

RESEARCH ARTICLE

Salinity and age induced upscaling of biochemical attributes in callus cultures of *Urena lobata* L.

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Abstract : The present study was founded on callus induction, antioxidant activity and thermostable secondary metabolite profiling in the callus. The experiment was conducted to study the effect of sodium chloride (NaCl) at concentrations of 50mM, 100mM, and 150mM on the callus culture of *Urena lobata*, an important wild medicinal plant. Callus cultures were initiated from stem explants on Murashige and Skoog (MS) medium supplemented with 1mg/l 2,4-D and 2mg/l kinetin. Callus was subcultured on the same medium with different salt concentrations for 4, 6, and 8 weeks. The studied growth parameters showed that mass productivity and Growth Index of callus decreased under increasing salt concentration. However, moisture content increased at 150mM salt compared to the control. The protein, proline, organic carbon, phenolic, sodium and calcium content too, increased under salt treatments, especially at higher concentrations, but total nitrogen and potassium decreased. Thus, Phenolics (non-nitrogenous secondary metabolites) accumulated at all the concentrations of the salts tested, as an induced protective response in *Urena lobata*. These results suggest that in *Urena lobata*, proline accumulation is an index of salinity tolerance through osmo protection along with accumulation of non-nitrogenous defence compounds, phenolics (antioxidant), which are up scaled during salinity tolerance response.

Keywords: Salinity, Phenolics, Proline, 2, 4-D and Callus.

Introduction

Abiotic factors like temperature, drought, salt stress, etc. result in the degradation or reduction in a large amount of food production in today's world (Miflin, 2000). Salinity is considered to be a major environmental factor limiting plant yield (Flowers and Flowers 2005), with the prediction that around 20% of the irrigated land in the world is affected by salinity, and it is expected that increase in salinization in agriculture fields will reduce the land available for cultivation by 30% by 2035, and up to 50% by the year 2050 (Rozema and Flowers 2008). Moreover, salinity is responsible for the degradation of 2 million ha of agricultural land every year (Cicek and Cakirlar 2008). High salt concentrations in the soil of the root zone limit the productivity of nearly 953 mha of productive land in the world alone (Singh 2009). Asia has the second-largest area under salinity in the world, with a 6.73 m ha area under salinity and sodicity in India

(Singh 2009). In India nearly 40% land can be classified under wasteland. Wastelands are considered high-soil salinity regions with low fertility and extremely limited water supply (Panghal and Soni 2013). An under-explored wild medicinal plants. *Urena lobata* L. (Malvaceae) is selected for study of salinity induced biochemical attributes. This is a herb, widely distribute annual herb annually throughout the world, including Africa, Asia, and South America (Babu *et al.* 2016) and has vast medicinal properties. The plant is commonly known as caesar weed or congo jute. The *U. lobata* has been used as a traditional medicinal plant in India and China (Gao *et al.* 2015), known for its diuretic and febrifuge properties. The plant contain anti-diarrhoeal, hepatoprotective, anti-diabetic, antioxidant, cytotoxic, and antifertility activity (Yadav and Tangpu 2007, Ali *et al.* 2013, Omonkhua and Onoagbe 2011, Lissy *et al.* 2006, Islam *et al.* 2017). Leaves of this plant contain active constituents like alkaloids, flavonoids, saponins, phenolic compounds, and tannins (Pharmacognosy 1962, Islam and Uddin 2017,

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Mathappan *et al.* 2010).

The use of plant cell and tissue culture methodology as a means of producing medicinal metabolites has a long history (Rout *et al.* 2000, Verpoorte *et al.* 2002). The study of stress/recovery response contributes to a better understanding of the plant's ability to adapt to different environments and climatic conditions. The plant tissue culture techniques can be used as an important tool to study the salt stress response of callus culture to salinity in controlled and uniform environment conditions (Bajji *et al.* 1998, Queiros *et al.* 2007), thus avoiding complications arising from physiological and structural variability of whole plant (Bajji *et al.* 1998, Elkahoui *et al.* 2005), and as a means of rapid selection and improvement for salinity tolerance.

Materials and methods

Plant material, callus induction, and growth media conditions

Stem (nodal) explants from plants grown in the Botanical Garden of the Department of Botany, CCSU, Meerut, were collected and surface sterilized by 0.1% mercuric chloride (Hi-media, Mumbai, India) along with 1% bavistin (BASF, India) for 4-5 minutes followed by three rinses in sterilized distilled water under a laminar airflow cabinet (Ansari and Vimala 2022). The sterilized explants were grown on callus induction medium consisting of Murashige and Skoog (1962) basal medium (Hi Media Laboratory Pvt Ltd, Nashik, India) adjusted at pH 5.8, supplemented with 2,4-D (1.0)mg/L and Kn (2.0) mg/L. (Sigma -Aldrich), and then autoclaved for 20 min at 121°C and 15 psi. The sterilized explants were placed on the medium and incubated in a growth chamber under 16/8 hr. light/dark PAR (Photosynthetically active radiation) at a temperature of 25±2°C for callus induction (Ansari and Vimala 2022).

