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Full Length Research Paper

Growth, proline and ion accumulation in sugarcane callus cultures under drought-induced osmotic stress and its subsequent relief

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Calli obtained from two sugarcane cultivars (R570 and CP59-73) were exposed to different osmotic stress intensities followed by a period of stress relief. Relative rate growth, callus water content and changes in organic and inorganic solutes were determined at the end of stress and relief periods. After the stress period, calli derived from both cultivars showed a decrease in RGR, but at lesser extent in R570 than CP59-73 cultivar. Same tendency was recorded in the callus water content under mannitol-induced osmotic stress. The inorganic solutes seemed to have no contribution in the osmotic adjustment in mannitol-stressed calli since K^+ and Ca^{2+} concentrations decreased drastically while Na^+ and Mg^{2+} concentrations were not affected. The accumulation of proline occurred in both cultivars and was more marked in CP59-73 than R570 cultivar. At the end of the relief period, we observed that all the considered parameters have recovered completely to reach the control levels. According to these results, we conclude that the drought stress-induced changes are reversible, at the least at the cellular level, in sugarcane cultivars.

Key words: Sugarcane, callus, drought stress, mannitol, recovery, ion content, proline.

INTRODUCTION

Drought stress remains an ever-growing problem that severely limits crop production worldwide and causes important agricultural losses particularly in arid and semi-arid areas (Boyer et al., 1982). Drought induced osmotic stress triggers a wide range of perturbations ranging from growth and water status disruption to the modification of ion transport and uptake systems (Lutts et al., 1996; Bajji et al., 2000; Santos-Diaz and Ochoa-Alejo, 1994). Upon exposure to water deficit, plants exhibit physiological, biochemical and molecular responses at both the cellular and whole plant levels (Greenway and Munns, 1980; Hasegawa et al., 2000). Generally, the plants accumulate some kind of organic and inorganic solutes in the cytosol to raise osmotic pressure and thereby maintain both turgor and the driving gradient for water uptake (Rhodes and Samaras, 1994). Among these solutes, proline is the most widely studied ((Delauney and Verma, 1993). It have been suggested that the increase of free proline levels is a symptom of injury that results from imbalances in other pathways (Bhaskaran et al., 1985; Perez-Alfocea and Lahrer, 1995). Also, the beneficial roles of proline in conferring osmotolerance have been widely reported (Kavi Kishor et al., 1995; Bajji et al., 2000).

However, water stress may be transient and can chan-

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Abbreviations: 2,4-D, 2,4-Dichlorophenoxyacetic acid; cv(s), cultivar(s); RGR, relative growth rate; MS medium, Murashige and Skoog medium; ANOVA, Analysis of variance.

ge with weather conditions and plants under such conditions are allowed to recover after rainfall period. Data on recovery proprieties have been studied at the whole plant in some species such as tomato (Torrecillas et al., 1995) and wheat (Kameli and Lösel, 1996). While, there are a few studies trying to evaluate physiological aspects of plant recovery after the alleviation of water stress at the cellular level (Bajji et al., 2000).

Tissue culture system is useful for the evaluation of tolerance to environmental stresses because the stress conditions can be easily controlled *in vitro.* Moreover, *in vitro* culture provides a uniform population of synchronously developing plant cells without involving regulatory mechanisms that naturally repaired at the whole plant level (Tal, 1983).

The present work was therefore performed in sight to gain information on the processes taking place at the cellular level in sugarcane (*Saccharum* sp.) after exposure to various osmotic stress intensities and its subsequent relief in relation with growth rate, callus water content, ion uptake and proline accumulation.

MATERIAL AND METHODS

Plant material

Cuttings of sugarcane (*Saccharum* sp.) cultivars R570 and CP59-73 were kindly provided by CTCS-Gharb (Centre Technique des Cultures Sucrières-Gharb, Morocco). Stalk segments were surface disinfected with 70% ethanol and sown in pots containing soil in greenhouse. Pots were irrigated every two days with tap water. After germination, sugarcane plants were grown in the same conditions until approximately 6 months. R570 (CERF, Reunion) is a leading commercial variety in several countries while CP59-73 cultivar (Canal Point, United States) is largely cultivated in Morocco.

