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Radiation-induced in vitro mutagenesis system for salt tolerance and other agronomic characters in sugarcane (*Saccharum officinarum* L.)

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ABSTRACT

Gamma ray-induced in vitro mutagenesis and selection for salt (NaCl) tolerance were investigated in sugarcane (*Saccharum officinarum* L.). Embryogenic callus cultures were irradiated (10 to 80 Gy) and subjected to in vitro selection by exposure of irradiated callus to NaCl (0, 50, 100, 150, 200, and 250 mmol L^{-1}). Increasing NaCl concentrations resulted in growth reduction and increased membrane damage. Salt-selected callus lines were characterized by the accumulation of proline, glycine betaine, and Na^+ and K^+ concentration. Higher accumulation of proline and glycine betaine was observed in NaCl stressed callus irradiated at 20 Gy. Na^+ concentration increased and K^+ concentration decreased with increasing salt level. Irradiated callus showed 50–60% regeneration under NaCl stress, and in vitro-regenerated plants were acclimatized in the greenhouse, with 80–85% survival. A total of 138 irradiated and salt-selected selections were grown to maturity and their agronomic performance was evaluated under normal and saline conditions. Of these, 18 mutant clones were characterized for different agro-morphological characters and some of the mutant clones exhibited improved sugar yield with increased Brix%, number of millable canes, and yield. The result suggest that radiation-induced mutagenesis offers an effective way to enhance genetic variation in sugarcane.

1. Introduction

Sugarcane (*Saccharum* spp.) is an important industrial crop, ranking among the ten most planted crops in the world. Besides being the major sugar contributor, accounting for more than 70% of the world's sugar, sugarcane is important as the raw material for sugar-producing and allied industries [1]. Conventional breeding has contributed greatly to the development of agronomically improved varieties; but limitations such as a narrow gene pool, a complex genome, poor fertility, and a long

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breeding/selection cycle make it difficult to undertake further improvement. Agronomically improved sugarcane varieties endowed with tolerance to biotic and abiotic stresses are highly beneficial, as unfavorable environmental factors can challenge cultivation and crop productivity. Although crops tolerant to biotic and abiotic stresses have been selected by conventional breeding programs, speeding up the pace of breeding is essential for developing improved varieties.

Soil salinity has become a major limiting factor that adversely affects crop production [2]. Worldwide, it is estimated that around 800 million hectares of land are affected by salinity, with salinity levels ranging from 2 to 4 dS m^{-1} [2]. Salinity affects plant cells, causing alterations in water relations, ionic and metabolic perturbations, generation of reactive oxygen species (ROS), and tissue damage [3]. Development of salt-tolerant cultivars by conventional, mutational, and biotechnological approaches can augment the utilization of salinity-affected regions. The availability and screening of large populations for mutagenesis are prerequisites to obtaining sufficient genetic variability. In vitro culture in combination with radiation-induced mutations has become an important method to induce genetic variability and rapidly multiply the selected mutants [4–6]. Methods of chemical- and/or radiation-induced in vitro mutagenesis have been successfully used to improve agronomic traits including salinity and drought tolerance in several crop plants [7–10]. Determinations of radiosensitivity and of optimal doses of ionizing radiation are important steps for undertaking induced mutagenesis for crop improvement. Their importance has been well demonstrated in plants such as rice [11], groundnut [12], sweet potato [10], banana [13], and *Zoysia* [14].

Although studies of salt selection are available for diverse plant species, limited research has been conducted in sugarcane. Sugarcane embryogenic callus has been shown to be sensitive to sodium chloride (NaCl) [15] and gamma radiation [16]. Saif et al. [17] reported the isolation of salt-tolerant mutants from irradiated sugarcane callus. Although these studies have demonstrated the application of mutagenesis and in vitro techniques to study radiosensitivity or isolation of mutants in sugarcane, little information on characterization of salt tolerant callus and progeny is available. Studies of the application of ionizing radiation for developing novel mutant germplasm in sugarcane will accordingly be beneficial for sugarcane improvement. The objective of the present study was to apply gamma ray-induced mutagenesis to isolate sugarcane mutants with improved tolerance to salinity, followed by morphological and agronomical characterization of selected mutants.

2. Materials and methods

2.1. Plant material and culture conditions

The commercial sugarcane variety Co86032 was used as the experimental material. The tops of mature canes were harvested from field-grown plants at the Vasantdada Sugar Institute, Manjari, Pune (India). The explant material was washed first in tap water and then for 5 min in sterile distilled water at least three times. Surface decontamination was performed with 80% ethanol (v/v) for 5 min and mercury chloride (0.1% w/v) for 4–5 min, followed by three washes with sterile distilled water for 15 min each. After removal of the outer leaves, the innermost leaf segments were cut into 2–3 mm pieces and aseptically inoculated onto MS [18] medium supplemented with 1 mg L^{-1} thymine HCl, 20 mg L^{-1} inositol, 3 mg L^{-1} 2,4-D, 10% coconut water and 2.0% sucrose. This medium is referred to as callus induction medium. Cultures were incubated in the dark at 25 ± 2 °C at relative humidity 70–80%. After 45 days of culture, the callus was subcultured onto modified MS medium with 1000 mg L^{-1} casein hydrolyzate, 1 mg L^{-1} thymine HCl, 20 mg L^{-1} inositol, 3 mg L^{-1} 2,4-D, 5% coconut water, and 2.5% sucrose to obtain embryogenic callus. This medium is referred to as callus maintenance medium.

2.2. Gamma ray radiation treatments

Embryogenic callus cultures were irradiated with 0, 10, 20, 30, 40, 50, 60, 70, and 80 Gy gamma rays using Gamma Cell 220 (a ^{60}Co source) at a dose rate of 9.6 Gy min^{-1}. Post irradiation, callus cultures were transferred to freshly prepared MS medium supplemented with 3 mg L^{-1} 2,4-D, 1000 mg L^{-1} casein hydrolyzate, 1 mg L^{-1} thymine HCl, 20 mg L^{-1} inositol, 5% coconut water, and 2.5% sucrose. Survival of the irradiated callus was determined using relative growth rate after four weeks of radiation treatment. The surviving callus was then subcultured for at least four passages on maintenance medium.

