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## IMPLICATION OF PROLINE, SOLUBLE SUGARS AND SOLUBLE PROTEINS IN SUGARCANE (*SACCHARUM SP.*) *IN VITRO* SALT-TOLERANCE

### Implication de la proline, des sucres solubles et des protéines solubles dans la tolérance à la salinité *in vitro* chez la canne à sucre (*Saccharum sp.*)

GANDONOU C.B.<sup>1,2,3\*</sup>, BADA F.<sup>2</sup>, ABRINI J.<sup>1</sup>, SKALI-SENHAJI N.<sup>1</sup>

1- Laboratoire de Biologie et Santé, Université Abdelmalek Essaâdi, Faculté des Sciences de Tétouan, B.P. 2121 Tétouan, Maroc

2- Laboratoire de Physiologie Végétale, Faculté des Sciences et Techniques (FAST/UAC), 01BP 526 Cotonou, République du Bénin

3- Unité de Recherche en Biotechnologies et Amélioration des Plantes/Laboratoire de Pharmacognosie, Centre Béninois de la Recherche Scientifique et Technique (CBRST); BP 06 Oganla, Porto-Novo, République du Bénin

\* Corresponding author: E-mail: ganchrist@hotmail.com

#### ABSTRACT

Salinity is one of the major environmental factors which limit agricultural production in the world. It affects plant growth either at whole plant level or at cellular level. In this study, callus cultures from two sugarcane cultivars NCo310 (salt-tolerant) and CP65-357 (salt-sensitive) were used to investigate the implication of proline, soluble sugars and soluble proteins in *in vitro* NaCl tolerance of sugarcane (*Saccharum sp.*). Leaf explant-derived calli obtained from the two sugarcane cultivars were exposed to four NaCl concentrations (0, 34, 68 and 102 mM). Relative fresh weight growth, proline, soluble sugars and soluble proteins content were quantified after 4 weeks of stress. NaCl effect resulted in calli growth reduction in the two cultivars; however, as expected, this growth inhibition was lower in calli issued from the salt-tolerant NCo310 than in calli obtained from the salt-sensitive CP65-357. Under salinity, proline and soluble sugars concentrations increased significantly in calli from the two cultivars while soluble proteins concentration increased significantly only in calli issued from the sensitive cultivar and decreased significantly in that issued from the tolerant cultivar. Calli issued from the salt tolerant NCo310 accumulated more soluble sugars than those from the sensitive CP65-357. The latter accumulated more proline and soluble proteins than those from NCo310. These results revealed an implication of soluble sugars in salt tolerance at cellular level in these genotypes and that proline and soluble proteins appeared not to play an important role in sugarcane *in vitro* salt-tolerance.

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**Key words:** Sugarcane (*Saccharum sp.*), *in vitro* salt-tolerance, callus cultures, proline, soluble sugars, soluble proteins.

#### RESUME

La salinité est l'un des facteurs environnementaux majeurs qui limitent la production agricole dans le monde. Elle affecte la croissance de la plante aussi bien à l'échelle de la plante entière qu'à l'échelle cellulaire. Dans cette étude, les callus provenant de deux variétés de canne à sucre NCo310 (tolérante à la salinité) et CP65-357 (sensible à la salinité) ont été utilisées pour évaluer l'implication de la proline, des sucres solubles et des protéines solubles dans la tolérance à la salinité *in vitro* chez la canne à sucre (*Saccharum sp.*). Des callus provenant d'explants foliaires obtenus à partir de deux variétés de canne à sucre sont exposés à quatre concentrations de NaCl (0, 34, 68 et 102 mM). La croissance relative en matière fraîche, la teneur en proline, en sucres solubles et en protéines solubles sont quantifiées après 4 semaines de culture sous stress salin. L'effet du NaCl se traduit par une réduction de la croissance des callus chez les deux variétés; toutefois, comme attendu, cette inhibition de la croissance est plus faible chez les callus issus de la variété tolérante NCo310 que chez ceux issus de la variété sensible CP65-357. Sous stress salin, les teneurs en proline et en sucres solubles augmentent significativement chez les callus provenant des deux variétés tandis que la teneur en protéines solubles n'augmente significativement que chez les callus issus de la variété sensible et diminuent significativement chez les callus provenant de la variété tolérante. Les callus issus de la variété tolérante NCo310 accumulent plus de sucres solubles que ceux issus de la variété sensible CP65-357; ces derniers accumulent plus de proline et de protéines solubles que ceux issus de NCo310. Ces résultats révèlent une implication des sucres solubles dans la tolérance à la salinité à l'échelle cellulaire chez la canne à sucre et que la proline et les protéines solubles ne semblent pas jouer un rôle important dans la tolérance à la salinité à l'échelle cellulaire chez la canne à sucre.