Culture conditions

Callus induction was carried out as described previously by Gandonou et al. (2005). After 6 weeks, calli were weighed and transferred to the same MS medium (absence of stress and osmotic pressure of 0.4 MPa) and to MS medium added with mannitol in order to obtain final osmotic pressures of about 0.62 MPa, 0.84 MPa and 1.08 MPa. Callus cultures were incubated in the same conditions as described above. After one month of stress, calli were divided in two sets. One set was weighed and further characterized for solutes accumulation. The second set was transferred to MS medium without mannitol for a subsequent period of one month.

For each period (stress and recovery), the callus relative growth rate (RGR) was expressed according to the formula RGR = [(Final weight - initial weight) x 100] / initial weight. The callus water content was calculated as (Fresh weight – dry weight)/dry weight.

Determination of proline concentration

Proline was extracted as described by Paquin and Lechasseur (1979). 200 mg of callus fresh matter was homogenized in 4 ml of methanol-chloroform-water (15:5:1 v/v/v) at 4°C and then was centrifuged at 20 000 x *g* for 30 min. Supernatants were then incubated at 4°C for 12 h in presence of 0.25 ml chloroform and 0.9 ml distilled water. Proline was quantified in the upper phase using ninhydrin acid reagent according to Bates et al. (1973). The chromophore containing proline was extracted in 4 ml of toluene and measured spectrophotometrically at 520 nm. L-proline was used as standard.

Determination of ion concentration

For ion measurements, calli were first rinsed for 5 min with cool distilled water and they were then oven-dried at 80 °C for 72 h and grounded. The dry matter obtained was used for mineral analysis. The major cations were extracted after digestion of dry matter with HNO_3 acid. The extract was filtered prior to analysis. Na^+ and K^+ concentrations were determined using a flame spectrophotometer (PHF 90D, France). Ca^{2+} and Mg^{2+} concentrations were quantified by atomic absorption spectrophotometer (Shimadzu AA-6200, Japan).

Statistical analyses

All the measurements were repeated on two sets of 30 to 40 calli and showed similar results. Data presented therefore are obtained from on single experiment. Each value is presented in the form of mean ± standard error with a reading of at least four samples per treatment. The analyses of the main effects of the stress intensity as well as the recovery data was based on the analysis of variance (ANOVA I). All statistical analyses were performed using SAS program (SAS Institute, 1992).

RESULTS AND DISCUSSION

Relative rate growth (RGR) appeared to be remarkably influenced by genotype, since a significant difference among cultivars was recorded even in the absence of stress. RGR was higher in R570 and lower in CP59-73 (Figure 1). Mannitol-induced osmotic stress impaired significantly RGR in both cultivars ($F= 349.45$, $P<0.001$) but at lesser extent in R570 than CP59-73 (Figure 1). Thus, at the osmotic pressure of 0.62 MPa, RGR decreased to approximately 29% and 34% in R570 and CP59-73, respectively. The callus water content decreased significantly ($F= 262.89$, $P<0.001$) and proportionally to the stress intensity in the medium, in similar manner to the RGR (Figure 2). Thus, at the highest osmotic pressure, water callus content declined to about 45 and 48% of the control in R570 and CP59-73, respectively. These findings indicated the important loss of water and they are in agreement with those reported in several other species such as *Triticum durum* (Bajji et al., 2000; Lutts et al., 2004), *Oryza sativa* (Lutts et al.,1996) and *Tagetes minuta* (Mohamed et al., 2000). Growth inhibition under osmotic conditions might be mainly due to the reduction in cytoplasmic volume and the loss of cell turgor as result of osmotic outflow of intracellular water (Rhodes and Samaras, 1994). Among the cultivars, our results suggested that R570 was slightly more drought-resistant than CP59-73 at the cellular level and that the drought-resistance is closely related to the maintenance

