J. Indian bot. Soc. (2023) Vol. 103 (1): 79-92
https://doi: 10.5958/2455-7218.2022.00053.5
e-ISSN:2455-7218, ISSN:0019 - 4468
www.indianbotsoc.org

RESEARCH ARTICLE

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

Farjana Ansari and Y. Vimala

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

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-diabeti, 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, Mathappan *et al.* 2010).

✉ Y. Vimala
yvimala17@gmail.com

Plant physiology and Biochemistry Lab, Department of Botany, C.C.S. University, Meerut, U.P, India-250004

Received : 22 August 2022 Accepted : 15 September 2022 Published online : 29 March 2023

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 8/16 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 with SD 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 [(callus fresh weight – callus dry weight)/callus dry weight × 100] (Lutts *et al.* 2004).

GI-Growth Index of the callus was calculated by the following formula as [(Weight/volume) × 100]. Moisture percentage was calculated as [(Fresh weight - Dry weight) / Fresh weight) × 100]; Callus samples of known fresh weight were dried in an oven set at 65ºC 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^{O}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 A_{520} against blank..

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 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 measuredat 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 of 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 1NHCl 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 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-molybdatewas added. A blue colour developed and the absorbance of sample was read at A_{660} against blank..

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 Nesseler'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} against blank..

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

1.0 g 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 color was developed and A_{660} was recorded using blank.

Estimation of Phosphorus (Olsen 1954)

1gm dried plant material 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 anther 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)

