

Provided for non-commercial research and education use.

Not for reproduction, distribution or commercial use.



This article was published in an Sjournals journal. The attached copy is furnished to the author for non-commercial research and education use, including for instruction at the authors institution, sharing with colleagues and providing to institution administration.

Other uses, including reproduction and distribution, or selling or licensing copied, or posting to personal, institutional or third party websites are prohibited.

In most cases, authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Sjournals's archiving and manuscript policies encouraged to visit:

<http://www.sjournals.com>

© 2018 Sjournals Publishing Company

Contents lists available at Sjournals

Scientific Journal of Crop Science

Journal homepage: www.Sjournals.com



Original article

In vitro plant regeneration, screening for salt and drought tolerance in sugarcane (*Saccharum Spp.*)

Mebrahtom Ftwi^{a,*}, Firew Mekbib^b, Eyasu Abraha^c

^aEthiopian Sugar Corporation, Research and Development Center, Wonji, Ethiopia.

^bHaramaya University, P.O. Box 138, Dire Dawa, Ethiopia.

^cTigray Agricultural Research Institute, P.O. Box 492, Mekelle, Ethiopia.

*Corresponding author: mebreftwi@gmail.com

ARTICLE INFO

Article history,

Received 16 December 2017

Accepted 17 January 2018

Available online 24 January 2018

iThenticate screening 18 December 2017

English editing 16 January 2018

Quality control 23 January 2018

Keywords,

Calli

Root regenerants

Shoots regenerants

Selection

Tissue culture

Variability

ABSTRACT

Soil salinity and drought are some of the major sugarcane production factors in Ethiopia. This investigation was conducted to develop, screen and select salt and drought tolerant genotypes under *in vitro* condition. Both direct and indirect pathways were employed in the development and selection of salt and drought tolerant lines from the commercial varieties. In all cultures, explants were inoculated to Murashige and Skoog (MS) medium supplemented with inhibitory levels of NaCl (0, 30, 100, 170 & 240 mM) and polyethylene glycol (0, 2.5, 5, 7.5 & 10%) concentrations. During callus induction, data for percent of explants induced callus and its morphological status were collected to evaluate varieties, and select healthy, salt and polyethylene glycol (PEG) tolerant calli. Moreover, shoot regeneration percentage, shoot Vigrouisity and color were taken as major criteria to select stress (salt and PEG) tolerant shoot while data for plantlet root attributes were collected to screen stress tolerant plantlets. Results of the study indicated that health and salt tolerant calli and shoots were identified at MS medium supplemented with 170 Mm while 10% PEG was the best selection medium to select healthy and PEG tolerant calli and shoots. Our screening results suggested that the *in vitro* developed regenerants were more discriminated at 240 mM

NaCl where S4SP-70-1284, S4 C0-501 and S2B-78-505 were selected as salt tolerant regenerants. Moreover, more variability was observed at MS medium induced with 10% polyethylene glycol where regenerants D4C-132-81, D5C-132-81, D3B52-298, D2B52-298 and D4B52-298 showed better degree of tolerance to drought. The dendrogram separated those regenerants developed through direct regeneration from those regenerants developed through indirect regeneration. It suggested the existence of somaclonal variation. Moreover, both dendrograms were capable of separating those regenerants exhibiting tolerance to the stresses in question thereby suggesting the existence of variability in relation to stress tolerance. We recommend these regenerants be used as a source of variability for future stress breeding studies. Moreover, these regenerants can be used as alternative varieties at salt affected and soil moisture limited fields of sugarcane production areas. However, it should be further evaluated for yield and yield quality before using for commercial purposes.

© 2018 Sjournals. All rights reserved.

1. Introduction

Abiotic stresses have undesirable effects on growth (Wang et al., 2001), production (Rengasamy, 2010) and physiology (Cha-um et al., 2012) of crops. Agronomically improved sugarcane varieties endowed with tolerance to biotic and abiotic stresses are highly beneficial, as unfavorable environmental factors challenging cultivation and crop productivity (Suprasanna et al., 2011). Globally, over one million hectares of the world sugarcane production is affected by salinity (Rozeff, 1995). Though sugarcane is classified as typical glycophyte, it exhibits stunted growth or no growth under saline soils, which results in up to 50% or even more of its true potential (Subbarao and Shaw, 1985). Besides, the development of drought tolerant varieties could be a plausible solution to sustain sugarcane productivity under such conditions. The drop in yield in such varieties grown under lower moisture regimes remains low, and they sustain productivity by yielding comparatively higher than under limited supply of water (Rajeswari et al., 2009).

Breeding of crops for stress tolerance would be the milestone to improve crops under such stresses. In many cases, however, these conventional breeding methods have failed to provide desirable results (Rai et al., 2011) especially in sugarcane owing to its inherent polyploidy and limited flowering rate and successful crosses. Such failures are related to many limitations such as complex genome, narrow genetic base, poor fertility, susceptibility to biotic and abiotic stresses and long duration to breed elite cultivars still impose a challenge (Jain, 2005; Vasantha et al., 2009; Ashok et al., 2011; Suprasanna et al., 2011). Moreover, multigenic nature of inheritance of the stresses, the lack of appropriate screening methodology, appropriate selection criteria for evaluation of germplasm and segregating materials further complicates the improvements to stress tolerance (Subbarao and Johansen, 1999).

Compared to field screening studies, plant tissue culture becomes an optimistic and best alternative tool to generate significant and useful genetic variability for stress tolerance in short period of time. Among the available biotechnological tools for crop breeding, *in vitro* selection through the application of selective pressure in culture conditions conducted for developing stress tolerant plants have been proved to be the most effective approaches (Sakhanokho and Kelley, 2009). Polyethylene glycol (PEG) has long been used in research programs to induce moisture stress in plants. PEG neither enters nor substantially degrades and is not absorbed by cells in culture and is used for selection environment under *in vitro* conditions. Studies conducted to see the effect of NaCl stress on sugarcane under *in vitro* conditions is inadequate. However, few attempts have been made for the selection of salt-tolerant cell lines in sugarcane (Gandonou et al., 2005). Ample variability has been observed in sugarcane for salt (Rajeswari et al., 2009; Shomeili et al., 2011; Mohammed et al., 2011; Munir and Aftab, 2013) and drought (Begum et al., 2011; Wagih et al., 2003; Hemarparabha et al., 2004; Cha-um et al., 2012) tolerances.

Soil salinity is the major sugarcane production constraints in Ethiopia, especially at Metahara Sugar Estate. A study made by Girma (1993) on salinity and sodicity status of the Metahara Sugar Estate showed the presence of salinity and sodicity problems in which the yield of certain fields is decreasing and a significant area of cultivated lands are abandoning, which could be attributed to the expansion of saline Lake Basaka (Megersa et al., 2009) and improper use of irrigation water (Tate, 2009). Moreover, the water level of the main streams that supply irrigation water for Metahara and Tendaho sugarcane plantations is declining from time to time due to climate changes and improper use of irrigation. The technical problems demand the use of tissue culture to develop and screen stress tolerant sugarcane regenerants under controlled condition. Despite the problems mentioned above, research works on development of sugarcane varieties tolerant to salt and drought stresses are very limited in Ethiopia. As a result, the development of sugarcane varieties tolerant to soil salinity and soil moisture stress received more research attention in recent years. However, the technical problems associated with the multigenic nature of the crop and field evaluation technical problems demand the use of tissue culture and *in vitro* selection to develop and screen sugarcane clones tolerant to different abiotic stresses. Therefore, the objectives of this study were to regenerate, screen and identify stress (salt and drought) tolerant regenerants under *in vitro* conditions and validate their tolerance under stressed field conditions.

2. Materials and methods

2.1. Explants selection and inoculation

Healthy young leaf sheath explants sampled from three commercial sugarcane test varieties grown at Wonji sugarcane research center and were used for indirect regeneration. Simultaneously, the explants sampled from meristem were also used for direct propagation. Two *in vitro* experiments (*In vitro* screening for salt and drought tolerant regenerants) were conducted separately and simultaneously at Mekelle Institute of Biotechnology Tissue Culture Laboratory in October-January, 2015. For the first *in vitro* experiment, explants were taken from sugarcane varieties B78-505 (tolerant), SP 70-1284 (moderate) and C90-501 (susceptible) while sugarcane varieties B52298 (susceptible), CO-449 (moderate) and C132-81 (tolerant) were source of explants for the second experiment. The commercial varieties were selected based on their local history related to their tolerance to soil salinity and moisture stresses. The leaf sheath explants were excised properly using knife and washed using drop of tween 20 and 10% NaOCl (barakina) and drop of soap solution for 10 minutes. Then the explants were immersed in to 0.25% of redimol and bayeloton /100 ml of water for 30 minutes was used to remove the fungal and bacterial contaminants. Then the explants were excised properly and some sheathes of the explants were pilled and immersed in to 20% of Na₂OCl for 5 minutes. After rinsed by using of DH₂O (deionized water) for 3 times, the explants were immersed in to 0.25% of HgCl₂ to kill microbes that were present in the explants.

2.2. Media preparation and culture methods

Murashige and Skoog (1962) or MS medium was used and four alternative media preparations MS+30g/l sugar+4g/l agar+1.5mg/l 2, 4-D, MS+30g/l sugar+4g/l agar+3mg/l 2, 4-D, MS+30g/l sugar+3mg/l 2, 4-D (without agar) and MS+30g/l sugar+4g/l agar+3mg/l BAP+3mg/l IBA were used for callus establishment. Out of the different media combinations used, the media MS+30g/l sugar+4g/l agar+3mg/l 2, 4-D was most convenient media combination as it relatively produced good callus induction both in quality and quantity. Thus, in the first culture, MS medium was supplemented with 30g/l sugar+4g/l agar+3mg/l 2 4-D was used for callus induction. In the second culture, callus was sub-cultured on fresh MS+30g/l sugar+4g/l agar+1mg/l BAP+0.5mg/l IBA for shoot regeneration and variants per treatment were selected and labeled. In the third culture, healthy initiated plantlets (variants) were separated and were sub-cultured again on fresh medium with ½MS+30g/l sugar+4g/l agar+5 mg/l NAA for root development. For comparison purposes, direct propagation was also performed simultaneously using meristem as explants type and with the same MS media composition but without 2, 4-D. In all cultures, MS medium was supplemented with different NaCl (salt screening *in vitro* experiment) and PEG (drought screening *in vitro* experiment) concentrations.