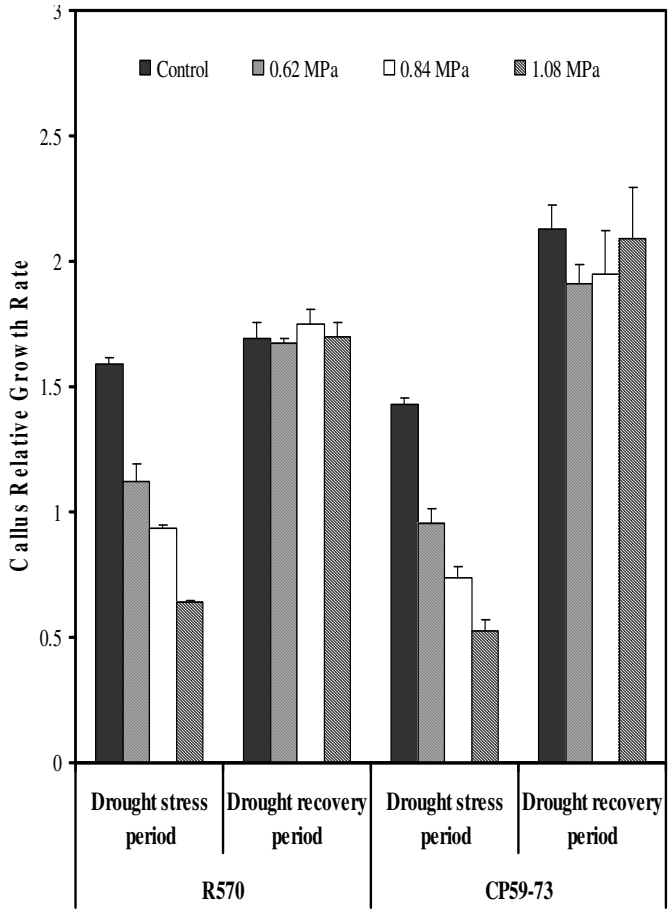

Figure 1. Callus relative growth of sugarcane (*Saccharum* sp.) R570 and CP59-73 calli after exposure to and recovery from mannitol-induced drought stress. Each value is the mean of six replicates and vertical bars represent ± standard error.

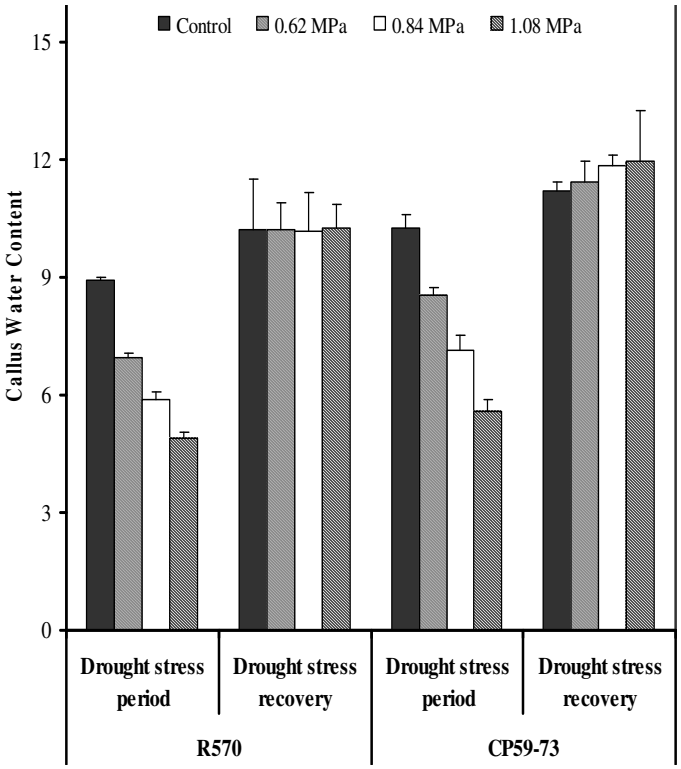

Figure 2. Changes in the water content of sugarcane (*Saccharum* sp.) R570 and CP59-73 calli after exposure to and recovery from mannitol-induced drought stress. Each value is the mean of six replicates and vertical bars represent ± standard error.

of an elevated water status under such conditions. No significant difference was recorded after the stress relief for the RGR and callus water content parameters. Thus, callus relative growth resumed once and both R570 and CP59-73 calli recovered growth to approximately 100% at all the stress intensities as shown in Figure 1. Similarly, the water status of both cultivars increased until reaching control levels at the end of the recovery period irrespective of the applied stress intensity (Figure 2). These results also indicated that the viability was maintained during the stress treatment as was reported in PEG-treated wheat calli (Bajji et al., 2000) and that the perturbations induced by the water deficit are reversible at the cellular level in sugarcane cultivars. However, these findings allow no discrimination between the cultivars used in this work since both cultivars recovered completely after the stress relief.