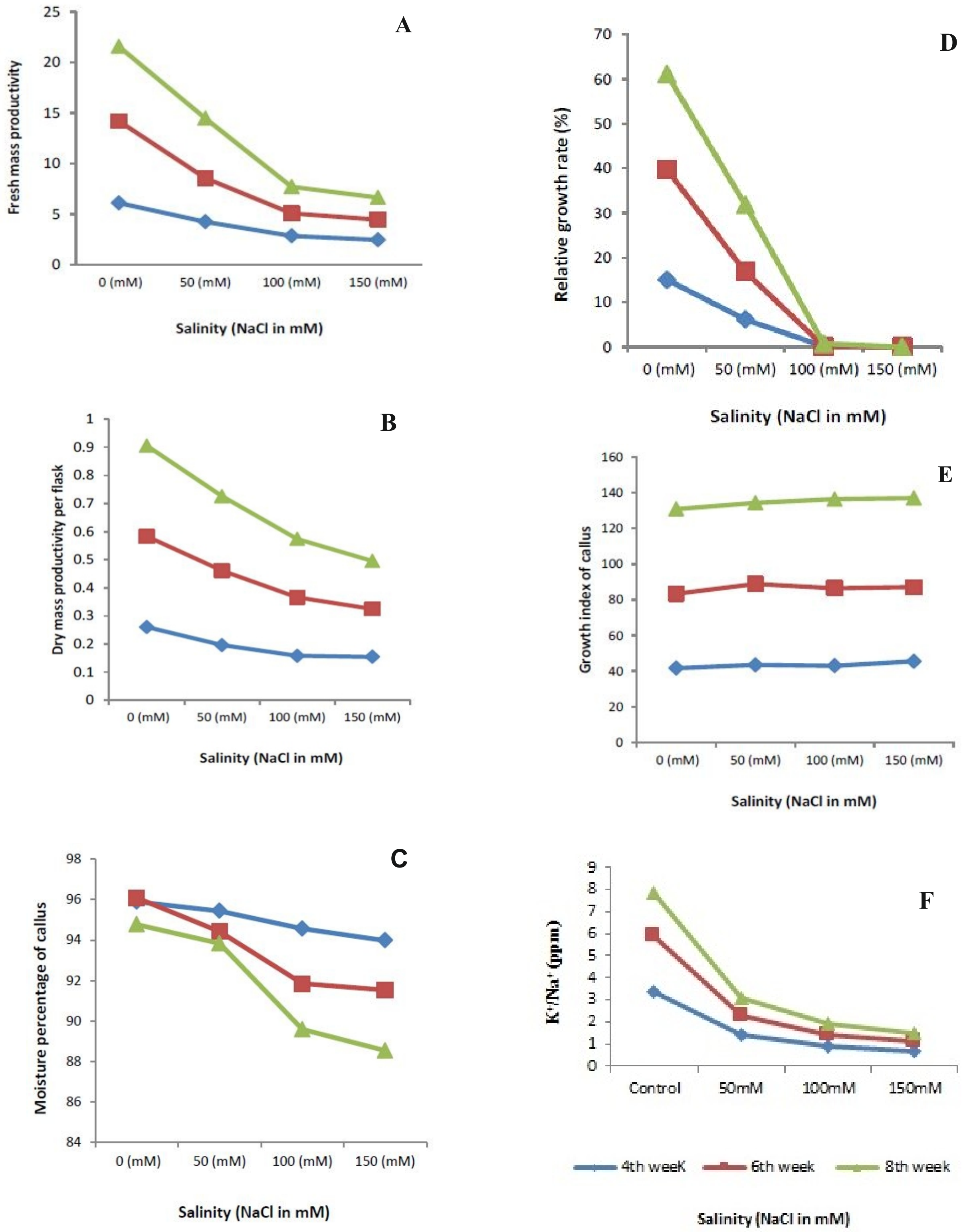

Figure 1 (A-F): Effect of *in vitro* NaCl concentrations on 4, 6, and 8 weeks callus growth in terms of **A** fresh mass **B.**Dry mass **C.** Moisture percentage **D.**

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^{O}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 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 (100ppm) and for calcium (10ppm) aginst $CaCl_2$ with the 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 1mg/L 2,4-D + 2mg/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 [Fig 1 (A-F)].

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. 1F).

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. 2A). 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 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. 2B). 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 8 week compared to the control in the 4 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. 2C). 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).

