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In vitro Response of Promising Sugarcane Varieties for Salinity Tolerance through Callus Culture

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A B S T R A C T

Present investigation was carried out to screen two promising sugarcane varieties CoN 13073 and CoN 09072 for salinity tolerance through callus culture. Various physiological and morphological parameters was studied, maximum shoot length was observed at 2.0 % NaCl concentration treated to callus at 65 days of culturing in variety CoN 13073, similar trend was observed for root length and leaf number per plant in variety CoN 13073 at 2.0 % NaCl concentration.. Very little differences observed in shoot: root ratio in variety CoN 09072, while there was much difference was observed in shoot : root ratio at various levels of NaCl in variety CoN 13073S. Leaf area, chlorophyll content and Na:K ratio were decreased with increased the concentration of NaCl in both the varieties Shoot regenerated under high concentrations of NaCl shown maximum chlorophyll content and leaf area in both the varieties. In comparatives studies, CoN 13073 responded positively to higher concentrations for all the characters studied.

Introduction

Sugarcane industry is the second largest agro-based industry in India. It is grown under varied agro-climatic conditions, hence it faces various biotic and abiotic stresses that impact the productivity in significant way. Salinity is one of the major abiotic stress which greatly affects the sugarcane productivity and recovery. The soils with electrical conductivity (EC) less than 4 dsm-1are generally considered as salt-free, whereas soil with EC range between 4-8 dsm-1are generally considered as salty soil. Salinity is a significant factor that affects crop production and agricultural sustainability worldwide, since about 10 % of the land surface and 50 % of all irrigated land in the world are prone to salinity (Flowers *et al.*, 2010). Salt stress affects several aspects of plant physiology by its osmotic and ionic components (Munns and Tester, 2008).

Sugarcane is a typical glycophyte and hence exhibits stunted growth or no growth under salinity, with its yield falling to 50% or less

than its true potential (Subbarao and Shaw, 1985). Salinity in the root zone of sugarcane decreases sucrose content, through its effect on both biomass and juice quality (Lingle and Wiegand, 1996). Salinity may interfere with sugar production in two major ways, first by affecting growth rate and yield of the cane secondly by affecting the sucrose content of the stalk (Rozeff, 1995). In any crop improvement program prime importance is given to yield and quality parameters. Development of high yields and high sugar recovery genotypes in sugarcane through conventional breeding program is the time consuming. So, In vitro screening and evaluation of sugarcane genotypes for salinity tolerance is a substitute improvement program. Sugarcane is an important cash crop of South Gujarat region. Gujarat has 1600 km costal area which is largest area among all states in India, so some area of sugarcane cultivation affected by salt accumulated through sea water as well as improper irrigation practices and sugarcane - paddy crop rotation is very common in the state which leads to excessive use of water causing soil salinity. This situation decreased the production as well as area of sugarcane cultivation in South Gujarat region. So, there is a need to develop resistant/tolerant *somaclones* of sugarcane varieties. So, present investigation was carried out to screen two promising sugarcane varieties CoN 13073 and CoN 09072 for salinity tolerance through callus culture at sugarcane tissue culture laboratories, Main Sugarcane Research Station, Navsari Agricultural University, Navsari. Various physiological and morphological parameters was studied.

Materials and Methods

The commercial cultivars of sugarcane CoN 13073 and CoN 09072 grown in Gujarat were used as the source of explants in this experiment. The explants were obtained from Main Sugarcane Research Station, Navsari Agricultural University, Navsari. The direct leaf whorl and meristem of sugarcane were used as explants and these explants were true to type, visually healthy and disease free. Disease-free and actively growing cane tops were selected from five months old sugarcane crop as an explant. Cane tops with the growing apices were cut approximately 10 cm long and washed thoroughly in running tap water for 30 minutes. Outer sheaths of cane tops were removed by wiping the sheath with rectified spirit. The shoots were then washed with soap water for about five to six minutes in a sterile 1 liter conical flask followed by cleaning the materials with distilled water. The shoots were rinsed in 5 per cent sodium hypochlorite for 10 minutes.