In vitro salt stress treatments

Callus was induced from stem nodal explants cultured on MS medium supplemented with 1.0 mg/L 2, 4-D, and 2.0 mg/L Kn (Ansari and Vimala, 2022). The calli were subsequently transferred onto MS medium containing the same concentration of growth regulators with four different

concentrations of NaCl: 0 mM (control), 50 mM, 100 mM, and 150 mM for 4, 6, and 8 weeks to record potential for salt tolerance. After 4, 6, and 8 weeks of salt treatment, various physiological (PGR, RWC, GI, Fresh & Dry weight, and Moisture %) and biochemical attributes (total protein, Proline, Phenolics, Organic carbon, Flavonoid, Nitrogen, Phosphorus, Sodium, Potassium, calcium, and Potassium/Sodium ratio) traits were measured for validating the findings. 15 replicates were used for each treatment, and all experiments were repeated three times. Data were recorded periodically and the results were expressed as mean values in the table.

Physiological traits

RGR- Relative Growth Rate of callus was calculated as $(W_1 - W_0) / W_0 \times 100$, where W_0 is the initial callus fresh weight and W_1 was considered as the final fresh weight of callus after 4, 6, and 8 weeks of salt treatment (Errabii *et al.* 2007); RWC- Relative Water Content was calculated as $[(\text{callus fresh weight} - \text{callus dry weight}) / \text{callus dry weight} \times 100]$ (Lutts *et al.* 2004).

GI-Growth Index of the callus was calculated by the following formula as $[(\text{Weight/volume}) \times 100]$. Moisture percentage was calculated as $[(\text{Fresh weight} - \text{Dry weight}) / \text{Fresh weight} \times 100]$; Callus samples of known fresh weight were dried in an oven set at 65°C for 48 h, after which they were reweighted and the difference in the initial and final mass were determined; Dry mass productivity of callus after 4, 6, and 8 weeks of salt treatments ; Fresh weight of callus after 4, 6, and 8 weeks of salt treatments.

Biochemical parameters

Determination of Total Protein (Bradford *et al.* 1951)

100 mg of fresh tissue was homogenized in 5.0 ml of tris buffer pH 7.0 and centrifuged at 5000 rpm at 4°C. The supernatant served as protein extract. To 1.0 ml of this plant protein extract, 5.0 ml of Coomassie brilliant blue dye was added. The absorbance was recorded at A_{595} against blank.

Estimation of Proline content (Bates *et al.* 1973)

50 mg of fresh plant material was extracted in 10.0 ml of 3% sulpho-salicylic acid. The homogenate was filtered through whatman No.1 filter paper. 2.0 ml glacial acetic acid and 2.0 ml acid ninhydrin were mixed in 2.0 ml filtrate. 2.0 ml aliquot of the reaction mixture was heated in boiling water bath for 1 hour. Placing the test tubes in ice bath terminated the reaction. 4.0 ml toluene was added to the reaction mixture and stirred well for 20-30 seconds. The toluene layer was separated and warmed to room temperature. Intensity of red colour was measured at 520 nm.

Estimation of Total Phenolic content (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 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 5 ml. Varying volumes of aliquots were dispensed and diluted with distilled water up to 1.0 ml. 0.5 ml 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 absorbance was measured at 650 nm against blank.

Determination of Reducing, Non-reducing and Total sugar (Nelson 1944)

50 mg plant material was homogenized in 80% ethanol kept on water bath till the smell of alcohol was completely removed. The extract was centrifuged to get clear supernatant. To this 5 ml of saturated lead acetate was added drop by drop to precipitate tannins, proteins and other substances that possibly interfere in the determination of sugars. The precipitate is removed by centrifugation and to the supernatant 6.0 ml of saturated Na_2HPO_4 was added to remove excess of lead. The clear supernatant obtained after centrifugation was made to 8.0 ml, out of which 3.0 ml of this extract was used for determination of reducing sugars. Total sugar was determined after hydrolysis of 5.0 ml extract with 1.0 ml 1N HCl kept in boiling water for 20 minutes. The hydrolysed was cooled, pH was brought to 7.0 with 1N NaOH and the volume was made up to 8.0 ml with distilled

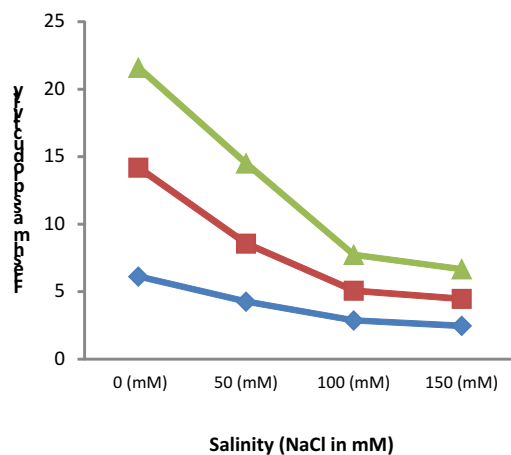


Figure 1: Effect of in-vitro NaCl concentrations on the fresh mass productivity of 4, 6, and 8 weeks callus

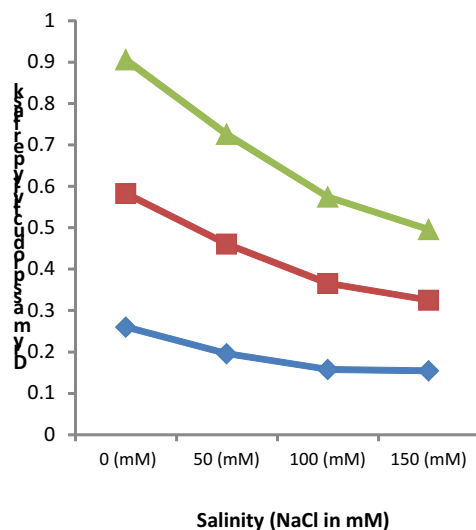


Figure 2: Effect of *in vitro* NaCl concentrations on the fresh mass productivity of 4, 6, and 8 weeks callus