2.3. Salt stress and its effects on irradiated callus

Irradiated and non-irradiated callus cultures were subjected to treatments with different concentrations of salt (NaCl) stress (0, 50, 100, 150, 200, and 250 mmol L^{-1}) to study salt stress effects and identify the optimal concentration of NaCl to be used in the selection medium. After four weeks of treatment, the response was recorded using several parameters: tissue water content (TWC), relative electrolyte leakage (REL), relative growth rate (RGR), accumulation of proline and glycine betaine (GB), protein content, and Na^+ and K^+ concentration.

Membrane damage was determined in terms of relative electrolytic leakage (REL) by the method of Sullivan [19]. For REL measurement, callus was incubated for 24 h in a test-tube (25 mm × 150 mm) containing distilled water (25 °C) and the initial electrical conductivity (EC_1) was measured after the incubation period. Samples were then autoclaved for 15 min at 121 °C to release the ions from the tissue, and the final electrical conductivity (EC_2) was measured after cooling to room temperature. The REL was calculated as: (EC_1/EC_2) × 100. TWC of the callus was determined as described by Lokhande et al. [20]. The percent tissue water content (TWC %) was determined using the following equation: TWC (%) = [fresh weight (FW) – dry weight (DW) / (FW)] × 100.

2.4. Free proline concentration

Proline concentration was evaluated by the method of Bates et al. [21] with minor modifications. Callus was ground in 3% sulfosalicylic acid and centrifuged at 4 °C. The filtrate was mixed with equal volumes of acid ninhydrin and glacial acetic acid, and then incubated at 100 °C in a hot water bath for 1 h. The reaction

was terminated in an ice bath and allowed to cool at room temperature. The reaction mixture was extracted with 4 mL toluene and mixed vigorously with a stirrer for 10–15 s. The chromophore-containing toluene was aspirated from the aqueous phase and warmed to room temperature. The optical density was measured at 520 nm using toluene as a blank. The amount of proline was determined from a standard curve using L-proline and expressed as proline μmol g^{-1} FW.

2.5. Glycine betaine (GB) concentration

GB concentration was determined by the method of Grieve and Grattan [22]. The callus was mechanically shaken with deionized water for 24 h at 25 °C. Samples were filtered and the filtrate was diluted (1:1) with 2 mmol L^{-1} H_2SO_4. The extract was cooled in ice and mixed with 200 μL of I_2-KI reagent (a mixture of 20% potassium iodide and 15.7% iodine). The tubes were gently mixed and stored at 4 °C for 16 h followed by centrifugation at 10,000 ×*g* for 15 min at 0 °C. Periodide crystals were dissolved in 9.0 mL of 1,2-dichloroethane, and after 2 h, absorbance was measured at 365 nm. GB concentration was determined from a standard curve prepared using standard glycine betaine and expressed as μg g^{-1} FW.

2.6. Lipid peroxidation

Oxidative damage to membrane lipids was estimated by measurement of the concentration of thiobarbituric acid-reactive substances (TBARSs), expressed as equivalents of malonyldialdehyde (MDA). The amount of MDA was calculated as described by Hichem et al. [23] with some modifications. Callus was ground in 0.25% thiobarbituric acid (TBA) in 10% trichloroacetic acid (TCA). The homogenate was centrifuged, and the absorbance was read at 532 and 600 nm with 10% TCA as a blank. The TBARS was expressed as μmol g^{-1} FW.

2.7. Na^+ and K^+ analysis

Na^+ and K^+ analyses were performed by the procedure of Basu et al. [24]. Callus was dried at 80 °C for 48 h and digested in nitric acid and perchloric acid (2:1, v/v) at 150 °C for 4 h. The residue was dissolved in distilled water and the final volume of the solution was adjusted to 1 mL. It was used for Na^+ and K^+ measurement using a flame photometer.

2.8. Regeneration of plants from selected callus cultures

Embryogenic callus cultures were placed on 100 mmol L^{-1} NaCl-supplemented MS medium and selection was performed for surviving callus. The irradiated and NaCl-selected calli were subjected to four passages on freshly prepared medium with 100 mmol L^{-1} to select for surviving tolerant callus. All the cultures were transferred onto MS basal medium supplemented with 1 mg L^{-1} thymine HCl, 20 mg L^{-1} inositol, 5% coconut water, and 2.5% sucrose without hormones to induce plant regeneration. The cultures were initially kept in the dark for 10 days and then the culture bottles were incubated under 16 h light illumination. Plantlets of about 5–6 cm in length were transferred to half-strength MS medium with NAA (4.0 mg L^{-1}) for root induction. Well-rooted plantlets were then transferred to poly bags in the green house for 40 days. Plant survival was determined after 45 days.

2.9. Agro-morphological characterization

To assess the agronomic performance of irradiated and NaCl-selected plants, evaluation was performed in the control, non-saline soil in a ground nursery trial. The plantlets were planted at distance 1.2 m × 1.0 m plant to plant along with parent variety Co86032. Selection of the promising mutants on the basis of stalk number per plant, diameter of canes, and Hand refractometer (HR) Brix% was recorded, and on the basis of high Brix% of cane at months 10 and 12 in comparison to the parent was used for selection of promising mutants. These selected mutants were field-transplanted for evaluation of agronomic performance in a clonal trial (augmented) under saline conditions. Data for cane and juice parameters were recorded in the months of 10 and 12 for total height of cane, millable height of cane, diameter of cane, number of internodes per cane, weight of single cane, Brix%, sucrose%, purity%, and commercial cane sugar (CCS%) and compared with parent and standard varieties.

The biological effect of the treatment was analyzed based on survival and variant characteristics (morphological and agronomical). Mutation frequency was calculated as percentage of selected promising mutants from the M1 generation. Different morphological and agronomic characters were compared between mutants and the parent variety. Mutagenic effectiveness and efficiency were calculated following Walther [25].