2.3. Experimental design and treatments application

The two *in vitro* experiments were arranged in Completely Randomized Design and replicated three times. The first *in vitro* experiment was conducted for screening of salt tolerant genotypes and variety (B78-505, SP70-

1284 and C90-501) and salt stress (NaCl treatments i.e. 0, 30, 100, 170 and 240 mM) were the two factors. The second *in vitro* experiment was conducted for screening of drought tolerant genotypes where variety (B52298 CO-449 and C132-81) and drought (PEG treatments 0, 2.5, 5, 7.5 and 10%) were factors. S1, S2, S3, S4 and S5 are stress level abbreviates for 0 mM, 30 mM, 100 mM, 170 mM, and 240 mM of NaCl concentrations, respectively while D1, D2, D3, D4 and D5 are stress level abbreviates for 0, 2.5, 5, 7.5 and 10% of PEG, respectively. At the end of shoot regeneration, healthy shoots from each treatment and experiment were selected, multiplied and labeled. Labeling of shoots/regenerants was given using a combination of stress level abbreviates (the stress level at which salt tolerant calli were selected) and full name of the variety from which the explants were taken (keeping the varietal pedigree). While screening regenerants under both *in vitro* experiments, the factor 'variety' was replaced by regenerant. During the field evaluation, RCBD design with two replications was used and each regenerant (genotype) was planted at plot size of 16m² (4m x 4m). The *in vitro* developed, and salt and drought tolerant regenerants were evaluated at salt affected and soil moisture limited field conditions along with the locally grown varieties with planting ratio of one plantlet to one double budded seed set.

2.4. Data collection

Forty days after inoculation, the calli were cut in to small pieces and re-inoculated on callus induction medium and data for percent of explants inducing callus (PEIC) was recorded, and calli that are yellow in color and showed vigorous growth were selected as salt and drought tolerant calli. After two consecutive subcultures, those salt and drought tolerant calli (Sixteen pieces of callus per treatment) were transferred in to shoot regeneration medium and data for Percent of calli regenerating shoots (PCRS) was collected for each treatment. After shoot multiplication was completed, 40 healthy, salt and drought shoots were selected for each labeled regenerant and transferred to rooting medium. Four weeks after inoculation, data for root attributes i.e. data for Percent of shoots regenerating roots (PSRR), Number of roots per plantlet (NRPP), Average root length per plantlet (ARLPS), number of shoots per plantlet (NSPP) and Plantlet length (PL) regenerants were recorded. Stress tolerance index (STI) was calculated as suggested by Goudarzi and Pakniyat (2008). The stress tolerance index was calculated as the ratio of the yield under stressed environment and the yield of the non stressed environment. STI was calculated as:

$$STI = \frac{Y_c}{Y_c'}$$

Where Y_s is yield under stressed and Y_c is yield under control or normal environment. The highest STI implies the most tolerance for the stress it exposed. Regenerated plants bearing well developed roots were transferred to greenhouse for acclimatization were grown in beds in greenhouse and survival % of the *in vitro* developed plantlets was recorded. After 20 days of acclimatization, the plantlets were transferred to salt affected and soil moisture limited fields. Data for tillering capacity (tiller number), millable stalk population and height were collected at 4, 13 and 13 months after planting, respectively.

2.5. Data analysis

Data was subjected to analysis of variance and interaction means were separated through Tukey's multiple mean comparison test and means of main effects were separated using LSD using SAS software package (SAS, 2009). For data that violated the assumption of ANOVA, transformed was carried out prior to statistical analysis as suggested by Steel and Torrie (Steel and Torrie, 1981) and the transformed data was subjected to analysis of variance and means separations. The calculated Jaccard similarity coefficients were used to construct Dendrogram based on average linkage method using SAS software package.

3. Results and discussion

3.1. Effects of salt (NaCl) on callus inducing and shoot regenerating potential of sugarcane varieties evaluated under *in vitro* condition

The analysis of variance for percent of explants inducing callus (PEIC) and percent of calli cultures regenerated shoots (PCRS) is provided in Table 1. Results revealed that variety and salt effects were highly significant (P≤0.01) for both PEIC and PCRS. The interaction between variety and salt effects was nonsignificant (P>0.05) for PEIC while highly significant (P≤0.05) interaction was observed between varietal and salt effects for SRP. For this reason, only the main effects (salt, PEG and variety) were reported for PEIC and are separated using

LSD (Table 2). Results indicated that the higher % of explants (75.17%) induced calli were from variety SP 70-1284 (Table 2) while the % of explants induced callus in variety C90-501 was lower (54%). Regarding to the effect of salt on PEIC, 74.44%, 71.83, 64.77%, 53% and 45% of explants induced calli at 0, 30, 100, 170 and 240 mM NaCl selection mediums, respectively.

In the absence of salt (0 mM NaCl), 78.67% of callus cultures from variety B78-505 regenerated healthy shoots which is significantly higher than shoots regenerated from callus cultures of other varieties while lower % (55%) of callus cultures from variety SP70-1284 regenerated shoots (Table not presented). At 100 mM NaCl selection medium, the higher % of callus cultures (60.34%) regenerated shoots were from variety C90-501 where lower % of callus cultures (45.30%) from variety B78-505 regenerated shoots. Moreover, 56, 39 and 53.50 % of callus cultures from varieties B78-505, C90-501 and SP70-1284 respectively regenerated healthy and vigorous shoots at 170 mM NaCl selection medium where salt tolerant shoot regenerants were identified. However, 38.72, 14.34 and 29.6% of callus cultures that regenerated shoots at 240 mM NaCl were from varieties B78-505, Co-501 and SP70-1284 respectively. Moreover, morphological and color changes were observed among the calli of all varieties treated with 170 mM NaCl (Fig. 1 a, b, c) and regenerate healthy shoots at 170mM salt concentration (Fig. 1 d, e, f). However, the growth of calli was sharply reduced at 240 mM NaCl (Figure not presented) (SP70-1284).

Results of the present study indicated varieties varied in inducing callus as salt concentration in the MS medium was increasing (Table 1). It suggested the callus induction was genotype dependent. Several workers (Gandonou et al., 2005; Shomeili et al., 2011; Avinash et al., 2012; Gadakh et al., 2015; Begum and Islam, 2015) reported similar results in which callus induction was genotype dependent. Like to callus induction, varieties were significantly variable in regenerating shoots. Moreover, when salt concentration in the MS medium was increased, the shoot regeneration potential of varieties was significantly affected inconsistently. Generally, both callus induction and shoot regeneration were variety dependent. Like to the above result, varieties evaluated for drought tolerance varied inducing callus (Table 1).

Table 1

Mean squares for sugarcane varieties evaluated under MS medium supplemented with different levels of PEG and NaCl concentrations.

Variety evaluation for drought tolerance					
Sources of variation	DF	PEIC (%)	Sources of variation	DF	PCRS (%)
Variety	2	27420**	Variety	1	76.8 ^{ns}
PEG	4	361.32**	PEG	4	462.28**
Variety*PEG	8	83.95 ^{ns}	Variety*PEG	8	48.05 ^{ns}
Error	28	77.32	Error	18	92.63
CV (%)		16	CV (%)		12
Variety evaluation for salt tolerance					
Sources of variation	DF	PEIC (%)	SRP (%)		
Variety	2	3409.3**	2686.9**		
Salt	4	1370.3**	1808**		
Var*salt	8	147.58 ^{ns}	127.27**		
Error	28	104.32	57.22		
CV (%)		16.5	16		
Mean		61.93	47.28		

**significant at 1%; *significant at 5%; ns=nonsignificant; PEIC=Percent of explants inducing callus; PCRS=Percent of calli cultures regenerated shoots.

3.2. *In vitro* selection of salt tolerant calli and shoots evaluated under different concentrations of NaCl

We have regenerated shoots from calli cultures. Our results were similar to results of Yasuda et al. (1982) and Tanvir et al. (2001) who worked on the development of salt tolerant plants from the salt-stressed callus cultures. When comparing the performances of varieties, explants from all varieties induced healthy calli at MS medium supplemented with 0-100 mM NaCl (Table 2). However, PEIC of varieties was drastically dropped when the salt concentration of the MS medium was shifted from 100 to 170 mM. At 170 mM NaCl selection medium, salt tolerant and healthy calli were identified and were selected for further evaluation. Moreover, at this level of salt concentration, the selected calli from varieties B78-505 and SP70-1284 were healthy and vigorous (Fig. 1 a, b)

while the calli cultures from variety C90-501 were somewhat compact (Fig. 1 c). Result of the present study was in close agreement with reports of Patade and Suprasanna (2009) where salt tolerant calli were isolated at MS medium supplemented with 171.5 mM NaCl. Other workers (Kenganal et al., 2008; Patade and Suprasanna, 2009; Shomeili et al., 2011; Gadakh et al., 2015) reported the possibility of identifying healthy calli at similar salt concentrations. Moreover, Ashraf et al. (2007) and Karpe et al. (2012) identified healthy and salt tolerant calli on MS medium supplemented with 100 mM NaCl. These different results indicated the ease of callus induction depends on genotype we worked with. When the concentration of NaCl was increased to 240 mM NaCl, morphological and color changes were observed among the calli of all varieties treated with and growth of calli sharply was reduced (Figure not presented). As a result, we found a difficulty of identifying salt tolerant and healthy calli under this stress level. Similar results were reported by Patade and Suprasanna (2009) in which all calli exhibited morphological and color changes at 256.7 mM NaCl. Similar to PEIC, callus cultures of the varieties studied showed considerable variation at the 170 mM and salt tolerant shoots were identified.

