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Evaluation of Differential Physiological and Biochemical Response of Sugarcane (*Saccharum* spp. Complex cv. Co 94012) Parent and Mutant's in Response to Salt Stress

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Abstract

The present investigation carried out at Main Sugarcane Research Station, Navsari Agricultural University, Navsari during 2011–2014 to develop salt tolerance lines in sugarcane. Sugarcane mutants against salt tolerance were derived using *in vitro* mutagenesis and regeneration under NaCl stress. Sugarcane cv. 94012 was used for callus development from meristimatic leaf whorl on MS medium supplemented with 4 mg/l 2,4-D+2% sucrose. At treatment of Ethyl Methane Sulphonate (0.5%) for 2 hours treatment 48.60% *calli* survived similarly out of four concentration of NaCl 100 mM concentration of NaCl give at least 50.20% survived of *calli*. So these concentrations were used for development of salt tolerant mutant in sugarcane. The *calli* were treated with EMS lethal dose (LD_{50}) placed on shoot regeneration media containing LD_{50} concentration of NaCl. The plants which regenerated from the tolerant *calli* were grown in pot culture system under five levels of salinity stress as compared to parental plants (source variety). With increasing supply of NaCl, root growth was more adversely affected. The growth of shoot, Chlorophyll content, photosynthesis rate, stomatal conductance and dry matter showed a decreasing trend but it shows significantly slow rate in tolerant mutants than parental plants. Mutant plants performed significantly well than parental plants in salt stress condition in all physiological and biochemical parameters. The result showed that high salt concentration Na^+ content in shoot of tolerant mutant and but was less than the normal plant. While, K^+ content in shoot was high in tolerant mutants than normal plants.

Keywords: Sugarcane, salt tolerance, *in vitro*, *calli*

1. Introduction

Sugarcane is the most important cash crops in India. Improvement in this crop through breeding is handicapped because of the complex flowering behavior under natural day-length conditions in India. The complexity and polygenic nature of salinity tolerance has seriously limited the efforts to develop the salt tolerant crop variety through conventional breeding practices. Tissue and cell culture techniques provide new methods for deriving genetic variation in relatively shorter duration. The use of plant cell culture techniques has expanded greatly particularly in the improvement of vegetatively propagated crops like sugarcane. In this crop genetic variation can be introduced through somaclonal variation (Heinz and Mee, 1960; Liu et al., 1972). It has been reported that *in vitro* irradiation and other mutagenic agents increase variation and the level of stress tolerance among regenerants (Choudhari et al., 1994). Somaclonal variation in combination with *in vitro* mutagenesis and selection has been applied for the isolation of agronomically useful mutants in sugarcane (Jain, 2000; Patade et al., 2006; Shomeili et al., 2011; Dalvi et al., 2012; Koch et al., 2012). Sugarcanes are extremely salt sensitive crop and soil salinity is a major constraint in the production of these crops in India. There are 6744 thousand ha of salt affected soil in the India (Anonymous, 2013). Majority of salt affected soils occur in the states of Gujarat, Uttar Pradesh, Maharashtra, Rajasthan and Tamilnadu. Keeping in view this situation, chemical induced mutations coupled with tissue culture based selection protocols were adopted for the development of salt tolerance in these crops. The present study was carried out to determine the effect of chemical mutation and salinity on callus growth and regeneration in sugarcane and growth of micro propagated plants in Sugarcane.

2. Materials and Methods

Present study was carried out in the Tissue Culture Laboratory, Main Sugarcane Research Station, Navsari Agricultural University, Navsari during 2011–2014. Healthy young leaf explants including apical meristems were obtained from the

shoot of commercial sugarcane variety Co 94012.