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**Mots-clés :** canne à sucre (*Saccharum sp.*), tolérance à la salinité *in vitro*, callus, proline, sucres solubles, protéines solubles.

#### INTRODUCTION

Salinity is a major environmental factor limiting the crop productivity in the arid and semi-arid areas of the world (Dasgan et al., 2002). This complex abiotic stress, which presents an osmotic and an ionic components, induces a wide range of metabolic perturbations in higher plants without always the possibility to distinguish those associated with

the osmotic component from those due to ion toxicity. These metabolic perturbations resulted in growth reduction and an alteration of nutritional imbalance. However, to survive in the presence of salt, plants have developed several adaptative mechanisms whose comprehension remains incomplete (Lutts et al., 1996b) and there is a substantial variation in salt-tolerance among different species (Munns et al., 2002)

and among cultivars of the same species (Watanabe et al., 2000; Al-Karaki, 2000; Ghoulam et al., 2002; Aghaei et al., 2008). The importance of proline and/or soluble sugars in osmotic adjustment and the increase of their synthesis under salt or water stresses has been reported in several plants (Solomon et al., 1994; Kavi Kishor et al., 1995; Ashraf and Fatima, 1995; Igarashi et al., 1997; Watanabe et al., 2000; Yokota, 2003; Ehsanpour and Fatahian, 2003; Amirjani, 2010; Shafi et al., 2011). These two organic solutes are generally reported to play a key role in plants osmotic stress tolerance (Delauney and Verma, 1993; Watanabe et al., 2000; Alvarez et al., 2003; Misra and Gupta, 2005; Sakhanokho and Kelley, 2009). Soluble proteins are also reported as organic solutes which concentration was modified by salt stress (Irigoyen et al., 1992; Perez-Alfocea et al., 1994).

It is known that in vitro tissue culture constitutes an important tool for the study of the physiological mechanisms of salt resistance at cellular level and several reports were interested in this field (Perez-Alfocea et al., 1994; Lutts et al., 1996a; Ehsanpour and Fatahian, 2003; Kashyap and Sharma, 2006).

As for sugarcane, there were only a few studies on the contributory role of organic solutes to the osmotic adjustment and salt tolerance at the cell level. In our previous study, we have shown that ions toxicity is implied in salt effect at cellular level and that  $K^+$  ion plays an important part in sugarcane salt-resistance at tissue level (Gandonou et al., 2005).

The aim of the present study was to assess the implication of proline, soluble sugars and soluble proteins in in vitro sugarcane salt tolerance using callus cultures of two sugarcane cultivars differing in their salt-tolerance.

## MATERIALS AND METHODS

### Plant material and culture conditions

The two sugarcane (*Saccharum* sp.) cultivars are obtained from the Technical Center of Sugar's Cultures (CTCS), Morocco. CP65-357 is American cultivar (Canal Point) largely cultivated in Morocco. It behaves as salt-sensitive in comparison to NCo310, an Indian variety selected in South Africa (NCo = Natal-Coimbatore), according to our previous results (Gandonou et al., 2005). Stalk segments were surface disinfected with ethanol 70° and sown in pots containing sand in greenhouse with a natural illumination where the day/night temperature was about 27°C/22°C. Daytime humidity was between 70 and 80%. Irrigation was done every two days; and after germination, sugarcane plants were grown in these conditions until approximately 6 months.