The healthy calli grown at under stressed MS medium were separated and identified as salt tolerant calli. Morphological status and color changes were used as indicators for selecting salt tolerant calli. The healthy calli selected from all varieties evaluated at MS medium treated with 170 mM NaCl are depicted (Fig. 1 a, b, c). However, the growth of calli was sharply reduced at 240 mM NaCl (Figure not presented). Results of the present study indicated the varieties evaluated varied in inducing callus as salt concentration in the MS medium was increased (Table 1). It suggested the callus induction was genotype dependent. Several workers (Gandonou et al., 2005; Shomeili et al., 2011; Avinash et al., 2012; Gadakh et al., 2015; Begum and Islam, 2015) reported similar results in which callus induction was genotype dependent. Like to callus induction, varieties were significantly variable in regenerating shoots. Moreover, when salt concentration in the MS medium was increased, the shoot regeneration potential of varieties was significantly affected inconsistently. Generally, both callus induction and shoot regeneration were variety dependent. The selected salt tolerant calli were inoculated in to shoot regeneration medium after weeks. Those healthy shoots with deep green were selected as salt tolerant shoots. More variability was observed at MS medium supplemented with 170 mM salt concentration, and salt tolerant and healthy shoots were selected from this selection medium (Fig. 1 d, e, f).

Table 2

Means of main effects (variety, PEG and NaCl) for percent of explants induced calli and percent of calli cultures regenerated shoots.

Variety evaluation for drought tolerance			Variety evaluation for salinity tolerance	
Variety	PEIC (%)	PCRS (%)	Variety	PEIC (%)
B52298	81.13 ^a		B78-505	67.82 ^a
CO-449	5.59 ^b		C90-501	44.80 ^b
C132-81	78.06 ^a		SP70-1284	75.17 ^a
LSD _{0.05}	6.57		LSD _{0.05}	7.64
PEG (%)			NaCl (mM)	
0	61.76 ^a	88.83 ^a	0	74.444 ^a
2.5	59.51 ^{ab}	84 ^{ab}	30	71.833 ^a
5	53 ^{ab}	80.34 ^{ab}	100	64.778 ^a
7.5	52.5 ^{ab}	53.80 ^c	170	53 ^b
10	45.61 ^c	26.67 ^d	240	45 ^b
LSD _{0.05}	8.49	11.67	LSD _{0.05}	9.86

*Means with same letters are not statistically significant based on least significant difference tests at $\alpha=0.05$; PEIC=Percent of explants induced callus and PCRS=Percent of calli regenerated shoots.

3.3. Effects of drought (PEG) on callus inducing and shoot regenerating potential of sugarcane varieties evaluated under *in vitro* condition

Both variety and PEG effects were highly significant ($P \leq 0.01$) for PEIC while the variety effect was not significant for PCRS (Table 1). The interaction between variety and PEG effects was nonsignificant for both traits. Mean separation presented in Table 2 suggested all varieties induce calli at 0% of PEG (control) and very few with poor quality callus (5.59%) were produced by variety CO-449. On the contrary, lower number of explants from this variety induce callus at PEG levels of 10% (Table not presented). Like to the above varieties evaluated for salt tolerance, varieties evaluated for drought tolerance varied in inducing callus (Table 1). At control (0% PEG), 61.76%

of explants produced callus, but this value decreased to 45.61% (which is a sharp drop) when the PEG level of the MS medium was increased to 10% (Table 2). The significant difference among the PEG levels suggested the increasing level of PEG brought differential physiological change on callus inducing and shoot regeneration potential of the varieties (Table 1). Mean separations for main effects (variety and PEG) revealed that 43.80% of the callus cultures shifted to MS medium with PEG levels of 7.5% regenerate shoots, indicating the effect of PEG on shoot regeneration potential of the callus cultures was high and significant. Sugarcane varieties (B52298 and C132-81) evaluated for drought tolerance showed better performance in inducing callus while little and unhealthy calli were obtained from variety Co-449 indicating callus inducing potential was variety dependent.

3.4. *In vitro* selection of drought tolerant calli and shoots evaluated under different levels of PEG (0-10%)

During callus induction, the explants inoculated at different level of PEG (0-10%) showed more variation in inducing calli at MS medium supplemented with 10% of PEG. For this reason, explants of different varieties were screened under this selection medium to increase the chance of identifying drought tolerant regenerants. Fortunately, healthy and tolerant calli were identified and selected from MS medium induced with 10% PEG level (Fig. 2 a, b, c, d, e, f). The selected healthy and drought tolerant calli were transferred in to shoot regeneration medium supplemented with different of PEG. Healthy and drought tolerant shoots were identified at MS medium induced with 10% PEG level and were selected as tolerant shoots regenerants. In both culture methods, both varieties induced quality calli (Fig. 2 a, b) and regenerate healthy shoots at PEG level of 10% (Fig. 2 f, g, h). The present results agreed with reports of many workers (Wagih et al., 2003; Gandonou et al., 2005; Ather et al., 2009; Raza et al., 2010; Begum et al., 2011; Kenganal et al., 2008; Patade and Suprasanna, 2006; Shomeili et al., 2011; Gadakh et al., 2015). Our result departed from reports of Srinath and Jabeen (2013) who found considerable healthy and tolerant calli at MS medium supplemented with 20%. Unlike to the callus induction, varieties did not significantly vary in regenerating shoots (SRP) which is similar to reports of Khan et al. (2009).

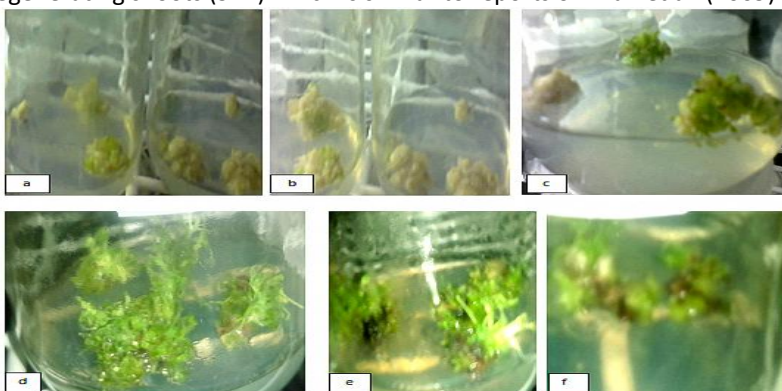


Fig. 1. Calli induced from variety (a) B78-505, (b) C90-501 and (c) SP70-1284 selected on MS medium with 170 mM; shoots regenerated from variety (d) B78-505 (e) C90-501 (f) SP 70-1284 at 170 m.

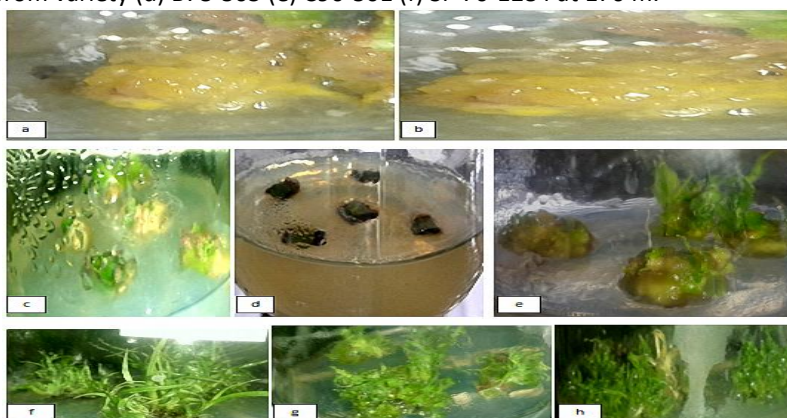


Fig. 2. Calli induced from variety (a) C132-81 at 7.5% PEG (b) B52298 at 10% PEG (c) C132-81 at 10% PEG (d) Co-449 at 7.5-10% PEG (e) B52298 at 10% PEG level; shoots regenerants of (f) C132-81 selected at 7.5% (g) B52298 selected at 10% (h) C132-81 selected at 10% PEG levels.

3.5. *In vitro* plantlet regeneration and screening for salt tolerance in sugarcane

The analysis of variance for root attributes of regenerants is presented (Table 3). Both regenerant and salt effects were highly significant ($P \leq 0.01$) for all traits studied. Similarly, the interaction between regenerant and salt effects was highly significant ($P \leq 0.01$) for all traits (except for number of shoots per plantlet). The highly significant difference among regenerants is an indication of remarkable variability among regenerants. The highly significant effect among the different salt concentrations suggested the increasing levels of NaCl in MS medium brought remarkable effect on the performance of the regenerants screened. Moreover, the highly significance of the interaction between salt and regenerant effects for percent of shoots regenerated roots (PSRR), whole plantlet length, root length per plantlet and root number per plantlet indicated regenerants showed inconsistent performance when the stress level of the MS medium was changed.

Means were separated using Tukey's test and compared at all levels of salt concentrations (Table 4). Moreover, the stress tolerance index (STI) was calculated for the same traits based on the performance of the regenerants screened at stressed (240 mM NaCl) and non-stressed (salt free) MS mediums. The screening results revealed that shoots from regenerants S2B-78-505, S1B-78-505 and S3SP-70-1284 showed better shoot regeneration potential at salt free MS medium. At stressed environment (240 mM NaCl), 52% of the shoots from S4 C90-501 regenerated roots better than others. In addition to this, regenerants S5SP-70-1284, S4 C90-501, S4SP-70-1284 and S3C90-501 had STI value of 0.89, 0.87, 0.84 and 0.82, respectively. The largest STI value (0.89) of S5SP-70-1284 suggested the rooting potential of this regenerant was least affected by the higher salt concentration with 11% reduction compared to its performance under non-stress selection medium. On the contrary, S1B-78-505, S3SP-70-1284 and S2SP-70-1284 showed lowest STI value of 0.45, 0.48 and 0.49, respectively and were sensitive to salt stress. S4SP-70-1284 showed significantly better performance in plantlet length than others under both non-stressed and stressed selection mediums. Relative to the others, regenerants S4SP-70-1284, S4 C90-501 and S3SP-70-1284 showed largest STI value of 0.91, 0.85 and 0.84, respectively. The STI value of 0.91 S4SP-70-1284 implied the plantlet length was reduced by 9% when the level of NaCl in the MS medium was increased from 0 mM to 240 mM. It showed better length tolerating the highest level of salt stress as the reduction which can be referred as yield penalty is lower and can be compromised. On the contrary, regenerants S5 C90-501, S1B-78-505, MSP-701284 and MC90-501 had lowest STI value of 0.46, 0.52, 0.52 and 0.53, respectively and their growth was highly affected.