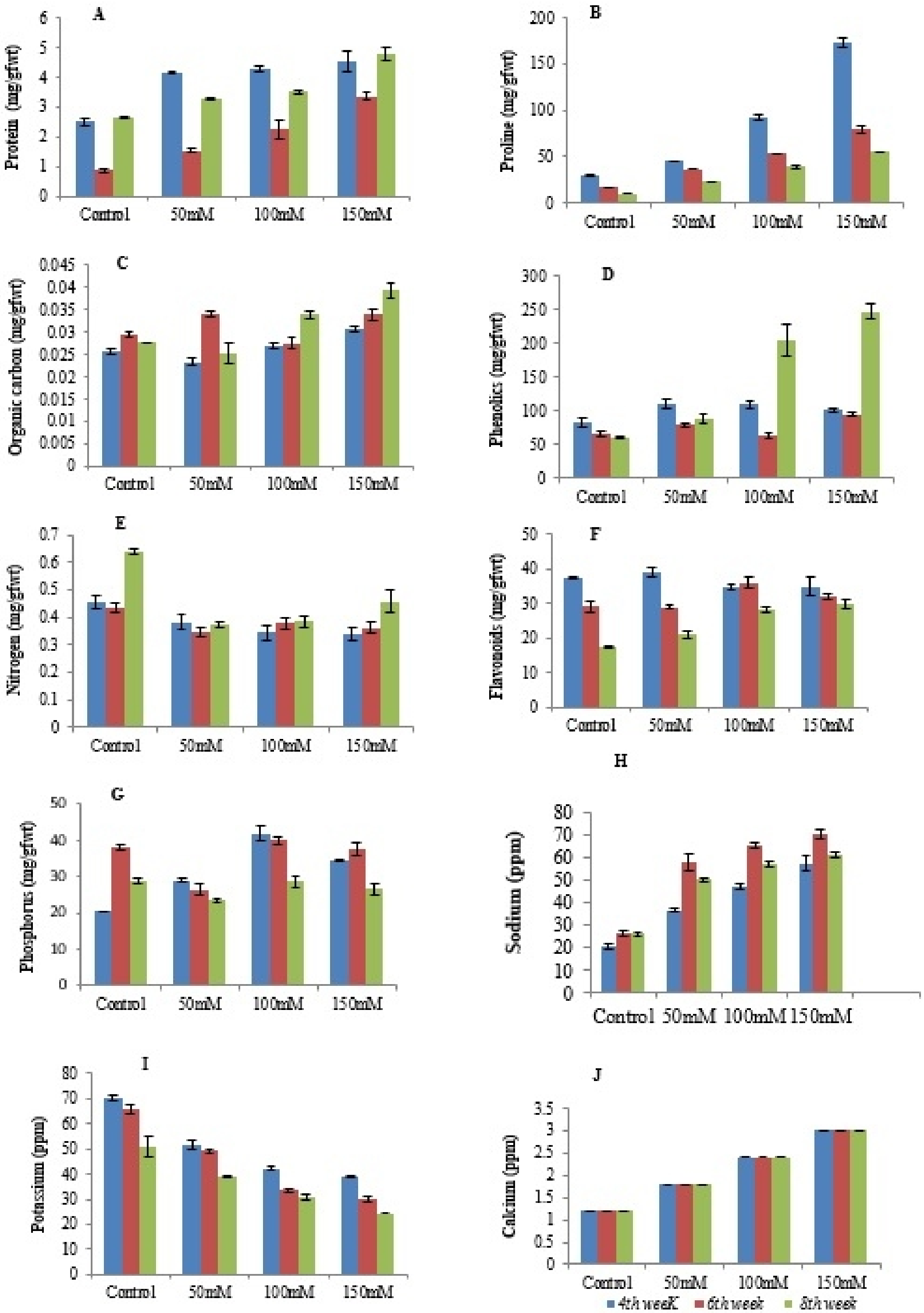

Figure 7: Effect of *in Vitro* NaCl Concentrations on protein in 4, 6, and 8 weeks callus of *U. lobata in* **A.** Protein **B.** Proline **C.** Organic **D.** Phenolics **E.** Nitrogen **F.** Flavonoids **G.** Phosphorus **H.** Sodium **I.** Potassium **J.** Calcium

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 to150 mM) phenolic content increased whereas dry biomass decreased continuously up to 8 week (Fig. 2D). 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 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. 2E). Some nitrogen containing compounds like aspartate, betaine, choline, glycine, glutamate, proline, proteins, putrescine, 4-gamma aminobutiric 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. 2F). 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, Chutipajit *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. 2G).

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; Soheilikhan *et al.* 2013). Other studies, however, have reported callus cultures to exhibit an initial 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. 2I).

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. 2H). The rising trend of Na^+ concentration observed in this study was similar to those reported for such other plants as safflower (Soheilikhar *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-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. 1D). 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

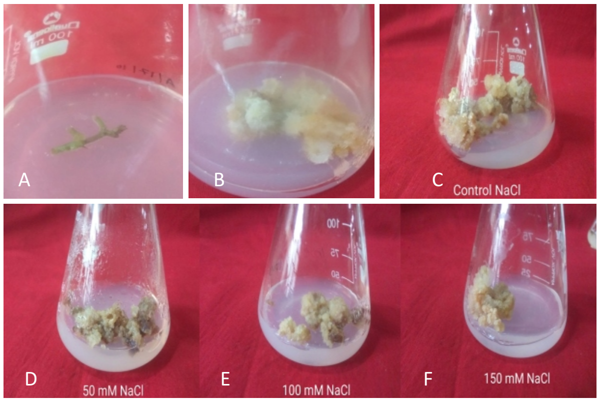

Figure 3: Effect of salt concentrations (0, 50, 100, 150 mM NaCl) on nodal callus of *Urena lobata* . A. Stem, B. Stem Callus, C-F. Nodal Callus subcultutres at various salt concentrations.

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 4, 6 and 8 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. 2J. 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 (Nevesand 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.

References

Abd EL-Azim W M and Ahmed S T 2009 Effect of salinity and cutting date on growth and chemical constituents of *Achillea fragratissima* Forssk, under Ras Sudr conditions. *Res. J. Agric. Biol. Sci.* **5(6)** 1121-1129.