2.1. In vitro mutagenesis and screening

Calli were established from the smaller pieces of explants, made on Murashige and Skoog medium, supplemented with 20 g l^{-1} sucrose, 7.5 g l^{-1} agar and 4 mg l^{-1} 2,4-D. The medium was adjusted to pH 5.8 with NaOH (0.1 N), autoclaved at 120 °C and 15 lbs psi for 15 min. After 4 weeks, embryogenic *calli* were separated from the explants and treated with different treatment of EMS (0.5%) *i.e*., 0, 1, 2, 3 and 4 hours. Lethal dose (LD_{50}) of EMS was decided on the basis of survival per cent of callus. After decide LD_{50} of EMS callus transferred to MS media supplemented with different levels of NaCl (0, 50, 100, 150 and 200 mM) and lethal dose (LD_{50}) of NaCl was decided on basis of survival per cent of callus. Salt tolerant plantlets were regenerated from callus treated with EMS (LD_{50}) on MS medium supplemented with the NaCl (LD_{50}).

2.2. Regeneration protocol

Cultures were grown in sterilize plastic petri-plates closed with paraffin wax strip. Plantlets were regenerated from treated callus and after 3 to 4 weeks of transfer selected healthy plantlets on root induction medium, i.e., ½ MS medium of the same composition as earlier mentioned, but with special hormones (Patel, 2007) and in a growth chamber under long-day conditions (16/8 hours light/dark cycle) at a temperature of 25 ± 2 °C and relative humidity of 55±5%. Light was provided by white fluorescent tubes (40 W) with approximately 2000 lux/m light intensity. After root formation plantlets were grown in poly-house for primary hardening then shed net for secondary hardening for one month each. The best and healthy plantlets were selected as tolerant mutants for the next evaluations.

2.3. Evaluation in pot experiment

Mutants rose from treatment EMS (0.5%) (LD_{50})+NaCl (LD_{50}) and Normal plants (somaclones from non-treated callus) were evaluate for salt tolerance in pot culture with five treatment of NaCl concentrations i.e., 0, 50, 100, 150 and 200 mM. So, healthy plant was transferred to pots with 50×30 cm^2 (height×width) specification. Holes were made in the bottom of pots and accommodate one plant pot^{-1}. Only 2/3 of the pot filled with the potting mixture i.e., Clay:Sand : FYM (1:1:1) to ensure the presence of adequate air inside the pots. Nutrient solutions were given to plant every 5 days together with addition of 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.

2.4. Morphological, physiological and biochemical analysis

Since the morphological features of the mutant plants were not sufficient, physiological and biochemical analysis was also used as compared to their parental variety. All the observation recorded 60 days after planting of plantlets. All measurements were conducted on five replicate plants per each treatment.

2.5. Morphological characters

Total number of green leaves on the plant from each treatment was counted. Leaf area was measured by leaf area meter (Model LI-3000, LI-COR, USA) and expressed as cm^2. Shoot length and root length measured of randomly selected plants. Fresh weight (g) and dry weight (g) also recorded. Dry matter accumulation was quantified by obtaining dry weights of plants at 70 °C for 48 hours in a dry oven.

2.6. Physiological characters

The chlorophyll content index was recorded with help of chlorophyll content meter (CCM-200 Plus manufactured by Apogee Instrument). It measures the absorbance of both wavelengths and calculates a Chlorophyll Concentration Index (CCI) value that is proportional to the amount of chlorophyll in the sample of each treatment. The photosynthetic rate, stomatal conductance and transpiration rate were measured using CIRAS-1A photosynthetic system. An open system of narrow rectangular chamber with window was used. Every observation was recorded with leaf covering full window of the system. Observations were made on fully expanded green leaves from 3rd and 4th from top and measured at 8.30 to 10.30 am. Each leaf was fully exposed and the open chamber was held at such an angle that the surface of the leaf directly faced the sun.

