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Evaluation of Sugarcane (*Saccharum officinarum* L.) Somaclonals Tolerance to Salinity Via *In Vitro* and *In Vivo*

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Tissue culture technique was used to obtain salt tolerant variants from embryogenic calluses of sugarcane (*Saccharum* sp. var. CP48-103) that cultured on a selective medium containing different levels of NaCl (0, 0.2, 0.4, 0.6, and 0.8% NaCl). A total of four plants regenerated from the tolerant calluses were selected but the best of them in vigor grown in *in vitro* and hydroponic systems under salinity stress to comparison with source variety. With increasing supply of NaCl in both systems, root growth was more adversely affected than was shoot growth. Chlorophyll contents showed a decreasing trend and dry matter yield of plants reduced but in a slow rate in tolerant somaclonal than source variety. The biochemical analysis showed that at high salt concentration, Cl^- and Na^+ content in shoot and root increased. With rising salt concentration from 0 to 0.8%, content of Cl^- in shoot and root of tolerant variant changed lower than parent showed that this variant had genetic lowest ratio of shoot/root chloride and had minimum transport of Cl^- to shoots. Also this variant had high content of Ca^{2+} in shoot and high K^+/Na^+ ratio at all salinity levels. Thus it probably has genetic potential to avoid harmful ions uptake.

Key words: sugarcane, salinity, somaclonal, *in vivo*, *in vitro*

INTRODUCTION

Soil or water salinity is considered to be the major environmental factor limiting plant growth and productivity, especially in arid and semi-arid irrigated regions including Iran. Salinity limits vegetative and reproductive growth of plants by inducing severe physiological dysfunctions and causing widespread direct and indirect harmful effects, even at low salt concentrations (Munns 2002; Altman 2003). Salt stress has been extensively investigated since soil salinity represents a major constraint for successful production and crop yielding (Munns 2002). The salt-affected lands extended to about 6% of the world surface and are becoming even more prevalent as the intensity of agriculture increases worldwide (Flowers & Yeo 1995).

Sugarcane (*Saccharum officinarum* L.) is a glycophyte considered as moderately sensitive to salinity stress and a crop of major economical value in tropical and subtropical developing countries where salinity is an ever-increasing problem (Wahid *et al.* 1997), due to it is estimated that about 1 million ha of land under sugarcane cultivation are affected by salinity or sodicity. In Iran, sugarcane is grown under irrigated systems and is seriously prone to soil salinization. This problem may be a serious handicap for the production and the yielding of this agricultural crop. Sugarcane growth may suppression to the accumulation of toxic ions. Salinity in the root zones of sugarcane decreases the sucrose yield, through its effect on both biomass and juice quality. Although the rate of canopy development and final size are an outcome of leaf and stem extension-growth, it has been shown that leaf injury and loss due to excess salt ion accumulation might be an important factor controlling the active size of the canopy (Lingle & Weigand 1996). Rozeff (1995) suggested that a steep decline in growth may take place once the ECe rises above 3 dSm^{-1}, although plants may survive up to 10-15 dSm^{-1} depending on cultivar. Many elite cultivars used in commercial production in Iran have superior agronomic performance but may have susceptibility to salinity which limits their cultivation. One such elite cultivar is CP48-103, which is agronomically superior on the clay-loam soils of the Khuzistan province but is susceptible to accumulation of abundant Cl^- in leaves (Soltani *et al.* 2008).

The complexity and polygenic nature of salinity tolerance has seriously limited the efforts to develop the tolerant crop variety through conventional breeding practices. Somaclonal variation in combination with *in vitro* mutagenesis and selection has been applied for the isolation of agronomically useful mutants (Jain 2000; Zhambrano *et al.* 2003). Many examples related to different vegetative propagated species, show that the combination of *in vitro* culture with selection is relatively inexpensive, simple, and efficient (Ahloowalia 1998).