The explants used for callus induction are leaf segments provided from the sheath of the youngest leaves. The in vitro culture procedure, the culture medium and the culture conditions were those reported by Gandonou et al., (2005). The pH was adjusted to 5.8 with NaOH (0.1 N) and all media were solidified with 8 g l<sup>-1</sup> agar before autoclaving during 20

min at 120°C. Five explants were cultivated per jar and the cultures were kept in darkness at 25±1°C.

### In vitro salt treatment and growth determination

After two subcultures (4 weeks each), the growing calli were transferred to callus culture medium containing either 0, 34, 68 or 102 mM NaCl. Calli were maintained on their respective treatments for 4 weeks in the same conditions as above. Calli were weighted before transferring to these medium (W0); they were weighted again after the 4 weeks of treatment (W1). Relative fresh weight growth of callus (RFGW) was calculated as (W1 - W0)/W0.

### Extraction and proline determination

For proline determination, 200 mg of callus were ground in a potter, homogenized in 4 ml of methanol-chloroform-water (67%-28%-5%) at 0°C and centrifuged at 1300 rpm for 30 min. Supernatants were then incubated at 4°C for 12 h in the presence of 0.25 ml of chloroform and 0.9 ml distilled water. Proline was quantified spectrophotometrically (515 nm) in the upper phase using ninhydrin acid reagent (Bates et al., 1973) and expressed on fresh matter basis. L-proline was used as standard.

### Extraction and soluble sugars determination

For soluble sugars determination, 100 mg of callus were ground in a mortar, homogenized in 1 ml of ethanol 80% and centrifuged at 5000 g for 10 min at 4°C. Supernatants were transferred in other tubes and the pellets were homogenized again in 0.5 ml ethanol 80% and centrifuged as above. The second supernatant was added to the first. Total soluble sugars were measured by a modified method of Watanabe et al., (2000). 1 ml of extract was reacted with 3 ml freshly prepared anthrone reagent (50 mg anthrone + 50 ml of H<sub>2</sub>SO<sub>4</sub> 95%) at 100°C for 10 min. After cooling, in ice, the total sugar content was determined at 620 nm by a spectrophotometer using glucose as standard.

### Extraction and soluble proteins determination

Soluble proteins determination was done using the method of Bradford (1976). 100 mg of fresh callus were ground in 1 ml of phosphate buffer 0.1 M, pH 7 and centrifuged at 9 000 g for 20 min at 4°C. 1 ml of supernatants was added to 3 ml of Bradford reactive. Soluble proteins were quantified spectrophotometrically (595 nm) and expressed on fresh matter basis. Bovin albumin serum was used as standard.

### Statistical analysis

All the experiments (growth, proline, soluble sugars and soluble proteins concentrations) were repeated twice independently with similar results. For each experiment, 30 to 35 calli were used (5 per jar). Each value is presented in the form of mean ± standard error with a reading of four (growth) or three (proline, soluble sugars and soluble proteins concentrations) independent samples per treatment. The analysis of the main effects of cultivars and stress intensity was based on 1 or 2-ways analysis of variance (ANOVA). All statistical analyses were performed by SAS 92 program (SAS Institute, 1992).

**RESULTS**

**Growth**

NaCl application induced a significant decrease of callus RFWG in all cultivars (table I). The growth reduction observed was about 38%, 45% and 53% of control for the sensitive

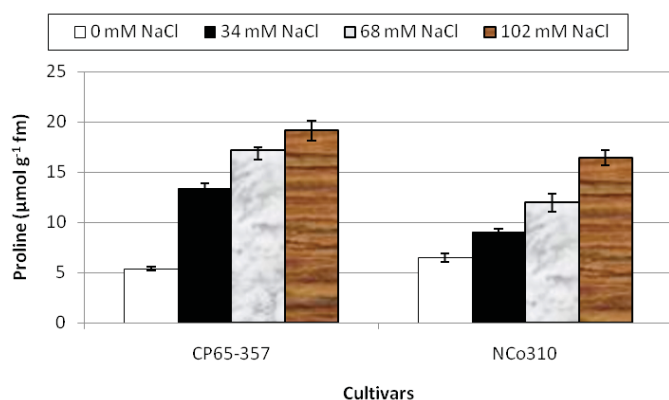
**Table I:** Relative fresh weight growth (RFG) of calli obtained from two sugarcane cultivars (CP65-357, salt-sensitive and NCo310, salt-tolerant) as affected by different concentrations of NaCl.

