. officinalis*

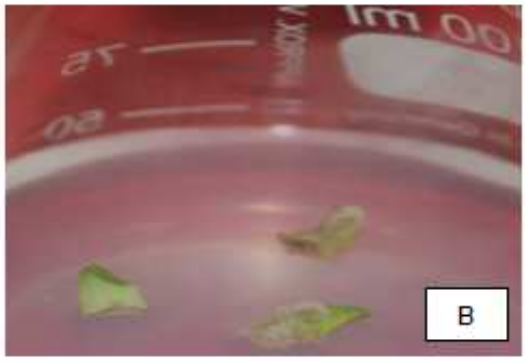
S.No.	MS + 3% sucrose + (mg/l) PGRs	SALT (NaCl)	Callus Growth	Morphology of callus
1.	1 2,4-D	-	Very good	Pinkish, friable
2.	1 2,4-D	50mM	Good	Pinkish, friable
3.	1 2,4-D	100mM	Good	Greenish, friable
4.	1 2,4-D	150mM	Good	Greenish, friable
5.	1 2,4-D	200mM	Good	Pinkish, friable

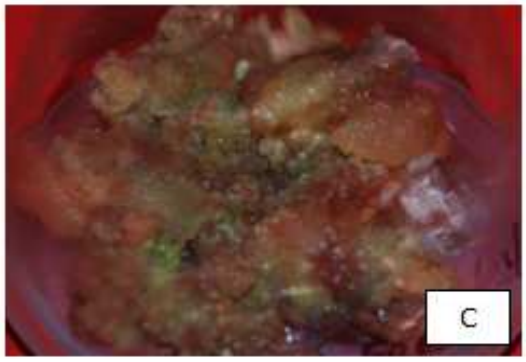
Effect of 2, 4-D on protein, proline, phenolics and ascorbic acid content of four weeks old callus of *E. officinalis* subcultured on NaCl supplemented MS medium

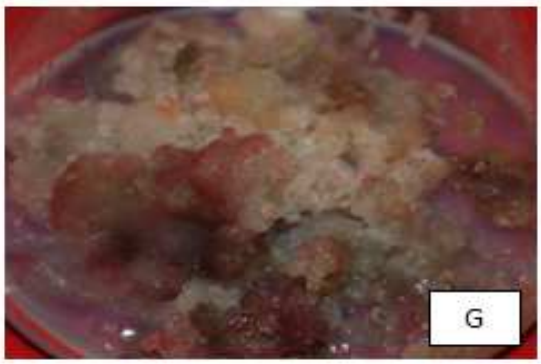
S.No.	MS + 3% sucrose + (mg/l) PGRs	NaCl	Protein (mg BSA eq/gfw±SD)	Proline (mg proline /gfw±SD)	Phenolics X1000(mg cinnamic acid eq/gfw±SD)	Ascorbic Acid (µg asc. acid eq/gfw±SD)
1.	1 2,4-D	-	2.830 ± 0.026	4.952±0.88	16.533 ± 1.760	0.460 ± 0
2.	1 2,4-D	50mM	1.717 ± 0.040	2.898±1.04	22.789 ± 0.716	1.173 ± 0.018
3.	1 2,4-D	100mM	3.235 ± 0.065	5.672±1.450	25.0633 ± 1.579	1.132 ± 0.021
4.	1 2,4-D	150mM	3.115 ± 0.020	7.312±0.224	58.258 ± 2.545	1.242 ±0.005
5.	1 2,4-D	200mM	3.359 ± 0.027	5.496±0.655	84.192 ± 5.55	1.547 ± 0

Plate 1: A, Callus induction from cotyledonary explant on MS + 2,4-D (1.0 mg/l)); B, callus on MS + 2,4-D (1.0 mg/l); C, callus on MS + 2,4-D (1.0 mg/l) + 50 mM NaCl; D, callus on MS + 2,4-D (1.0 mg/l) + 100 mM NaCl; E, callus on MS+2,4-D (1.0 mg/l)+150 mM NaCl; and F, callus on MS + 2,4-D (1.0 mg/l) + 200 mM NaCl.









Results and Discussion

Callus formation appeared by swelling of explants and followed by proliferation of cells. The sub-epidermal cells were stimulated to show hypertropical growth. Later, inner cells were also involved in the formation of callus. Best callus initiation and growth were observed on 2, 4-D (1.0 mg/l) alone (with colours like pink and green). Callus was transferred from 1.0 mg/l 2, 4-D to different

salt concentrations i.e. 50, 100, 150, 200 mM NaCl. The effect of salt stress on proline, protein, phenolics and ascorbic acid were observed after about four weeks.