The mean separation for average root length per plantlet (ARLPP) suggested that regenerants S1B-78-505, S1SP-70-1284 and S2B-78-505 were significantly ($P < 0.05$) better than others in root growth in the salt free environment while S4SP-70-1284 showed better root growth in the saline MS medium (240 mM NaCl). The STI calculated for RLPP indicated regenerants S4SP-70-1284, S4 C90-501, S2SP-70-1284 and S3B-78-505 scored 0.98, 0.88, 0.84 and 0.82, respectively thereby suggesting the effect of salt concentration on root length was not drastic because of their tolerance to salt stress. For the same trait, 0.35, 0.39 and 0.49 STI values recorded for S1SP-70-1284, S5SP-70-1284, S3C90-501 and MSP-701284, respectively. Root length of plantlets (regenerants) was highly affected by the increased salt concentration. Regenerants S2SP-70-1284 and S4 C90-501 produced higher NRPP at the stress free environment while S4 C90-501 produced relatively higher number of roots in the saline environment. Moreover, regenerants S4B-78-505, S4SP-70-1284, S4 C90-501, S5SP-70-1284, MSP-701284, MC90-501 and MB-78-505 had largest STI value of 1.37, 1.2, 0.91, 0.90, 0.89, 0.85 and 0.82, respectively indicating their tolerance to salt stress while producing roots at the MS medium supplemented with higher NaCl. The lowest value of STI (0.40) was recorded by S2SP-70-1284 and the number of roots produced from this regenerant was highly reduced when the salt concentration of the MS medium was increased to 240 mM.

Generally, the above screening results indicated that regenerants were more discriminated at the MS medium supplemented with 240 mM NaCl concentration where above 50% reductions due salt stress were recorded and salt tolerant regenerants were identified (Table 4). We have observed that plantlets regenerated from callus responded differently to salt effects regardless of the background of the respective source varieties, which is an indication of the existence of somaclonal variation. Our result is in favor of the findings reported by Munir and Aftab (2013) and Begum and Islam (2015).

3.6. *In vitro* plantlet regeneration and screening for drought tolerance in sugarcane

The regenerant, PEG and the interaction effects were highly significant ($P \leq 0.01$) for all traits studied except for the latter effect was non-significant for plantlet length (Table 3). As the interaction effect between regenerant

and PEG levels was highly significant for PSRR, RL, NRPP and NSPP, multiple comparisons were made to identify drought tolerant regenerants (Table 5). In the control treatment (0% PEG), regenerants were not significantly different ($P>0.05$) in regenerating roots. While the PEG level in the MS medium was increased to 10%, their performance was decreased.

Table 3

Mean squares for sugarcane regenerants evaluated MS medium supplemented with different levels of NaCl and PEG concentrations.

<i>In vitro</i> plantlet regeneration and screening for salt tolerance						
Sources of variation	DF	PSRR (%)	NRPP	ARLPP (cm)	PL (cm)	NSPP
Salt level	4	0.008 ^{ns}	0.12 ^{**}	0.26 [*]	40.88 [*]	0.55 ^{**}
Regenerant	17	0.09 ^{**}	0.03 ^{**}	0.08 ^{**}	0.09 ^{**}	0.33 ^{**}
Regenerant*salt	68	0.05 ^{**}	0.033 [*]	0.04 ^{**}	0.15 ^{**}	0.03 ^{ns}
Error	178	0.012	0.013	0.010	0.0029	0.011
<i>In vitro</i> plantlet regeneration and screening for drought tolerance						
Regenerants	11	0.098 ^{**}	0.38 ^{**}	0.08 ^{**}	0.11 [*]	0.14 ^{**}
PEG level	4	0.022 ^{**}	1.93 ^{**}	0.28 ^{**}	16.07 ^{**}	0.62 ^{**}
Regenerants*PEG level	44	0.058 [*]	0.29 ^{**}	0.04 ^{**}	0.03 ^{ns}	0.07 ^{**}
Error	114	0.0102	0.008	0.002	0.021	0.009
CV (%)		6.19	10	7.2	13.5	10.5

**=Highly significant at 1%, *=Significant at 5%; PSRR=Percent of shoots regenerated roots; NRPP=Root number per plantlet; ARLPP=Root length per plantlet; NSPP=Number of shoots per plantlet and PL=Plantlet length.

Table 4

Interactions means of indirect (1-15) and direct (16-18) regenerated plantlets screened at MS medium supplemented with different levels of NaCl (0-240 mM).