NaCl concentrations (mM)	Cultivars	
	CP65-357	NCo310
0	1.192 ±0.027 a	2.032±0.133 a
34	0.734±0.042 b	1.883±0.086 a
68	0.66±0.093 b	1.215±0.089 b
102	0.56±0.057 bc	1.163±0.058 b

CP65-357 respectively at 34, 68 and 102 mM NaCl; and about 8%, 40% and 43% of control at the same NaCl concentration for the salt-tolerant NCo310. There were significant ( $p < 0.001$ ) differences between the two cultivars (table II) with the salt-tolerant NCo310 which maintained relative high calli growth under salt-stress in comparison with the salt-sensitive CP65-357. A significant ( $p < 0.05$ ) interaction was observed between cultivars and stress intensity (table II).

**Free proline accumulation**

No significant differences in proline concentration were recorded in the absence of stress (fig. 1). In the presence



**Fig. 1:** Proline concentration [in µmol g<sup>-1</sup> (fm)] of sugarcane calli obtained from two cultivars (CP65-357, salt-sensitive and NCo310, salt-tolerant) as affected by different concentrations of NaCl: vertical bars are S.E.

of NaCl, free proline concentration increased significantly ( $p < 0.001$ ) in calli obtained from the two sugarcane cultivars (fig. 1). A significant ( $p < 0.001$ ) difference was recorded between cultivars (table II); which indicated that calli issued from the salt sensitive cultivar CP65-357 accumulated more proline than those obtained from NCo310 on stress media. A significant ( $p < 0.001$ ) interaction between cultivar and stress intensity was noted (table II).

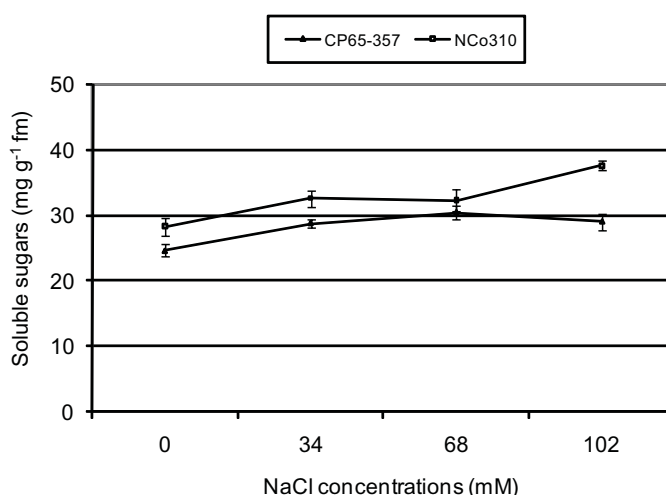
**Table II:** Results of 2-ways variance analysis for RFWG, ion content and proline accumulation of sugarcane calli; F- ratios are given for the main effects of the following levels of classification: stress intensity (i.e. NaCl concentration of stressing media) and cultivars and interaction between these levels of classification

Parameter	Stress intensity	Cultivar	Interaction (Stress intensity x Cultivar)
RFG	28.27***	144.73***	4.32*
Proline	142.24***	43.12***	10.68***
Soluble sugars	11.58***	27.31***	2.55ns

ns: not-significant; \*: significant at  $p = 0.05$ ; \*\*\*: significant at  $p = 0.001$

**Soluble sugars accumulation**

Salt-tolerant NCo310 showed higher soluble sugars content in calli compared to salt-sensitive CP65-357 in the absence of stress (fig. 2). In the presence of NaCl, soluble sugars concentrations increased significantly ( $p < 0.001$ ) (fig. 2) in calli issued from both sugarcane cultivars with a



**Fig. 2:** Soluble sugars concentration [in mg g<sup>-1</sup> (fm)] of sugarcane calli obtained from two cultivars (CP65-357, salt-sensitive and NCo310, salt-tolerant) as affected by different concentrations of NaCl: vertical bars are S.E.

very highly significant difference between cultivars (table II). It appears that salt-tolerant cultivar accumulated more soluble sugars in calli under salt stress than the salt-sensitive cultivar. A non significant interaction was observed between cultivar and stress intensity (table II).