Proline accumulation is a common metabolic response of higher plants when subjected to salt stress, and has been the subject of numerous works. In the present study addition of salt (50 mM) led to decrease in proline content. But further addition of 100 mM, 150mM and 200 mM NaCl led to increase in proline content. Proline content has been reported to increase under NaCl stress in *Phaseolus aureus* (Misra and Gupta, 2005), *Morus Alba* (Ahmad *et al*., 2007), *Sesamum indicum* (Koca *et al.,* 2007). Proline has several functions during stress: e.g. osmotic adjustment (Voetberg and Sharp 1991), osmoprotection (Kishor *et al*., 2005), free radical scavenger and antioxidant activity (Sharma and Dietz, 2006). A positive correlation between magnitude of free proline accumulation and salt tolerance has been suggested as an index for determining salt tolerance potentials between mulberry cultivars (Ramanjulu and Sudhakar, 2000).

Plant antioxidant defense systems and their roles in protecting plants against stresses have attracted considerable interest. The present study showed that AsA content of the *Emblica officinalis* callus notably increased upon salinity stress treatments, especially at 200 mM NaCl. The highest values were recorded in salt treated calli, while the lowest values were in case of non-treated calli. These results seem to support the hypothesis that tolerance to oxidative stress plays an important role in adapting plants to adverse environmental conditions (McKersie *et al.,* 1996; Smirnoff, 1998). The same trend was reported by Yu and Liu (2003) also in wild soybean. The increased AsA content is a stress-protecting mechanism of plants under salinity conditions (Shalata *et al.,* 2001). A high level of endogenous AsA is essential for maintaining the non-enzymatic scavenging system that protects plants from oxidative damage due to salinity stress (Shigeoka *et al.,* 2002).

Proteins that accumulate in plants under saline conditions may provide a storage form of nitrogen that is re-utilized later (Singh *et al.,* 1987) and may play a role in osmotic adjustment. Protein was accumulated at increasing concentration of NaCl in all concentrations except 50 mM NaCl compared to control. A higher content of soluble proteins has been observed in salt tolerant cultivars of barley, sunflower, finger millet, and rice (Ashraf and Harris, 2004). They may be synthesized *de novo* in response to salt stress or may be present constitutively at low concentration (Pareek-Singla and Grover, 1995). It has been concluded that a number of proteins induced by salinity are cytoplasmic which can cause alterations in cytoplasmic viscosity of the cells (Hasegawa *et al*., 2000).

The effect of NaCl on total phenol is shown in Figure 1, NaCl at 200mM resulted in the highest content of total phenol which was 4-fold and 3-fold higher than control. Phenol accumulation could be a cellular adaptive mechanism for scavenging oxygen free radicals during stress (Mohamed and Aly, 2008). Several studies have reported that total phenol production is stimulated by NaCl (Hanen *et al*., 2008; Muthukumarasamy *et al*., 2000).

Conclusion

Undebatable increase in all the four biochemical compounds indicates their variable contributions in defense against salt stress. Whereas protein and proline create an environment in defense pathways to function, phenolics and ascorbate perform the function of neutralizing the effect of the free radicals and other oxidants in different compartments of the cell.

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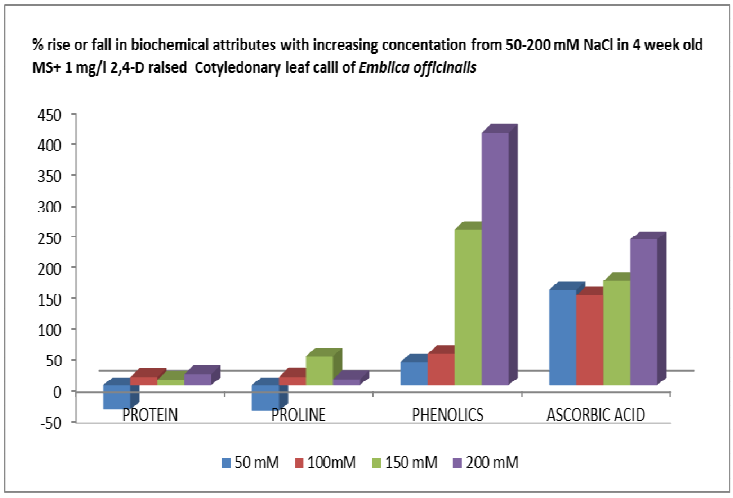

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