2.10. Statistical analysis

The experiment was laid out in a completely randomized design (CRD). The analyses were repeated with three independent biological samples. The data were statistically analyzed by two-way analysis of variance (ANOVA) and Scott–Knott group significance at 5% probability [26] using Windostat software (version 8.5, http://www.windostat.org/). The results were presented as means with standard error of three replicates and different levels were compared by Scott–Knott group test, with significant group indicated by a horizontal line. A graphical presentation showed changes in colors of bar graphs, indicating significant differences at 5% probability by ANOVA. Field evaluations of cane and juice and biochemical analysis were analyzed by an augmented method. Data are reported as mean values, and standard check varieties and mutants with least significant difference (LSD) in CD (≤0.05) values are reported. Mutants were compared with parent and standard varieties and also with the moderately salt-tolerant CoM0265 sugarcane variety.

3. Results

3.1. Gamma irradiation and salt-stress response

Fresh, actively proliferating embryogenic callus showed more sensitivity to gamma radiation than the control (Fig. 1-a, b, c). RGR decreased with increasing doses (10–80 Gy) of gamma rays (Fig. 2). Callus exposed to low doses of 10 and 20 Gy

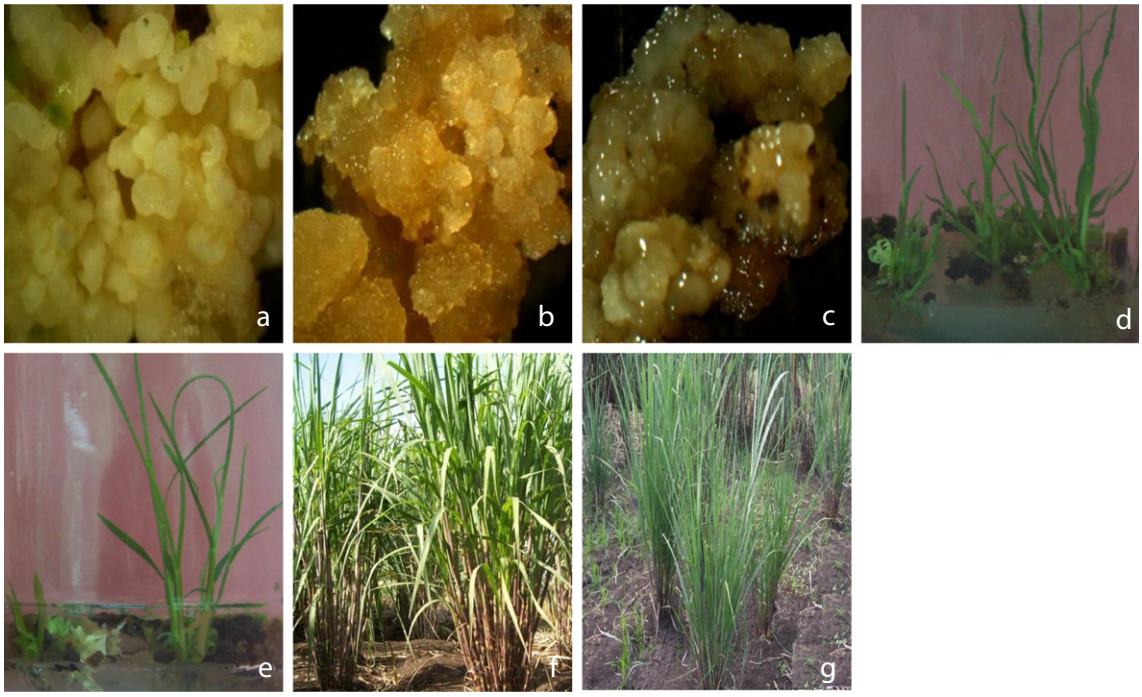

Fig. 1 – Effects of radiation and salinity (NaCl) on callus growth, plant regeneration, and field plants of sugarcane variety Co86032. (a) Embryogenic control callus; (b) embryogenic callus irradiated with 20 Gy on 100 mmol L^{-1} NaCl; (c) embryogenic callus irradiated with 30 Gy on 150 mmol L^{-1} NaCl; (d) regeneration from callus irradiated with 20 Gy on 100 mmol L^{-1} NaCl; (e) regeneration from callus irradiated with 30 Gy on 100 mmol L^{-1} NaCl; (f) plants from 20 Gy and 100 mmol L^{-1} NaCl treatments; (g) plants from 30 Gy and 100 mmol L^{-1} NaCl treatments growing in the field.

showed 50% reduction over control callus, whereas 70% reduction in RGR was observed in treatments with 30 to 40 Gy and >70% reduction at higher doses (50 to 80 Gy). The extent of browning also increased with radiation dose, and the callus turned completely brown at doses above 40 Gy. Exposure of embryogenic callus to 100–150 mmol L^{-1} NaCl inhibited callus growth (Fig. 3-a). Lower RGR was observed with increase in salt concentration and radiation dose. A reduction in TWC was also observed with increase in radiation and NaCl treatment (Fig. 3-b). Significant reduction up to 81.9% or 77.8% was observed at 30, 40 Gy, respectively, as compared to higher TWC (86.0%) at 20 Gy. Similarly, a significant reduction in TWC was observed in irradiated callus treated with 100 mmol L^{-1} and higher NaCl (150, 200, and 250 mmol L^{-1}). The pooled data of irradiated and NaCl stress callus showed a gradual reduction in percent tissue water content with increase in irradiation and NaCl concentration.

