Acta Scientific AGRICULTURE (ISSN: 2581-365X)

Volume 5 Issue 3 March 2021

Research Article

Regeneration of Sugarcane genotypes Under Different Level of Sodium Chloride Salt

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Received: January 12, 2021

Published: February 27, 2021

Abstract

The effects of Murashige and Skoog medium supplemented with different Sodium Chloride salt (NaCl) concentrations (0, 50, 100, 150, 200, 250 and 300 mM) containing 2, 4-D (3 mg/l) along with green coconut water (10%) on explants (leaf sheath) of four sugarcane genotypes were aimed for their callus formation ability and production of embryogenic callus. Besides, regeneration potentiality of sugarcane genotypes including shooting and rooting ability under different levels of NaCl concentrations (0, 50, 100, 150, 200, 250 and 300 mM) was evaluated. MS medium supplemented with 50 mM NaCl produced the highest callus (83.33%) in Isd 16 variety followed by BSRI Akh 41 (83%) and Mutant CC 37 M_5 produced the lowest (66.66%)). But no callus was produced supplemented with 300 mM NaCl in all genotype. The highest shoot and root regeneration (91% and 92%) were obtained from MS medium fortified with 50 mM NaCl containing shooting media combination BAP (6-Benzylaminopurine) 2 mg/l + KN (Kinetin) 1 mg/l and rooting media NAA (1-Naphthaleneacetic acid) 5 mg/l respectively in BSRI Akh 41. On the other hand, little or no shoot and root were initiated from Isd 16, CC37 M_5 and BSRI Akh 42 under above mentioned shooting and rooting media. Callus, shoot and root as well as regeneration ability of plant is highly correlated with callus, shoot and root formation. Significant differences were observed for callus, shoot and root regeneration capacity among all the four genotypes. It revealed that callus, shoot and root regeneration performance ability was decreased by increasing of NaCl concentration levels.

Keywords: Sugarcane; Callus; 2, 4-D; NaCl; Regeneration and Salt Tolerant

Abbreviations

mM: Milli Mole; 2, 4-D= 2,4-Dichlorophenoxyacetic Acid; BSRI: Bangladesh Sugarcrop Research Institute; CC: Clone; MS Medium: Murashige and Skoog Medium; BAP: 6-Benzylaminopurine; KN: Kinetin; NAA: 1-Naphthaleneacetic Acid; NaOH: Sodium Hydroxide; kg cm^{-2}: Kilogram Per Centimeter Square; ANOVA: Analysis of Variance

Introduction

Sugarcane (*Saccharum officinarum* L.) is considered as an important agro-industrial, semi-perennial sugar producing monocot crop which cultivation occurs in the more than 80 tropical and subtropical regions [1-3]. About 70% of the world's sugar production is contributed by this complex polyploidy crop. Globally, about 2.6 M ha of land are being occupied, a little about 2% of the total cropped area, producing 1907 million MT of cane [4]. Nearly 60 countries

over the world's are being cultivated sugarcane as a valuable commercial crop for producing sugar and bioethanol [5]. Unfortunately, several diseases and abiotic stresses such as salinity, drought and freezing are responsible for decreasing the production of this crop [6]. However, being a typical glycophyte, it exhibits stunted growth or no growth, chlorophyll contents and represents the toxic damages, i.e. wilting, chlorosis, necrosis with its yield falling up to 50% especially sugar content when cultivated in the salt-affected soils [7-9]. Salt-affected soil is enriched with a variety of salts, which are quickly dissolved in water to produce toxic ions, especially sodium ion [10]. The improvement of sugarcane plant resistance to salt stress is of great importance for Bangladesh. Callus culture is a supplementary tool to traditional breeding for the production of stress-resistant plants in sugarcane using a multitude of biochemical, physiological and morphological indices [8,9,11-14]. *In vitro* selection program of a given genotype depends on its aptitude to *in vitro* culture, essentially to induction of embryogenic callus and regeneration of plantlets. A variation observed among the four genotypes which regenerated from tissues termed somaclonal variation [15] has been considered as a source of new plant genotype for further crop improvement. In vitro mutagenesis can be beneficial for the development of Somaclonal variation to the isolation of salinity tolerant lines in a short duration [16-18]. However, several variants are often uncertain or non-heritable being epigenetic changes rather than genetic changes. Such epigenetic alterations may result in false-positive signals if one seeks mutational change in a particular phenotype [19,20].