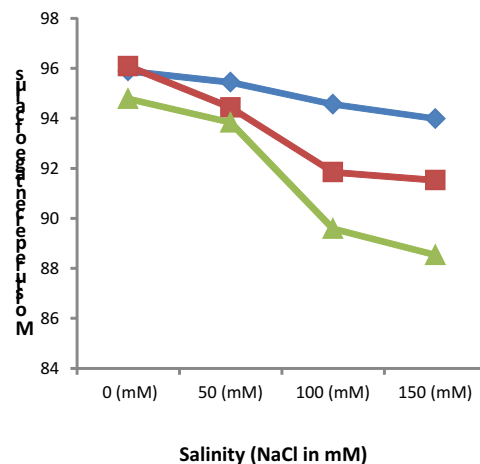


Figure 3: Effect of in vitro NaCl concentrations on the fresh mass productivity of 4, 6, and 8 weeks callus

water. 1.0 ml hydrolysate was mixed with 1.0 ml alkaline Copper tartarate. The tubes were kept in water bath for 20 minutes, cooled and 1.0 ml Arseno-molybdate was added. A blue colour developed and the absorbance of sample was read at 660 nm.

Estimation of Total Nitrogen (Snell and Snell 1967)

50mg of sample was taken and digested with appropriate amount of digestion mixture. The test tubes were kept on hot plate for 30 min. for digestion, test tubes were cooled at room temperature and 3.0 ml of 30% H_2O_2 was again added to it and kept for future digestion for 60 min. or till the digest became clear. Made total volume upto 10 ml with DDH_2O (double distilled water) and to 1.0 ml of digest 3.0 ml of Nessler's reagent and 1.0 ml distilled water were added. Nitrogen was calculated using Koch and Mc Meakin's formula and the colour developed was recorded at A_{425} .

Estimation of Organic Carbon (Datta *et al.* 1962)

1.0 g of sieved soil as well as the dried plant sample were taken in 100 ml conical flask and homogenized with 10 ml of 1 N $K_2Cr_2O_7$ solution followed by 20 ml of acidified silver sulphate and swirled again. Allowed the flask to stand for 30 min. and then centrifuged the contents to clear state. The supernatant was made to a constant volume with DW. A green chromium sulphate colour was developed and A_{660} was recorded using blank.

Estimation of Phosphorus (Olsen 1954)

1gm dried plant material or soil was taken in 100 ml flask a little Draco-G or activated charcoal was added followed by 20 ml, 0.5 M $NaHCO_3$. The flask was shaken for 30 minutes on a platform type shaker and the contents were filtered immediately through dry filter paper. From the filtrate 5.0 ml was used for further estimation, 5.0 ml filtrate was taken out in another flask, to this 5.0 ml molybdate reagent was added. Made total volume to 20 ml with DW and shake the contents thoroughly and added 1.0 ml $SnCl_2$ working solution. Made total volume to 25 ml with DW. After 10 minutes, absorbance of the samples were measured at A_{660} against blank.

Estimation of Total Flavonoids (Chang *et al.* 2002)

The total flavonoids content of the sample was determined using ammonium chloride colorimetric method with slight modifications. 500 mg sample was extracted with 10 ml 80% methanol in ultrasonicator at room temperature. The sample extract was filtered through Whatman No. 1 filter paper. The residue was re-extracted under the same condition. The combination of filtrates were evaporated on water bath at $60^\circ C$. 1 ml extract was separately mixed with 1.0 ml of methanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1M potassium acetate and 1.0 ml of distilled water. The mixture was allowed to stand at room temperature for 45 minutes. The absorbance of mixture was measured

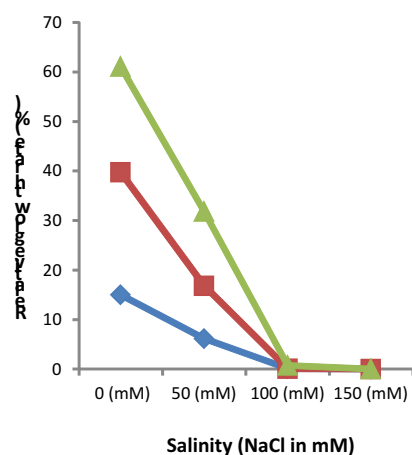


Figure 4 : Effect of in vitro NaCl concentrations on the fresh mass productivity of 4, 6, and 8 weeks callus

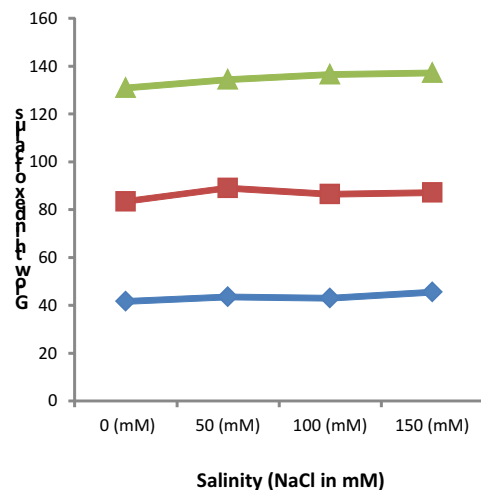


Figure 5: Effect of in vitro NaCl concentrations on the fresh mass productivity of 4, 6, and 8 weeks callus

at 415 nm using spectrophotometer.