Code	Regenerant	PSRR (%)					STI	PL (cm)					STI
		0 mM	30 mM	100 mM	170 mM	240 mM		0 mM	30 mM	100 mM	170 mM	240 mM	
1	S1SP-70-1284	64.87 ^{af}	43.9 ^{af}	65.03 ^{af}	27.97 ^f	37.5 ^{bf}	0.58	15.71 ^{ab}	13.31 ^{bh}	14.91 ^{ba}	8.84 ^{ah}	9.63 ^{ah}	0.61
2	S2SP-70-1284	54.77 ^{af}	58.1 ^{af}	58.4 ^{af}	38.07 ^{bf}	26.9 ^f	0.49	14.75 ^{af}	12.64 ^{bh}	11.47 ^{ah}	10.7 ^{ah}	10.7 ^{ah}	0.74
3	S3SP-70-1284	74.43 ^{abc}	42.7 ^{af}	57.03 ^{af}	28.9 ^{af}	35.73 ^{cdaf}	0.48	11.44 ^{ah}	13.25 ^{bh}	13.63 ^{ae}	11.5 ^{ah}	9.6 ^{ah}	0.84
4	S4SP-70-1284	53.03 ^{af}	45.8 ^{af}	44.7 ^{af}	50 ^{af}	44.73 ^{af}	0.84	15.8 ^a	14.74 ^{af}	8.237 ^{ah}	14.8 ^{af}	14.3 ^{af}	0.91
5	S5SP-70-1284	48 ^{af}	60.83 ^{af}	40.43 ^{af}	31.37 ^{af}	42.53 ^{af}	0.89	14.44 ^{af}	15.14 ^{af}	12.3 ^{ah}	7.49 ^{af}	9.55 ^{ah}	0.66
6	S1B-78-505	75.43 ^{ab}	60.17 ^{af}	72.6 ^{abc}	55.73 ^{af}	34.07 ^{bf}	0.45	11.51 ^{ah}	14.16 ^{af}	7.84 ^{ch}	8.87 ^{ah}	5.97 ^{ah}	0.52
7	S2B-78-505	79.77 ^a	79.13 ^a	50.8 ^{af}	40.67 ^{bf}	54.37 ^{af}	0.68	15.38 ^{abc}	13.54 ^{ae}	15.34 ^{abcd}	12.1 ^{ah}	10.8 ^{ah}	0.70
8	S3B-78-505	55.63 ^{af}	67.97 ^{ba}	57.5 ^{af}	41.8 ^{af}	43.4 ^{def}	0.78	13.77 ^{af}	11.68 ^{ae}	14.63 ^{af}	9.58 ^{ah}	9.07 ^{ah}	0.66
9	S4B-78-505	51.43 ^{af}	37.67 ^{ba}	55.27 ^{af}	40.93 ^{af}	32.5 ^{def}	0.63	10.56 ^{ah}	11.41 ^{ah}	10.35 ^{ah}	8.57 ^{ah}	7.72 ^{ch}	0.74
10	S5B-78-505	60.47 ^{af}	62.1 ^{af}	44.73 ^{af}	46.43 ^{af}	38.3 ^{bf}	0.63	10.84 ^{ah}	13.87 ^{af}	13.61 ^{ae}	11.7 ^{ah}	7.91 ^{ch}	0.73
11	S1C90-501	49.47 ^{af}	37.33 ^{ba}	46.77 ^{af}	40.9 ^{af}	37.5 ^{bf}	0.76	11.83 ^{ah}	12.85 ^{ah}	13.66 ^{ae}	10.2 ^{ah}	7.8 ^{ch}	0.66
12	S2C90-501	67.77 ^{af}	56.13 ^{af}	38.93 ^{af}	39 ^{af}	36.57 ^{bf}	0.54	13.13 ^{ah}	14.26 ^{af}	8.49 ^{ah}	9.53 ^{ah}	7.61 ^{ch}	0.58
13	S3C90-501	47.57 ^{af}	58.1 ^{af}	64.53 ^{af}	46.57 ^{af}	39.2 ^{bf}	0.82	12.28 ^{ah}	13.48 ^{ae}	8.673 ^{ah}	11 ^{ah}	7.61 ^{ch}	0.62
14	S4C90-501	59.93 ^{af}	34.9 ^{cdaf}	48.87 ^{af}	54.53 ^{af}	52 ^{af}	0.87	14.84 ^{abc}	12.78 ^{bh}	13.107 ^{ah}	13.7 ^{af}	12.4 ^{ah}	0.85
15	S5C90-501	52.23 ^{af}	35.23 ^{cdaf}	35 ^{cdaf}	46.1 ^{af}	33.27 ^{daf}	0.64	12.37 ^{ah}	7.447 ^{af}	7.97 ^{ah}	7.07 ^{ah}	5.67 ^h	0.46
16	MB-78-505	46.8 ^{af}	47.8 ^{af}	35 ^{cdaf}	35 ^{cdaf}	35 ^{cdaf}	0.75	10.38 ^{ah}	10.63 ^{ah}	12.5 ^{ah}	7.7 ^{ah}	6.33 ^{ah}	0.61
17	MSP-701284	56.17 ^{af}	56.67 ^{af}	36 ^{bf}	36 ^{bf}	36 ^{bf}	0.64	10.9 ^{ah}	12.5 ^{ah}	12.9 ^{ah}	5 ^h	5.64 ^h	0.52
18	MC90-501	47.83 ^{af}	48 ^{af}	32 ^{af}	42 ^{af}	32 ^{af}	0.67	10.2 ^{ah}	11.53 ^{ah}	13.47 ^{ae}	7.93 ^{ch}	5.43 ^h	0.53
Code	Regenerant	ARLPP (cm)					STI	RNPP					STI
		0 mM	30 mM	100 mM	170 mM	240 mM		0 mM	30 mM	100 mM	170 mM	240 mM	
1	S1SP-70-1284	7.5 ^a	5.07 ^{ae}	5.83 ^{ae}	4.13 ^{ae}	2.6 ^{fe}	0.35	6.94 ^{abcd}	5.45 ^{ae}	6.36 ^{ae}	5.04 ^{ae}	4.02 ^{ae}	0.58
2	S2SP-70-1284	5.6 ^{ae}	4.57 ^{ae}	4.57 ^{ae}	5.43 ^{ae}	4.7 ^{ae}	0.84	7.82 ^a	5.53 ^{ae}	5.61 ^{ae}	5.37 ^{ae}	3.13 ^a	0.40
3	S3SP-70-1284	5.33 ^{ae}	5.57 ^{ae}	3.8 ^{ae}	4.77 ^{ae}	3.5 ^{ae}	0.66	6.18 ^{ae}	5.59 ^{ae}	5.7 ^{ae}	5.28 ^{ae}	4.42 ^{ae}	0.72
4	S4SP-70-1284	7.27 ^{abcd}	4.57 ^{ae}	4.9 ^{ae}	6.44 ^{ae}	7.1 ^{abc}	0.98	5.24 ^{ae}	5.63 ^{ae}	6.0 ^{ae}	4.34 ^{ae}	6.3 ^{abc}	1.20
5	S5SP-70-1284	6.37 ^{af}	3.07 ^{de}	2.47 ^{ae}	2.43 ^{ae}	2.51 ^{ae}	0.39	7.70 ^{ab}	6.51 ^{ae}	4.82 ^{ae}	4.81 ^{ae}	6.82 ^{abc}	0.90
6	S1B-78-505	7.67 ^a	5.77 ^{ae}	4.42 ^{ae}	4 ^{ae}	3.9 ^{ae}	0.51	7.12 ^{abcd}	5.73 ^{ae}	5.82 ^{ae}	4.11 ^{ae}	3.82 ^{de}	0.54
7	S2B-78-505	7 ^{abc}	5.8 ^{ae}	5.6 ^{ae}	4.57 ^{ae}	5 ^{ae}	0.71	7.23 ^{abcd}	5.86 ^{ae}	6.51 ^{ae}	4.52 ^{ae}	3.92 ^{ae}	0.54
8	S3B-78-505	6.17 ^{ae}	5.67 ^{ae}	2.87 ^{de}	7.05 ^{ab}	5.03 ^{ae}	0.82	7.56 ^{abc}	5.83 ^{ae}	5.76 ^{ae}	4.69 ^{ae}	4.22 ^{ae}	0.56
9	S4B-78-505	6.67 ^{abcd}	4.71 ^{ae}	5.13 ^{ae}	5.67 ^{ae}	3.53 ^{ae}	0.53	5.09 ^{ae}	5.38 ^{ae}	4.45 ^{ae}	3.75 ^{ae}	6.97 ^{abcd}	1.37
10	S5B-78-505	4.3 ^{ae}	4.07 ^{ae}	5.53 ^{ae}	3.07 ^{de}	2.67 ^{fe}	0.62	5.96 ^{ae}	4.89 ^{ae}	3.6 ^{de}	3.94 ^{ae}	4.08 ^{ae}	0.68
11	S1C90-501	5.1 ^{ae}	5 ^{ae}	3.4 ^{ae}	5.92 ^{ae}	2.6 ^{fe}	0.51	5.88 ^{ae}	6.19 ^{ae}	6.08 ^{ae}	5.31 ^{ae}	4.67 ^{ae}	0.79
12	S2C90-501	4.7 ^{ae}	5.27 ^{ae}	3.37 ^{ae}	5.77 ^{ae}	2.57 ^{fe}	0.55	5.40 ^{ae}	6.28 ^{ae}	4.75 ^{ae}	5.73 ^{ae}	4.12 ^{ae}	0.76
13	S3C90-501	5.57 ^{ae}	5.07 ^{ae}	3.83 ^{ae}	6.57 ^{bd}	2.5 ^{fe}	0.45	6.44 ^{ae}	4.75 ^{ae}	4.23 ^{ae}	4.36 ^{ae}	3.86 ^{ae}	0.60
14	S4C90-501	6.72 ^{ae}	5.57 ^{ae}	2.79 ^{fe}	6.7 ^{abcd}	5.89 ^{ae}	0.88	7.73 ^a	4.29 ^{ae}	4.03 ^{ae}	4.06 ^{bcde}	7.07 ^{abcd}	0.91
15	S5C90-501	4.33 ^{ae}	3.07 ^{de}	2.87 ^{de}	2.5 ^{fe}	2.72 ^{fe}	0.63	5.92 ^{ae}	5.41 ^{ae}	5.19 ^{ae}	3.77 ^{bcde}	3.84 ^{de}	0.65
16	MB-78-505	4.47 ^{ae}	3.92 ^{ae}	2.87 ^{ae}	4.02 ^{ae}	2.6 ^{fe}	0.58	3.57 ^{bcde}	3.64 ^{ae}	4.27 ^{ae}	2.82 ^f	2.92 ^f	0.82
17	MSP-701284	5.37 ^{ae}	3.43 ^{ae}	4.28 ^{de}	4.86 ^{ae}	2.61 ^{fe}	0.49	3.04 ^a	3.07 ^a	2.76 ^f	3.67 ^{ae}	2.7 ^f	0.89
18	MC90-501	3.9 ^{ae}	2.96 ^{de}	2.47	3.26 ^{de}	2.17 ^{fe}	0.56	2.63 ^f	1.3 ^f	3.16 ^g	2.73 ^f	2.24 ^f	0.85

*Means with different letters across columns of a trait represent significant difference based on Tukey's multiple comparison tests at $\alpha=0.05$; M=Mother plant; STI (%)=Stress tolerant index; PSRR=Percent of shoots regenerated roots; PL=Plantlet length; ANRPP=Average number of roots per plantlet; RLPP=Root length per plantlet.

Table 5
Interactions means of indirect (1-10) and direct (11 & 12) regenerated plantlets screened at MS medium supplemented with different levels of PEG (0-10%).