The productivity of sugarcane is worldwide subjected to increasing environmental constraints, predominantly to drought and salinity. salinity is a serious limitation for the production of sugarcane in southern region of Bangladesh [21]. Sugarcane is moderate sensitivity to salinity stress and confined to tropical and sub-tropical irrigated regions, where salinity is an ever-increasing problem. Germination, growth rate, cane yield as well as sucrose content in cane are highly affected by the soil containing high level of salts [22]. Salinity is one of the most serious menaces to crop production. Sugarcane has been categorized as a glycophytic (salt susceptible); because it exhibits toxic symptoms including limited sprout emergence, nutritional imbalance and growth reduction, leading to low productivity, especially sugar content when cultivated in the salt-affected soils [10]. The severe sensitivity of sugarcane to salinity at various growth stages is manifested by a considerable reduction in growth rate [12].

Aim of the Study

We aimed to observe the in vitro response to identify the salt tolerant and susceptible genotypes through *in vitro* micropropagation based on *in vitro* phenotypic performance. Therefore, the experiment was conducted to find out salt tolerant callusing, shooting and rooting performance ability as well as developing salt tolerant variety for the southern region in Bangladesh.

Materials and Methods

Plant material

Four sugarcane genotypes (Isd 16, CC37M_5, BSRI Akh 41 and BSRI Akh 42) were evaluated for their response to callus induction capacity, shooting and rooting performance under control, 50 mM NaCl, 100 mM NaCl, 150 mM NaCl, 200 mM NaCl, 250 mM NaCl, 300 mM NaCl were arranged in completely randomized design (CRD) with five replications.

Callus induction

The explants used for callus induction are leaf cylinders provided from the sheath of the four youngest genotypes. The basal part of the stem (constituted by the sheath of leaves) was surface sterilized for 10 mins in 0.03% mercuric chloride supplemented with Tween 80 followed by three rinses with sterile distilled water (10 min each). After drying on sterile filter paper, leaf cylinders were aseptically placed on based MS medium [23] supplemented with 4 mg/L 2,4-D and 30 g/L sucrose using different levels of Sodium Chloride salt (0 mM, 50 mM, 100 mM, 150 mM, 200 mM, 250 mM and 300 mM). The pH was adjusted to 5.8 with 0.1 N NaOH before autoclaving at 121°C and 1.2 kg cm^{-2} for 30 minutes and all media were solidified with 6.5 gm/L agar before autoclaving during 20 mins at 120°C. Each ex-plant per 36 test tubes was cultivated and cultures were kept in dark at 25 ± 1°C. Callus induction percentage was determined after 4 weeks [24]. For the salt shock treatment, the callus was transferred on medium to culture medium with the concentrations with different saline condition.

Shooting initiation

After two subcultures (4 weeks each), callus were transferred in test tubes containing the shooting medium (BAP 2 mg/L and Kinetin 1 mg/L) of regeneration (MS modified) with 30 g/L of sucrose using different levels of NaCl salt. Cultures were incubated in growth cabinet at 25 ± 1°C under 16-h photoperiod Callus that regenerate plant shoots were recorded after 5 weeks [22] and the

data were expressed as a percentage of regenerated callus and total number of callus transferred for regeneration.

Rooting initiation

After shooting initiation, plantlets were transferred in test tubes containing the rooting medium (NAA 5 mg/L) of regeneration (MS modified) with 30 gm/L of sucrose using different levels of NaCl salt. Cultures were incubated in a growth cabinet at 25 ± 1°C under 16-h photoperiod for rooting that regenerate roots were recorded after 3 to 4 weeks [24].