To achieve salt tolerance, plant cells evolve several biochemical and physiological pathways. These processes are thought to operate additively to ensure plants and cells survival and they include the exclusion of Na^+ ions and their compartmentation into vacuoles as well as the accumulation of compatible solutes such as

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proline, glycinebetaine and polyols (Parida & Das 2005). Although there are numerous reports on selection and physiological characterization of salt-tolerant clones using whole plants in diverse plant species, limited data are available on this manner in Iran condition, and in sugarcane there has not been concerted effort in this direction. In an earlier report, Patade *et al.* (2006) studied the effects of salt and drought stresses on irradiated cells of sugarcane and obtained plants tolerant to higher salt stress. Gandonou *et al.* (2006) studied the effects of salt stress by exposing the callus to a single level of 68 mM NaCl, and observed that physiological and biochemical indicators could play a crucial role in salt tolerance. Radiation induced mutagenesis followed by *in vitro* selection was employed for salt tolerance in popular Indian sugarcane (Patade *et al.* 2008).

MATERIALS AND METHODS

Plant Material. The research was carried out in the Biotechnology-Tissue Culture Lab, Department of Sugarcane Research Center, Karun Agro-industrial Co., Iran. Healthy young leaf explants included apical meristems were obtained from the shoot of commercial sugarcane variety CP48-103. These sections washed thoroughly under running tap water for 20 min followed by sterilized in a 1.5% NaClO solution for 20 min then washed with sterile distilled water and transferred to laminar air flow cabinet. The explants cut into thin smaller pieces of 1 to 1.5 cm length so prepared for cultured.

***In Vitro* Performance.** Calli were established from the smaller pieces of explants on callus induction, made on Murashige and Skoog (1962) medium, supplemented with 30 g l^{-1} sucrose, 8 g l^{-1} agar, and 3 mgl^{-1} 2,4-D. Constituents of all media were products of Sigma Co., USA. The medium was adjusted to pH 5.8 with NaOH (0.1 N), autoclaved at 120 °C and 1 bar for 20 min. After 4 weeks embryogenic calli were separated from the explants and transfer to MS media supplemented with different levels of NaCl (0, 0.2, 0.4, 0.6, and 0.8%) during serial subculture (in a step-wise manner). Cultures were grown in 100 ml glass jars containing 25 ml of culture medium closed with aluminum foil caps. Plantlets were regenerated and then rooted after 3-4 weeks of transfer of high healthy callus on regeneration and root medium, i.e., MS medium of the same composition as above but with special hormones (Barba *et al.* 1977) and none 2,4-D in a growth chamber under long-day conditions (16/8 h light/dark cycle) at a temperature of 25 $\pm$ 2 °C and relative humidity of 60 -70%. Light was provided by white fluorescent tubes (60 W, photon flux density 50 µmol $m^{-2}s^{-1}$). The best and healthy plantlets were selected as tolerant somaclonal variants for next evaluations.

***In Vivo* Performance.** Four weeks-old selected variants that come from tissue culture were used for salinity tolerance evaluation under *in vivo* condition including the $^1/_4$ strength modified Hoagland's solution (Hoagland & Arnon 1950). So healthy plant was transferred to dark plastic boxes with 50 × 30 × 20 cm^3 (length × width × depth) specification. Holes were made in the boxes lids used, in 30 × 20 cm spacing to accommodate five plants per box. Only $^2/_3$ of the boxes were filled with the solution to ensure the presence of adequate air inside the box so that a special aeration mechanism system have used. Nutrient solutions were renewed every 14 days along with added the salinity levels. The solution was tested every week to regulate the pH and EC and distilled water was added daily to replace transpiration losses. The growth room used for growing plants to cover approximately twelve and half hours of daylight with mean irradiance value of 800 Wm^{-2}. The mean temperature and relative humidity values were 27.5 $\pm$ 3.5 °C and 60 $\pm$ 5%, respectively.

Morphological and Biochemical Analysis. Since the morphological features of the somaclonal plants were not sufficient, biochemical analysis were also used as compared to their parental variety. Fully expanded green leaf number (as long as 75% of the leaf was still green, it was considered as a green leaf) and plant height were determined weekly. Transpiration rates of nutrient solution-grown plants were determined every two day by weighing of the pots at 2.5 h intervals between 10:00 and 15:00 h and from different weight of pots include plant with no plant pot as control. The acceptable agreement between the methods (Calculated transpiration with a photosynthesis meter, $r^2 = 0.82$), gave confidence in the method.