Then shoots were thoroughly rinsed in 70 per cent ethanol for 30 seconds followed by sterilize double distilled water for four to five times till ethanol was completely washed out from the surface of material. Surface sterilization was performed by using 0.1 per cent mercuric chloride solution. Shoots were shaken vigorously for 5 minutes. Then the container was taken to a laminar clean air station. They were rinsed three to four times with sterile double distilled water to remove all traces of chemicals. The isolation of shoot apex was done by carefully removing the two to three outer whorls of the developing leaves with the help of a sterile sharp blade. The second innermost whorls of developing leave cut in to small pieces of approximately one centimeter length with the help of a sterile sharp blade and utilized as explant for callus induction on MS medium supplemented with different concentrations of 2,4-D (0,1, 2, 3, 4, and 5 mg/l) in different treatments along with 2 mg/l NAA as constant for callus induction Good quality callus generated from leaf whorl and meristem was selected for further experimentation to impose different levels of NaCl concentration and to check the response

to salinity tolerance on the basis of *in vitro* evaluation procedure in both the varieties. In regeneration medium, NAA 2 mg/l + BAP 1mg/l kept constant in all the treatments.

Transfer the regenerated shoots on rooting medium i.e., MS + NAA (2mg/l) + BAP (1mg/l) + different concentration of NaCl. Incubate the culture either in the incubator or growth room maintained at temperature 25± 2^0C, with florescent light (3000-5000 Lux), 16 hrs light/ 8 hrs dark regimes and possessing good relative humidity (60-80 %). The best and healthy plantlets were selected as tolerant somaclonal variants for the next evaluations. After successful regeneration of multiple shoots at different levels of NaCl concentration in MS medium. The plantlets were shifted to polythene bags with sand + soil + compost (1:1:1) at primary hardening. Polythene bags were irrigated at alternate days and the irrigation water was incorporate with different levels of NaCl concentration to evaluate salinity tolerance at primary hardening considering morphological and physiological parameters.

Observation recorded during experiment

Length of regenerated shoots from callus developed on different treatments medium, measured in centimeter after 25 days for fourth subculturing and length of shoot was measured from collar region to the tip of top most leaf. Root length of *in vitro* plantlets was measured in centimetre. These observations were recorded at the 30 day after inoculation on rooting media. The length of the root was measured from collar region down to tip of the longest root. Plant height (cm) was recorded under primary hardening after 25 days of planting. 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 was proportional to the amount of chlorophyll in the sample of each treatment. The leaves from plants selected from each treatment were used for the estimation of leaf area after 25 days of planting. Leaf area was measured by leaf area meter (Model LI3000, LI-COR, USA) and expressed as cm2. Total number of green leaves on the plant from each treatment were counted at 60 days after planting and recorded. Shoot root ratio was estimated by dry weight basis. The potassium and sodium contents were estimated by flame photometer (Jenway PFP 7, ELE Instrument Co. Ud.) method and expressed as ratio on the basis of dry weight. Survival per cent was calculated on the basis of number of plantlets survived from the total number at each treatment combination.

Statistical analysis

The data generated from the various *in vitro* experiments were subjected to statistical analysis in Completely Randomized Design (CRD).

Results and Discussion

Shoot length (cm)

In variety CoN 13073 maximum shoot length was observed in MS medium supplemented with 2 mg/l NAA + 1 mg/l BAP + 2.0 % NaCl in treatment T_5 (5.8 cm), followed by MS medium without NaCl in treatment T_1 (5.2 cm) and treatment T_2, MS + 0.5 % NaCl (4.8 cm). Whereas minimum shoot length was observed in treatment T_6 MS supplemented with 2.5 % NaCl (3.5 cm).

In variety CoN 09072 maximum shoot length was observed in MS medium supplemented with 0.5 % NaCl in treatment T_2 (4.8 cm), followed by MS + 1.0 % NaCl in treatment T_3 (4.6 cm) and MS + 1.5 % NaCl in treatment T_4

and without NaCl in treatment T_1 (4.4 cm). Whereas minimum shoot length was observed in MS medium supplemented with 2.5 % NaCl concentration in treatment T_6 (3.4 cm). MS medium without NaCl concentration registered above to average shoot length.