Estimation of Potassium, Sodium and Calcium (Pratt and Fathi-Ettai 1990)

100 mg soil was homogenised in 10 ml of 1N/1M HCl, centrifuged, collected the supernatant and made total volume to 10 ml with 1N/1M HCl and was analysed for potassium against KCl solution (20ppm KCl) for sodium against NaCl solution (ppm) and for calcium against CaCl_2 with help of a Systronic Flame Photometer # 125.

Results and discussion

The callus induction took place after 45 days of inoculation on basal MS medium supplemented with 2mg/L 2,4-D + 1mg/L Kn. The callus was subcultured on the same medium just after callus initiation for 21 days for attaining suitable growth, followed by transfer over the same medium supplemented with NaCl (0, 50, 100, and 150 mM). The response to salt stress was noted in terms of fresh and dry weight, PGR, GI, and K^+/Na^+ ratio. The proliferation of callus exhibited a reduction in fresh and dry weight under maximum salt concentration (150 mM) as against non-salt-supplemented control sets. Maximum fresh and dry weight loss of fresh weight (upto 75%) and dry weight (upto 47%) occurred in 6-week-old callus. Interestingly, 4-week-old calli raised on added salt remained low in growth in terms of fresh and dry weight, RGR, GI, and K^+/Na^+ ratio but retained maximum moisture content (Figs. 1-6).

The decline in fresh and dry weight of callus in the presence of added NaCl may be due to osmotic stress-causing dehydration and reduction of growth due to the ionic toxicities, as also reported in several other plants (Murillo *et al.* 2006, Aqeel Ahmad *et al.* 2007, Mokhberdoran *et al.* 2009, Daneshmand *et al.* 2010, Zhao *et al.* 2007 and Lokhande *et al.* 2010).

The decline in fresh & dry weight of calli was found to be associated with reduced RGR and GI at high salt concentrations, i.e. 100 mM NaCl onward. K^+/Na^+ ratio was noted to be favourable in the absence of added NaCl and even an addition of 50 mM NaCl, led to a sharp decline in the K^+/Na^+ ratio which indicates induction of osmotic stress and

severe dehydration (Fig. 6).

Biochemical analysis

Protein content

Data analysis presents a significant difference between the protein content of salt-treated callus compared with untreated callus (control). The variations have been recorded after 4, 6, and 8 weeks of callus culture under 0, 50, 100, and 150 mM NaCl supplementation in the medium.

Salt leads to salting out of protein depending on ionic strength. In the present study, increasing the concentration of salt (NaCl) resulted in the accumulation of soluble protein and proline. The highest (4.79 mg/g FW) and the least (0.87 mg/g FW) contents of total proteins were observed in control at the 6th week and 150 mM NaCl in the 8 week, respectively. However, the amount of protein declined with the passage of time except in control by contrast (Fig. 7). A higher content of soluble proteins has been observed in salt-tolerant cultivars of barley, sunflower, finger millet, and rice (Ashraf and Harris 2004). Similar results were observed in terms of an increase in protein content in *B. juncea* (Mukhtar and Hasnain 1994), *Acanthophyllum* (Niknam *et al.* 2011), and *Broussonetia papyrifera* (Zhang *et al.* 2013), with an increase in the salinity.

In all the 12 independent experiments, the total protein content in control and stressed callus

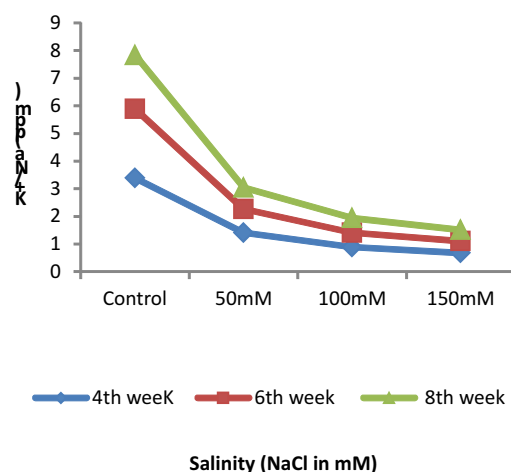


Figure 6: Effect of in vitro NaCl concentrations on the fresh mass productivity of 4, 6, and 8 weeks callus

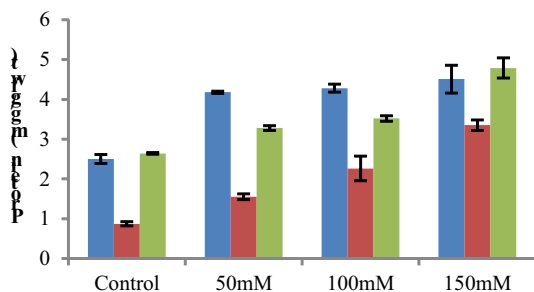


Figure 7: *In Vitro* effect of NaCl on protein in 4, 6, and 8 weeks old callus of *Urena lobata*

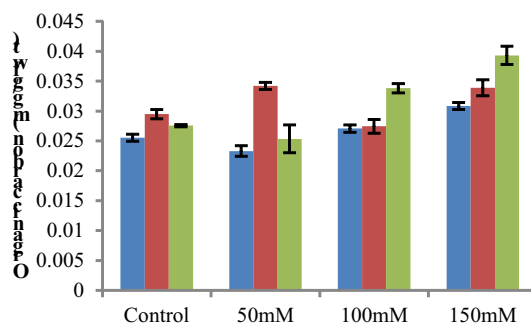


Figure 9: *In Vitro* effect of NaCl on organic carbon in 4, 6, and 8 weeks old callus of *Urena lobata*