Abraham EGR, Szekely G, Nagy R, Koncz C and Szabados L (2003) Light-dependent induction of proline biosynthesis by abscisic acid and salt stress is inhibited by brassinosteroid in Arabidopsis. *Plant Mol Biol.* **51**: 363-72.

Ahamd P and Jhon R (2005) Effect of salt stress on growth and biochemical parameters of *Pisum sativum* L. *Arch. Agro. Soil Sci.* **51(6)** 665-672.

Al-Amier H and Craker LE 2007 *In vitro* selection for stress tolerant spearmint. reprinted from: Issues in New Crops and New Uses. J Janick and A Whipkey (eds.). ASHS Press, Alexandria, VA. 306-10.

Ali M S, Faruq K O, Rahman M A A and Hossain M A 2013 Antioxidant and cytotoxic activities of methanol extract of *Urena lobata* (L) Leaves. *The Pharma Innovation -J.* **2** 2. 5.pdf (thepharmajournal.com)

Ali R M and Abbas H M 2003 Response of salt stressed barley seedlings to phenyl urea. *Plant Soil Environ.* **49:** 158-162.

Ali R M, Abbas H M and Kamal R K 2007 The effects of treatment with polyamines on dry matter, oil and flavonoid contents in salinity stressed chamomile and sweet marjoram. *Plant Soil Envir.* **53** 529-43.

AL-Khayri JM (2002) Growth, proline accumulation, and ion content in sodium chloride-stressed callus of date palm. *In Vitro Cell Dev Biol Plant.* **38** 79-82.

Ansari F and Vimala Y 2022 Optimized protocol for in vitro callus induction and micropropagation of *Urena lobata* L.: A fast-vanishing important medicinal plant. J. *Indian bot. Soc.* **102 (2)** 156-163.

Aqeel A M S, Javed F and Ashraf M 2007 Iso-osmotic effect of NaCl and PEG on growth, cations and free proline accumulation in callus tissue of two *indica* rice (*Oryza sativa* L.) genotypes. *Plant Growth Regul.* **53** 53-63

Arzani A and Ashraf M 2016 Smart engineering of genetic resources for enhanced salinity tolerance in crop plants. *Crit. Rev. Plant Sci.* **35** 146-189.

Ashraf M and Harris P J C 2004 Potential biochemical indicators of salinity tolerance in plants. *Plant Sci.* **166** 3–16.

Ashraf M and Orooj A 2006 Salt stress effects on growth, ion accumulation and seed oil concentration in an arid zone traditional medicinal plant ajwain (*Trachyspermum ammi* [L.] Sprague). *J Arid Env.* **64(2)** 209-20.

Babu S S, Madhuri D B and Ali S L 2016 A Pharmacological review of *Urena lobata* plant. *Asia J. Pharm. Clin. Res.* **9** 20-22.

Bajii M, Kinet J M and Lutts S 1998 Salt stress on the roots and leaves of *Atriplex halmus* L. and their corresponding callus culture. *Plant Sci.* **137** 131-142.

Basu S, Gangopadhyay G and Mukherjee B B 2002 Salt tolerance in rice *in vitro*: implication of accumulation of Na^+, K^+ and proline. *Plant Cell Tiss. Org. Cult.* **69** 55-64.

Bates CA, Waldern R P and Taleve I D 1973 Rapid determination of free proline or water stress studies. *Plant Soil*. **39** 205-207.

Bradford M M 1976 A rapid and sensitive method for the quantitative of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72** 248-254.

Bray H G and Thorpe W V T 1954 Analysis of phenolic compound of interest in metabolism. *Methods Biochem Anal.,* **1** 27-52. doi: 10.1002/9780470110171.ch2.

Chang C C, Yang M H, Wen H M and Chem J C 2002 Estimation of total flavonoid content in propoils by two complementary colorimetric methods. *J. food Drug Analysis* **10 (3)** 178-182.

Chaudhary M T, Merrett M J and Wainwright S J 1997 Growth, ion content and proline accumulation in NaCl-selected and non-selected cell lines of lucerne cultured on sodium and potassium salts. *Plant Sci* **127** 71-79.