Plants were sampled when they were 60 and 150 days old for tissue cultures and hydroponics, respectively. Each harvested plant was partitioned into stem, leaf and root for analysis. Morphological aspects i.e. leaf area, dry matter accumulation, dry matter partitioning and total chlorophyll rates were analyzed in these components. Leaf area was measured using a leaf area meter (LI-3050A, LI-COR, USA) in square centimeters. Dry matter accumulation was quantified by obtaining dry weights of plants at 70 °C for 48 hours in a dry oven. Dry matter partitioning to shoots and roots was estimated by calculating shoot:root and leaf:stem ratios in dry weight basis. Total chlorophyll rates were measured using the chlorophyll meter (SPAD 502, Minolta, Japan).

Tissue chloride content was determined by coulometric-amperometric titration (Soltani *et al.* 2008) on water extracts of samples taken from dried and ground plant material. The potassium and sodium content was estimated by flame photometer (Jenway PFP 7, ELE Instrument Co. Ud.) method (Yoshida *et al.* 1976). Ca was estimated by versene titration method as described by Jackson (1973). The concentration of K^+ and Na^+ in the digested solution was determined by flame photometer (Jenway PFP 7, ELE Instrument Co. Ud.). All measurements were conducted on three replicate plants per each treatment.

Statistical Analysis. The experiment was a factorial experiment of two factors, with three replications and arranged in Randomized Completely Block Design. The first factor was one sugarcane variety, CP48-103, and 4 derivation salinity tolerant variants. The second factor was five salinity levels (0, 0.2, 0.4, 0.6, and 0.8% NaCl).

The data were subjected to analysis of variance (ANOVA), and comparisons between the mean values of treatments were made by the least significant difference (LSD) test calculated at a confidence level of $P \leq 0.05$ using the statistical software SAS (SAS Institute 1992).

RESULTS

Morphological Aspects. Under salt stress condition of main variety (i.e. CP48-103) showed that root growth of tolerant variants was found to be not reduced significantly by an increasing supply of NaCl than was that of shoots. Both root length and the mean number of rooted shoots (Figure 1a) decreased with increasing salt concentration in main variety but not in the tolerant variants. In culture conditions, tolerant variants kept the normally growth, and elevated NaCl concentrations in showed no inhibitory effect on shoot growth (Figure 1b).

With increasing salt concentrations the total dry weight decreased sharply in main variety than new tolerant variants. Maximum total dry matter produced at higher salinity was 1.9 g plant^{-1} in tolerant variant and 1.1 g plant^{-1} in source variety in hydroponic system (Table 1). It was indicated that higher amounts of Na^+ in plant tissues significantly reduced dry matter production (Figure 2).

All the Morphological aspects expect leaf number (Table 1) were significantly higher ($P < 0.05$) in the best variant than control under both cultivation systems at high salinity, 0.8% NaCl. Chlorophyll contents decreased with a slowly slope (20%) and rapidly slope (59%) with increasing NaCl supply up to 0.8% in tolerant variants and source variety, respectively.

The rate of salt accumulation in shoots of salt tolerance plants can be determined by the rate of transpiration. Our results showed that the salt tolerant somaclonal variant have able to transport lower of harmful salt ions (e.g. Na^+ and Cl^-) to shoot tissues (Table 2) and then had a higher transpiration than source variety (Table 1).

Biochemical Aspects. In this study shoot Na^+ concentration increased to 0.345 in tolerant somaclonal variant and to 0.580% of dry weight in source variety with the application of 0.8% NaCl compare to without salt, cases respectively (Table 2). Similarly, increase was found in root Na^+ concentration but in high amounts than shoot Na^+ (Table 3), so that increased to 0.655 in tolerant somaclonal variant and to 1.105% of dry weight in source variety, respectively. It was interesting to note that tolerant somaclonal variant had lower ratio of shoot Na^+ to root Na^+, absorbed and also transport lower rate of Na^+ from root to shoot tissues, an important characteristic of salt tolerant genotypes, compared to parents' variety. While the salt increased, root and shoot Cl^- content also

