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Original article

Callus induction and regeneration in sugarcane under drought stress

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ABSTRACT

Tissue culture methods are useful in assessing the tolerance of various stresses due to the ease of controlling stress under *in vitro* conditions. This study aimed to investigate the response of sugarcane genotyps to drought stress using calli as a model system. For inducing sugarcane callus, the medium of Murashige and Skoog (MS) was used with different mannitol concentrations (100, 200, and 300 mM) to measure their effects on callus frequency, the day of callus initiation, embryogenic potential, relative growth rate (RGR), water and proline contents, K^+ and Na^+ contents, as well as the formation of shoot and roots for three sugarcane genotypes (e.g., GT 54-9, G 84-47, and pH 8013). The RAPD-PCR analysis was carried out using five oligonucleotide primers to identify the genetic variation among sugarcane genotypes. The results indicated that the degree of callus proliferation varied from 70 – 86%. The highest value of callus proliferation, PGR, shoot formation was recorded for the genotype GT 54-9 compared to the other two genotypes (G 84-47 and pH 8013). Calli treated with 100 mM mannitol showed the highest RGR, proline and waer contents for the genotype GT 54-9, while, those treated with 300 mM recorded the lowest values of these parameters for the genotype pH 8013. The genotype G 84-47 collected highest Na^+ content, while the genotype pH 8013 collected highest K^+ content. The results of this study recommend preference for GT 54-9 genotype, which is considered the most promising genotype, showing more tolerance to drought stress based on all studied traits.

1. Introduction

Sugarcane is the fundamental material for white sugar production worldwide (Arif et al., 2019). The present area of sugarcane (*Saccarum officinarum*) is about 14.2 million ha with a total commercial world production of about 1374.9 million ton per year cane or 60.5 million ton per year sucrose. (FAOSTAT, 2019). Optimum temperature for sprouting (germination) of stem cuttings is 32 to 38 °C. Optimum growth is achieved with mean daily temperatures between 22 and 30 °C. Minimum temperature for active growth is approximately 20 °C. For ripening, however, relatively lower temperatures in the range of 20 to 10 °C are desirable, since this has a noticeable influence on the reduction of vegetative growth rate and the enrichment of sucrose in the cane. Sugarcane juice is used as a fresh drink and is rich in vitamins, carbohydrates, and amino acids. About four hundred and ninety-three megagrams (Mg; metric ton) of bagasse from about one thousand six hundred Mg of sugarcane have been obtained in the sugar industry (Khattab et al., 2019). About 2,500 years ago, sugar began to be commercially produced from sugarcane in India and China, and in the eighteenth century spread to Western Europe (Leal, 2007; Kaur, 2014). Today, sugarcane is used as raw material for fuel production, chemicals, biofertilizers, paper, and pulp. Sugarcane contributes about 70% of sugar production worldwide, so it is an important crop for agricultural industries (Arruda, 2011; Aguilar-Rivera, 2019). It is a tribe

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that is part of Andropogoneae under the Poaceae family (grasses) (Selman-Housein et al., 1999). As polyploids and aneuploids, modern sugarcane cultivars are originated from crosses of *S. officinarum* L. (2n = 80) with *S. spontaneum* L. (2n = 40:128) and backcrossing interspecific hybrids with the *S. officinarum* L. parent (Ming et al., 2006).

(Rady et al., 2021)As one of the most limiting abiotic stresses, drought (water deficit) minimizes the efficiency of crop plants in terms of all morphological, physiological, and biochemical performances and thus the final productivities (Abd El-Mageed et al., 2016, 2017; Al-Elwany et al., 2020; Alharby et al., 2020; Rady et al., 2020; Taha et al., 2020; Desoky et al., 2021; Semida et al., 2021). Under a prolonged period of water scarcity, most crop plants suffer from drought and eventually die. Drought-stressed plants tend to have higher solute concentrations in their cells due to reduced cell turgor (Desoky et al., 2020a). Drought affects plant nutrition and metabolism, which leads to a minimization of the assimilate partitioning into organs, which is reflected in the reduction of leafy areas (Merwad et al., 2018). Due to the complexity of responding to water scarcity, plants evolve and/or adopt different mechanisms by which to confront this opponent. Among these mechanisms, escaping (by prompt development), avoidance (by maximizing water uptake and minimizing its loss through reducing stomatal conductance and leafy areas), tolerance (by keeping cell turgor through efficient osmotic adjustment), and resistance (by other survival mechanisms), all of which allow plants to perform well to terminate their life cycles (Rady et al., 2019a; Desoky et al., 2020b).