Regenerant	Code	ARLPP (cm)					STI	PSRR (%)					STI
		0	2.5	5	7.5	10		0	2.5	5	7.5	10	
D1B52298	1	7.45 ^a	4.9 ^{def}	4.9 ^{def}	3.8 ^{kl}	2.6 ^{uvv}	0.35	65.16 ^{abc}	35.5 ^{bcd}	70.16 ^{abc}	30.6 ^{cde}	24.9 ^{gh}	0.38
D2 B52298	2	4.5 ^{ehi}	4.7 ^{efg}	4.5 ^{ehi}	6.4 ^{abc}	2.84 ^{stu}	0.63	87 ^a	27.23 ^{efg}	68.03 ^{abc}	60.96 ^{abc}	20.03 ^{gh}	0.23
D3 B52298	3	7.11 ^{ab}	4.9 ^{def}	4.19 ^{hij}	4.13 ^{jk}	3.5 ^{mno}	0.49	74.7 ^{abc}	39.73 ^{bc}	45.67 ^{abc}	55.9 ^{abc}	47 ^{abc}	0.63
D4 B52298	4	4.6 ^{fgh}	3.5 ^{lmn}	6.29 ^{abc}	3.2 ^{opq}	3.7 ^{klm}	0.80	39.93 ^{bcd}	35.06 ^{bcd}	27.8 ^{efg}	50.70 ^{abc}	31 ^{cde}	0.78
D5 B52298	5	4.1 ^{feh}	3.1 ^{lmn}	6.3 ^{abc}	4.5 ^{ehi}	2.4 ^{uvv}	0.59	41.1 ^{bcd}	35 ^{bcd}	26.1 ^{efg}	48.51 ^{abc}	33 ^{bcd}	0.80
D1C-132-81	6	5.84 ^{cde}	5.92 ^{cd}	5.39 ^{cde}	4.4 ^{ghi}	3.6 ^{mno}	0.62	79.70 ^{abc}	45.8 ^{abc}	44.36 ^{abc}	37.1 ^{bcd}	29 ^{efg}	0.36
D2 C-132-81	7	5.39 ^{cde}	5.7 ^{cde}	5.02 ^{def}	4.3 ^{ghi}	3.13 ^{qrs}	0.58	82.34 ^{ab}	68.17 ^{abc}	51.46 ^{abc}	65.93 ^{abc}	27.3 ^{efg}	0.33
D3 C-132-81	8	5.32 ^{cde}	4.69 ^{efg}	3.75 ^{klm}	6.26 ^{bc}	3.67 ^{lmn}	0.69	43.90 ^{abc}	53 ^{abc}	36.5 ^{bcd}	62.84 ^{abc}	39.2 ^{bcd}	0.89
D4 C-132-81	9	4.16 ^{opqr}	3.924 ^{ijk}	3.4 ^{nop}	3.1 ^{qrs}	3.47 ^{nop}	0.83	54.65 ^{abc}	37.3 ^{bcd}	34.86 ^{bcd}	48.7 ^{bc}	48.10 ^{bc}	0.88
D5 C-132-81	10	4.2 ^{pqr}	2.9 ^{stuv}	3.8 ^{klm}	2.7 ^{qrs}	3.40 ^{nop}	0.81	53.65 ^{abc}	37.30 ^{bcd}	26.9 ^{efg}	47.70 ^{abc}	29.1 ^{cde}	0.54
MP B52298	11	4.2 ^{pqr}	2.82 ^{stuv}	3.5 ^{mno}	2.96 ^{rst}	2.74 ^{uvv}	0.65	45.17 ^{abc}	42.26 ^{abc}	26 ^{efg}	29.5 ^{def}	25.90 ^{gh}	0.57
MP C-132-81	12	4.31 ^{pqr}	3.82 ^{kl}	3.10 st	2.7 ^{uvv}	2.40 ^{uvv}	0.56	45.60 ^{abc}	77.53 ^{abc}	17.84 ^h	43.36 ^{abc}	21.89 ^{gh}	0.48
		NRPP						NSPP					
D1B52298	1	8.76 ^{bcd}	7.34 ^{cde}	7.29 ^{cdef}	5.5 ^{hijkl}	3.71 ^{nop}	0.42	5.4 ^{hijkl}	6.3 ^{defg}	7.3 ^{abcd}	7.29 ^{abc}	3.1 ^{nop}	0.57
D2 B52298	2	8.29 ^{bcd}	6.72 ^{def}	8 ^{bcd}	4.47 ^{mno}	3.4 ^{pqr}	0.41	6.09 ^{defg}	7 ^{abcde}	5.97 ^{defg}	5 ^{hijklm}	4.44 ^{mno}	0.73
D3 B52298	3	8.70 ^{bcd}	7.09 ^{cde}	8.56 ^{bcd}	4.56 ^{lm}	3.8 ^{nopq}	0.44	4.96 ^{klm}	7.1 ^{bcd}	6.87 ^{defg}	5.09 ^{klm}	4.7 ^{mno}	0.95
D4 B52298	4	9.13 ^{bc}	6.5 ^{efgh}	9.24 ^{bc}	4.1 ^{nopq}	3.8 ^{nopq}	0.42	7.023 ^{cde}	4 ^{nop}	8.2 ^{bc}	5.2 ^{hijklm}	3.86 ^{nop}	0.55
D5 B52298	5	9.0 ^{bc}	6.4 ^{efgh}	8.9 ^{bc}	4.2 ^{nopq}	4.6 ^{mno}	0.51	6.80 ^{defg}	4.1 ^{nop}	8.1 ^{abc}	5 ^{klm}	3.48 ^{lmno}	0.51
D1C-132-81	6	8.98 ^{bcd}	9.75 ^{ab}	6.79 ^{defg}	4.5 ^{mno}	3.9 ^{rs}	0.43	6.63 ^{defg}	7.21 ^{abcd}	5.5 ^{ehij}	4.7 ^{lmno}	4.6 ^{mno}	0.69
D2 C-132-81	7	11.66 ^a	8.16 ^{bcd}	11.73 ^a	7.71 ^{bcd}	5.40 ^{ijkl}	0.46	8.53 ^{ab}	8.62 ^a	7.3 ^{abcd}	6.13 ^{defg}	4.94 ^{klmn}	0.58
D3 C-132-81	8	6.7 ^{efgh}	7.53 ^{bcd}	5.54 ^{hijk}	4.1 ^{nopq}	3.26 ^{qrs}	0.49	6.5 ^{defg}	8.23 ^{abc}	6.95 ^{cdef}	5.22 ^{hijkl}	3.86 ^{op}	0.59
D4 C-132-81	9	7.2 ^{cdef}	5.77 ^{efgh}	5.1 ^{klmn}	3.7 ^{nopq}	4.68 ^{lmn}	0.65	6.33 ^{defg}	5.29 ^{hijk}	5.4 ^{hijkl}	5.70 ^{efgh}	4.94 ^{klmn}	0.78
D5 C-132-81	10	7 ^{cdef}	5.5 ^{bcd}	6.9 ^{defg}	4.5 ^{mno}	3.8 ^{nopq}	0.54	6.41 ^{defg}	8.47 ^{ab}	7 ^{abc}	6.2 ^{defg}	4.92 ^{klmn}	0.77
MP B52298	11	7.4 ^{cdef}	5.77 ^{hijk}	5.39 ^{ijkl}	4.2 ^{nopq}	2.53 ^d	0.34	5.66 ^{hijkl}	3.68 ^p	4.9 ^{klmn}	5.15 ^{ijklm}	3.85 ^{op}	0.68

*Means with different letters across columns of a trait represent significant difference based on Tukey's multiple comparison tests at $\alpha=0.05$; STI(%)=stress tolerant index at MS medium supplemented with 10% PEG level relative to MS medium with 0% of PEG; PSRR=Percent of shoots regenerated shoots; NRPP=Number of roots per plantlet; ARLPP=Average root length per plantlet; NSPP=Number of shoots per plantlet.

Shoots from D4 C-132-81, D3 B52298, D3 C-132-81, D5 B52298 and D4 B52298 performed better than the others ($P \leq 0.05$) in regenerating roots. Similar to the above screening procedures, the stress tolerance index (STI) was calculated to identify the drought tolerant regenerants (Table 5). Generally, the highest STI value indicates the lower reduction while the smaller value of the STI suggested higher reduction in performance. Shoot regenerants D4 C-132-81 and D3 B52298 had large STI value of 0.89 and 0.88, respectively while D2 B52298, D2 C-132-81 and D1B52298 showed smaller STI value of 0.23, 0.33 and 0.38, respectively. At non-stressed MS medium (0 PEG), regenerants D1B52298 and D3 B52298 showed better root growth of 7.45 and 7.11 cm, respectively. However, the root length of D1B52298 was significantly decreased when the PEG level was increased with small STI value of 0.35. When the MS medium was supplemented with 10% of PEG indicating it was sensitive to the higher level of PEG. Relatively, regenerants D4 C-132-81, D5 C-132-81 and D4 B52298 showed little root growth reduction in the stressed selection environment.

Moreover, under the same stressed environment, regenerants D4 C-132-81, D5 C-132-81 and D4 B52298 showed better root growth and had relatively large STI value of 0.83, 0.81 and 0.80, respectively while the smallest value of STI (0.35) was recorded in regenerant D1B52298. Regenerant D2 C-132-81 produced significantly ($P \leq 0.05$) higher NRPP at both non-stressed (0% PEG) and stressed (10% PEG) MS medium. However, D4 C-132-81 had relatively largest STI value of 0.65 indicating the NRPP showed little reduction though the stress concentration in the medium increased. For number of shoots per plantlet (NSPP), regenerants D2 C-132-81, D4 B52298 and MP C-132-81 produced large numbers of shoots per plantlet at the PEG free MS medium. Of these regenerants, D2C-132-81 produced significantly ($P \leq 0.05$) higher NSPP while D3 B52298 produced the lowest number of shoots. At MS medium supplemented with 10% PEG agent, the NSPP was highly reduced. Relative to others, highest NSPP were produced by D4 C-132-81, D5 C-132-81, D3 B52298 and D2 B52298. At the MS medium supplemented with 10% of PEG, regenerant D3 B52298 followed by D4 C-132-81, D5 C-132-81 and D2 B52298 showed minimum reduction in regeneration of roots with largest STI value of 0.95. Regenerants D5 B52298, D4 B52298 and MP C-132-81 showed lowest STI value of 0.51, 0.55 and 0.55, respectively, indicating the NSPP of these regenerants was drastically affected by the imposed stress.

Generally, the performance of regenerants screened reduced while the level of PEG in the MS medium increased (0-10%). In all traits, more variability was observed when MS medium induced with 10% PEG and our screening focused to select tolerant lines screened under this selection medium. Our result is in close agreement with reports of Begum et al. (2011) who found drought tolerant sugarcane lines at similar level of PEG. Far from our results, Hemarparabha et al. (2004) and Gadakh et al. (2015) developed drought tolerant lines at 2% of PEG as lethal dose. Such discrepancies of results could happen as a result of using different genotypes. Under both *in vitro*

experiments, the stress index (%) calculated at the highest stress level supported the above screening results and was helpful in identifying stress tolerant regenerants (Tables 4 and 5).

3.7. Clustering of direct and indirect regenerated plantlets

Data obtained from indirectly (1-15) and directly (16-18) regenerated plantlets (Table 4) was subjected to cluster analysis. The dendrogram was divided in six clusters within the range of 2.2-3.04 Euclidean distance where regenerants developed *via* direct regeneration (16-17) classified in to separate clusters and salt tolerant regenerants were explicitly clustered in one group (Fig. 3 a). In between the Euclidean distances of 3.04-3.4, the dendrogram was divided in to two major clusters the where the salt tolerant regenerants 4, 8, 14 and 7 were separated from the others (Fig. 3 a). For the same purpose, we used data from 12 regenerants (Table 5) developed through indirect (1-10) and direct (11 and 12) regeneration pathways cluster analysis. The Dendrogram classified the regenerants in to five clusters at 0.96 (Fig. 3 b). Accordingly, regenerants 1 and 7 classified in cluster I while regenerants namely, 2, 3, 4, 5 and 6 grouped in cluster II. Moreover, regenerants namely; 9 and 10 classified in cluster III while regenerants 11 and 12 classified in separate groups. Lastly, regenerant 8 was not grouped to any other and classified in the last fifth group. At 0.86 similarity coefficient, the dendrogram classified the regenerants in to two major clusters: drought tolerant (cluster II and III) and sensitive (cluster I) ones which is somewhat similar to the classification based on mean comparison provided in Table 5. Mother plants (MPB52-298 and MPC13-81) regenerated using direct regeneration pathway classified in to another cluster (cluster IV).

Both dendrograms examined the nature and pattern of the variability existed. This is because considering a number of parameters at a time using multivariate analysis increased the chance of identifying appropriate stress tolerant genotypes. Different workers (Begum et al., 2011; Cha-um et al., 2012; Gadakh et al., 2015) benefited from use of similar analysis to classify regenerants. In both cases (Fig. 3 a, b), the dendrogram separated the regenerants based on the regeneration pathways and tolerance to stresses under investigation. The separations of regenerants based on the regeneration pathway indicated the existence of somaclonal variation. From the perspective of stress tolerance, the dendrogram was able to separate the sensitive and tolerant regenerants in to separate groups; an indication of the accumulation of new variability with respect to stress tolerance. Results of the present study were similar to results reported by Patade et al. (2006).