2.7. Biochemical characters

Na^+ and K^+ content in shoot were assayed by the procedure earlier reported (Basu et al., 2002). Na^+ and K^+ contents of shoot were quantified with an Atomic Absorption Spectrophotometer (GBC 904 AA, GBC Scientific Equipment PTY LTD, Australia) and expressed as µmol g^{-1} fresh weight

2.8. Statistical analysis

The experiment was a factorial experiment of two factors, with five replications and arranged in a Completely Randomized Design. The first factor was sugarcane Normal plants and derived salinity tolerant mutants of variety Co 94012. The second factor was five salinity levels (0, 50, 100, 150 and 200 mM NaCl). The data generated from the experiments were subjected to statistical analysis in Factorial Completely Randomized Design (FCRD) whenever, necessary as prescribed by Panse and Sukhatme (1985). Transformation of data was carried out prior to statistical analysis as suggested by Steel and Torrie (1981).

3. Results and Discussion

3.1. In vitro mutagenesis

The results on survival per cent of callus to different EMS (0.5%) treatments are given in Table 1. Culture exposed to 2 hour treatment almost 50% survival response compared to control non-treated culture (Table 1). Similar result noticed by Mallikarjun et al. (2008) in sugarcane. Koch et al. (2009) exposed callus to 8 mM and 16 mM EMS for development of tolerant cell.

3.2. In vitro salinity screening

The results on survival per cent of callus to different NaCl

Table 1: Survival per cent of callus after 30 days to different EMS (0.5%) treatments to determine lethal dose (LD_{50}) of EMS

Geno-type	EMS 0.5% (Hour)	% Callus survive					
		1	2	3	4	5	Mean
Co 94012	1	69	70	70	69	70	69.60
	2	49	48	49	48	49	48.60
	3	12	12	13	13	13	12.60
	4	3	3	2	3	3	2.80
	0 (control)	100	100	100	100	100	100.00

treatments are given in Table 2. Culture exposed to 100 mM treatment almost 50% survivals as compared to control non-treated culture (Table 2). Similar results obtained by Patade and Suprasanna (2009) for 171.1 mM and Munir and Aftab (2009) for 120 mM concentration of NaCl. These results are in close agreement with those reported by Mallikarjun et al. (2008); Shomeili et al. (2011); Gandonou et al. (2005b).

Table 2: Survival per cent of callus after 30 days to different treatments of NaCl to determine lethal dose (LD_{50}) of NaCl

Geno-type	Conc. of NaCl (mM)	% Callus survive					
		1	2	3	4	5	Mean
Co 94012	50	88	87	88	89	88	88.00
	100	51	50	49	50	51	50.20
	150	24	23	22	24	23	23.20
	200	3	4	3	4	3	3.40
	0 (control)	100	100	100	100	100	100.00

3.3. Evaluation in pot experiment

In the treatment T_3 leaf area of mutant plants (334.9 cm^2) was significantly higher as compared normal plants (305.8 cm^2) (Table 3). 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; Shomeili et al., 2011). There was significantly decrease in number of leaves in treatment T_5 in mutant plants (5.84) as compared to treatment T_1 (7.44) (Table 3). Rapid and transient reductions in leaf expansion rates after a sudden increase in salinity have been recorded in maize (Cramer and Bowman, 1991; Neumann, 1993), rice (Yeo et al., 1991) and wheat and barley (Passioura and Munns, 2000).

Numerous works comparing general responses of some plant species with different salinity levels, reported growth reduction under salt stress conditions (Altman, 2003; Barba et al., 1977; Jain, 2000; Shomeili et al., 2011). Under this experiment conditions, contrary to the normal plants (Co 94012), shoot length significantly higher in tolerant mutants (51.98 cm) as compared to normal plants (35.07 cm) in treatment T_4 (Table 3). Root length was significantly decreased from 50.23 cm to 24.20 cm and 50.12 cm to 20.84 cm in tolerant mutants and normal plants respectively with increase in salinity concentration. Both root length and shoots length decreased with increase in salt concentration in normal plants but not in the tolerant variant. These results are in agreement with results obtained previously, which also indicated that roots were among the first plant organs affected by salt stress and the most sensitive ones (Bhatnagar et al., 2008). According to Neumann (1993) report, salinity can rapidly inhibit root growth and hence the capacity for uptake of water and essential mineral nutrients from the soil. In culture conditions, tolerant variant kept normal growth at elevated NaCl concentrations and showed no inhibitory effect on shoot growth.