The plantlets regenerated from callus supplemented with MS medium + different levels of NaCl showed significant differences in shoot length. Among both the varieties CoN 13073 exhibited maximum shoot length at higher NaCl concentration compared to variety CoN 09072. Very little differences in shoot length were observed in variety CoN 09072. The MS medium with 2% NaCl showed maximum shoot length in variety CoN 13073, followed by MS medium without NaCl. Whereas decline in shoot length was observed with the increase in NaCl concentration in variety CoN 09072. At higher concentration of NaCl 2.5% both the varieties responded negatively and resulted reduced shoot length. These results are agreement with Wahid and Ghazanfar (2006) and Ather *et al.,* (2009). The minimum shoot length was observed in plantlets treated with 2.5% NaCl. The saline solution may be at a higher water potential like sea water. Plants challenged by this magnitude of water potential developed through medium, so the leaf is unable to meet the transpirational demands. *Somaclonal* variation appears due to various biological phenomena like, chromosomal aberration, cytoplasmic changes, mitotic crossing over and genetic rearrangement. Variation might have been created during callus formation. The same was reflected in one of the sub culture where surprisingly more shoot and root length was observed even under higher NaCl concentration. The present finding is in accordance with Shomeili *et al.,* (2011).

Root length (cm)

The multiple shoots developed from regeneration media were shifted to rooting media, ½ MS medium was supplemented with NAA 2 mg/l + IBA 2 mg/l kept constant and different NaCl concentrations were imposed to the culture as per treatment combination.

In variety CoN 13073 maximum root length was observed in treatment T_5 (4.4 cm), where ½ MS medium was supplemented with 2.0 % NaCl concentration along with NAA and IBA standard, followed by ½ MS + NaCl 1.0 % in treatment T_3 (4.2 cm). Moderate root length was observed in ½ MS medium without NaCl whereas ½ MS medium supplemented with 2.5 % NaCl concentration in treatment T_6 (2.2 cm) registered minimum root length.

The response of both the varieties to different NaCl concentration was highly differential. Variety CoN 13073 responded positively to higher NaCl concentration up to 2 % for root length. Whereas variety CoN 09072 responded positively to NaCl concentration up to 1.0 % then after there is reduction in root length was observed. In both the varieties minimum root length was observed when plantlets developed from callus supplemented with 2.5 % NaCl. Among both the varieties CoN 13073 exhibited moderate in root length to higher NaCl concentration. These results are in agreement with Akhtar *et al.,* (2003) and Shomeili *et al.,* (2011). According to Mathur *et al.,* (2008) root growth is the prime parameter that affected by salinity condition. At high saline condition rapid inhibition of root growth was observed and hence reduction in uptake of water and essential nutrients can be seen at field condition.

Plant height (cm)

Maximum plant height was observed in treatment T_5 (21.4) where rooting mixture was supplemented with 2.0 % NaCl concentration in variety CoN 13073 followed by treatment T3 (20.6 cm). While minimum plant height was registered in rooting mixture supplemented with 2.5 % NaCl concentration

in treatment T_6 (10.6 cm). Whereas average plant height was observed in rooting mixture without NaCl concentration in treatment T1 (16.5 cm).

In variety CoN 09072, rooting mixture without NaCl concentration registered maximum plant height in treatment T_1 (14.4 cm) followed by rooting mixture with 2.0 % NaCl concentration in treatment T_5 (13.5 cm) and rooting mixture with 0.5 % NaCl concentration in treatment T_2 (12.6 cm). Whereas minimum plant height was recorded in rooting mixture with 2.5 % NaCl concentration in treatment T6 (8.2 cm).

Both the varieties responded poorly to higher NaCl concentration at primary hardening level. Similar results were noticed by Shomeili *et at*., (2011). Among both the varieties effect of salinity in the plant height was not significant in CoN 13073 that indicate at high sodium levels up-taking of nutrients from the rooting mixture were not inhibited and water potential retained at cellular level at saline condition.