Chauhan V and Prathapasena G 2000 Growth characteristic and ion contents of rice callus under the influence of NaCl and hydroxyproline. *Acta Physiol Plant*. **22** 39-44.

Cherian S and Reddy M P 2003 Evaluation of NaCl tolerance in callus cultures of *Suaeda nudiflora* Moq. *Biol. Plant.* **46**193–198.

Chutipaijit S, Cha-Um S and Sompornpailin K 2009 Differential accumulations of proline and flavonoids in indica rice varieties against salinity. *Pakistan J Bot* **45:** 2497-2506.

Cicek N and Cakirlar H 2008 Effect of the salt stress on some physiological and photosynthetic parameters at three different temperatures in six soybean (*Glycine max* L.) cultivars. *J. Agron. Crop Sci.* **194** 34-46.

Cik J K, Klejdus B, Hedbavny J and Bačkor M 2009 Salicylic acid alleviates NaCl-induced changes in the metabolism of *Matricaria chamomilla* plants. *Ecotoxicol.* **18(5)** 544-54.

Daneshmand M, Arvin M J and Kalantari K M 2010 Physiological responses to NaCl stress in three wild species of potato *in vitro*. *Acta. Physiol. Plant.* **32** 91-101

Datta N P, Khera M S and Saini T R 1962 A rapid colorimetric procedure for the determining of organic carbon in the soil. *J. India Soil Sci*. **10** 67-74.

Ehsanpour A A and Fatahian N 2003 Effects of salt and proline on *Medicago sativa* callus. *Plant Cell Tiss Org Cult* **73** 53-56.

Elkahoul S, Hemandez J A, Abdelly C, Ghrir R and Limam F 2005 Effects of salt on lipid peroxidation and antioxidant enzyme activities of *Catharanthus roseus* suspension cells. *Plant Science* **168** 607-613.

Enteshari S, Alishavandi R and Delavar K 2011 Interactive effects on silicon and NaCl on some physiological and biochemical parameters in *Borago officinalis* L. *Iranian J. Plant Physiol.* **2(1)** 315-320.

Errabil T, Gandonou C B, Essalmni H, Abrini J, Idaoma M and Senhaji NS (2007) Effect of NaCl and mannitol induced stress on sugarcane (*Saccharum* sp.) callus culures. *Acta Physiol. Plant.* **29** 95-102.

Farag M A, Huhman D V, Lei Z and Sumner LW 2007 Metabolic profiling and systematic identification of flavonoids and isoflavonoids in roots and cell suspension cultures of *Medicago truncatula* using HPLC-UV-ESI-MS and GC-MS. *Phytochemistry* **68** 342–54.

Flowers T J and Flowers SA 2005 Why does salinity pose such a difficult problem for plant breeders? *Agricultural Water Management* **78** 5-24.

Gandonou C, Errabii T, Abrini J, Idaomar M and Senhaji N 2006 Selection of callus cultures of sugarcane (*Saccharum* sp.) tolerant to NaCl and their response to salt stress. *Plant Cell Tiss. Org. Cult.* **87** 9-16.

Gao XL, Liao Y, Wang J, Liu X Y, Zhong K, Huang Y N, Gao H, Gao B and Xu Z J 2015 Discovery of a potent anti-yeast triterpenoid saponin, clematoside-S from *Urena lobata* L. *Int. J. Mol. Sci.* **16** 4731-4743.

Gliszczyńska-Swigło A, Szymusiak H and Malinowska P 2006 Betanin, the main pigment of red beet: molecular origin of its exceptionally high free radical-scavenging activity. *Food Addit. Contam.* **23**1079–87.

Grattan S R and Grieve C M 1998 Salinity–mineral nutrient relations in horticultural crops. *Scientia Horticulturae* **78** 127–157.

Gupta B and Huang B 2014 Mechanism of salinity tolerance in plants: physiological, biochemical, and molecular characterization. *Int. J. Genom.* 701596.