In present study, at higher salinity level significantly maximum photosynthesis rate 1.54 µmol CO_2 m^{-2} s^{-1} was recorded in tolerant mutants as compared to normal plants (0.83 µmol CO_2 m^{-2} s^{-1}) (Table 4). In tolerant mutants stomatal conductance was significantly decreased from 20.04 to 8.28 (mol CO_2 m^{-2} s^{-1}) and in normal plants stomatal conductance decreased from 20.06 to 6.95 (mol CO_2 m^{-2} s^{-1}) with increase in salinity level (Table 4). In present investigation it is reported that photosynthesis rate and stomatal conductance reduced with increase in salinity level but, reduction rate was less in tolerant mutants as compared to normal plants. Present finding are in line with Vasantha et al., 2010; Shomeili et al., 2011 in sugarcane. The reasons for reduced photosynthesis include stomatal closure and feedback inhibition due to reduced sink activity. Further, a reduction in stomatal conductance may results from the osmotic effect of salinity.

In tolerant mutants of chlorophyll content index was maximum in treatment T_1 (45.20) and minimum in T_5 (42.40) (Table 4). Similarly in normal plants CCI was maximum in treatment T_1 (45.23) and minimum in T_5 (41.19). Reductions of chlorophyll content under elevated salinity conditions were observed in some salt-sensitive land species (Munns, 2002). In contrast, chlorophyll content in salt tolerant plants either do not decline or rise 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 (Don et al., 2010). The rate of salt accumulation in shoots of salt tolerant plants can be determined by the rate of transpiration. Transpiration rate generally tend to decline with increasing rhizospheric salinity in both sensitive and tolerant plants (Michael et al., 1997; Shomeili et al., 2011). It might be due to salt accumulation in the mesophyll which reduced stomatal aperture (Flowers and Yeo, 1995). Our results showed that the salt tolerant variant have been able to transport lesser harmful salt ions (Na^+) to shoot tissues and then had a higher transpiration than normal

Table 3: Physiological response of sugarcane mutants (cv. Co 94012) to salt stress in pot culture

	Treat.	Salt conc. (mM)	Leaf area (cm^2)	No. of leaves	Shoot length (cm)	Root length (cm)	Fresh weight (g)	Dry weight (g)
Mutant plants	T_1	0	359.8	7.44	88.11	50.23	35.42	12.49
	T_2	50	346.6	7.47	68.97	37.42	28.27	8.88
	T_3	100	334.9	7.17	60.51	32.38	19.96	6.88
	T_4	150	326.2	6.83	51.98	29.81	15.11	5.10
	T_5	200	313.4	5.84	36.57	24.20	11.10	4.70
Normal plants	T_1	0	360.2	7.48	88.23	50.12	35.00	12.61
	T_2	50	340.8	7.31	61.08	35.82	18.96	7.36
	T_3	100	305.8	6.53	51.55	30.13	16.52	5.95
	T_4	150	282.9	6.08	35.07	24.12	10.47	4.81
	T_5	200	268.3	5.45	31.22	20.84	9.89	3.50
SEM±		M	0.881	0.045	0.235	0.236	0.201	0.070
		N	1.394	0.071	0.371	0.373	0.318	0.110
		M×N	1.971	0.101	0.525	0.528	0.450	0.156
CD (*p*=0.05)		M	2.618	0.134	0.697	0.701	0.597	0.207
		N	4.139	0.212	1.102	1.109	0.944	0.328
		M×N	5.854	0.300	1.558	1.568	1.336	0.463
CV %			1.217	2.992	1.830	3.151	4.482	4.319