Acclimatization of regenerated plants

The regenerated plantlets were aseptically transferred to the same rooting medium after 4 weeks. Plantlets with at least five well-developed roots were transferred to a pot containing soil with coco dust under high humidity (> 90%) by covering the plants with plastic envelops after cutting their leaves [25]. For acclimatization, pots were placed in hardening shed.

Statistical analysis

All the data were statistically analyzed by Statistix 10 (Tallahassee, FL 32312, USA) as ANOVA with Multiple comparison of number of explants that induced callus percentage, callus weight, shooting initiation percentage, shoot number per plant, shoot height per plant, rooting initiation percentage, root number per plant, root height per plant.

Results

Callus induction performance

Callus induction rate varied from 0 to 100% under all the treatments. High callus induction percentages revealed the high capacity of the sugarcane genotypes grow under saline conditions. In control, all the genotypes produced 100% callus induction. But when applied 50 mM NaCl almost forty to fifty percent callus induction reduced among the genotypes. The highest callus induction percentage was found in Isd 16 under all treatments. The lowest callus induction percentage was found in CC 37 M_5 under all NaCl concentrations. However, among the saline treatments, highest callus observed in 50 mM NaCl and the lowest found in 200 mM NaCl concentration. Callus induction performance drastically reduced from above 150 mM NaCl concentration. In contrary from the four genotypes and BSRI Akh 41 performed better callus induction in different saline conditions. These results indicated that the ability of callus induction greatly influenced by the genotype in different saline conditions.

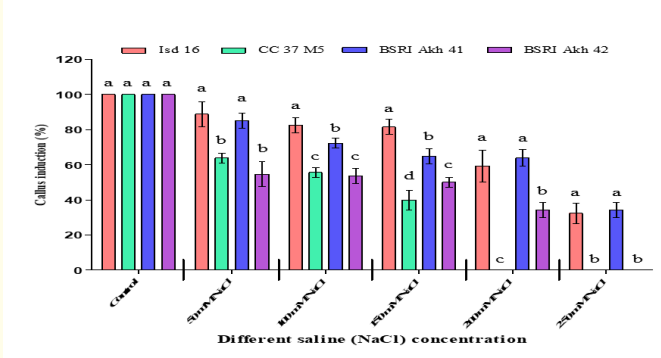

Figure 1: Effects of salt (NaCl) on callus induction percentage (%) among four sugarcane genotypes.

Callus weight performance

Callus weight rates were varied from 0 to 2.94 gm (Table 1) under all treatments. A significant difference ($p < 0.01$) was observed among genotypes which is supported the result of Gandonou., *et al*. 2005 [18].

Genotype	Control	50 mM NaCl	100 mM NaCl	150 mM NaCl	200 mM NaCl	250 mM NaCl
Isd 16	2.01b	1.36a	0.84b	1.20a	0.16a	0.01a
CC 37 M_5	1.92b	0.58b	0.24d	0.00c	0.00b	0.00a
BSRI Akh 41	2.94a	1.37a	1.20a	0.11b	0.02b	0.00a
BSRI Akh 42	1.92b	1.38a	0.47c	0.19b	0.00b	0.00a
Level of Significance	**	**	**	**	**	**
CV (%)	7.49	23.79	23.79	20.98	59.19	47.21

Table 1: Performance of callus weight of four sugarcane genotypes under different saline conditions.
CV: Co-Efficient of Variation; mM: Millimole; NaCl: Sodium Chloride; CC: Somaclone.

The results indicated that callus weights were varied significantly under saline conditions. The highest callus weight was found in Isd 16 (2.01g 1.36g, 1.20g, 0.16g) under control, 50 mM NaCl, 150 mM NaCl, 200 mM NaCl conditions respectively. The lowest callus weight was observed in CC 37 M_5 (1.92, 0.58, 0.24, 0.00g) under control condition with four treatments including 50 mM NaCl, 100 mM NaCl, 150 mM NaCl, 200 mM NaCl.