Chlorophyll content index

The plantlets developed from callus culture in MS medium supplemented with different NaCl (0 to 2.5%) concentration registered maximum chlorophyll content index in treatment T_2 (5.40), followed by treatment T_1 (5.27) and T_3 treatment (4.87). While plantlets registered from callus culture and rooting mixture supplemented with NaCl 2.5% concentration resulted minimum chlorophyll content index in treatment T_6 (3.70) in variety CoN 13073.

In variety CoN 09072 maximum chlorophyll content index was observed in plantlets developed from rooting mixture without NaCl (%) and registered maximum chlorophyll content (4.86) followed by treatments T_2 (4.54) and T_3 (4.27). while minimum chlorophyll content index was observed in the plantlets developed from the rooting mixture with 2.5 % NaCl in treatment T_6 (3.67).

The plantlets regenerated from callus culture were imposed to different NaCl concentration in rooting mixture and observed low differences among chlorophyll content in both varieties. In variety CoN 09072 control showed maximum chlorophyll content. Increased in the level of NaCl the chlorophyll content was decreased gradually. These results are agreement with Wahid and Ghazanfar (2005) and Shomeili *et al.*, (2011). The minimum chlorophyll content was observed in plantlets treated with 2.5 % NaCl. Chlorophyll content can be used as a sensitive indicator of the cellular metabolic state, thus its decrease signifies toxicity in tissues due to accumulation of ions.

Leaf area (cm^2/plant)

In variety CoN 13073, rooting mixture without NaCl registered maximum leaf area T_1 (41.6 cm^2), followed by treatment with NaCl concentration 0.5 % in treatment T_2 (40.7 cm^2) and with 2.0 % NaCl concentration in treatment T_5 (40.4 cm^2). Minimum leaf area was registered in treatment with 2.5 % NaCl concentration T_6 (32.6 cm^2).

In variety CoN 09072 maximum leaf area was registered in rooting mixture without NaCl concentration in treatment T_1 (40.4 cm^2), followed by 0.5 % NaCl concentration in treatment T_2 (38.5 cm^2) and with 2.0 % NaCl concentration in treatment T_5 (36.4 cm^2). Whereas minimum leaf area was registered with 2.5 % NaCl concentration in treatment T_6 (32.3 cm^2).

High leaf area was observed in control condition (0 % NaCl) while, minimum leaf area was observed where plantlets treated with

2.5 % NaCl in both the varieties. Similar results were observed by Wahid (2004), Shomeili *et al.,* (2011) and Reena *et al.,* (2017).

Leaf number per plant

Rooting mixture supplemented with 2.0 % NaCl registered maximum number of leaves in treatment T_5 (3.4), followed by the rooting mixture supplemented with 0.5 % NaCl in treatment T_2 (3.0) and rooting mixture with 1.5 % NaCl in treatment T_4 (2.8) in variety CoN 13073. Whereas moderate leaf number was observed in rooting mixture without NaCl and minimum leaf number was registered in rooting mixture supplemented with 2.5 % NaCl in treatment T_6 (2.0).

In variety CoN 09072, rooting mixture supplemented with 0.5 % NaCl registered maximum number of leaves in treatment T_2 (2.6) followed by rooting mixture with 2.0 % NaCl in treatment T_5 (2.4) and treatment T_3 (2.2). Whereas rooting mixture with 2.5 % NaCl registered minimum number of leaves in treatment T_6 (1.5).

The plantlets regenerated from MS medium supplemented with different NaCl concentration showed narrow differences among different treatment for leaf number (Table 1) in variety CoN 09072. Whereas plantlets regenerated from MS medium supplemented with different NaCl concentration showed wide difference among different treatment for leaf number in variety CoN 13073. Higher numbers of leaves were observed in 2.0 % NaCl while minimum leaf number observed in 2.5 %NaCl in CoN 13073. Whereas higher leaf number were observed in control condition (0 % NaCl) while minimum leaf number were observed when plantlets were treated with 2.5 % NaCl in CoN 09072. A similar result was observed by Shomeili *et al.,* (2011) as the increase in concentration of NaCl leaf number decreased.