The increase in radiation dose and NaCl concentration resulted in significant increase in percent relative electrolyte leakage (Fig. 3-c). Pooled data of irradiated and control callus showed significantly higher RELs of 83.2%, 84.1%, and 87.0% at NaCl concentrations of respectively 150, 200, and 250 mmol L^{-1},

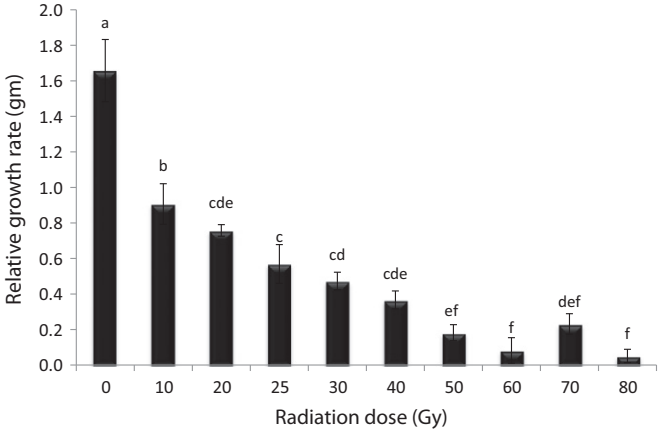

Fig. 2 – Effect of gamma radiation on relative growth rate (RGR) of Co86032 callus after 30 days of radiation. Error bars indicate SE and letters above bar indicate significant differences at 5% probability.

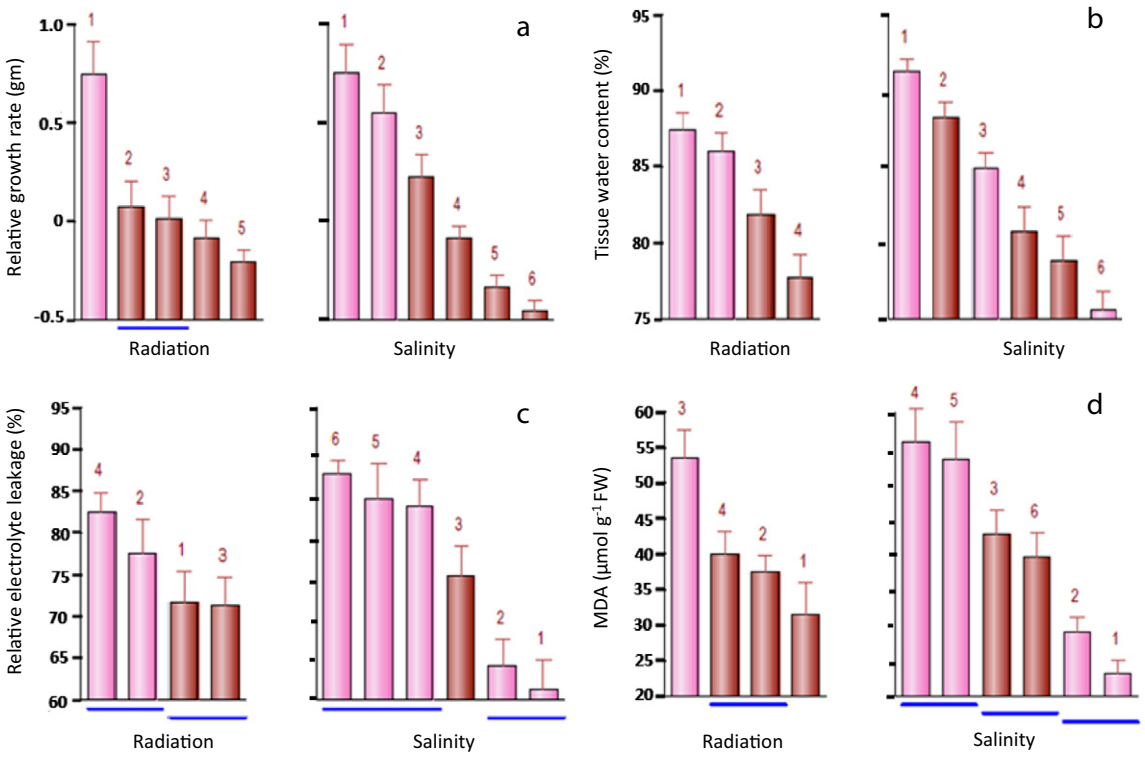

Fig. 3 – Effect of radiation and salinity on sugarcane callus: RGR (a), tissue water content (TWC%) (b), relative electrolyte leakage (REL%) (c), and lipid peroxidase (MDA) (d). Number above bar indicates radiation dose in Gy: 1(0), 2(20), 3(30), 4(40), and NaCl (mmol L^{-1}): 1 (0), 2 (50), 3 (100), 4 (150), 5 (200), 6 (250) mmol L^{-1} NaCl. Graph color change indicates significance at 5% probability level and horizontal line on X-axis indicates group significance level by Scott–Knott test. The error bars show standard errors of means.

with the differences showing group significance by Scott–Knott test. However, the 0 and 50 mmol L^{-1} NaCl concentrations led to significantly lower REL than the 100 mmol L^{-1} NaCl treated callus (Fig. 3-c). In contrast, 40 Gy-irradiated callus showed maximum REL under NaCl concentration.

Higher MDA content was observed in irradiated callus exposed to NaCl stress (Fig. 3-d). The 30 Gy radiation treatments showed significant higher MDA (53.5 μmol g^{-1} FW) than other treatments and non-irradiated callus (Fig. 3-d). Irradiated callus exposed to 150 and 200 mmol L^{-1} NaCl showed significantly higher MDA contents of 55.8 and 53.3 μmol g^{-1} FW, respectively.

Pooled data of control and 20 Gy-irradiated callus showed significantly higher proline (4.9 and 4.7 μmol g^{-1} FW) than 30 and 40 Gy-irradiated callus (3.1 and 2.9 μmol g^{-1} FW) (Fig. 4-a). Irradiated callus on 100 mmol L^{-1} NaCl showed the highest proline accumulation (5.5 μmol g^{-1} FW). Irradiated callus exposed to 50 and 150 mmol L^{-1} NaCl stress showed group significance, whereas control and 250 mmol L^{-1} NaCl-stressed callus showed the lowest accumulation of proline (Fig. 4-a). Glycine betaine (GB) accumulation was significantly higher in 30 Gy irradiated callus (10.8 μg g^{-1} FW). Higher levels of GB (13.9 μg g^{-1} FW) were observed in irradiated callus grown under 150 mmol L^{-1} NaCl stress. As in the case of proline, the lowest accumulation of GB was observed in the control and 250 mmol L^{-1} NaCl treated callus (Fig. 4-b).