Cellular adaptation to such stress might be improved towards the restoration of intracellular volume and cell turgor. It have been widely reported that plant cells achieve their osmotic adjustment by the accumulation of some kind of compatible solutes such as proline, betaïne and polyols to protect membranes and proteins (Delauney and Verma, 1993). Consequently, there occurs an osmotic re-inflow of water into the cells.

Compatible solutes are overproduced under osmotic stress aiming to facilitate osmotic adjustment (Hasegawa et al., 2000). These compounds accumulated in high amounts mainly in cytoplasm of stressed cells without interfering with macromolecules and behaved as osmoprotectants (Yancey, 1994). It has been shown that proline also have a key role in stabilizating cellular proteins and membranes in presence of high concentrations of osmoticum (Rudolph et al., 1986; Yancey, 1994). In our results, a highly significant difference was recorded in proline concentration ($F= 912.94$, $P<0.001$). Thus, proline accumulation rate in callus culture increased drastically and proportionally to the increasing mannitol concentration (Figure 3). R570 calli accumulated less proline than CP59-73 ones. At osmotic pressure of 1.08 MPa, proline concentration increased by 10 fold in R570 calli and by 13 fold in CP59-73 calli. Although, these statements suggested that the proline is not directly involved in the drought-resistance, from a quantitative point of view, in sugarcane cultivars at the least in the cellular level and corroborated with those reported in rice (Lutts et al., 1996) and wheat (Lutts et al., 2004).

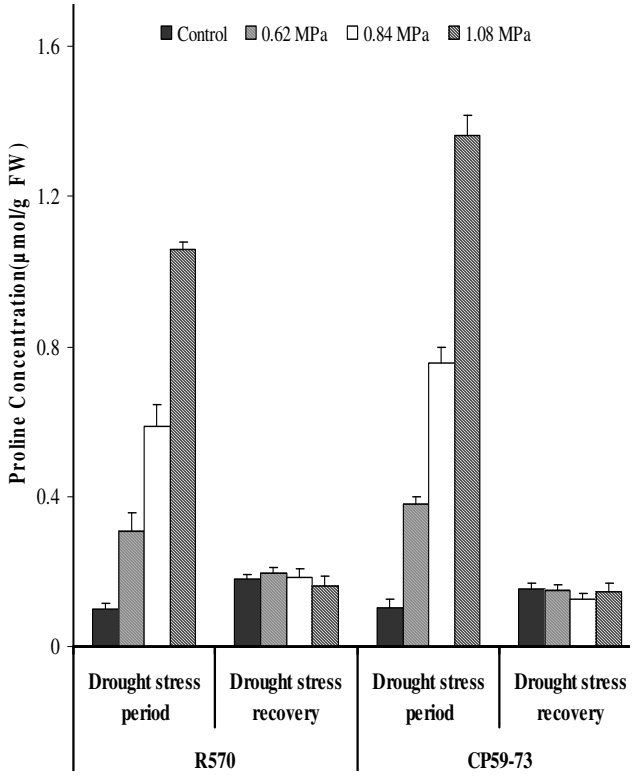

Figure 3. Changes in proline concentration of (*Saccharum* sp.) R570 and CP59-73 calli after exposure to and recovery from mannitol-induced drought stress. Each value is the mean of six replicates and vertical bars represent ± standard error.