Heidari M and Sarani S 2012 Growth, biochemical components and ion content of Chamomile (*Matricariachamomilla* L.) under salinity stress and iron deficiency. *J. Saudi Soc. Agricul. Sci.* **11(1)** 37-42.

Hendawy S F and Khalid K H A 2005 Response of sage (*Salvia officinalis* L.) plants to zinc application under different salinity levels. *J. Appl Sci. Res.* **1** 147-155.

Islam M T and Uddin M A 2017 A revision on *Urena lobata* L. *Int. J. Med*. **5 (1)** 126-131.

Kanner J, Harel S and Granit R 2001 Betalains-a new class of dietary cationized antioxidants. *J. Agric. Food Chem.* **49** 5178–85.

Kavi K P B 1988 Effect of salt stress on callus cultures of *Oryza sativa* L. *J. Exp Bot.* **39** 235-240.

Khorami R and Safarnejad A 2011 *In vitro* selection of *Foeniculum vulgare* for salt tolerance. Not. *Sci. Biol.* **3** 90-97.

Kinnersley A M and Turano F J 2000 Gamma amino butyric acid (GABA) and plant responses to stress. CRC Crit. *Rev. Plant Sci*. **19** 479–509.

Lissy K P, Simon T K and Lathab M S 2006 Antioxidant potential of *Sida retusa*, *Urena lobata* and *Triumfetta rhomboidea*. *Ancient Sci. Life* **25 (3-4)** 10-15.

Liu E, Zong H, Fei Z and Yong-Chao L G M 2000 Effects of drought, salt and chilling stresses on proline accumulation in shoot of rice seedlings. *J. Trop. Subtrop. Bot.* **8** 235-238.

Liu Q, Dixon R A and Mabry T J 1993 Additional flavonoids from elicitor-treated cell cultures of *Cephalocereus senilis*. *Phytochemistry* **34**167–70.

Lokhande V H, Nikam T D and Penna S 2010 Biochemical, physiological and growth changes in response to salinity in callus cultures of *Sesuvium portulacastrum* L. *Plant cell Tiss. Organ Cult*. **102** 7-25.

Lutts S, Almansouri M and Kinet J M 2004 Salinity and water stress have contrasting effects on the relationship between growth and cell viability during and after stress exposure in durum wheat callus. *Plant Sci.* **167** 9-18.

Mansour M M F 2000 Nitrogen containing compounds and adaptation of plants to salinity stress. *Biol Plant.* **43:** 491-500.

Mathappan R, Joe V F, Prasanth V V and Varirappan K 2010 Pharmacog-nostical and preliminary phytochemical studies of *Urena lobata* linn. *Int. J. Phytomedicine* **2** 408-411.

Matkowski A 2004 Isoflavonoids in callus cultures from different organs of *Pueraria lobata* (Wild.) Ohwi. *J. Plant Physiol.* **161** 343–6.

Matkowski A 2008 Plant *in vitro* culture for the production of antioxidant-A review. *Biotechnol. Adv.* **26** 548–560.

Miflin B 2000 Crop improvement in the 21st century. *J. Exp. Bot.* **51** 1-8.

Mittal S, Kumari N and Sharma V 2012 Differential response of salt stress on *Brassica juncea*: photosynthetic performance, pigment, proline, D1 and antioxidant enzymes. *Plant Physiol. Biochem.* **54** 17-26.

Mokhberdoran F, Nabavi K S M and Sadrabadi H R 2009 Effect of temperature, iso-osmotic concentrations of NaCl and PEG agents on germination and some seedling growth yield components in rice (*Oryza sativa* L.). *Asian J. Plant Sci.* **8** 409-416

Mukhtar CZ and Hasnain S (1994) Effects of NaCl stress on DNA, RNA and soluble protein contents of callus cultures of *Brassica oleracea* . *Pakistan J. Agric. Res.* **15** 233–238.

Munns R S, Husain A R, Rivelli R A, James A G, Condon M P, Lindsay E S, Lagudah D P S and Hare R A 2002 Avenues for increasing salt tolerance of crops, and the role of physiologically based selection traits. *Plant Soil* **247** 93–105.