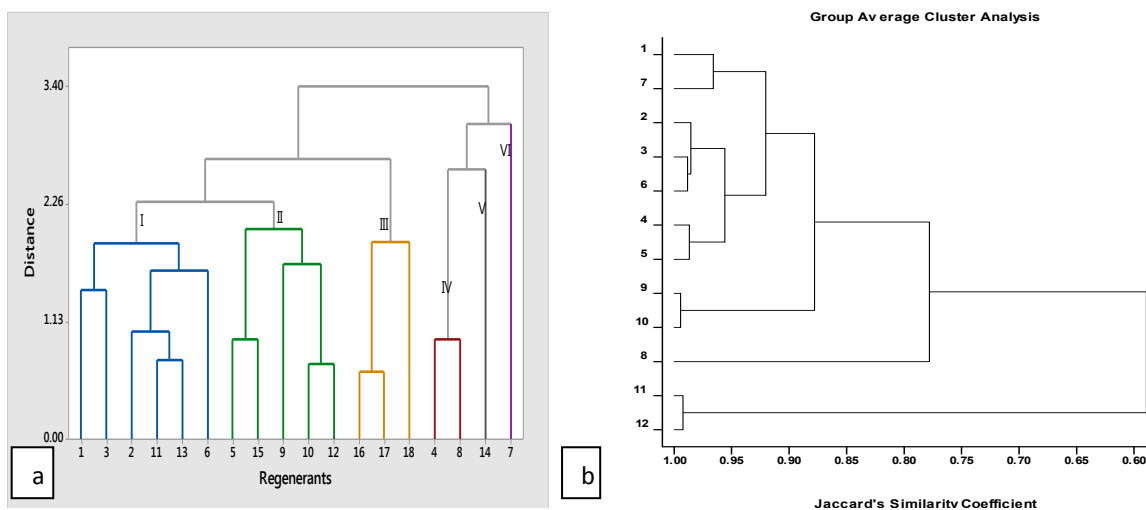


Fig. 3. Dendrogram of sugarcane regenerants screened for (a) for salt tolerance (b) drought tolerance.

3.8. Performance of *in vitro* developed regenerants at salt affected and soil moisture limited field conditions

The *in vitro* developed and stress tolerant regenerants were transferred to field (Tendaho Sugar Project) for further evaluation after the greenhouse acclimatization was completed. The healthy regenerants were selected and transferred to saline field for field validation and were evaluated for some morphological characters (numbers of tillers, stalk population and plant height). Salt tolerant regenerants were planted along with the respective varieties planted using conventional planting (C90-501, B78-505 and SP-70-1284). Based on the field evaluation at

Tendaho Sugar Project, regenerants S4 C90-501, S4SP-70-1284 and S2B-78-505 survived under field condition and performed better than the conventionally grown varieties (Table not presented) and showed high vigor in growth (Fig. 3 a, b, c).

Similarly, the drought tolerant regenerants D3B52-298, D4B52-298, D4 C-132-81 and D3C132-81 transferred to soil moisture limited field were evaluated for some morphological characters. Like to salt tolerant regenerants, regenerants that tolerated higher level of PEG under *in vitro* condition showed better performance than the locally grown varieties and showed better stand and high vigor (Fig. 4 d, e, f, g). In conclusion, those regenerants identified as salt and drought tolerant under *in vitro* condition survived well at greenhouse and field conditions; an indication of the validity of our *in vitro* experiments. Similar field validation studies reported by many workers who identified salt (Shah et al., 2004; Patel, 2007; Gadakh et al., 2015) and drought (Islam et al., 2010; Dalvi et al., 2012; Gadakh et al., 2015) tolerant superior somaclones over donor parents.

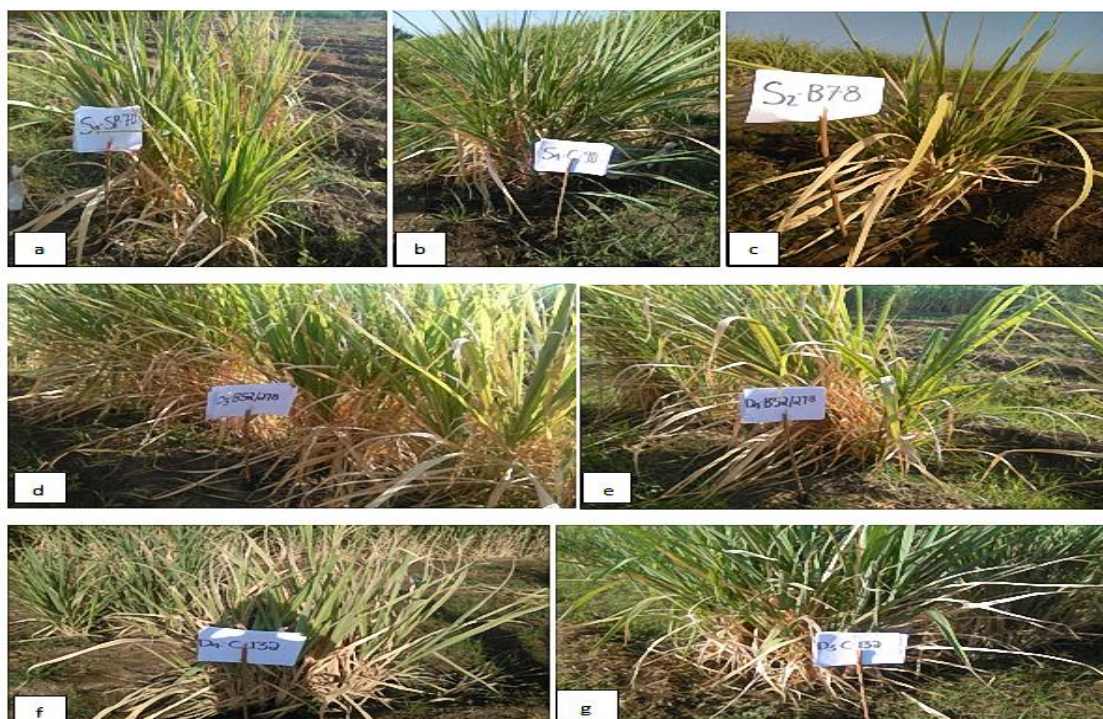


Fig. 4. Field of evaluation of sugarcane salt and drought tolerant regenerants (a) S4C0-501, (b) S4SP-70-1284, (c) S2B-78-505, (d) D3B52-298, (e) D4B52-298, (f) D4C-132-81 and (g) D3C132-81 grown at Tendaho Sugar Project.

4. Conclusion

Our investigation was successful in detecting variability among varieties and the developed regenerants with respect salt tolerance, and isolating salt and drought tolerant regenerants. Among the regenerants screened, regenerants S4SP-70-1284, S4 C0-501 and S2B-78-505 showed considerable tolerance for salt stress under *in vitro*, greenhouse and field conditions while D4C-132-81, D4B52-81, D3B52-81 and D3C132 identified as drought tolerant regenerants. The cluster analysis conducted to assess the existed variability among regenerants demonstrated the existence of ample genetic variability among regenerants under both *in vitro* experiments. The dendrograms separated those regenerants developed through direct regeneration from those regenerants developed through indirect regeneration. It suggested the existence of somaclonal variation. Moreover, both dendrograms were capable of separating those regenerants exhibiting tolerance to the stresses in question thereby suggesting the existence of variability in relation to stress tolerance. The regenerants selected under *in vitro* and field conditions should be further evaluated for yield and quality traits. Furthermore, the stress (salt and PEG) tolerant regenerants would be valuable in enhancing the diversity of sugarcane varieties with high degree of salt and drought tolerance suitable for soil moisture prone sugarcane production areas in Ethiopia.

Acknowledgements

We acknowledge the Ethiopian Sugar Corporation for its full financial support. Moreover, we wish to acknowledge the Mekelle Institute of Biotechnology Tissue Culture Laboratory for providing necessary inputs on time. Our great appreciation goes to Mr. Birhanu Debessay and Mr. Jemal Aman, for their kind cooperation during the execution of the *in vitro* experiments. Finally, we extend our great gratitude to Mr. Abera Bedada who helped us in managing the field evaluations conducted at Tendaho Sugar Project.