The concentration of sodium (Na^+) was significantly higher in gamma-irradiated and NaCl-treated callus than in control callus, and Na^+ concentration increased with NaCl concentration (Fig. 4-c). In contrast, K^+ concentration was significantly higher in control callus than in irradiated and NaCl-treated samples (Fig. 4-d). The maximum sodium (468.4 μmol g^{-1} FW) and lowest potassium (44.4 μmol g^{-1} FW) accumulations were observed in 250 mmol L^{-1} NaCl-stressed callus.

3.2. *Regeneration and selection for salt tolerance*

Irradiated embryogenic callus cultures on NaCl (salt) selection media showed pronounced browning with increasing salt concentration, compared to control. Embryogenic callus placed on 100 mmol L^{-1} NaCl-supplemented MS medium showed surviving callus, which was selected for further selection. The irradiated, NaCl-selected callus was subcultured for four passages on 100 mmol L^{-1} and surviving tolerant callus was selected (Fig. 1-c, d). The regeneration response of the selected callus was affected by increase in radiation dose and NaCl treatment (Fig. 1-d). Irradiated callus showed 50–60% regeneration under NaCl stress (Fig. 1-e). Some (2–3%) albino plants were also observed during regeneration of irradiated callus in salt selected medium (data not shown). The 30 Gy-irradiated callus showed induction of shoots after an initial necrotic effect, but only slight proliferation was observed on incubation of selected cultures for a long period (50–60 days) on regeneration medium. Control, non-irradiated callus cultures showed no regeneration on media supplemented with higher NaCl

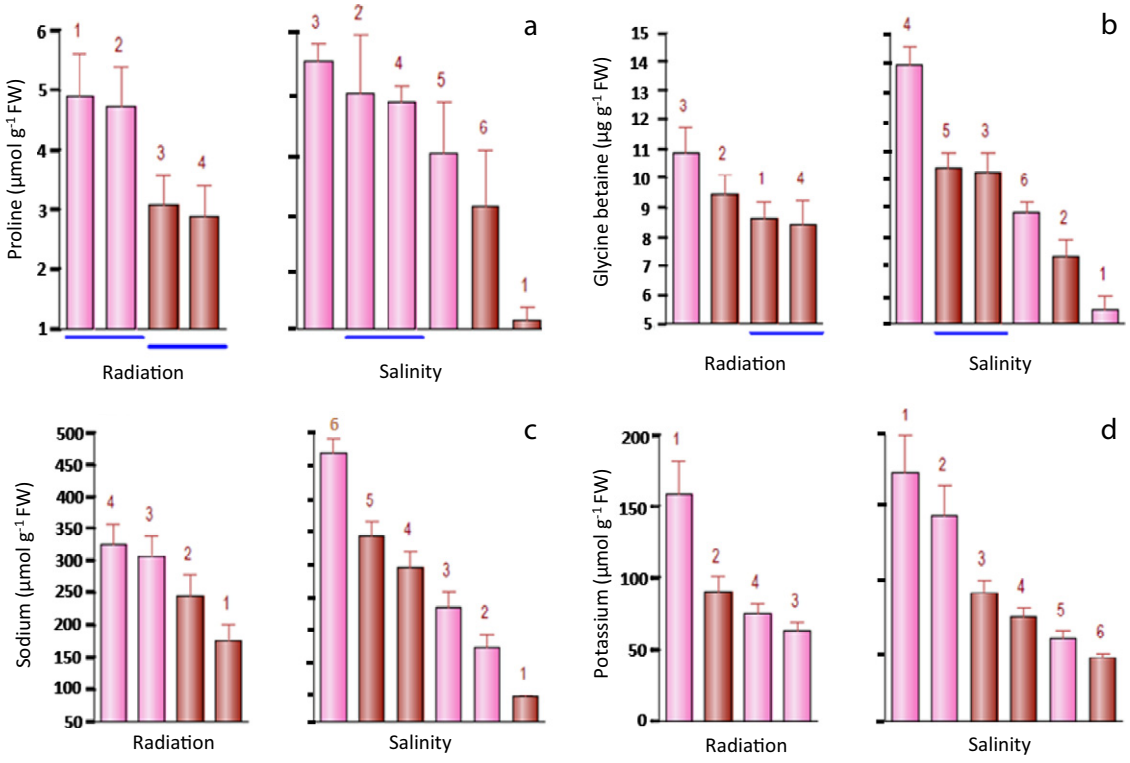

Fig. 4 – Effect of radiation and salinity on sugarcane callus: proline (a), glycine betaine (b), sodium (Na^+) (c), and potassium (K^+) (d) concentrations. Number above bar indicates radiation dose in Gy: 1(0), 2(20), 3(30), 4(40) and NaCl (mmol L^{-1}): 1 (0), 2 (50), 3 (100), 4 (150), 5 (200), 6 (250). Graph color change indicates significance at 5% probability, and horizontal line on X-axis indicates group significance level by Scott–Knott test. Error bars show standard error of mean.

concentration, whereas 50 and 100 mmol L^{-1} NaCl-selected callus cultures showed good regeneration of plantlets. In contrast, irradiated callus selected on 150 mmol L^{-1} NaCl regenerated few shoots even after prolonged culture. The in vitro regenerated plants were found to acclimatize with 80–85% survival in the greenhouse. A total of 138 plants that showed normal phenotype and growth were subsequently field-planted (Fig. 1-f, g).

3.3. *Field evaluation*

Irradiated and salt-selected plants developed normally under field conditions, with few exceptions. One mutant clone developed under 20 Gy + NaCl stress showed improved cane growth, with some changes in phenotypic characters including broad leaf lamina, increased leaf length, and increased waxiness compared to control, whereas a few 30 Gy-irradiated and NaCl-selected clones showed stunted growth with narrow, spiny leaves and waxy leaf sheath (data not shown).