Tissue culture methods help assess the tolerance of various stresses due to the ease of controlling stress under *in vitro* conditions. Besides, a standardized set of synchronously developing plant cells is furnished in *in vitro* culture whose regulatory mechanisms (which are naturally fixed on the whole plant basis) are not accompanied (Evans et al., 1981; Ammirato (1983)). In vitro tissue culture comprises a pivotal tool to examine the physio-biochemical mechanisms, which stimulate response to stress conditions at the level of the plant cell (Lerner, 1985; Lushchak, 2011; Rai et al., 2011). Additionally, tissue culture permits plants to control stress homogeneity and characterization of cellular behaviors under stress conditions, autonomously of regulatory systems, which occur at the level of the whole plant (Lutts et al., 2004).

Many *in vitro* studies were done to assess sugarcane response to stress (Karpe et al., 2012). The authors assessed sugarcane's responses to salinity stress (up to 150 mM) by *in vitro* plantlets, and thus, *in vitro* sugarcane plantlets could be incorporated into a screening system for selecting salinity- and/or drought-resistant sugarcane genotypes. Comparing with stress-sensitive genotypes, stress-resistant sugarcane genotypes aggregate a lower proline content (Singh, 2003) and succeed in conferring regeneration from tissue culture. This qualify sugarcane to be incorporated into breeding programs for clones' immediate screening for drought resistance, early maturity, and high sugar yield. The tissue culture-identified new sugarcane variety (regenerated somatic embryogenesis on the MS medium complemented with 3 mg NAA L^{-1}) with high sugar content and stress resistance should be available to the producers for commercial interest (María et al., 1997). Nine sugarcane genotypes have been evaluated for their ability to induce callus, to produce embryogenic callus, and to regenerate (Gandonou et al., 2005). The authors reported a high rate of 69 to 95% for inducing callus and 60 to 100% for embryogenic callus.

Genetic variation has been displayed in individual species or organisms by genetic markers (Jones et al., 1997). It is usually visually characterized phenotypic characters (Sumarani et al., 2004). The markers of DNA are exceedingly welcomed as a worthwhile tool for many crop improvements (Mackill et al., 1999; Koebner and Summers, 2003; Tuberosa et al., 2003; Williams, 2003; Abdelsalam et al., 2019). The RAPD (**R**andom **A**mplified **P**olymorphic **D**NA) is an uncomplicated and time-efficient technique (Garcia et al., 2004), its results in the amplification of few random segments of DNA, allowing for variation in length and number of amplified segments when the sequence of the segments is altered. The RAPDs have been highly welcomed for genetic variance analyses and recommended for plant somaclonal variance analyses (Ngezahayo et al., 2007; Cuesta et al., 2010; Babu et al., 2014; Teama, 2018).

A reasonable number of studies have not been performed to evaluate the improvement of drought tolerance of sugarcane genotypes such as GT 54-9, G 84-47, and pH 8013. This study hypothesized that at least one genotype of sugacane (from GT 54-9, G 84-47, and pH 8013) would be generated from tissue culture technique to select as the most drought-tolerant for cultivation under drought expected, due to climate change, at the open field level. Therefore, the objectives of this investigation are to evaluate three sugarcane genotypes for their capabilities to callus induction and their drought tolerance under different mannitol concentrations, besides, identify the genetic variation among sugarcane genotypes *via* RAPD-PCR.

2. Materials and methods

2.1. The source of genotypes

For this study, the sugarcane genotypes found in Table S1 as supplementary material are from complex hybrids *Saccharum officinarum* and other species under the genus *Saccharum*.