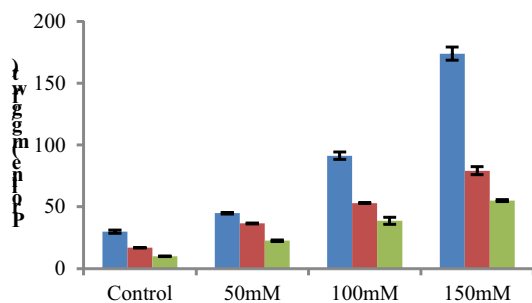


Figure 8: *In Vitro* effect of NaCl on proline in 4, 6, and 8 weeks old callus of *Urena lobata*

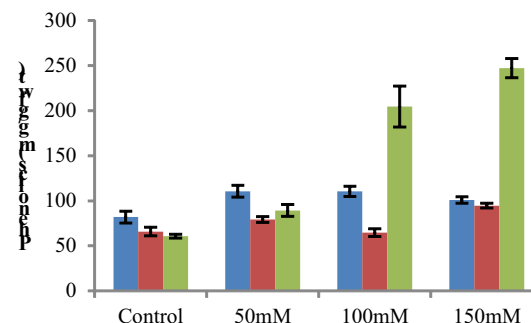


Figure 10: *In Vitro* effect of NaCl on phenolic in 4, 6, and 8 weeks old callus of *Urena lobata*

cultures was higher after the 8 week in comparison to the 4 and 6 weeks after inoculation.

Proline content

The analysis of variance showed a significant difference between time intervals, and salt treatments for proline content. In *U. lobata* callus, average proline content was found to be remarkably high at 150 mM NaCl compared to controls but it declined with time in all the sets maintaining a difference of 6 times from control. The highest proline content practically occurred at 150 mM NaCl, which indicates that salt stress was problematic for cellular functions at this point. Surprisingly, the concentration of proline increased 5 to 6 times at an elevated salt concentration. With the advancement of time (in weeks), the concentration of proline declined to 1/2 to 1/3 (Fig.8). From this, it is concluded that proline concentration decreases with increasing week, but increases with increasing salt treatment. At the lowest salt conc. used even 150 mM NaCl, shows need for osmoprotection. Proline acts as an osmotic agent; a protective agent of enzyme, cellular structure, and a storage compound for reducing

nitrogen for rapid regrowth after stress is relieved. It was also found that proline could react with hydroxyl radicals, thereby protecting lipids, DNA, proteins, and macromolecular structures from degradative reactions leading to cell destruction during salinity stress (Orthen et al., 1994). As observed earlier, proline supported the growth of salt-adapted callus. The positive effect on the growth with low levels of NaCl could be due to an increase in free proline as reported earlier in Indica rice callus cultures (Kavi, 1988, Reddy and Vaidyanath 1986). The lower levels of proline in the control callus may be due to an increased rate of degradation (Pandey and Ganapathy 1985). For instance, in calli of *Solanum nigrum*, exposure to the salinity stress levels resulted in a correlated enhancement of production of solasodine and proline for its tolerance (Sutkovic et al., 2011).

Our results are in agreement with those of Abraham et al., (2003), who reported that proline occurs widely in higher plants and accumulates in larger amounts than other amino acids. The enhanced proline level in the cultures may be due to an alteration in the amino acid pool (Yoshiba et al.,

1997). The saline growth conditions *in vitro* or *in vivo* include osmotic adjustment that involves the production and accumulation of cellular osmolytes (polyols, proline, sugar alcohols, pinitol, glucosinolates, and glycine betaine, etc.), soluble sugars (glutamate, sorbitol, mannitol, oligosaccharides, fructans, and sucrose, etc.) and amino acids (alanine, arginine, glycine, serine, leucine, and valine) together with the amino acid, proline, and the non-protein amino acids, citrulline, and ornithine). Amides (such as glutamine and asparagines) have also been reported to accumulate in plants subjected to salt stress (Mansour 2000; Parihar *et al.* 2015, Sytar *et al.* 2018, Omamt *et al.* 2006, Sujata and Kshitija 2013). Our results are in agreement with the finding in *Salvia officinalis* (Hendawy and Khalid 2005), *Trachyspermum ammi* (Ashraf and Orooj, 2006), Spearmint (Al-Amier and Craker, 2007), Chamomile and Sweet marjoram (Ali *et al.* 2007), *Catharanthus roseus* (Osman *et al.* 2007), *Achillea fragratissima* (Abd EL-Azim and Ahmed 2009), *Matricaria chamomilla* (Cik *et al.* 2009), *Sweet fennel* (Zaki *et al.* 2009) and *Satureja hortensis* (Najafi *et al.* 2010), *Pisum sativum* (Ahmad and Jhon 2005), *Portulaca oleracea* (Rahdari *et al.*, 2012), *Matricaria chamomilla* (Heidari and Sarani 2012), *Borago officinalis* (Enteshari *et al.* 2011), *Brassica juncea* var. Bio902, *Brassica juncea* var. Urvashi (Mittal *et al.* 2012), *Suaeda maritima* (Rajaravindran and Natarajan 2012) *Medicago sativa* (Ehsanpour and Fatahian, 2003), safflower (Soheilikhah *et al.* 2013), and *Oryza sativa* (Lui *et al.* 2000).