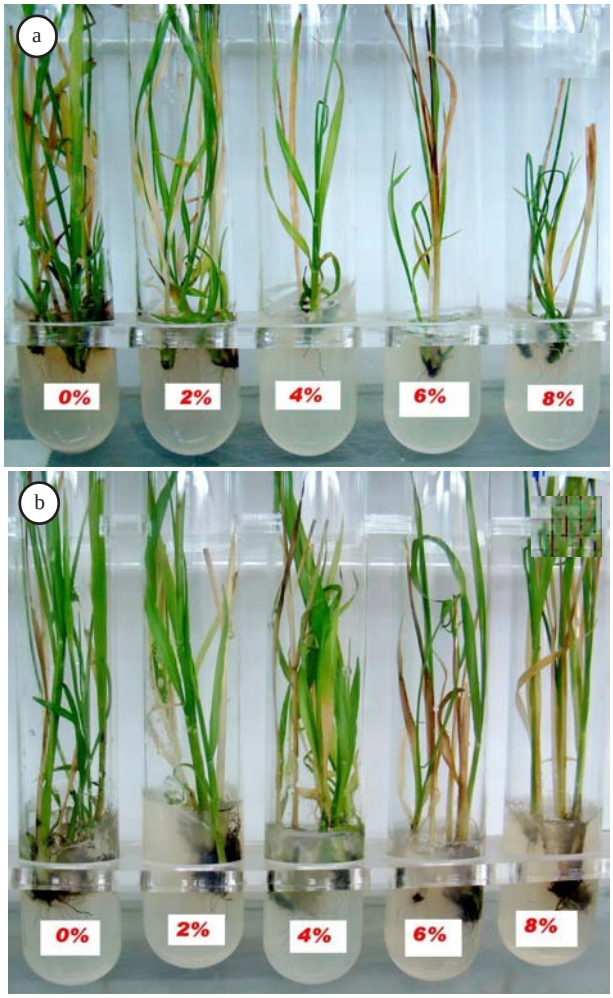

Figure 1. (a) Source variety and (b) its best somaclonal variant growth response to different salinity levels (NaCl %) in, *in vitro* system.

Table 1. Means of some determined morphological aspects of *in vitro* and *in vivo* source sugarcane variety and its best somaclonal variant grown in response to salinity stress. Means sharing same latter are non-significantly ($P > 0.05$) different

Plant type	Salinity (%)	Plantlet height (cm)		Chlorophyll content (mg g^{-1})		Leaf area (cm^2 plant^{-1})		Leaf number (per plant)		Mean leafl dry weight (g plant^{-1})		Total dry weight (g plant^{-1})		Shoot:root ratio		Leaf:stem ratio	Transpiration (l d^{-1} plant^{-1})
		in vitro	*in vivo*	*in vitro*	*in vivo*	*in vitro*	*in vivo*	*in vitro*	*in vivo*	*in vitro*	*in vivo*	*in vitro*	*in vivo*	*in vitro*	*in vivo*	*in vivo*	*in vivo*
Best soma-clone	0	12.2b	24.4ab	2.3a	2.6a	25.1ab	257.1b	3.8a	7.8ab	0.6a	2.4a	0.9a	3.6a	2.3f	1.2d	3.8ef	0.202a
	0.2	12.0b	22.4b	2.3a	2.5a	25.0b	246.7c	3.5b	7.3c	0.5ab	2.2b	0.8ab	3.2b	2.3f	1.2d	4.2d	0.186a
	0.4	11.3bc	20.1c	2.2a	2.4ab	24.5b	236.4de	3.1c	6.7e	0.4bc	1.8c	0.7bc	2.7c	2.4f	1.2d	4.8c	0.140b
	0.6	9.5de	18.3c	2.0b	2.2bc	24.1bc	229.9e	2.8d	6.2f	0.4bc	1.4d	0.6cd	2.1d	2.5f	1.3d	5.7b	0.113c
	0.8	8.6e	15.8d	1.7c	2.0c	23.3c	218.5f	2.1e	5.4g	0.3cd	1.1e	0.5de	1.9de	2.5f	1.3d	6.2a	0.058e
Source variety	0	13.5a	24.9a	1.9b	2.2bc	26.5a	266.3a	3.8a	7.9a	0.6a	2.5a	0.8ab	3.5ab	3.1e	1.4 d	3.0g	0.201a
	0.2	10.6cd	18.1c	1.5c	2.0c	23.3c	241.3cd	3.6ab	7.6b	0.4bc	2.1b	0.7bc	2.8c	3.6d	1.7c	3.6f	0.149b
	0.4	5.3f	10.9e	1.0d	1.3d	19.8d	200.3g	3.5b	7.2c	0.3cd	1.5d	0.5de	2.1d	4.1c	2.0b	4.0ed	0.110c
	0.6	3.6g	7.7f	0.8de	1.1d	17.1e	186.4h	2.9cd	7.0cd	0.2de	1.0e	0.4e	1.6e	4.8b	2.3a	4.8c	0.076d
	0.8	2.2h	4.7g	0.7e	0.9e	15.0f	174.5i	2.8d	6.8de	0.1e	0.5f	0.2f	1.1f	5.3a	2.5a	5.4b	0.045e