2.2. The techniques of tissue culture

2.2.1. The contents of the used medium

The medium of Murashige and Skoog (MS) (Ahloowalia and Maretzki, 1983) was used (from Duchefa Biochemical, Duchefa, Postbus 809, Haarlem, Netherlands). The medium was supplemented with sucrose (3%) solidified with 8 g agar per L. For induction of callus, the MS medium was provided by 4 mg 2,4-D L^{-1}, 0.5 g Casein hydrolysate L^{-1}, and 10% coconut water as previously recommended (Mohashweta et al., 2011). The medium pH value was adjusted to pH 5.7–5.8 by adding HCl (0.1 M) or NaOH (0.1 M). The medium was poured into a jar (150 ml) and then autoclaved (for sterilization) for ⅓ h at 121 °C under the pressure of 1.1 kg cm^{-2}. The medium also contains the following plant growth regulators and other components; NAA (1-Naphthaleneacetic acid), 2,4-D (2,4-Dichlorophenoxy acetic acid), kinetin, casein hydrolysate, sucrose, agar-agar, coconut water, mannitol, and HNO_3.

2.2.2. The production of callus

From each genotype, stem sections including two lateral buds were established in soil-containing plastic containers under controlled conditions for six months. Then, healthy and disease-free explants were used to cut shoot tips for use in this study (Fig. 1).

2.2.3. The culture preparation and conditioning

Using a pair of secateurs, the sugarcane stalks were harvested by cutting them off at the base. The stalk was then cleaned from the immature leaf roll by removing it above the natural breaking point and the apical stalk section leaves were pruned. In *in vitro*, cutting (to 100–150 mm) was performed transversely for the leaf roll on the upper end (Fig. 1) and then disinfected by wiping the surface with ethanol (95 %, v/v). Thereafter, in the laminar flow under aseptic conditions, the outer leaf sheaths of the stalk apices