Shoot : root ratio

In variety CoN 13073 maximum shoot : root ratio was observed in rooting mixture with 0.5 % NaCl concentration in treatment T_2 (2.4), followed by rooting mixture with 1.5 %

NaCl concentration in treatment T_4 (2.2) and rooting mixture with 1.0 % NaCl concentration in treatment T_3 (2.0). Whereas minimum shoot : root ratio was observed in treatment T_6 (1.3) with 2.5 % NaCl concentration.

In variety CoN 09072 maximum shoot : root ratio was observed in rooting mixture with 0.5 % NaCl concentration in treatment T_2 (1.8), followed by rooting mixture with 1.5 % NaCl concentration in treatment in treatment T_4 (1.6). Whereas minimum shoot root ratio was observed in treatment T_5 (1.2) with 2.0 % NaCl concentration.

The plantlets regenerated from callus culture taken to primary hardening and imposed with NaCl levels in rooting mixture exposed very little differences in shoot root ratio in variety CoN 09072. While there was much differences was observed in shoot : root ratio at various levels of NaCl in variety CoN 13073.

Lower concentration of NaCl 0.5 % resulted higher shoot:root ratio. Whereas higher concentration of NaCl 2.5 % resulted lower shoot:root ratio in both the varieties.

In the comparative study, variety CoN 13073 showed superior for shoot: root ratio at higher concentration of NaCl, at primary hardening. The increase in value of the shoot:root dry weight ratio at high NaCl indicates that root was positively affected by salinity than shoots.

These results are in agreement with Akhtar *et al.,* (2003) and Shomeili *et al.,* (2011).

Table.1 Response of sugarcane varieties CoN 13073 and CoN 09072 to salt stress

S.No	Shoot length (cm)		Root length (cm)		Plant height (cm)		Chlorophyll content index		Leaf area (cm^2/plant)		Leaf number per plant		Shoot : root ratio		Na : K ratio		Survival per cent	
	Variety 13073	Variety 09072	Variety 13073	Variety 09072	Variety 13073	Variety 09072	Variety 13073	Variety 09072	Variety 13073	Variety 13073	Variety 13073	Variety 09072	Variety 13073	Variety 09072	Variety 13073	Variety 09072	Variety 13073	Variety 09072
T_1	5.20	4.40	2.60	3.00	16.50	14.40	5.27	4.86	41.60	40.40	2.80	2.00	1.80	1.41	0.86	0.78	87.50	70.40
T_2	4.80	4.80	3.20	3.20	19.40	12.60	5.40	4.54	40.70	38.50	3.00	2.60	2.40	1.80	0.80	0.84	80.60	64.60
T_3	4.20	4.60	4.20	2.80	20.60	10.50	4.87	4.27	34.30	36.10	2.40	2.20	2.00	1.40	0.76	0.80	72.30	58.50
T_4	4.40	4.40	3.60	2.40	15.70	10.40	4.62	4.14	36.50	34.50	2.80	2.00	2.20	1.60	0.82	0.68	64.10	54.43
T_5	5.80	4.00	4.40	2.10	21.40	13.50	4.20	3.86	40.40	36.40	3.40	2.40	1.80	1.20	0.84	0.74	84.60	50.60
T_6	3.50	3.40	2.20	1.80	10.60	8.20	3.70	3.67	32.60	32.30	2.00	1.50	1.30	1.40	0.62	0.70	50.40	36.20
SEm	0.12	0.12	0.10	0.01	0.44	0.21	0.01	0.01	0.45	0.56	0.01	0.02	0.01	0.01	0.01	0.01	0.57	0.58
CV	0.37	0.37	0.33	0.05	1.36	0.67	0.05	0.04	1.39	1.72	0.04	0.06	0.05	0.04	0.05	0.05	1.75	1.79
CD	4.47	4.87	5.55	1.16	4.41	3.26	0.69	0.59	2.08	2.67	0.95	1.83	1.80	1.88	4.17	4.17	1.34	1.80

T_1 = 0 % NaCl, T_2 = 0.5 % NaCl, T_3 = 1.0 % NaCl, T_4 = 1.5 % NaCl, T_5 = 2.0 % NaCl, T_6 = 2.5 % NaCl

Na : K ratio

In variety CoN 13073 plantlets developed without NaCl in the rooting mixture registered maximum Na : K ratio in treatment T_1 (0.86) followed by plantlets developed from rooting mixture with 2.0 % NaCl concentration in treatment T_5 (0.84) and plantlets developed from 1.5 % NaCl concentration in treatment T_4 (0.82). Whereas minimum Na : K ratio was observed in rooting mixture with 2.5 % NaCl concentration in treatment T_6 (0.62).