Shooting initiation performance

Shooting initiation varied from 0 to 100% under all treatments (Figure 2). High shooting initiation percentages revealed the maximum number of the sugarcane genotypes. In the control condition, all genotypes produced 100% shooting initiation except BSRI Akh 42 (83.33%).

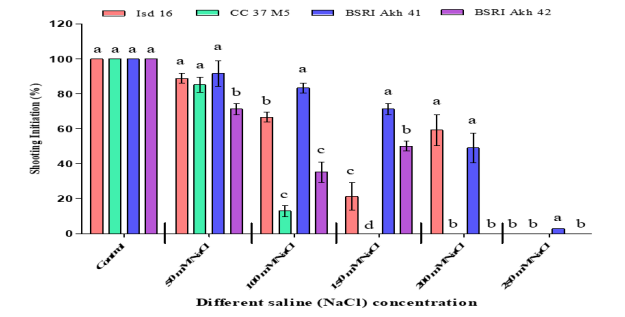

Figure 2: Effects of salt (NaCl) on shooting initiation percentage (%).

But when applied NaCl almost shooting initiation were reduced. From the four genotypes highest shooting initiation was Isd 16 (100.00%, 88.80%, 66.60%, 21.20%, 7.20%) and the lowest was CC37M_5 (100.00%, 85.80%, 13.10%, 0). These results indicated that the highest rate of shoot differentiation was greatly influenced by the genotype and different saline conditions.

Shoot height performance

Shoot height varied from 0 to 37.99 cm under different treatments. The effects of genotypes on shoot height are shown in table 2. The results indicated that shoots height varied significantly under saline condition.

Genotype	Control	50 mM NaCl	100 mM NaCl	150 mM NaCl	200 mM NaCl	250 mM NaCl
Isd 16	22.52b	19.14a	14.58a	12.46a	1.46a	0.02a
CC37M_5	23.10b	17.06ab	2.68c	0.00c	0.00b	0.00a
BSRI Akh 41	37.99a	20.00a	16.58a	11.68a	0.32b	0.00a
BSRI Akh 42	22.80b	13.96b	10.56b	1.94b	0.00b	0.00a
Level of Significance	**	**	**	**	**	**
CV (%)	10.45	14.50	24.76	13.18	37.03	44.21

Table 2: Performance of shoot height (cm) of four sugarcane genotypes under different saline conditions.
CV: Co-Efficient of Variation; mM: Millimole; NaCl: Sodium Chloride; CC: Somaclone.

From the four genotypes, the highest shoot height was found in BSRI Akh 41 but the tolerance level highest in Isd 16 than BSRI Akh 41 and the lowest was CC 37 M_5 (17.06 cm, 2.68 cm, 0 cm). Significant difference ($p < 0.01$) was observed among genotypes (Table 2).

Shoot number per plant performance

Shoot number varied from 0 to 16.58 under all treatments. The results indicated that shoot numbers were varied significantly under saline conditions.

Genotype	Control	50 mM NaCl	100 mM NaCl	150 mM NaCl	200 mM NaCl	250 mM NaCl
Isd 16	10.40b	9.00b	14.58a	3.80b	2.60a	0.40a
CC37M_5	6.40c	6.80b	2.68c	0.00d	0.00b	0.00a
BSRI Akh 41	10.00b	9.20b	16.58a	4.60a	2.20a	0.00a
BSRI Akh 42	15.40a	15.40a	10.56b	2.60c	0.00b	0.00a
Level of Significance	**	**	**	**	**	NS
CV (%)	22.43	24.85	19.71	20.73	51.03	47.21

Table 3: Performance of shoot number per plant of four sugarcane genotypes under different saline conditions.
CV: Co-Efficient of Variation; mM: Millimole; NaCl: Sodium Chloride; CC: Somaclone.

Isd 16 produced significant shoot number up to 200 mM NaCl and $CCR_5 37M_5$produced shoot number up to 100 mM NaCl.

Rooting initiation performance under different saline conditions

From four sugarcane genotypes up to 88.88% rooting initiation percentage observed (Figure 3).