References

- Arzani, A., 2008. Improving salinity tolerance in crop plants: A biotechnological view. *In Vitro Cell Development Biology Plant*, 44, 373-383.
- Ashok, K., Shrivastava, A., Srivastava, K., Solomon, S., 2011. Sustaining sugarcane productivity under depleting water resources. *Curr. Sci.*, 101, 748-754.
- Ashraf, M., Rahmatullah, K.S., Tahir, M.A., Sarwar, A., Ali, L., 2007. Differential salt tolerance of sugarcane genotypes. Directorate of Land Reclamation, Irrigation & Power Department, Punjab, Pakistan. *Pakistan J. Agr. Sci.*, 44, 85-89.
- Ather, A., Khan, S., Rehman, A., Nazir, M., 2009. Optimization of the protocol for callus induction, regeneration and acclimatization of sugarcane. CV. Thatta- 10. *Pakistan J. Botany*, 41(2), 815-820.
- Avinash, L., Prashant, N., Bharose, A.A., 2012. *In vitro* screening of sugarcane cultivar Co 86032 for salinity tolerance. College of Agricultural Biotechnology Marathwada Krishi Vidyapeeth Latur, India. *J. Sugar Res.*, 2, 25-31.
- Begum, M.K., Islam, M.O., Miah, M.A.S., Hossain, M.A., Islam, N., 2011. Production of somaclone: *In vitro* for drought stress tolerant plantlet selection in sugarcane (*Saccharum officinarum* L.). Bangladesh Sugarcane Research Institute, Ishurdi-6620, Pabna, Bangladesh. *Agr.*, 9, 18-28.
- Begum, M.S., Kohinoor, T., Islam, M.D.O., 2015. Selection of salt tolerant somaclones for development of salt stress tolerant varieties. *Plant*, 3, 37-46.
- Biradar, S., Biradar, D.P., Patil, V.C., Patil, S.S., Kambar, N.S., 2009. *In vitro* plant regeneration using shoot tip culture in commercial cultivar of sugarcane. *Karnataka J. Agr. Sci.*, 22, 21-24.
- Cha-um, S., Chuencharoen, S., Mongkolsiriwatana, C., Ashraf, M., Kirdmanee, C., 2012. Screening sugarcane (*Saccharum sp.*) genotypes for salt tolerance using multivariate cluster analysis. National Center for Genetic Engineering and Biotechnology. *Plant Cell, Tissue Organ Culture*, 110, 23-33.
- Gadakh, S.S., Patel, D.U., Patil, A.B., 2015. Evaluation of sugarcane (*Saccharum officinarum* L.) mutants for yield, yield contributing traits and quality parameters. Department of Genetics & Plant Breeding, N.M. College of Agriculture, Navsari Agricultural University, Navsari. *I.J.A.B.R.*, 5, 220-228.
- Gandonou, C.H., Errabini, T., Abrinij, J., Idaomar, M., Chibi, F., Skali, S.N., 2005. Effect of genotype on callus induction and plant regeneration from leaf explants of sugarcane. *Afr. J. Biotechnol.*, 4, 1250-1255.
- Garcia, A.A., Kido, E.A., Meza, A.N., Souza, H.M.B., Pinto, L.R., Pastina, M.M., Leite, C.S., Silva, D., Ulian, E.D., Figueira, A., Souza, A.P., 2006. Development of an integrated genetic map of a sugarcane (*Saccharum spp.*) commercial cross; based on maximum likelihood approach for estimation of linkage and linkage phases. *Theoret. Appl. Genet.*, 112, 298-314.
- Girma, A., 1993. Assessment of salinity effect and sodicity status of Metahara sugar estate. MSc Thesis. Alemaya University, College of Agriculture, Ethiopia.
- Gower, J.C., 1971. A general coefficient of similarity and some of its properties. *Biometr.*, 27, 857-871.
- Hadi, M.Z., Bridgen, M.P., 1996. Somaclonal variation as a tool to develop pest resistant plants of *Torenia fournieri* 'Compacta Blue'. *Plant Cell, Tissue Organ Culture*, 46, 43-50.
- Hemarparabha, G., Nagarajan, R., Alarmelu, S., 2004. Response of sugarcane genotypes to water deficit stress. *Sugar Technol.*, 6, 165-168.
- Islam, M.W., Miah, M.A.S., Hossain, M.A., Begum, M.K., Islam, M.S., 2010. Selection of drought stress tolerant somaclones of sugarcane under field condition. *Int. J. Sustain. Agr. Technol.*, 6, 52-60.
- Kenganal, M., Hanchinal, R.R., Nadaf, H.L., 2008. Ethyl methane sulphonate (EMS) induced mutation and selection for salt tolerance in sugarcane *in vitro*. *Indian J. Plant Physiol.*, 13, 405-410.

- Khan, I.A., Dahot, M.U., Seema, N., Yasmin, S., Bibi, S., Raza, S., Khatri, A., 2009. Genetic variability in sugarcane plantlets developed through *in vitro* mutagenesis. *Pakistan J. Bot.*, 4, 153-166.
- Larkin, P.J., Scowcroft, W.R., 1981. Somaclonal variation: A novel source of variability from cell cultures for plant improvement. *Theoret. Appl. Genet.*, 60, 197-214.
- Maas, E.V., 1986. Salt tolerance in plants. *Appl. Agr. Res.*, 1, 12-26.
- Mahmoud, S., Majid, N., Mosa, M., Hamid, R., 2011. Evaluation of sugarcane (*Saccharum officinarum* L.) somaclonals tolerance to salinity via *in vitro* and *In vivo* culture. *Afr. J. Biotechnol.*, 10, 9337-9343.
- Megersa, O., Willibald, L., Josef, F., 2009. Effect of lake Basaka expansion on the sustainability of Matahara SE in the Awash River basin, Ethiopia. *Water, sanitation and hygiene: Sustainable development and multisectoral approaches*. 34th WEDC International Conference, Addis Ababa, Ethiopia.
- Munir, N., Aftab, F., 2013. Effect of salt stress on callus morphology and growth of sugarcane callus cultures. Department of Biotechnology, Lahore College for Women University, Lahore. *Pakistan J. Sci.*, 65, 473-477.
- Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant*, 15, 473-497.
- Naidoo, S., Van, R.A., Parmmenter, N.W., 2004. Quantifying the effect of soil salinity on the physiology of three South African sugarcane varieties. Council for Scientific and Industrial Research, South Africa. *Proceeding South African Sugar Technology Association*, 78, 1-19.
- Patade, V.P., Suprasanna, P., 2009. An *in vitro* radiation induced mutagenesis selection system for salinity tolerance in sugarcane. Functional Plant Biology Section, Nuclear Agriculture and Biotechnology Division, Bhabha. *Sugar Technol.*, 42-44.
- Patade, V.Y., Suprasanna, P., Bapat, V.A., Kulkarni, U.G., 2006. Selection for abiotic (salinity and drought) stress tolerance and molecular characterization of tolerant lines in sugarcane. *Bhabha Atomic Research Centre Newsletters*, 27, 244-257.
- Patel, S.R., 2007. Induction of variability through callus culture in sugarcane (*Saccharum officinarum* L.) variety CoN 95132. Ph.D. Thesis submitted to N.A.U., Navsari.
- Perez-Clemente, R.M., Gomez-Cadenas, A., 2012. *In vitro* tissue culture, a tool for the study and breeding of plants subjected to abiotic stress conditions. Department of Agricultural Sciences. Universidad Jaume I, Spain. *Recent Advances in Plant in Vitro Culture*.
- Rai, M.K., Kalia, R.K., Singh, R., Gangola, M.P., Dhawan, A.K., 2011. Developing stress tolerant plants through *in vitro* selection: An overview of the recent progress. *Experimental Botany*, 71, 89-98.
- Rajeswari, S., Krishnamurthi, M., Shinisekar, S., Prem, A.S., Thirugnana, K.S., 2009. Performance of somaclones developed from intergeneric hybrids of sugarcane. *Sugar Technol.*, 11, 258-261.
- Raza, G., Ali, K., Mukhtar, Z., Mansoor, S., Arshad, M., Asad, S., 2010. The response of sugarcane (*Saccharum officinarum* L.) genotypes to callus induction, regeneration and different concentrations of the selective agent (geneticin-418). *Afr. J. Biotechnol.*, 9, 8739-8747.
- Rengasamy, P., 2010. Soil processes affecting crop production in salt affected soil. School of Agriculture, Food and Wine, University of Adelaide, Australia. *Funct. Plant Biol.*, 37, 613-620.
- Rozeff, N., 1995. Sugarcane and Salinity: A review paper. *Sugarcane*, 5, 8-19.
- Sakhanokho, H.F., Kelley, R.Y., 2009. Influence of salicylic acid on *in vitro* propagation and salt tolerance in *Hibiscus acetosella* and *Hibiscus moscheutos* (cv 'Luna Red'). *Afr. J. Biotechnol.*, 8, 1474-1481.
- Shah, J.K., Khan, M.A., Ahmad, H.K., Khan, R.D., Zafar, Y., 2004. Somaclonal variation in sugarcane through tissue culture and subsequent screening for salt tolerance. *Asian J. Plant Sci.*, 3, 330-334.
- Shomeili, M., Majid, N., Mosa, M., Rajabi, M., 2011. Evaluation of sugarcane (*Saccharum officinarum* L.) somaclonal variants tolerance to salinity *in vitro* and *In vivo* cultures. *Afr. J. Biotech.*, 10, 9337-9343.
- Srinath, R., Jabeen, F.T.Z., 2013. *In vitro* selection and characterization of polyethylene glycol (PEG) tolerant callus lines and regeneration of plantlets from the selected callus lines in sugarcane (*Saccharum officinarum* L.). *Physiol. Mol. Biol. Plant*, 19, 261-268.
- Statistical Analysis System Institute, 2009. *SAS Users Guide; Version 9.2*. SAS Ins. Inc. Cary North Carolina, USA.
- Steel, R.G.D., Torrie, J.H., 1981. In: *Principles and procedures of statistics*. McGraw Hill International Book Company, London (2nd Edition), 254-238.
- Subbarao, G.V., Johanssen, C., 1999. Strategies and scope for improving salinity tolerance in crop plants. In: *Advanced Life Support Systems Group*. John F. Kennedy Space Center Kennedy Space Center, Florida.

- Subbarao, M., Shaw, M.A.E., 1985. A review of research on sugarcane soils of Jamaica. Proceeding of Meeting West Indies Sugar Technology, 2, 343-355.
- Tate, B., 2009. Re-evaluation of the plantation soils at Metahara sugar factory, Ethiopia.
- Vasantha, S., Venkataramana, P.N., Gururaja, R., Gomathi, R., 2009. Long term salinity effect on growth, photosynthesis and osmotic characteristics in sugarcane. Biol. Life Sci., 12, 5-8.
- Wagih, M.E., Aal, A., Musa, Y., 2003. Regeneration and evaluation of sugarcane somaclonal variants for drought tolerance. Sugarcane Technol., 2, 35-40.

How to cite this article: Ftwi, M., Mekbib, F., Abraha, E., 2018. *In vitro* plant regeneration, screening for salt and drought tolerance in sugarcane (*Saccharum Spp.*). Scientific Journal of Crop Science, 7(1), 265-279.

Submit your next manuscript to Sjournals Central and take full advantage of:

- Convenient online submission
- Thorough peer review
- No space constraints or color figure charges
- Immediate publication on acceptance
- Inclusion in DOAJ, and Google Scholar
- Research which is freely available for redistribution

Submit your manuscript at
www.sjournals.com



The Academic and Scholarly Research Publication Center Ltd. (ASRPC), a corporation organized and existing under the laws of the England country with No., 10401338. Established in 2016, Academic and Scholarly Research Publication Center Ltd. is a full-service publishing house. We are a leading international publisher as well as distributor of our numerous publications. Sjournals Publishing Company is published under cover of ASRPC Publishing Company Ltd., UK.

<http://asrpc.co.uk>