Mutant plants (M): *in vitro* screened tolerant mutant plant; Normal plants (N): Normal plants of variety

Table 4: Physiological response of sugarcane mutants (cv. Co 94012) to salt stress in pot culture

	Treat.	Salt conc. (mM)	Chloro-phyll content Index	Photosynthesis rate (µmol CO_2 m^{-2} s^{-1})	Stomatal conductance (mol CO_2 m^{-2} s^{-1})	Transpiration rate (m mol H_2O m^{-2} s^{-1})	K+ (µmol g^{-1} FW)	Na+ (µmol g^{-1} FW)
Mutant plants	T_1	0	359.8	7.44	88.11	50.23	35.42	12.49
	T_2	50	346.6	7.47	68.97	37.42	28.27	8.88
	T_3	100	334.9	7.17	60.51	32.38	19.96	6.88
	T_4	150	326.2	6.83	51.98	29.81	15.11	5.10
	T_5	200	313.4	5.84	36.57	24.20	11.10	4.70
Normal plants	T_1	0	360.2	7.48	88.23	50.12	35.00	12.61
	T_2	50	340.8	7.31	61.08	35.82	18.96	7.36
	T_3	100	305.8	6.53	51.55	30.13	16.52	5.95
	T_4	150	282.9	6.08	35.07	24.12	10.47	4.81
	T_5	200	268.3	5.45	31.22	20.84	9.89	3.50
SEM±		M	0.881	0.045	0.235	0.236	0.201	0.070
		N	1.394	0.071	0.371	0.373	0.318	0.110
		M×N	1.971	0.101	0.525	0.528	0.450	0.156
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		N	4.139	0.212	1.102	1.109	0.944	0.328
		M×N	5.854	0.300	1.558	1.568	1.336	0.463
CV %			1.217	2.992	1.830	3.151	4.482	4.319

Mutant plants (M): *in vitro* screened tolerant mutant plant; Normal plants (N): Normal plants of variety

plants. Sodium and chloride concentration in shoots and roots of sugarcane differently increased with salinity genotypically (Patade et al., 2006; Karpe et al., 2012).

Under salinity stress, results showed that total dry matter production highly correlated with K^+ and Na^+ (Table 1 and 2). The Na^+ content in shoot was significantly higher in normal plants (3.15 µmol g^{-1} FW) as compared to tolerant mutants (1.96 µmol g^{-1} FW) at highest salinity level (Table 4). It was indicated that higher amounts of Na^+ in plant tissues significantly reduced dry matter production. Similar results reported by Patade et al. (2008). In the absence of stress, K^+ concentration showed a non-significant difference among the two experimental plant types. But with increased in salinity, it changed adversely and sharply in tolerant variant though, this was lower in normal plants than tolerant mutants. Results showed that, high correlation between dry weight and K^+ at all salinity levels (Table 1 and 2). These findings are in contrast with some previous results of (Patade et al., 2006; Wahid et al., 2006) and are in agreement with others (Gandonau et al., 2008; Shomeili et al., 2011; Karpe et al., 2012).

With increasing salt concentrations, total dry weight decreased sharply in normal plants than new tolerant variant. At highest salinity level significantly maximum dry weight was recorded in tolerant mutants (4.70 g $plant^{-1}$) as compared to normal plants (3.50 g $plant^{-1}$) (Table 3). The decrease in value of the dry weight at high NaCl concentrations indicates that plantlets were affected positively by salinity, especially in normal plants of variety.

Salinity still remains the major abiotic stresses that limit and pose a threat to agricultural production in many parts of the world (Altman, 2003; Don et al., 2010). While, numbers 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 et al., 2008).

4. Conclusion

In vitro mutagenesis with 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. Salt tolerance seems to be related to the efficiency of a tissue to absorb, deposit and transport the levels of inorganic solutes in response to salt stress. The results indicated that, the physiological parameters have a positive role to play in tolerance of salinity by the generated plant.

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