Murashige T and Skoog F A 1962 A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant.* **15** 473-479

Murillo A B, Troyo D E, García H J L, López A l R, Ávila S N Y, Zamora S S, Rueda P E O and Kaya C 2006 Effect of NaCl salinity in the genotypic variation of cowpea (*Vigna unguiculata*) during early vegetative growth. *Scientia Horticulturae* **108** 423-431

Najafi F, Khavari-Nejad R A and Ali M S 2010 The effects of salt stress on certain physiological parameters in summer savory (*Satureja hortensis* L.) plants. *J. Stress Physiol. Bioch.* **6(1)** 13-21.

Nelson N 1944 A photometric adaptation of the somogyi methods for the determination of glucose. *J. Biochem.* **153** 375-380.

Neves-Piestun BG and Bernstein N 2001 Salinity-induced inhibition of leaf elongation in maize is not mediated by changes in cell wall acidification capacity. *Plant Physiol.* **125** 1419–1428.

Niknam V, Meratan A A and Ghaffari S M 2011 The effect of salt stress on lipid peroxidation and antioxidative enzymes in callus of two *Acanthophyllum* species. *In Vitro Cell Dev. Biol. - Plant* **47** 297-308.

Olsen 1954 Estimation of phosphorus. *In*: Prayogik Mrida Urwarta Evam Urwarak. Singh, A., Eds., 1973. P. 56-58. U.P. Hindi Granth Academy, Lucknow.

Omonkhua A A and Onoagbe I O 2011 Evaluation of the long-term effects of *Urena lobata* root extracts on blood glucose and hepatic function of normal rabbit. *J. Toxicology Environmental Health Sci*. **3(8)** 204-213.

Orthen B, Popp M and Smirnoff N 1994 Hydroxyl radical scavenging properties of cyclitols. *Proc. Roy. Soc Edin.* **102B** 269–272.

Osman M E H, Elfeky S S, Abo El-Soud K and Hasan A M 2007 Response of *Catharanthus roseus* shoots to salinity and drought in relation to vincristine alkaloid content. *Asian. J. Plant. Sci.* **6** 1223-1228.

Pandey R and Ganapathy P S 1985 The proline enigma: NaCl-tolerant and NaCl-sensitive callus lines of *Cicer arietinum*. *Plant Sci.* **40** 13-17.

Panghal S and Soni S S 2013 *In vitro* studies on effect of different concentration of NaCl on *Jatropha curcas. J. Environ. Biol.* **35** 709-712.

Parihar P, Singh S, Singh R, Singh V P and Prasad S M 2015 Effect of salinity stress on plants and its tolerance strategies: a review. *Environ. Sci. Poll. Res.* **22 (6)** 4056–75.

Pharmacognosy of Ayurvedic drug. Department of Pharmacognosy, University of Kerala, 1962 **5**108-112.

Pratt D and hi-Ettai R A 1990 A variation in organic and inorganic mineral components in young Eucalyptus seedlings under salinity stress. *Physiol. Plant.* **79** 479-486.

Qiu Q S, Barkla B J, Vera-Estrella R, Zhu J K and Schumaker K S 2003 Na^+/H^+ exchange activity in the plasma membrane of Arabidopsis thaliana. *Plant Physiol.* **132** 1041–1052.

Queiros F, Fidalgo F, Santos l and Salema R 2007 In vitro selection of salt tolerant cell lines in *Solanum tuberosum* L. *Biologia Plantarum.* **51** 728-734.

Rahdari P, Tavakoli S and Hosseini S M 2012 Studying of salinity stress effect on germination, proline, sugar, protein, lipid and chlorophyll content in Purslane (*Portulaca oleracea* L.) leaves. *J. Stress Physiol. Biochem.* **8(1)** 182-193.

Ramakrishna A and Ravishankar G A 2011 Influences of abiotic stress signals on secondary metabolites in plants. *Plant Signal Behav*. **6(11)**1720–1731.