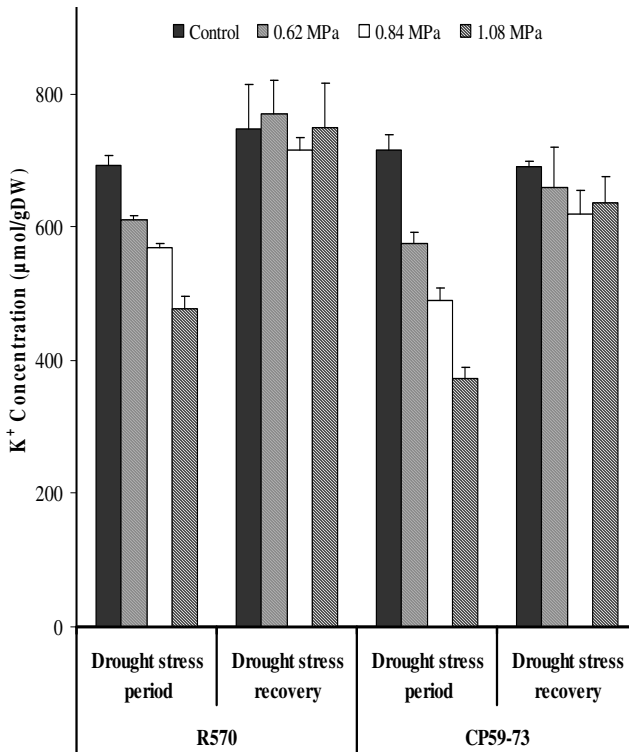

Figure 4. Changes in K^+ concentration of sugarcane (*Saccharum* sp.) R570 and CP59-73 calli after exposure to and recovery from mannitol-induced drought stress. Each value is the mean of six replicates and vertical bars represent ± standard error.

Calli growth recovery was accompanied with a reduction in proline concentration (Figure 3). The proline concentration decreased in both cultivars after the stress relief and returned to control levels. These findings suggested that organic solutes accumulated during the stress period are likely to be utilised for growth after stress relief. The proline has been suggested to play a key role after the stress relief. Thus, Ahmad and Wyn Jones (1979) reported that the decrease in proline concentration during the recovery period was related to tissue rehydration. As well, it has been suggested to serve as an organic nitrogen reserve ready to be used after stress relief to sustain both amino acid and protein synthesis (Trotel et al., 1996; Sairam and Tygai, 2004).

K^+ is a major cation in cell organization and it was reported to be a major contributor to osmotic adjustment under stress conditions in several species (Santos-Diaz and Alejo-Ochoa, 1994; Bajji et al., 2001). Under mannitol-induced osmotic stress, K^+ concentration declined significantly ($F= 282.49$, $P<0.001$) and it reached about 31 and 48% of the control in R570 and CP59-73 calli, respectively at 1.08 MPa (Figure 4). Thus, R570 accumulate more K^+ than CP59-73. Similar results were reported earlier in mannitol-treated wheat callus (Trivedi et al., 1991) and in mannitol-treated rice callus (Lutts et al., 1996), while just slight decrease in K^+ content was observed in wheat callus (Bajji et al., 2000) with the increasing PEG concentration in medium. In contrast, Santos-Diaz and Alejo-Ochoa (1994) reported that cell cultures of chilli pepper increased their amounts in K^+ than the control when exposed to PEG-induced osmotic stress. Similarly, The Ca^{2+} concentration decreased significantly in the presence of mannitol-supplemented medium ($F= 238.03$, $P<0.001$) and it reached to about 32 %, 50 % of the control in R570, CP59-73, respectively, at osmotic pressure of 1.08 MPa (Figure 5). Among the cultivars, R570 calli seemed to retain more Ca^{2+} than CP59-73 ones. The decrease of Ca^{2+} content under osmotic stress has been previously reported in other species (Lutts et al., 1996, 2004). Besides, the role of Ca^{2+} in ensuring membrane integrity and in the ion regulation within plant cells is well known (Hirschi, 2004). While, Na^{2+} and Mg^{2+} concentrations were not significantly affected by osmotic stress whatever the mannitol concentration (Table 1). Similar statements have been reported previously in some other species (Bajji et al., 2000) while an important increase of Na^+ concentration in chilli pepper cell cultures exposed to PEG-induced stress was reported (Santos-Diaz and Alejo-Ochoa, 1994). After stress relief, Ca^{2+} and K^+ concentrations returned to the control levels at all the experimented mannitol doses in both cultivars (Figures 4 and 5). Comparative data on physiological changes in sugarcane cultivars under drought stress and its

Table 1. Na^+ and $Mg2^+$ concentrations in sugarcane (*Saccharum sp)* R570 and CP59-73 calli exposure to mannitol-induced drought stress.