In variety CoN 09072 plantlets developed from rooting mixture with 0.5 % NaCl registered maximum Na : K ratio in treatment T_2 (0.84) followed by rooting mixture with 1.0 % NaCl concentration in treatment T_3 (0.80) and rooting mixture without NaCl concentration in treatment T_1 (0.78). Minimum Na : K ratio was observed in rooting mixture with 1.5 % NaCl concentration in treatment T_4 (0.68).

The plantlets regenerated through callus culture were taken for primary hardening and in the rooting mixture different NaCl (0 to 2.5 %) concentrations were imposed and observed much differences in Na : K ratio among the treatments and among the varieties. Higher Na : K ratio was observed in control condition (0 % NaCl) in CoN 13073 while (0.5 % NaCl) in CoN 09072. Whereas minimum Na : K ratio was observed when plantlets treated with 2.5 % NaCl in both the varieties. These findings are in agreement with (Ahsarf, 2007; Karpe*et al.,* (2012) and Reena*et al.,* (2017).

Survival per cent

Maximum survival per cent was registered in rooting mixture without NaCl concentration in treatment T_1 (87.50 %) followed by rooting mixture with 2.0 % NaCl concentration in treatment T_5 (84.60) and rooting mixture with 0.5 % NaCl concentration in treatment T_2 (80.60 %) in variety CoN 13073. Whereas minimum survival per cent was observed in rooting mixture supplemented with 2.5 % NaCl concentration in treatment T_6 (50.40 %).

In variety CoN 09072, rooting mixture without NaCl registered maximum survival per cent in treatment T_1 (70.40 %) followed by rooting mixture with 0.5 % NaCl concentration in treatment T_2 (64.60 %) and rooting mixture with 1.0 % NaCl concentration in treatment T_3 (58.50 %).Whereas minimum survival per cent at primary hardening was observed in rooting mixture with 2.5 % NaCl in treatment T_6 (36.20 %).

As the leaf provides the platform for photosynthesis. Leaf area indicates the strength of the source of energy of a crop. Photosynthesis and dry matter production of a plant is proportional to leaf number and shoot root ratio of a plant. Prolonged and high intensity abiotic stress leads to plasmolysis and retention of moisture content in plant body which isgoverned by physiological expression and genetic nature of a particular variety. Plantlets regenerated from callus culture were taken to primary hardening and different NaCl concentrations imposed in rooting mixture, observed that maximum survival per cent was recorded in rooting mixture without NaCl concentration in both the varieties. Increase in NaCl concentration resulted poor survival per cent at 25 days after primary hardening. Whereas at 2 % NaCl imposition optimum survival per cent was noticed in variety CoN 13073 that indicate particular variety is tolerant to salinity levels up to 2.0 %. Variety CoN 09072 responded poorly to high salinity levels at primary hardening that indicate Sensitivity of that particular variety to saline condition. Similar results at *in vitro* condition registered by Akhtar *et al.,* (2011).

Form the study it is concluded that *in vitro* selection can be used to identify salt tolerance clones in sugarcane and also to study physiological and biochemical parameters. Salt tolerance seems to be related to the efficiency of an individual varietal genetic constitution at cellular and molecular level to absorb, deposit and transport elements in both available and unavailable forms in response to salt stress. Clones derived from variety CoN 13073 showed higher tolerance towards NaCl up to 2.0% than variety CoN 09072. The study also suggests that in vitro cultured tissue or cell and plantlets can be useful as a system to screen for salinity stress in sugarcane. Overall, Variety CoN 13073 showed better performance in respect of all the characters in the study as compare to Variety CoN 09072.

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