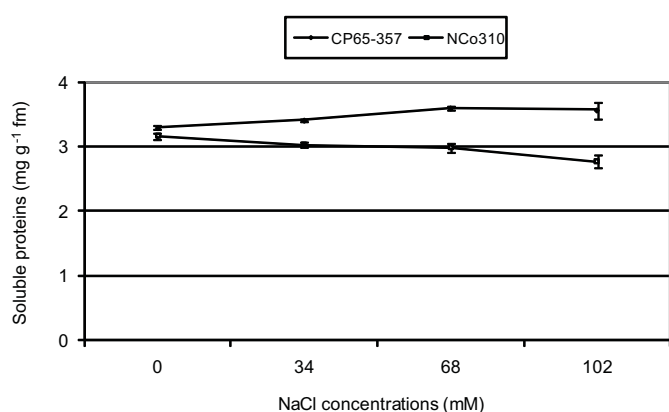
**Soluble proteins accumulation**

No significant differences in soluble proteins concentration were recorded in the absence of stress (fig. 3). Soluble proteins concentration decreased in calli issued from the salt-tolerant cultivar NCo310 while it increased in calli issued from the salt-sensitive CP65-357 (fig. 3). The decrease and increase observed are significant ( $p < 0.05$ ) as shown by a one-way ANOVA (table III). It seems that the salt-sensitive variety accumulated more soluble proteins in calli under salt stress than the salt-tolerant one.

**Table III:** Results of 1-way variance analysis for soluble proteins content of calli obtained from two sugarcane cultivars (CP65-357, salt-sensitive and NCo310, salt-tolerant) as affected by different concentrations of NaCl; F- ratios are given for the main effect of stress intensity (i.e. NaCl concentration of stressing media).

	Cultivars	
	CP65-357	NCo310
Number of observations (n)	16	16
Degree of freedom (df)	3	3
F values	4.11*	5.96*
Probability (p)	0.032	0.010
Least significant difference (LSD)	0.2148	0.2089

\*: significant at  $p = 0.05$ .



**Fig. 3:** Soluble proteins concentration [in mg g<sup>-1</sup> (fm)] of sugarcane calli obtained from two cultivars (CP65-357, salt-sensitive and NCo310, salt-tolerant) as affected by different concentrations of NaCl: vertical bars are S.E.

## DISCUSSION

Relative fresh weight growth (RFWG) of calli obtained from the two cultivars decreased as NaCl concentration increased in the medium. Salt-induced RFWG decreased was larger and detectable at lower dose in the salt-sensitive CP65-357 than in the salt-tolerant NCo310. These data confirmed the *in vitro* salt-tolerance of NCo310 in comparison with CP65-357 as reported in our previous study (Gandonou et al., 2005).

Proline accumulation was frequently reported in salt-stressed calli and whole plants. Most usually, it was considered to act as a compatible osmoticum and therefore to be involved in salt resistance mechanisms (Igarashi et al., 1997; Alvarez et al., 2003; Ehsanpour and Fatahian, 2003; Sakhanokho and Kelley, 2009). Our results reveal that it is not the case in the sugarcane stressed calli because proline accumulation was higher and occurred at lower stress intensities in the salt-sensitive variety CP65-357 than in the salt-tolerant cultivar NCo310. These results are in agreement with those reported by Liu and Zhu (1997), Yokota (2003) and Prajuabmon et al., (2009) who have reported a larger accumulation of proline in salt-hypersensitive plants than in salt-tolerant ones.