Of 138 plants screened for phenotype and growth characters, 18 mutants were selected and field-transplanted for evaluation of agronomic performance in a clonal trial of augmented design under saline conditions. Agronomic data of the mutants were compared with those of the parent variety and standard check varieties (Tables 1 and 2). Clone 8270 showed the highest germination (52.1%) compared to standard varieties (Table 1). Clone 8151 showed higher tillering (11.1%), followed by 8188 (9.0%) and 8149 (8.1%), than the standard check varieties (5.3%) at 45 days after planting. The mutant clone 8151 showed a higher number of millable canes (92,400 ha^{-1}) than the standard varieties (62,800 ha^{-1}). There was a significant reduction in leaf length and leaf width in mutant clones (82.8 and 3.2 cm) in comparison to the standard varieties (114.0 and 4.8 cm). The leaf length of the clones 8151, 8182, 8268, and 8271 was significantly greater, at 127.3, 115.3, 111.7, and 118.7 cm, respectively, than that of the parent Co86032 (95.0 cm) under salt stress, whereas clones 8162, 8168, 8174, 8188, 8193, 8197, 8209, 8210, 8219, 8236, and 8270 showed significant reductions in leaf length (Table 1).

Clones 8147 and 8188 showed higher Brix% (22.0 and 20.2, 22.0 and 21.4) at months 10 and 12, respectively (Table 2). However, clones 8151, 8209, 8268, 8270, and 8271 showed higher Brix% (20.0, 19.7, 20.0, 20.2, and 20.4) only at month 10 than the parent Co86032 (19.0). Clone 8188 and standard CoC671 showed the highest sucrose% (19.3 and 19.8, 19.5 and 19.7) at months 10 and 12 respectively. Among the mutants, clone 8151 showed higher cane yield (101.9 t ha^{-1}) and CCS (12.4 t ha^{-1}).

In general, mutagenic frequency increased with dose of gamma rays up to 30 Gy (Table 3). An increasing trend in lethality percentage was observed with increasing dose of gamma radiation in sugarcane calli. The 30-Gy dose of gamma radiation produced the maximum (90.5) lethality. The biological damage efficiency observed was highest under the 20-Gy treatment. The dose giving maximum mutation efficiency for sugarcane calli of Co86032 may thus be considered as 20 Gy of gamma radiation.

Table 1 – Field performance of sugarcane mutant clones of Co86032 under salinity stress.

	Germination % 45 DAP	Tillering ratio at 120 DAP	Total height (cm)	Millable height (cm)	NMC ($\times 10^3$ ha^{-1})	Cane weight (kg)	Diameter (cm)	Internodes/ cane	Leaf length (cm)	Leaf width (cm)
Parent, standard varieties										
Co86032	41.6	5.8	215.6	184.4	64.4	1.2	2.6	17.8	95.0	4.1
CoC671	42.4	5.8	251.1	211.7	59.2	1.4	3.0	21.9	128.2	5.7
CoM0265	38.9	5.2	192.2	163.3	65.6	1.4	3.3	17.1	126.7	5.5
Co740	51.4	4.2	176.7	148.9	62.0	0.6	2.4	15.0	106.2	3.9
Mean (C)	43.6	5.3	208.9	177.1	62.8	1.1	2.8	17.9	114.0	4.8
Mutant										
8147	33.3	7.2	213.3	188.3	62.4	0.8	2.5	18.3	103.3	4.0
8149	25.0	**8.1**	193.3	158.3	79.2	0.5	1.7	15.7	98.3	4.4
8151	27.1	**11.1**	216.7	188.3	**92.4**	1.1	2.8	20.7	**127.3**	4.7
8162	25.0	4.6	186.7	158.3	68.4	0.4	1.7	13.7	65.0	2.8
8168	25.0	4.6	150.0	133.3	70.8	0.5	1.9	14.3	78.3	3.4
8174	35.4	6.1	156.7	130.0	30.0	0.4	1.9	15.3	55.0	2.0
8182	10.4	**9.0**	210.0	176.7	79.2	0.9	2.6	17.0	**115.3**	4.7
8188	43.8	3.1	200.0	178.3	78.0	0.7	2.0	17.7	58.3	2.5
8193	20.8	5.4	156.7	140.0	70.8	0.5	1.9	16.0	57.0	1.9
8197	35.4	3.1	133.3	103.3	27.6	0.6	1.8	17.3	58.3	2.0
8209	22.9	1.6	100.0	86.7	6.0	0.2	1.7	18.0	63.3	2.7
8210	27.1	1.2	90.0	66.7	9.6	0.2	1.8	17.7	61.7	2.5
8219	31.3	1.7	170.0	136.7	21.6	0.5	1.8	18.3	75.0	2.6
8236	35.4	1.0	128.3	106.7	43.2	0.4	2.6	15.0	70.3	3.0
8249	25.0	3.4	140.0	101.7	70.8	0.5	2.7	15.3	105.0	3.9
8268	41.7	3.8	113.3	93.3	7.2	0.4	2.7	16.7	**111.7**	3.6
8270	52.1	2.7	100.0	83.3	60.0	0.4	2.4	14.3	68.3	2.7
8271	35.4	1.3	130.0	110.0	12.0	0.4	1.9	12.0	**118.7**	3.6
Mean (V)	30.7	4.4	154.9	130.0	49.4	0.5	2.1	16.3	82.8	3.2
CD1	7.6	2.4	7.6	53.3	20.4	0.5	0.4	3.8	14.8	0.7
CD2	22.8	7.2	22.8	159.9	61.1	1.5	1.2	11.5	44.5	2.0
CD3	18.6	5.9	18.6	130.6	49.9	1.2	1.0	9.4	36.4	1.7

Values in bold represent significant superiority over parent variety. Values in italics represent significant superiority over CoM0265 (moderately salt-tolerant variety).
CD1: critical difference for parent and standard varieties (C); CD2: critical difference for mutants (V); CD3: critical difference between standard varieties (C) and mutants (V); NMC: number of millable cane; DAP: days after planting.