Organic carbon content

In *U. lobata* callus, organic carbon was found to increase dramatically with increasing salt and time. The highest organic carbon content practically occurred at 150 mM NaCl in the 8th week compared to the control in the 4th week. The concentration of organic carbon increased 1.5 times at an elevated salt concentration compared to control. In a comparison study, callus contains around half the quantity of organic carbon in plants. There was a significant difference between weeks and salt treatment for organic carbon (Fig. 9). The amount of soluble carbon assayed in extracts depends among others on the kind of extractant, time of extraction, sample moisture, the soil-extractant ratio as well as the method of organic

carbon assessing in the extracts (Zsolnay and Gorlitz 1994).

Phenolic content

In comparison to the control, the lower concentration of salt lowered growth, while the higher concentration enhanced phenolic content. However low NaCl treatment such as 50 mM, led to a decline in dry biomass and also phenolic content compared to control indicating promotion of primary metabolism and growth and as the NaCl concentrations were increased (100 to 150 mM) phenolic content increased whereas dry biomass decreased continuously up to 8 week (Fig. 10). In general, secondary metabolites have been reported to increase under salinity stress (Matkowski 2008). However, among phenolics, flavonoids in the present study have been found to decline. Thus, *Urena lobata* a wild medicinal plant has a strategy to accumulate phenolics other than flavonoids.

Fortunately, many of the stress-induced compounds are useful secondary metabolites. If added in appropriate concentration to culture

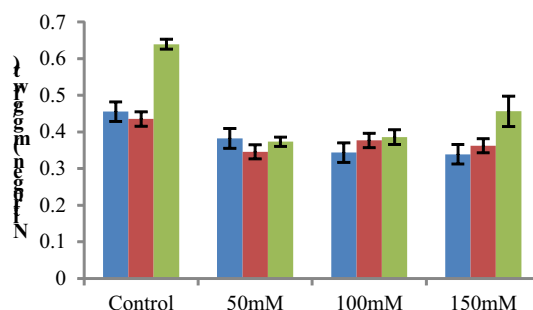


Figure 11: *In Vitro* effect of NaCl on nitrogen in 4, 6, and 8 weeks old callus of *Urena lobata*

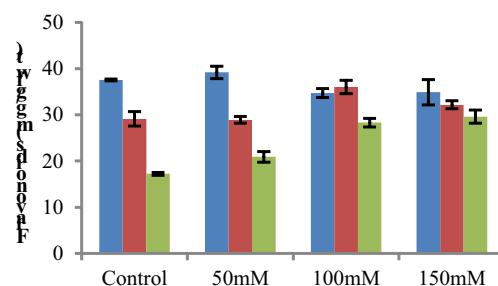


Figure 12: *In Vitro* effect of NaCl on flavonoids 4, 6, and 8 weeks old callus of *Urena lobata*

media, may enhance secondary metabolite accumulation in callus cultured *in vitro* and hence, can be used as factors for improving secondary metabolite production *in vitro*. For example, *in vitro* cultured plant cells were observed to synthesize extra amounts of soluble phenolics (Grace and logn, 2000), flavonoids (Chutipaijit *et al.* 2009) in response to various abiotic stress factors. Many studies have shown that changes in the levels of secondary metabolites, including phenolic compounds, enhance plant defence mechanisms against stress, particularly against oxidative stress induced by high salt concentrations (Matkowski 2008).

Nitrogen content

With increasing concentration of salt treatment, the amount of nitrogen in the callus dropped, while the amount of organic carbon increased. With passage of time from 4 to 8 weeks, the effect on nitrogen content in presence or absence of added salt was insignificant, however in control and in 150 mM NaCl treated sets the nitrogen content starkly increased which are in concurrence with increase in dry biomass (Fig. 11). Some nitrogen containing compounds like aspartate, betaine, choline, glycine, glutamate, proline, proteins, putrescine, 4-gamma aminobutyric acid (Kinnersley and Turano, 2000) although widely used as non-toxic food colorants, remain understudied in terms of their antioxidant potential (Gliszczynska-Swiglo *et al.* 2006; Kanner *et al.* 2001). Nearly 25% of the cultivable land around the world contains excessive amounts of salt, mainly NaCl (Shannon and Grieve 1999). Nitrogen, Phosphate, Potassium, and Sulfur-induced stresses influence the biosynthesis of phenylpropanoids and phenolics is reported in several plant species (Ramakrishna and Ravishankar 2011). The glutathione synthases are involved in nitrogen metabolism via ammonium assimilation which catalyzes the ATP-dependent biosynthesis of glutamine from glutamate and ammonia (Tullius *et al.* 2003).

Flavonoids

The total flavonoid content varied with different salinity treatments. In the 4 week, the highest flavonoids were observed at 50 mM, in the

6-week maximum at 100 mM, and in the 8-week maximum at 150 mM NaCl, suggesting that the flavonoid concentration adopts to salt level with time and hence rises with passage of time in higher concentration of salt treatment. Overall, in every treatment of salt the flavonoids were highest in 4 week callus compared to 8 week, at the same time in 8 week, concentration wise flavonoid contents were higher in higher salt concentration (150 mM NaCl). This is indicative of reducing requirement of flavonoids with passage of time in general for protection of callus. However, such decline in flavonoids is insignificant with passage of time, if the salt stress is high (Fig. 12). Flavonoids have defence and pigmentation functions in plant systems (Kondo *et al.* 1992, Halbrock and Scheel, 1989). These findings are in agreement with Ali and Abbas (2003) in Barley, Chutipaijit *et al.* (2009) in Indian rice, Louis *et al.* (1993) in *Cephalocereus senilis*, Matkowski (2004) in *Puaria lobata*, Farg *et al.* (2007) in *Medicago truncatula*.