Mannitol-induced drought stress	Na^+ Concentration (µmol/g dry weight)		Mg^{2+} Concentration (µmol/g dry weight)	
	R570	CP59-73	R570	CP59-73
Control	66.67 ± 8.17	159 ± 6.64	236.99 ± 5.09	285.78 ± 10.15
0.62 MPa	72.5 ± 9.58	155 ± 12.91	239.39 ± 4.26	278.74 ± 17.44
0.84 MPa	80 ± 16.32	148,75 ± 6.29	241.53 ± 5.68	282.1 ± 8.14
1.08 MPa	70 ± 14.14	150 ± 10.80	238.91 ± 4.88	280.09 ± 12.95

Each value is the mean of six replicates ± standard error

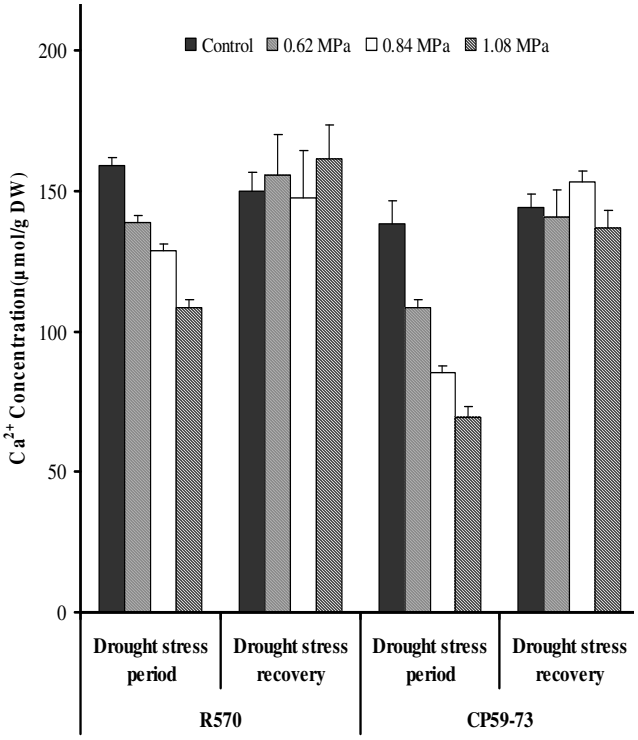

Figure 5. Changes in Ca^{2+} concentration of sugarcane (*Saccharum* sp.) R570 and CP59-73 calli after exposure to and recovery from mannitol-induced drought stress. Each value is the mean of six replicates and vertical bars represent ± standard error

subsequent relief at the cellular level are scarce. Based cultivars at the cellular level. Such disruptions were mainly due to the water outflow and the leakage of essential ions such as K^+ and Ca^{2+} since that callus RGR showed to be strongly correlated with both K^+ and Ca^{2+} contents. Besides, the mineral fraction seemed to have no contribution in the osmotic adjustment as a strong decrease was observed in K^+ and Ca^{2+} contents while Na^+ and Mg^{2+} remained unchanged. In sugarcane cultivars, proline accumulation is weakly involved in the osmotic adjustment at the cellular level. Among the cultivars, the data allowed no distinction for drought resistance trait among the cultivars, since a slight variation in callus growth under osmotic-induced stress was recorded. It appears that the drought stress-induced changes are reversible, at the least at the cellular level, in sugarcane cultivars since most of the investigated parameters returned to control levels in both cultivars irrespective of the stress intensity according to the statistical analyses at the end of the recovery period. As well, our findings suggested the presence of cellular machinery that controls the recovery from the osmotic induced stress in sugarcane cultivars.

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REFERENCES

Ahmad N, Wyn Jones RG (1979). Glycinebetaine, proline and inorganic ion levels in barley seedlings following transient stress. Plant. Sci. Lett. 15: 231-237.

Bajji M, Lutts S, Kinet JM (2000). Physiological changes after exposure to and recovery from polyethylene glycol-induced water deficit in callus cultures issued from durum wheat (*Triticum durum* Desf.) cultivars differing in drought resistance. J. Plant. Physiol. 156: 75-83.

Bates LS, Waldren RP, Teare ID (1973). Rapid determination of free proline for water-stress studies. Plant and Soil. 39: 205-207.

Bhaskaran S, Smith RH, Newton RJ (1985). Physiological changes in cultured sorghum cells in response to induced water stress. Plant. Physiol 79: 266-269.

Boyer JS (1982). Plant productivity and environment. Science. 218: 443-448.

Delauney AJ, Verma DPS (1993). Proline biosynthesis and osmoregulation in plants. Plant J. 4: 215-223.