increased in both type of experiments plant but the trend was slow and had a low rate in tolerant somaclonal variant than the its parent. Although, the root:shoot ratio of Cl^- content in the source variety was higher than the variant (2.10 against 1.25) but the Cl^- content in the shoot and root of tolerant variant was lower than parent variety (Table 2 & 3).

In the absence of stress, K^+ concentration showed a low differed significant rate among the two experimental type plants, and was lower in salt tolerance somaclonal variant, but with increased the salinity this manner changed adversely and sharply for the benefit of tolerant variant, resulting in a very differ changed K^+/Na^+ ratio (Figure 3), though this ratio was more reduced in source

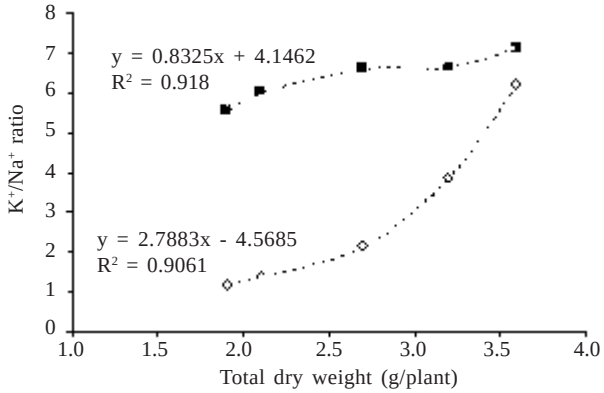

Figure 2. Trend of total dry weight Changes with changed in K^+/Na^+ content in the shoots and roots of Sugarcane salt tolerance somaclonal under increased salinity. ■: shoot, ◇: root.

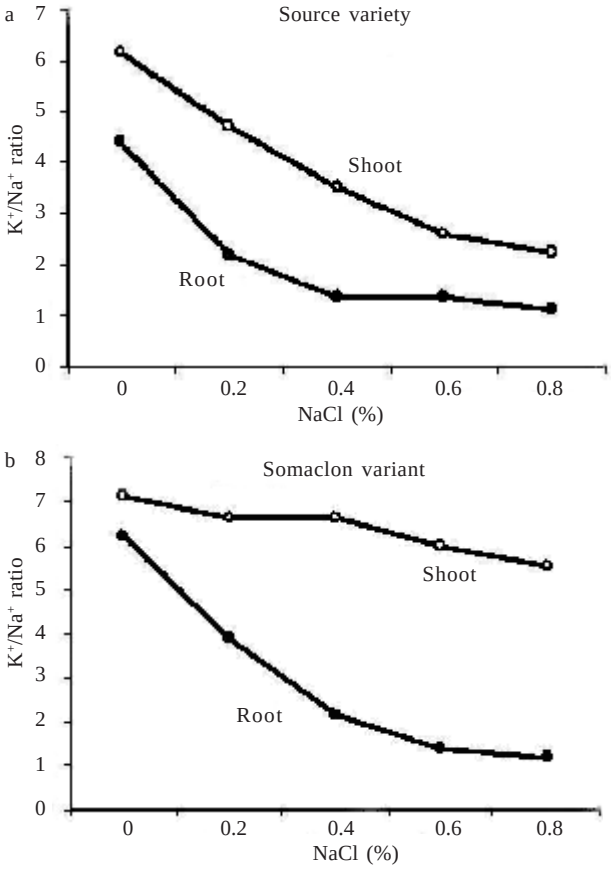

Figure 3. Changes in K^+/Na^+ content in the shoots and roots of (a) Source variety and (b) its best somaclonal sugarcane variant under increased salinity.