Phosphorus

The total phosphorus content varies with different salinity treatments. Highest phosphorus concentration at 100 mM NaCl compared to control, 50mM and 150mM NaCl was recorded. Phosphorus decreased when salt levels increased. The lowest phosphorus accumulation was recorded in the 4 week in controls (Fig. 13).

Ionic content (Na⁺ and K⁺)

K⁺ ion reportedly play an important role in enzyme activation (Tester and Davenport 2003) although the relationship between K⁺ content and salt stress may vary from one species to another (Al-Khayri 2002). The results of the analysis of variance showed significant differences among the weeks and the significant effects of salt treatments on ion content (K⁺, Na⁺, and K⁺/Na⁺).

In the present study, increased salt at 150 mM (NaCl) led to a significant decrease in K⁺ concentration. Similar to these findings, K⁺ reportedly declined steadily in response to the increasing salt concentration of callus cultures (Chauhan and prathapasenan 2000, Basu *et al.* 2002, Gandonou *et al.* 2006, Lokhande *et al.* 2010; Soheilikhani *et al.* 2013). Other studies, however, have reported callus cultures to exhibit an initial

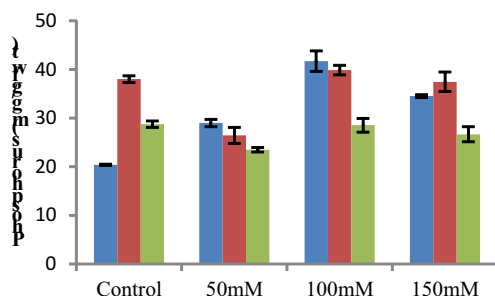


Figure 13: *In Vitro* effect of NaCl on phosphorus in 4, 6, and 8 weeks old callus of *Urena lobata*

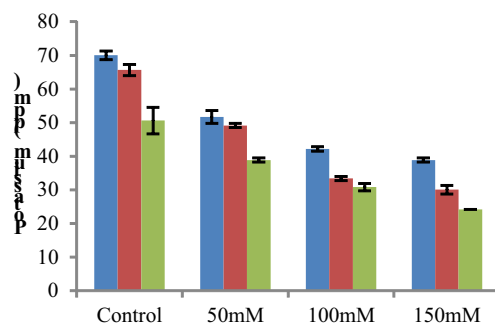


Figure 15: *In Vitro* effect of NaCl on potassium in 4, 6, and 8 weeks old callus of *Urena lobata*

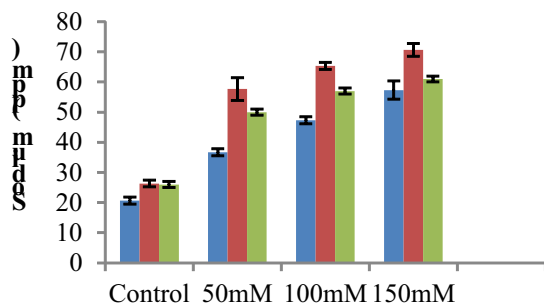


Figure 14: *In Vitro* effect of NaCl on sodium in 4, 6, and 8 weeks old callus of *Urena lobata*

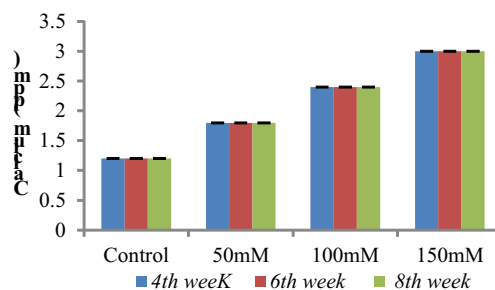


Figure 16: *In Vitro* effect of NaCl on calcium in 4, 6, and 8 weeks old callus of *Urena lobata*

increase in their K^+ levels in response to low NaCl levels (50 mM) which later declined steadily at higher NaCl levels (AL-Khayri 2002). The reduction in K^+ concentration in callus cells under salt stress could be explained by the alterations in expression and/or function of transporters as well as the ion channels especially those related to K^+ (Arzani and Ashraf 2016).

The patterns of K^+ content in response to increasing NaCl levels were parallel to the trends of callus growth and callus water-related traits. For instance, the highest callus growth was achieved with a culture medium with no NaCl, that is, the same concentration at which the highest K^+ uptake was observed. Furthermore, the inhibitory concentration of *Urena lobata* callus growth was identified to be 100mM NaCl, which is the same concentration at which potassium concentration significantly reduced relative to the control (Fig. 15).

Na^+ concentration in the callus was observed to increase significantly with increasing salt concentration. The sharpest increase in Na^+

concentration was observed when the callus was cultured on a medium containing 100 mM (Fig. 14). The rising trend of Na^+ concentration observed in this study was similar to those reported for such other plants as safflower (Soheilikhari *et al.* 2013), *Foeniculum vulgare* (Khorami and Safarnejad 2011), and date palm (Al-Khayri 2002).