It could be suggested that proline overproduction is a response to the osmotic component of salt stress. Other functions have been attributed to proline accumulation in stress tissue: it could be a protective agent of enzyme and membranes (Solomon et al., 1994; Van Rensburg et al., 1993), a free radical scavenger (Smirnoff and Cumbes, 1989), a storage compound of carbon and nitrogen (Jäger and Meyer, 1977) or it could be involved in the regulation of cytosolic pH (Venekamp, 1989). Our results have shown that proline accumulation was higher in salt-sensitive genotype; this observation tends to demonstrate that proline is a symptom of stress injury rather than an indicator of tolerance as reported in rice (Lutts et al., 1996b), *Lycopersicon* species (Perez-Alfocea et al., 1994; Ruz-Alvarez and Guerrier, 1994), maize (Ibarra-Caballero et al., 1988), soybean (Moftah and Michel, 1987) and sorghum (Bhaskaran et al., 1985).

NaCl induced an increase in soluble sugars content in calli of both cultivars. Tolerant variety NCo310 accumulated more soluble sugars than the sensitive variety CP65-357. Salt stress effects result mainly in an increase of soluble sugars content in both leaves and the roots in many plants and the most tolerant genotypes accumulated more soluble sugars. Thus Watanabe et al., (2000) reported that in the genus *Populus*, most salt-resistant species accumulated more soluble sugars in leaves than sensitive ones in the presence of salt stress. Similar results were found in sunflower (Ashraf and Tufail, 1995). On the other hand, in *Carthamus tinctorius*, Ashraf and Fatima (1995) reported that two salt resistant accessions showed different responses: one of them accumulated more soluble sugars than the sensitive accessions, whereas the other accession accumulated similar amount of sugars than the sensitive accessions though it is more tolerant. Soluble sugars are known for their role in the osmo-regulation in plants exposed to osmotic stress. According to Cram (1976), among the organic osmotica, sugars contribute with more than 50% to the total osmotic potential in glycophytes subjected to salt stress. In our previous study, we have demonstrated that soluble sugars are one the main option used by sugarcane calli selected *in vitro* for salt-tolerance to counteract the negative effects of salt stress (Gandonou et al., 2006). The fact that the most tolerant variety accumulated more soluble sugars in calli indicated that soluble sugars play an important role as osmo-protector in salt stressed sugarcane calli.

In general, salt stress results in a reduction of soluble proteins contents which is often due to an inhibition of their synthesis and/or the increase of their hydrolysis (Irigoyen et al., 1992). Thus, Perez-Alfocea et al. (1994) reported that salinity caused a reduction in soluble proteins of tomato species independently from their level of salinity tolerance. Our results revealed that soluble proteins content increased significantly in the salt-sensitive cultivar while it decreased in the salt-tolerant one. This observation indicated that soluble proteins would not play a key role in the NaCl tolerance of cultivar NCo310 *in vitro*.

## CONCLUSION

The present study revealed that proline and soluble proteins are not directly involved in salt tolerance of sugarcane calli issued from cultivars, and that soluble sugars play a key role in sugarcane *in vitro* salt-tolerance.