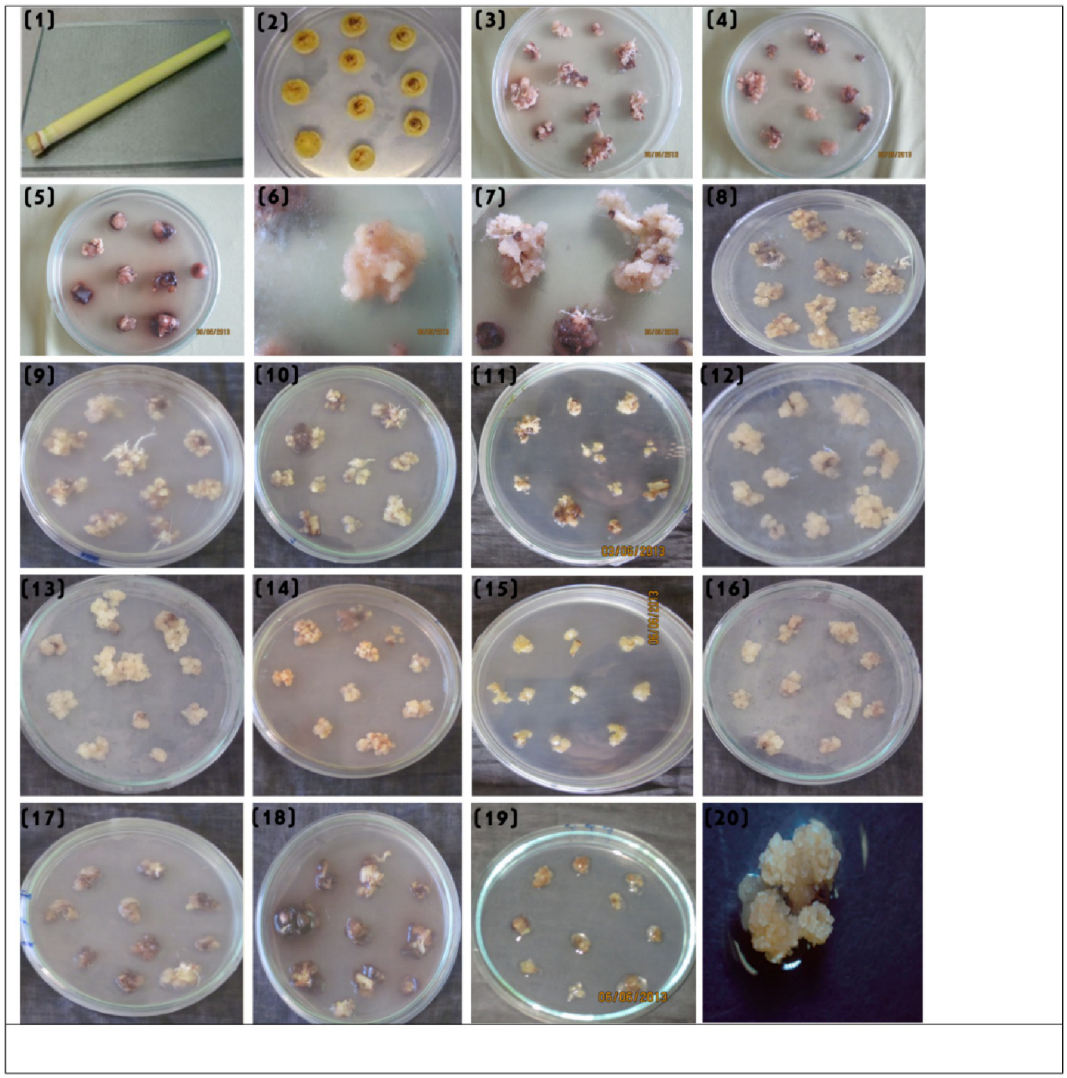

Fig. 1. Showing the following i.e. (1) material of sugarcane used in the current study (sugarcane leaf roll), (2) leaf sections on solidified medium, (3–5) different callus induction in three sugarcane genotypes (3) GT 54-9, (4) G 84-47, (5) ph 8013, (6) Embryogenic, (7) non embryogenic callus induced from leaf explant of sugarcane genotypes, (8–11) different concentrations of mannitol on callus relative growth rate of GT 54-9 genotype as (8) control, (9) 100 mM mannitol, (10) 200 mM mannitol, (11) 300 mM mannitol, (12–15) effect of different concentrations of mannitol on callus relative growth rate of G 84-47 genotype; (12) control, (13) 100 mM mannitol, (14) 200 mM mannitol, (15) 300 mM mannitol, (16–19) effect of different mannitol concentrations on callus relative growth rate of pH 8013 genotype; (16) control, (17) 100 mM mannitol, (18) 200 mM mannitol, (19) 300 mM mannitol, and (20) effect of different concentrations of mannitol on percentage of callus water content.

were removed using a sterile scalpel until the inner immature leaf roll was exposed.

The stem sections were disinfected with ethanol (75%, v/v) then dipped in a 0.03% (w/v) solution of $HgCl_2$ for ½ h and rinsed in sterile distilled water for 10 min three times as recommended (Errabii et al., 2007). The leaf roll was sliced into 30 transverse sections, each approximately 2 mm thick, from the tip of the leaf roll towards the base of the apical meristem. The leaf roll sections were then placed on a solidified medium in Petri dishes 10 sections per plate (Fig. 1) with the ventral surface of each disk in contact with the medium. Petri dishes each with 25–30 ml of the medium were established to perform the experiments. The ten sections were cultured on the surface of the agar-solidified medium. Each dish was considered as one replication that resulted in five replicates for each genotype. Plates were sealed with parafilm and incubated at 25 ± 2 °C in the dark. Every two weeks, the subculture was carried out on a fresh medium. The flowing variables were recorded for each petri dish in this investigation as the day number required to callus initiation and percentage of embryogenic callus derived from the three genotypes.

2.2.4. In vitro-inducing drought states using mannitol ($C_6H_8OH_6$)

Immediately before sterilization of the culture medium, mannitol was added in final concentrations of 0, 100, 200, and 300 mM according to Errabii et al. (2007).