Experimental NaCl concentrations (150 mM) concurrently led to an increase in plant Na^+ content although no significant changes were observed in the values of callus RGR. This suggests that the elevated uptake of Na^+ and K^+ might have led to water retention in the callus (Chaudhary *et al.*, 1997). Maintaining the cellular K^+/Na^+ homeostasis is pivotal for plant survival in a saline environment (Arzani and Ashraf 2016). As a result of increasing, Na^+ ions during salt stress for the transporter as they both share the same transporter mechanisms, thereby decreasing the uptake of K^+ . Accordingly, the K^+/Na^+ ratio was observed in this study to decrease significantly from the control to 150 mM NaCl. This result is in agreement with those reported elsewhere (Chaudhary *et al.* 1997, Al-

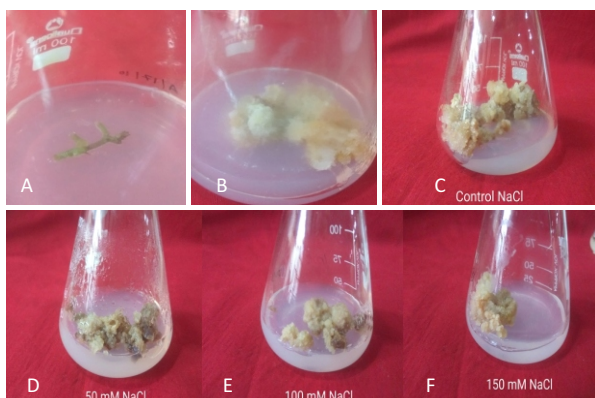


Figure 17: Effect of salt concentrations (0, 50, 100, 150 mM NaCl) on nodal callus of *Urena lobata*. A. Stem, B. Stem Callus, C-F. Nodal Callus subcultures at various salt concentrations.

Khayri *et al.* 2002). Since the K^+/Na^+ ratio is critical for salt tolerance, increasing this ratio to 150 mM NaCl in *Urena lobata* will be a promising area of future research. The genotypes examined were found to differ with respect to their intracellular ions under control conditions; all the genotypes, however, accumulated more Na^+ ions than did the control.

The imposition of NaCl-shock caused an injury to the tissue leading to excessive leaching and poor retention of K^+ in the callus in genotypes with lower salt stress tolerance, demonstrating a very sharp reduction in K^+ content with increasing salt from control to higher levels. The K^+ content and K^+/Na^+ ratio were found to be correlated with proline content in callus affected by salt stress. These results were in agreement with those of other researchers who reported a positive and significant correlation between K^+ content with K^+/Na^+ ratio (Basu *et al.* 2002, Ehsanpour and Fatahian 2003). According to Cherian and Reddy (2003), the reduction in K^+ concentration is capable of inhibiting growth as a result of reducing plant capacity for osmotic adjustment and turgor maintenance or by adversely affecting metabolic functions. In the current study, no significant relationship was found between K^+ and another physiological trait RGR (Fig. 4). On the other hand, a positive relationship was observed between Na^+ with some of these traits. It is expectable that the enhanced Na^+ content would not only disturb plant nutrient balance and osmotic regulation but also reduce growth (Gupta and Huang 2014).

Calcium

In *U. lobata* callus, average calcium content was found to increase dramatically with increasing salt at 4th, 6th, and 8th weeks respectively. The highest calcium content practically occurred at 150 mM NaCl. Surprisingly, the concentration of calcium increased 2 to 2.5 times at with elevated salt concentration every week. There was a significant difference between weeks and salt treatment for calcium accumulation, as reported in Fig. 11. Clearly, calcium content increased sharply in all weeks with the elevated concentration of NaCl. Plant exposed to NaCl salinity lose calcium as a particularly important nutrient because of its role in reducing Na^+ uptake and increasing both K^+ and Ca^{2+} uptake, thus increasing plant growth (Grattan and Grieve 1998, Munns *et al.* 2002). High Ca^{2+} concentrations can reduce the permeability of the plasma membrane to Na^+ and change cell wall properties that lead to reducing the Na^+ accumulation by passive influx (Vitart *et al.* 2001). Calcium is an important determinant for plant's salt tolerance that is particularly relevant to sodium and potassium homeostasis. Calcium plays an essential role in processes that preserve the structural and functional integrity of plant membranes (Tuna *et al.* 2007), stabilize cell wall structures (Neves and Bernstein, 2001), regulate ion transport and selectivity, and control ion-exchange behaviour as well as cell wall enzyme activities (Qiu *et al.* 2003, Ashraf and Harris 2004, Zhao *et al.* 2007).

Conclusion

The current investigation of salinity induced upscaling of proline and phenolic callus cultures of *Urena lobata* L. revealed that stem nodal callus cultures combat salinity in two ways: first, by osmo-protection of the cellular osmotic potential for maintenance of metabolic functions through the accumulation of phenolic content; and second, by reducing toxic principles such as ROS through the accumulation of phenolics with accruing age and salinity both. However, flavonoids maintain a high level during the 4 week and decline upto the 8 week, yet salinity-induced accumulation above controls is significantly high indicating UV-protection and other functions of flavonoids too. Thus, the major antioxidant role might be played by phenolics other than flavonoids in *Urena lobata* calli, making them

uniquely medicinally important. A higher growth index from the 4 to 8 week, which is not significantly affected by salt treatment, also makes phenolics play a major role in fighting detoxification of salt- induced toxicants without inhibiting growth.

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