Reddy P J and Vaidyanath K 1986 *In vitro* characterization of salt stress effect and the selection of salt tolerant plant in rice (*Oryza sativa* L.). *Theor. Appl. Genet.* **71** 757-760.

Rout G R, Samantaray S and Das P 2000 *In vitro* manipulation and propagation of medicinal plants. *Biotechnol. Adv.* **18** 91-120.

Rozema J and Flowers T 2008 Crops for a salinized world. Science **322** 1478-1480.

Shannon M C and Grieve C M 1999 Tolerance of vegetable crops to salinity. *Sci Hortic.* **78** 5-38.

Singh G 2009 Salinity-related desertification and management strategies : Indian experience *Land Degradation Development* **20 (4)** 367-385.

Snell F D and Snell C T 1967 Colorimetric methods of analysis including photometric methods. **IV A** 331-332.

Soheilikhah Z, Karimi N, Ghasmpour H R and Zebarjadi A R 2013 Effects of saline and mannitol induced stress on some biochemical and physiological parameters of *Carthamus tinctorius* L. varieties callus cultures. *Aust. J. Crop Sci.* **7** 1866-1874.

Sujata B and Kshitija S 2013 Drought stress adaptation: metabolic adjustment and regulation of gene expression. *Plant Breed*. **132(1)** 21–32.

Šutković J, Lerl D and Ragab M G A 2011 *In vitro* production of solasodine alkaloid in *Solanum nigrum* under salinity stress. *J. Phytol.* **3(1)** 43–9.

Sytar O, Barki S, Zivcak M and Brestic M 2018 The involvement of different secondary metabolites in salinity tolerance of crops. *In*: Kumat V, editor. Salinity responses and tolerance in plants, vol. 2. Berlin: Springer Int Publishing AG, part of *Springer Nature* 21-48.

Tester M and Davenport R 2003 Na^+ tolerance and Na^+ transport in higher plants. *Ann. Bot.* **91** 503-527.

Tuna A L, Kaya C, Ashraf M, Altunlu H, Yokas I and Yagmur B 2007 The effects of calcium sulphate on growth, membrane stability and nutrient uptake of tomato plants grown under salt stress. *Environ. Exp. Bot*. **59** 173–178.

USDA-ARS 2012 Germplasm Resources Information Network (GRIN). Online Database. Beltsville, Maryland, USA: National Germplasm Resources Laboratory.

Verpoorte R, Contin A and Memelink J 2002 Biotechnology for the production of plant secondary metabolites. *Phytochem. Rev.* **1** 13-25.

Vitart VI, Baxter P, Doerner and Harper JF 2001 Evidence for a role in growth and salt resistance of a plasma membrane H^+-ATPase in the root endodermis. *The Plant J.* **27** 191–201.

Yadav A K and Tangpu V 2008 Antidiarrheal activity of *Lithocarpus dealbata* and *Urena lobata* extracts : Therapeutic implications. *Pharmaceutical Biol.* **45(3)** 223-229**.**

Yoshiba Y, Kiyosue T, Nakashima K, Shinozaki K Y and Shinozakj K 1997 Regulation of levels of proline as an osmolyte in plants under water stress. *Plant Cell Physiol.* **38** 1095-1102.

Zaki M F, Abou-Hussein S D, Abou El-Magd M M and El-Abagy H M H 2009 Evaluation of some sweet fennel cultivars under saline irrigation water. *Eur. J. Sci. Res.* **30(1)** 67-78.

Zhang M, Fang Y, Ji Y, Jiang Z and Wang L 2013 Effects of salt stress on ion content, antioxidant enzymes and protein profile in different tissues of *Broussonetia papyrifera*. *South African J. Bot.* **85** 1-9.

Zhao M G, Tian Q Y and Zhang W H 2007 Nitric oxide synthase-dependent nitric oxide production is associated with salt tolerance in Arabidopsis. *Plant Physiol*. **144** 206–217.

Zsolnay A and Gorlitz H 1994 Water extractable organic matter in arable soil: Effects of drought and long-term fertilization. *Soil Biol Biochem*. **26** 12571261.