4. Discussion

The application of radiation-induced mutagenesis with in vitro culture has proved effective in the induction of genetic variation, selection, and multiplication of mutant clones [6,9,27]. Assessment of radiosensitivity is a prerequisite for identifying a suitable dose for in vitro mutagenesis of a particular cultivar. In the present study, sugarcane embryogenic callus was exposed to different doses of gamma radiation and post-irradiation survival based on RGR showed significant reduction in growth rate with increasing doses of gamma radiation. The 20 Gy radiation dose was found to be the optimum LD_{50} for Co86032 sugarcane callus. Taras et al. [28] reported 20 Gy as the LD_{50} for *Brassica* and characterized radiosensitivity using LD_{50} as the criterion. However, LD_{20} or LD_{30} are also used as optimum doses, as these levels of mutagens showed no toxicity to plant tissues [29]. In sugarcane, Patade et al. [16] suggested 20 Gy as the optimum dose for embryogenic callus and also observed reduced regeneration frequency with increasing gamma radiation. In our study, mutagenic frequency also increased with increasing dose of gamma rays (Table 3) and based upon the results, the dose giving maximum mutation efficiency for calli of Co86032 may thus be considered as 20 Gy of gamma radiation.

In this study, in vitro selection was applied to embryogenic callus culture by inclusion of growth-inhibitory levels of NaCl in the selection medium. Screening of mutagenized cultures during dedifferentiation and differentiation stages could be very useful for selection of salt tolerance, as described earlier [14]. The present study indicated a significant reduction in the growth rate of sugarcane embryogenic callus exposed to 100 to 250 mM NaCl stress. This reduction may have resulted from reduced water availability to callus cells due to increased NaCl stress in the medium. In sugarcane a significant decline in callus growth rate occurred with 150 mmol L^{-1} NaCl [30] and 171 mmol L^{-1} NaCl [16]. The results also showed that reduction in callus growth with increasing salt concentration resulted from lower percent tissue water content and membrane damage to cells. This effect presumably arises from dehydration of cells through low water potential or nutritional imbalance because of interference of salt ions with essential nutrients [31]. In our study, we observed an increase in REL and reduction in water content with increasing salt and radiation concentrations. Correlation of REL and water content has also been observed in salt-stressed wheat [31,32],

Table 2 – Field performance of sugarcane mutant clones of Co86032 under salinity stress.

	Yield (t ha^{-1})	CCS (t ha^{-1})	Brix%		Sucrose%		Purity%		CCS%	
			10th month	12th month	10th month	12th month	10th month	12th month	10th month	12th month
Parent, standard varieties										
Co86032	81.6	11.4	19.1	19.0	18.0	18.9	94.2	99.5	13.0	14.0
CoC671	82.9	11.3	21.7	22.1	19.5	19.7	90.2	89.1	13.8	13.9
CoM0265	94.6	11.9	19.1	20.2	18.0	18.0	94.8	89.1	13.0	12.6
Co740	36.4	4.8	19.0	19.2	16.9	18.4	89.4	95.5	11.9	13.3
Mean (C)	73.9	9.9	19.7	20.1	18.1	18.7	92.1	93.3	12.9	13.5
Mutant										
8147	50.1	6.9	**21.6**	**20.2**	18.6	19.1	86.1	**94.7**	12.9	13.8
8149	37.5	4.9	**20.8**	18.9	18.7	17.9	89.7	**94.8**	13.2	13.0
8151	101.9	12.4	17.1	**20.0**	14.8	17.5	86.3	87.3	10.2	12.2
8162	26.4	3.7	20.0	**19.6**	16.9	19.2	84.6	**98.4**	11.6	**14.1**
8168	37.5	4.7	18.7	18.5	15.6	17.3	83.2	**93.8**	10.6	12.5
8174	12.0	1.5	18.6	**20.6**	16.3	18.0	87.8	87.1	11.4	12.5
8182	69.7	8.9	18.5	**20.0**	17.0	18.0	91.8	90.1	12.1	12.7
8188	56.7	8.0	**22.4**	**21.4**	**19.3**	**19.8**	85.9	**92.4**	13.3	**14.1**
8193	33.3	4.1	16.8	18.9	13.7	17.3	81.4	91.3	9.2	12.3
8197	15.6	2.1	17.8	19.2	16.9	18.5	94.7	**96.7**	12.2	13.5
8209	1.4	0.2	17.5	**19.7**	15.2	18.5	87.0	**93.7**	10.6	13.3
8210	1.9	0.3	18.4	18.5	16.4	18.6	89.2	**100.3**	11.6	13.7
8219	11.6	1.6	15.0	19.0	11.5	18.9	76.8	**99.4**	7.5	**14.0**
8236	17.6	2.4	17.5	18.5	15.7	18.6	89.8	**100.2**	11.1	13.8
8249	32.8	4.4	18.0	17.8	15.0	18.1	83.5	**101.9**	10.2	13.5
8268	2.9	0.4	19.4	**20.9**	17.3	18.4	89.2	88.1	12.2	12.9
8270	22.2	3.1	18.0	**20.2**	15.5	**19.3**	85.8	95.6	10.7	**14.0**
8271	4.8	0.6	19.3	**20.4**	16.5	17.7	85.7	86.5	11.4	12.2
Mean (V)	29.8	3.9	18.6	19.6	16.2	18.4	86.6	94.0	11.2	13.2
CD1	42.0	5.5	1.3	0.6	1.1	0.6	6.1	2.8	1.0	0.5
CD2	126.0	16.4	3.9	1.8	3.2	1.7	18.3	8.5	2.9	1.6
CD3	102.9	13.4	3.2	1.5	2.6	1.4	15.0	6.9	2.4	1.3

Values in bold represent significant superiority over parent variety. Values in italics represent significant superiority over CoM0265 (moderately salt-tolerant variety).
CD1: critical difference for parent and standard varieties (C); CD2: critical difference for mutants (V); CD3: critical difference between standard varieties (C) and mutants (V); CCS: commercial cane sugar.

tobacco [33], and *Sesuvium* [20]. NaCl stress results in oxidative damage to membranes and peroxidation of membrane lipids [34]. The degradation of membranes due to lipid peroxidation also leads to leaching of cellular electrolytes, a response used as an indicator of disturbance of membrane integrity. In soybean, MDA content increased significantly at 50 and 100 mmol L^{-1} NaCl [35]. In our study, MDA rapidly increased under NaCl stress treatments (100 to 200 mmol L^{-1} NaCl) as well as under higher doses of radiation.