Table 2. Nutrient composition (% of dry weight) of shoot of source sugarcane variety and its best somaclonal variant under increased salinity in two culture systems. Means sharing same latter are non-significantly (P > 0.05) different

Plant type	Salinity (%)	Na^+		Cl^-		K^+		Ca^{2+}	
		in vitro	*in vivo*	*in vitro*	*in vivo*	*in vitro*	*in vivo*	*in vitro*	*in vivo*
Best soma clone	0	0.24c	0.20a	0.19a	0.18a	1.47b	1.43ab	0.11a	0.12c
	0.2	0.28b	0.22a	0.20a	0.19a	1.52a	1.46a	0.12a	0.14ab
	0.4	0.31b	0.24b	0.31b	0.26b	1.53a	1.48a	0.15b	0.15a
	0.6	0.35a	0.27c	0.43c	0.41c	1.53a	1.47a	0.16bc	0.15a
	0.8	0.38a	0.31c	0.49c	0.45c	1.53a	1.47a	0.17c	0.14ab
Source variety	0	0.27c	0.24a	0.24a	0.20a	1.52a	1.48a	0.07a	0.09c
	0.2	0.35c	0.31b	0.29a	0.26a	1.50a	1.46a	0.07a	0.10bc
	0.4	0.44b	0.39c	0.37b	0.35b	1.41ab	1.37b	0.09b	0.11b
	0.6	0.56a	0.49c	0.47c	0.40b	1.32c	1.28c	0.10b	0.11b
	0.8	0.61a	0.55d	0.53d	0.48c	1.27d	1.23c	0.15c	0.13a

Table 3. Nutrient composition (% of dry weight) of root of source sugarcane variety and its best somaclonal variant under increased salinity in two culture systems. Means sharing same latter are non-significantly (P > 0.05) different

Plant type	Salinity (%)	Na^+		Cl^-		K^+		Ca^{2+}	
		in vitro	*in vivo*	*in vitro*	*in vivo*	*in vitro*	*in vivo*	*in vitro*	*in vivo*
Best soma clone	0	0.28a	0.24a	0.16a	0.12a	1.58a	1.49a	0.10a	0.08a
	0.2	0.34a	0.31a	0.19a	0.16a	1.54a	1.35b	0.10a	0.09b
	0.4	0.45b	0.41b	0.33b	0.29b	1.02b	1.25c	0.11b	0.11c
	0.6	0.60c	0.55c	0.53c	0.48c	0.85bc	1.20cd	0.12c	0.11c
	0.8	0.69c	0.62c	0.62c	0.56c	0.77c	1.20cd	0.11b	0.11c
Source variety	0	0.35a	0.32a	0.20a	0.15a	1.48a	1.41a	0.03a	0.04a
	0.2	0.67b	0.62b	0.28a	0.24a	1.42b	1.35b	0.04a	0.04a
	0.4	0.98c	0.95c	0.54b	0.49b	1.36c	1.30bc	0.04a	0.05ab
	0.6	1.03c	0.95c	0.93c	0.87c	1.30d	1.28c	0.06b	0.06bc
	0.8	1.15d	1.06c	1.11c	1.04c	1.25d	1.21d	0.09c	0.10c

variety than tolerant somaclonal variant, especially in the shoot than root tissues. High correlated observed between dry weight and K^+/Na^+ ratio of shoot and root at all NaCl salinity from the hydroponic and tissue culture study, r = 0.90 for root and r = 0.92 for shoot, respectively (Figure 2).