Gandonou C, Errabii T, Abrini J, Idaomar M, Chibi F, Skali Senhaji N (2005). Effect of genotype on callus induction and plant regeneration from leaf explants of sugarcane (*Saccharum* sp.). Afr. J. Biotechnol. 4 (11): 1250-1255.

Greenway H, Munns R (1980). Mechanisms of salt tolerance in nonhalophytes. Annu. Rev. Plant Physiol. 31: 149-190.

Hasegawa P, Bressan RA, Zhu JK, Bohnert HJ (2000). Plant cellular and molecular responses to high salinity. Annu. Rev. Plant Mol. Biol. 51: 463-499.

Hirschi D (2004). The Calcium conundrum. Both versatile nutrient and specific signal. Plant Physiol. 136 (1): 2438-2442.

Kameli A, Lösel DM (1996). Growth and sugar accumulation in durum wheat plants under water stress. New Phytol. 132:57-62.
Kishor PBK, Hong Z, Miao GH, Hu CAA, Verma DPS (1995). Overexpression of [delta]-Pyrroline-5-Carboxylate Synthetase Increases Proline Production and Confers Osmotolerance in Transgenic Plants. Plant. Physiol. 108 (4): 1387-1394.
Lutts S, Kinet JM, Bouharmont J (1996). Effects of various salts and of mannitol on ion and proline accumulation in relation to osmotic adjustment in rice (*oryza sativa L.*) callus cultures. J. Plant. Physiol. 149: 186-195.
Lutts S, Almansouri M, Kinet JM (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.
Mohamed MAH, Harris PJC, Henderson J (2000). *In vitro* selection and characterisation of a drought tolerant clone of *Tagetes minuta*. Plant. Sci. 159: 213-222.
Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant. 15: 473-497.
Paquin R, Lechasseur P (1979). Observations sur une méthode de dosage de la proline libre dans les extraits de plante. Can. J. Bot. 57: 1851-1854.
Perez-Alfocea F, Larher F (1995). Sucrose and proline accumulation and sugar efflux in tomato leaf discs affected by NaCl and polyethylene glycol 6000 iso-osmotic stresses. Plant. Sci. 107: 9-15.
Rhodes D, Samaras Y (1994). Genetic control of osmoregulation in plants. In cellular and molecular physiology of cell volume regulation. Strange K. Boca Raton: CRC Press. pp: 347-361.
Rudolph AS, Crowe JH, Crowe LM (1986). Effects of three stabilizing agents: proline, betaine, and trehalose on membrane phospholipids. Arch Biochem Biophys. 245: 134 -143.
Sairam RK, Tygai A (2004). Physiology and molecular biology of salinity stress tolerance in plants. Curr. Sci. 86: 407-421.
Santos-Diaz MS, Ochoa-alejo N (1994). PEG-tolerant cell clones of chilli pepper (*Capsicum annuum* L.): Growth, osmotic potentials and solute accumulation. Plant Cell Tiss. Org. Cult. 37: 1-8.
SAS Institute Inc (1992). SAS/STATJ User's Guide. Release 6.03 edn. SAS Institute Inc. Cary, NC., 1028.
Tal M (1983). Selection for stress tolerance. In Evans DA, Sharp WR, Ammirato PV, Yamada Y (Eds): Handbook of plant cell culture (Vol.1). Techniques for propagation and breeding, Macmillan publishing Co., New York. pp 461-488.
Torrecillas A, Guillaume C, Alarcon JJ, Ruiz-Sanchez MC (1995). Water relations of two tomato species under water stress and Recovery. Plant. Sci. 105: 169-176.
Trivedi S, Galiba G, Sankhla N, Erdei L (1991). Responses to osmotic and NaCl stress of wheat varieties differing in drought and salt tolerance in callus cultures. Plant. Sci.73: 227-232.
Trotel P, Bouchereau A, Niogret MF, Larher F (1996). The fate of osmoaccumulated proline in leaf discs of rape (*Brassica napus* L.) incubated in a medium of low osmolarity. Plant. Sci. 118: 31-45.
Yancey PH (1994). Compatible and counteracting solutes. In Cellular and Mol. Physiol. of Cell Volume. Edited by Strange K. Boca Raton: CRC Press; pp: 81-109.