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## REFERENCES

- Aghaei K., Ehsanpour A.A., Balali G., Mostajeran A. 2008. In vitro screening of potato (*Solanum tuberosum* L.) cultivars for salt tolerance using physiological parameters and RAPD analysis. *American-Eurasian Journal of Agricultural and Environmental Sciences*, **3** (2): 159-164.
- Al-Karaki G.N. 2000. Growth, water use efficiency and sodium and potassium acquisition by tomato cultivars grown under salt stress. *Journal of Plant Nutrition*, **23** (1): 1-8.
- Alvarez I., Tomaro L.M., Bernavides P.M. 2003. Changes in polyamines, proline and ethylene in sunflower calluses treated with NaCl. *Plant Cell, Tissue and Organ Culture*, **00**: 1-9.
- Amirjani M.R. 2010. Effect of Salinity Stress on Growth, Mineral Composition, Proline Content, Antioxidant Enzymes of Soybean. *American Journal of Plant Physiology*, **5** (6): 350-360
- Ashraf M. and Fatima H. 1995. Response of some salt tolerant and salt sensitive lines of safflower (*Carthamus tinctorius* L.). *Acta Physiologiae Plantarum*, **17**: 61-71.
- Ashraf M., Tufail M. 1995. Variation in salinity tolerance in sunflower (*Helianthus annuus* L.). *Journal of Agronomical and Soil Sciences*, **174**: 351-362.
- Bhaskaran S., Smith R.H., Newton R.J., 1985. Physiological changes in cultured sorghum cells in response to induced water stress. I. Free proline. *Plant Physiology*, **79**: 266-269.
- Bradford M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein binding. *Anal of Biochemistry*, **72**: 248-253.
- Cram W.J. 1976. Negative feedback regulation of transport in cells. The maintenance of turgor, volume and nutrient supply. In: Luttge U., Pitman M.G., Editors, *Encyclopedia of Plant Physiology*, Berlin: Springer-Verlag, 284-316.
- Dasgan H.Y., Aktas H., Abak K., Cakmak I. 2002. Determination of screening techniques to salinity tolerance in tomatoes and investigation of genotype responses. *Plant Science*, **00**: 1-9.
- Delauney A.J., Verma D.P.S. 1993. Proline biosynthesis and osmoregulation in plants. *Plants Journal*, **4**: 215-223.
- Ehsanpour A.A. and Fatahian N. 2003. Effects of salt and proline on *Medicago sativa* callus. *Plant Cell, Tissue and Organ Culture*, **73**: 53-56.
- Gandonou Ch., Abrini J., Idaomar M., Skali Senhaji N. 2005. Response of sugarcane (*Saccharum sp.*) varieties to embryogenic callus induction and *in vitro* salt stress. *African Journal of Biotechnology*, **4** (4): 350-354.
- Gandonou Ch., Errabii T., Abrini J., Idaomar M., Skali Senhaji N. 2006. Selection of callus cultures of sugarcane (*Saccharum sp.*) tolerant to NaCl and their response to salt stress. *Plant Cell, Tissue and Organ Culture*, **87**: 9-16.
- Ghoulam C., Foursy A., Fares K. 2002. Effects of salt stress on growth, inorganic ions and proline accumulation in relation to osmotic adjustment in five sugar beet cultivars. *Environmental and Experimental Botany*, **47**: 39-50.
- Ibarra-Caballero J., Villanueva-verduzco C., Molina-Galan J., Sanchez-de Jimenez E. 1988. Proline accumulation as a symptom of drought stress in maize: a tissue differentiation requirement. *Journal of Experimental Botany*, **39**: 889-897.
- Igarashi Y., Yoshiba Y., Sanada Y., Yamaguchi-Shinozaki K., Wada K., Shinozaki K. 1997. Characterization of gene for L-pyrroline-5- carboxylate synthetase and correlation between the expression of gene and salt tolerance in *Oryza sativa* L. *Plant Molecular Biology*, **33**: 857-865.
- Irigoyen J.J., Emerich D.W., Sanchez-Diaz M. 1992. Water stress induced changes in concentrations of proline and total soluble sugars in nodulated alfalfa (*Medicago sativa*) plants. *Physiologiae Plantarum*, **84**: 55-60.
- Jäger H.J. and Meyer H.R. 1977. Effect of water stress on growth and proline metabolism of *Phaseolus vulgaris* L. *Oecologia*, **30**: 83-96.
- Kashyap S. and Sharma S. 2006. *In vitro* selection of salt tolerant *Morus alba* and its field performance with bioinoculants. *Horticultural Sciences. (Prague)*, **33** (2): 77-86.
- Kavi Kishor P.B., Hong Z., Miao G.H., Hu C.A.A., Verma D.P.S. 1995. Overexpression of L-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. *Plant Physiology*, **108**: 1387-1394.
- Liu J. and Zhu J-K. 1997. Proline accumulation and salt-stress-induced gene expression in a salt-hypersensitive mutant of *Arabidopsis*. *Plant Physiology* **114**: 591-596.
- Lutts S., Kinet J.M., Bouharmont J. 1996a. Effects of salt stress on growth, mineral nutrition and proline accumulation in relation to osmotic adjustment in rice (*Oryza sativa* L.) cultivars differing in salinity resistance. *Plant Growth Regulation*, **19**: 207-218.
- Lutts S., Kinet J.M., Bouharmont J. 1996b. Effects of various salts and of mannitol on ion and proline accumulation in relation to osmotic adjustment in rice (*Oryza sativa* L.) callus cultures. *Journal of Plant Physiology*, **149**: 186-195.
- Misra N., Gupta K.A. 2005. Effect of salt stress on proline metabolism in two high yielding genotypes of green gram. *Plant Science*, **169**: 331-339.
- Moftah A.E., Michel B.E. 1987. The effect of sodium chloride on solute potential and proline accumulation in soybean leaves. *Plant Physiology*, **83**: 238-240.