DISCUSSION

Salinity still remains the major abiotic stresses that limit and pose a threat to agricultural production in many parts of the world (Altman 2003). While a number of mechanisms relating to improved stress adaptation in crops have been suggested, the fact remains that their association with genetic gains for yield and their relative importance in different salinity-prone environments are still only partially defined. Therefore, a well-focused approach combining the molecular, physiological, and metabolic aspect of abiotic stress tolerance is required (Bhatnagar-Mathur *et al*. 2008). Numerous works comparing general responses of some plant species to different salinity levels report growth reduction under salt stress conditions (Barba *et al.* 1977; Jain 2000; Altman 2003). In our experiment, on the contrary, tolerant variants kept the normally growth, and elevated NaCl concentrations in showed no inhibitory effect on shoot growth.

With increasing salt concentrations the total dry weight decreased sharply in main variety than new tolerant variants. The increases value of the shoot/root dry weight ratio at high NaCl concentrations indicates that roots were affected positively by salinity than were shoots especially in main variety than its tolerant variant. Under salinity stress, results showed that total dry matter production high correlated with K^+/Na^+ ratio (r = 0.90 for root and r = 0.92 for shoot).

Carbon partitioning depends on the strength of both source and sink. As the leaf provides the platform for photosynthesis leaf area indicates the strength of the source of a crop. Photosynthesis and dry matter production of a plant is proportional to the amount of leaf area on the plant (Padmathilake *et al.* 2007). Reductions of chlorophyll content under elevated salinity conditions were observed for some salt-sensitive plant species (Munns 2002). In contrast, chlorophyll content in salt tolerant plants either does not decline or else rises with increasing salinity (Patade *et al.* 2006). Chlorophyll concentration can be used as a sensitive indicator of the cellular metabolic state; thus, its decrease signifies toxicity in tissues due to accumulation of ions.

The rate of salt accumulation in shoots of salt tolerance plants can be determined by the rate of transpiration. Transpiration rate generally tend to decline with increasing rhizospheric salinity in both sensitive and tolerance plant (Michael *et al.* 1997). It might be due to by salt accumulation in the mesophyll which reduced stomatal aperture (Flowers *et al.* 1995).

Subclonal variations play an important role in sugarcane varietals improvement. It is proven that some tissue culture variants are superior than the donor clones in terms of higher biomass, sugar yield and disease resistance. Plant tissue culture is recognized as an important tool to generate useful genetic variability for crop improvement. Wide differences in the salt tolerance of germplasm of a number of crops have been reported (Arzani 2008) but new genetic variability induced by *in vitro* culture was first reported in sugarcane (Rajeswari *et al.* 2009).

In this study, the subclonal variants of intergeneric hybrids showed significant differences for various characters. The statistical analysis of the data from the present studies showed that the changes are genetic. Sodium and chloride concentration in shoots and roots of sugarcane differently increased with salinity, as genotypically (Patade *et. al.* 2006). Thus tissue culture system can be applied in sugarcane breeding programs as a complimentary system for the development of subclones for commercial purpose, parental lines and energy cane. Similarly in the results of the tissue culture with hydroponic techniques showed that hydroponic should be useful for initial screening of the many commercial and new sugarcane variants before final field testing and release new variety. The present study highlights the importance of the effects of both ionic and physiological component of the salt stress on sugarcane. Our results lead us to suggest that the physiological mechanisms that mediate the response to salt stress different. We also provide evidence that the growth inhibition is mainly due to the build up of Na^+ and Cl^- ions in the activate tissues under salt stress. Moreover, we demonstrated that the ion status is closely related to the nature of the stress factor applied in the medium. We revealed that stress resistance in sugarcane somaclonal variants is closely related to the retention of a high amount of K^+ and Ca^{2+} and a low level of Na^+ and Cl^-.

In conclusion, this study demonstrates that *in vitro* selection techniques can be used to generate salt-tolerant plant lines in sugarcane and also to study physiological and biochemical indicators of salinity tolerance in this plant. That seemed salt tolerance to be related to the efficiency of a tissue to absorb, deposit and transport the level of inorganic solutes in response to salt stress. Study results indicated that some of minerals solutes i.e K^+ and Ca^{2+} have a positive role to excuse the tolerant to generated plant.

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