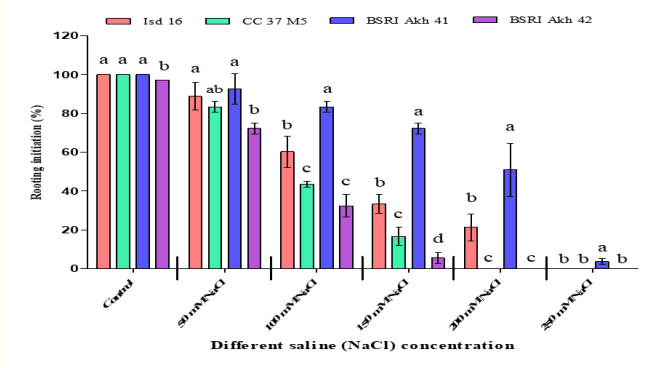

Figure 3: Effects of salt (NaCl) on rooting initiation percentage (%).

The highest rooting initiation was found in BSRI Akh 41 (92%, 83.33%, 72.22%, 50%, 2.77%) and lowest was CC 37 M_5 (72.22%, 30.55%, 5.5%). These results indicated that rooting initiation differentiation was vary from genotype to genotype in different saline conditions.

Root height

The results indicate that root height varied significantly with the varieties and it ranged from 0 to 4.46. In 100 mM NaCl the highest root height found in Isd 16 (3.16cm, 3.84 cm, 4.46 cm) and the lowest found in CC37M5 (2.02 cm, 1.78 cm, 0.14 cm). From 150 mM NaCl to 200 mM NaCl, the highest root height found BSRI Akh 41 (0.74 cm, 0.14 cm) and the lowest found in $CC37M_5$ (0 cm, 0 cm). BSRI Akh 41 performed better root height than other three genotypes.

Root number per plant

The results indicate that root numbers were varied significantly with the varieties and it ranged from 0 to 16.00. Upto 100 mM NaCl the highest root number per plant found in Isd 16 (15.80, 14.6, 6.60) which is statistically similar to BSRI Akh 41 and the lowest found in $CC37M_5$ (10, 11.60, 1.80).

Genotype	Control	50 mM NaCl	100 mM NaCl	150 mM NaCl	200 mM NaCl	250 mM NaCl
Isd 16	3.16a	3.84a	4.46a	0.40b	0.06b	0.00
$CC37M_5$	2.02c	1.78c	0.14c	0.00c	0.00c	0.00
BSRI Akh 41	3.02ab	2.76b	1.16b	0.74a	0.14b	0.00
BSRI Akh 42	2.34bc	1.14c	0.30c	0.16c	0.00c	0.00
Level of Significance	**	**	**	**	**	M
CV (%)	20.59	21.59	18.79	52.40	77.46	M

Table 4: Performance of root height (cm) of four sugarcane genotypes under different saline conditions.
CV: Co-Efficient of Variation; mM: Millimole; NaCl: Sodium Chloride; CC: Somaclone.

Figure 4: Callus induction performance (A), Shoot initiation performance (B), Root initiation performance (C), Plantlets (D) of Isd 16 in vitro saline conditions (150 mM NaCl).

Genotype	Control	50 mM NaCl	100 mM NaCl	150 mM NaCl	200 mM NaCl	250 mM NaCl
Isd 16	15.80a	14.60a	6.60a	2.40b	1.20a	0.00
CC 37 M_5	10.00b	11.60a	1.80b	0.00c	0.00b	0.00
BSRI Akh 41	12.00b	13.00a	5.00a	3.40a	1.80a	0.00
BSRI Akh 42	16.60a	5.60b	2.60b	2.00b	0.00b	0.00
Level of Significance	**	**	**	**	**	M
CV (%)	12.47	24.00	38.12	19.86	18.88	M

Table 5: Performance of root number per plant of four sugarcane genotypes under different saline conditions.
CV: Co-Efficient of Variation; mM: Millimole; NaCl: Sodium Chloride; CC: Somaclone.