To avoid oxidative damage, plants have evolved various defensive mechanisms to counteract the effect of reactive oxygen species in cellular compartments [36]. These defenses include modulated expression in the metabolic and defensive pathways and synthesis of osmolytes [37]. The results of this study revealed increase in proline concentration in irradiated and NaCl-stressed callus cultures. A change in proline concentration has been correlated with its capacity to tolerate and adapt to salinity conditions [38]. Gandonou et al. [15] reported that proline accumulation increases in salt-tolerant callus under salinity. The accumulation of proline is widely used as a selection criterion for salinity and drought tolerance [39]. Salt-sensitive barley plants synthesized more proline and glycine betaine than did salt-tolerant plants [40] and salt-tolerant rice cultivars accumulated less proline under NaCl stress [41]. Our results indicated significant proline accumulation in 100 mmol L^{-1} NaCl stress callus and higher proline in 20 Gy-irradiated callus under

Table 3 – Mutagenic effectiveness and efficiency of gamma radiation in sugarcane callus of variety Co86032.

Gamma ray (Gy)	Plantlet	No. of variants	Mutagenic frequency (%) (Mf)	Biological damages (%)		Mf*100/dose	Effectiveness	
				Survival reduction	Variant		Lethality Mf*100/lethal	Biological Mf*100/damage
10	40	5	12.5	20 (8)	30 (12)	125.0	62.5	41.7
20	64	12	18.8	28 (18)	9.4 (6)	93.8	67.0	199.5
30	34	8	23.5	26 (21)	21 (7)	78.4	90.5	112.0

NaCl stress, indicating the salt tolerance of irradiated callus. Significantly higher accumulation of GB was also observed in callus with 150 mmol L^{-1} NaCl-stressed callus, and in 30 Gy-irradiated callus exposed to NaCl, according to Scott–Knott group significance. Higher accumulation has been reported in salt-tolerant species, whereas moderately tolerant species accumulate intermediate levels and sensitive species accumulate low or no levels [34].

The study of Na^+ and K^+ levels revealed that the sodium (Na^+) concentration increased in irradiated callus exposed to salt stress, a response that may be due to osmotic adjustment of cells. The reduction of callus growth may be due to nutritional imbalance resulting from interference by Na^+. However, increasing amounts of Na^+ destabilize osmotic potential, creating a highly toxic environment to plant cells, even with the aid of defense mechanism of antioxidant enzymes, and leaving callus slimy or dead [16,30]. Our results revealed that Na^+ concentration was higher in NaCl-treated callus than in control callus, in contrast to K^+ concentration, which was higher in control than in NaCl-treated callus. It is important to note that growth retardation is often associated with increase in Na^+ but decline in K^+ concentration, demonstrating the typical glycophyte nature of sugarcane [16,30]. The result indicates that the increase in accumulation of sodium in plant cells adversely reduced the uptake of potassium. The combined accumulation of salt ions (Na^+ and K^+) and osmolytes (proline, glycine betaine, and MDA) may play an important role in osmotic adjustment in sugarcane cells under NaCl stress.

Plant tissue culture techniques have been used in conjunction with induced mutagenesis to create genetic variation, and gene mutations can occur more frequently in tissue-culture-derived plants [42]. In vitro mutagenesis of cultured explants, cells, and tissue cultures represents a feasible method for induction of genetic variation, which can be subjected at the cellular level to selection for desirable traits [43,44]. However, success of in vitro mutagenesis programs will depend on evaluation of mutant clones under field conditions to confirm their performance for the selected trait of interest. The performance of selected salt-tolerant genotypes of durum wheat under saline and non-saline field conditions indicated that genetic variation for traits such as number of grains per spike, grain weight per spike, 1000-grain weight, number of spikes per m^2, grain yield, and harvest index could be induced by mutagenic treatment [45]. In our study, agronomic traits showed a wide range of genetic variation for leaf length, tillering ratio, total height, number of millable canes/ha, CCS, Brix%, sucrose%, purity%, and CCS% (Tables 1 and 2). Number of millable canes is the most important character, contributing directly to higher yield [46]. Number of stalks has also been considered as a major contributing factor for cane yield [47]. We could obtain mutant lines (8151) with improved number of millable cane (NMC) (92.4) over the parent variety (64.4) and other standard check varieties. This is a significant observation, given that the mutant also had high tillering ratio and leaf length. Zhou [48] recommended a focus on the tiller development and leaf development parameters that influence cane yield or its components. Such studies are likely to be useful in identifying potentially high-yielding varieties during the early stages of sugarcane selection. In a study of genetically diversified sugarcane clones tested for yield stability, sugar yield showed significant positive correlation with tillers/plant, cane length, weight/stool, and cane yield [49]. In the present study, irradiated and salt-selected (mutant) clones were evaluated for their performance based on morphological and agronomic characters, resulting in the selection of 18 mutants. Among these, mutants, 8151, 8209, 8268, 8270, 8188, and 8271 are the best performing and promising for further studies. The results suggest that in vitro-induced mutagenesis followed by in vitro selection can be applied to induce genetic variation for salt tolerance, besides improving agronomic characteristics in sugarcane.

5. Conclusion

This study has successfully demonstrated that in vitro mutagenesis and selection can be used to generate mutants and salt-tolerant lines in sugarcane and to study the physio-biochemical basis of salinity tolerance. Our results suggest that the accumulation of salt ions (Na^+ and K^+) and osmolytes (proline and glycine betaine) plays an important role in osmotic adjustment in sugarcane cells under NaCl stress. Agronomically superior mutants can be useful in sugarcane improvement programs.

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