- Munns R., Husain S., Rivelli A.R., James R.A., Condon A.G., Lindsay M.P., Lagudah A.S., Schachtman D.P., Hare R.A. 2002. Avenues for increasing salt tolerance of crops, and the role of physiologically based selection traits. *Plant and Soil*, **247**: 93-105.
- Prajuabmon A., Theerakulpisut P., Kijwijan B., Muangsan N. 2009. In Vitro Investigation on Salt Tolerant Characteristics of Rice Seedlings (*Oryza sativa* L.). *Research Journal of Agriculture and Biological Sciences*, **5**(4): 423-427.
- Perez-Alfocea F., Santa-Cruz A., Guerrier G., Bolarin C. 1994. NaCl stress-induced organic solute changes on leaves and calli of *Lycopersicon esculentum*, *L. pennellii* and their interspecific hybrid. *Journal of Plant Physiology*, **143**: 106-111.
- Rus-Alvarez A. and Guerrier G., 1994. Proline metabolic pathways in calli from *Lycopersicon esculentum* and *L. Pennellii* under salt stress. *Biologia Plantarum*, **36** (2): 277-284.
- Sakhanokho H.F. and Kelley R.Y., 2009. Influence of salicylic acid on in vitro propagation and salt tolerance in *Hibiscus acetosella* and *Hibiscus moscheutos* (cv' Luna Red'). *African Journal of Biotechnology*, **8** (8):1474-1481.
- SAS Institute. 1992. SAS/STAT user's guide, Vol. 1; Release 6.03, ed. SAS Institute Inc. Cary, NC. USA
- Shafi M., Bakht J., Khan M.J., Khan M.A. and Raziuddin. 2011. Role of abscisic acid and proline in salinity tolerance of wheat genotypes. *Pakistan Journal of Botany*, **43**(2): 1111-1118.
- Smirnoff N. and Cumbes Q.J. 1989. Hydroxyl radical scavenging activity of compatible solutes. *Phytochemistry*, **28**: 1057-1060.
- Solomon A., Beer S., Waisel Y., Jones G.P., Paley L.G. 1994. Effects of NaCl on the carboxylating activity of rubisco from *Tamarix jordanis* in the presence and absence of proline-related compatible solutes. *Physiologiae Plantarum*, **90**: 198-204.
- Van Rensburg L., Krüger G.H., Krüger H. 1993. Proline accumulation as drought-tolerance selection criterion: its relation-ship to membrane integrity and chloroplast ultrastructure in *Nicotiana tabacum* L. *Journal of Plant Physiology*, **141**: 188-194.
- Venekamp J.H. 1989. Regulation of cytosol acidity in plants under conditions of drought. *Physiologiae Plantarum*, **76**: 112-117.
- Watanabe S., Kojima K., Ide Y., Sasaki S. 2000. Effects of saline and osmotic stress on proline and sugar accumulation in *Populus euphratica* in vitro. *Plant Cell, Tissue and Organ Culture*, **63**: 199-206.
- Yokota S. 2003. Relationship between salt tolerance and proline accumulation in Australian acacia species. *Journal of Forest Research*, **8**: 89-93.