From 150 mM NaCl to 200 mM NaCl, the highest root number found BSRI Akh 41 (2.40, 1.20) and the lowest found in CC 37 M_5 (0, 0). So BSRI Akh 41 performed a better number of roots than other three genotypes.

Discussion

Callus induction parameters

These results are in agreement with an earlier report in sugarcane where a significant decline in callus growth rate occurred with 150 mM NaCl [14,26]. Nutritional imbalance is the main factor for declining callus growth upon (NaCl) salt stress due to an interference of salt ions, such as Na^+ and Cl^- with essential nutrients involved in both uptake and translocation processes [25]. The decreased cell viability in salt-stressed calli may be associated with toxic effects of increased Na^+ and reduced K^+ contents. A reduction in cell viability was also reported in tobacco in response to salt stress [27]. Salt stress compromised membrane integrity in all the genotypes for callus induction. Furthermore, membrane damage as well as tissue injury caused by NaCl application to fail callus formation [28].

Shotting initiation parameters

In addition, shooting initiation was slower and the yellowish of older leaves arises due to the osmotic effect of salt stress because Organic solutes (sugars, proline, polyols, quaternary ammonium compounds like glycine betaine, and other low molecular weight metabolites) serve a function in cells to lower or balance the osmotic potential of intracellular and extracellular ions to tolerate osmotic stresses [24]. Inorganic ions mainly Na^+, K^+, Ca^{2+} and Cl^- also make great contribution in osmotic adjustment [29]. But the toxicity of Na^+, K^+, Ca^{2+} decreased the plant growth. The leaves of plants showed succulence under salt stress condition mainly attributed to increased vacuole size in leaves that accumulate salt [24,30].

Shoot heights were drastically decreased due to increase of NaCl concentrations. Salinity imposes diffusive and metabolic limitations to photosynthesis, affects cell growth by restricting water uptake and cell turgor, resulting in increasing accumulation of Na^+ and Cl^- ions inside the cell. As a result, shoot height become stunted [4].

Rooting initiation parameters

The saline stress leads to the increase of Na^+ with the consequent decrease of K^+ in the plant, both on the leaves and in the roots [31]. K^+ is the most abundant inorganic cation in plant cells, which composed with nitrogen (N) and phosphorus (P) is fundamental for crop yield [32]. When NaCl was gradually added to the culture medium, the cation concentration in the tissue of the salt-treated plants was lower than that of the non-stressed plants and inhibits root development [33].

Restriction of shoot and root development is the basic response to the stress (Bars., *et al*. 1962). Inhibition of root growth by salinity decrease the uptake of water and essential minerals, diminished supply of water and nutrients to shoot, which might contribute to growth reduction (Bars., *et al*. 1962).

Conclusion

Our findings showed that Sugarcane genotypes were significantly different in their capacity of the callus induction, production of the embryogenic callus and regeneration of plantlets when grown in the presence of NaCI. The genotypes Isd 16 had the lowest performance in root system under different saline conditions but revealed the highest performance in case of callus initiation percentage, callus weight, shooting initiation percentage and shooting height under salinity stress. So Isd 16 genotype appeared to possess the highest potential of making better growth among four sugarcane genotypes. On the other hand, genotypes CC 37 M_5 had the lowest performance in all parameters under different salt stress. CC 37 M_5 genotype appeared to possess salt susceptible

among four sugarcane genotypes. Wide differences in the growth of sugarcane genotypes observed in this experiment encourage screening of more genotypes against salinity, especially in the field, to identify salt tolerant genotypes in Bangladesh.

Acknowledgements

Authors are grateful to Ministry of Agriculture of Bangladesh for providing financial support for the project "Development of Salt Tolerant Sugarcane Somaclones and Their Field Evaluation in Southern Region in Bangladesh" under special budget of Ministry of Agriculture and Bangladesh Sugarcrop Research Institute (BSRI) and also Bangladesh Agricultural Research Council (BARC) authority for their cooperation.

Conflict of Interest

There is no competing interest among the authors of this research.

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