2.2.5. Determination of callus relative growth rate and water content

Ten weeks after the start of the drought treatments, each callus was weighed and then applied to the medium of MS performed or not with stressors. For each treatment (genotype), calli were utilized at counts of approximately 30–35. Four weeks after the start of the drought treatments, evaluation of calli and their ion and proline contents was performed.

Based on the weight of fresh callus (FW), its relative growth rate (RGR) was assessed applying the following formula (Patade et al., 2011):

$$RGR = [(final\ FW - - initial\ FW) \div initial\ FW]$$

Based on the weight of fresh and dried callus (FW and DW, respectively), the water content of callus was computed applying the formula:

$$Water\ content = [(callus\ FW - - callus\ DW) \div callus\ DW]$$

2.2.6. Assessment of the contents of K^+ and Na^+ ions, and proline

Using an electric oven set at 80 °C, calli were kept for three days or until weight consistency. After grinding the dried calli, K^+ and Na^+ ions were analyzed for their contents, utilizing a Flame Spectrophotometer (PDF 90D, France), in the filtered extract of the digested dried calli (using HNO_3) (Lutts et al., 1996). The content of proline was assessed applying the method of Bates et al. (1973).

2.2.7. Shoot regeneration

Somatic embryos of calli were selected for plant regeneration and transferred to a full-strength medium of MS supplement with 1 mg KIN L^{-1}. Illumination (2,000 μmol s^{-1} m^{-2} of light fluorescent tubes) was applied at 20 °C for 16 h daily to incubate calli for four weeks. The numbers of calli with the green shoot (shoot formation) were recorded.

2.2.8. Root formation

Shoot plantlets were grown until reaching 5 cm in height. They were then transferred to half-strength MS medium with another supplement plus 0.2 mg NAA L^{-1}. They were then subjected to illumination (2,000 μmol s^{-1} m^{-2} of light fluorescent tubes) that was applied at 20 °C for 16 h daily for incubation to record the numbers of days to indicate the root. After four weeks, the number of roots was recorded.

2.3. Molecular examination

With applying the analysis of **R**andom **A**mplified **P**olymorphic **D**NA (RAPD-PCR), five oligonucleotide primers (Table S2) were selected from Operon Kit (Operon Technologies Inc., Alabameda CA).

2.3.1. Total genomic DNA extraction

For each sample, genomic-DNA from sugarcane leaves were extracted through a DNA isolation kit (Gene JET™, plant genomic DNA purification mini kit. Fermentas). To purify DNA from polyphenol, Lysis Buffer was supplemented with polyvinyl pyrrolidone (PVP) at 2% (W/V) final concentration. DNA was quantified by Gene quant at 260/280 nm.

2.3.2. Analysis with RAPD- PCR

Quality was further checked on 0.1% agarose gel. RAPD-PCR analysis was implemented applying the Williams et al. (1990) method.

2.3.3. Data analysis

Phoretix electrophoresis gel image analysis, ID software was used for scanogram tracing of DNA bands of tissues. Data matrices were submitted into the NTSYS program, version 2.1, Applied Biostatistics Inc. UK (Rohlf, 2000). Similarity coefficients were applied to generate dendrograms applying UPGMA software.

2.4. Statistical analysis

The data generated in this study were tested applying four-way ANOVA for randomized complete design and error variance homogeneity throughout the procedure applying COSTAT software (CoStat Version 6.303, Berkeley, CA, USA). For each treatment, analysis of combined data was implemented with LSD at $p \leq 0.05$ applying Duncan's Multiple Range Test.

3. Results

3.1. Callus induction in sugarcane

The resulting data indicate a variable level of callus proliferation of 70–86%. Analysis of variance indicates a highly significant difference between the three genotypes concerning the percentage of callus induction. The highest frequency was recorded to GT 54-9 compared to the other two genotypes in respect, as shown in Table 1 and Fig. 1. Although the two GT 54-9 and G 84–47 genotypes had the same day to 280 callus initiation in an average of 10 days, the pH 8013 was the highest compared with the others. While the highest day to callus, initiation was 14 days for pH 8013 and showed the second value in callus induction (Table 3, Fig. 1).

The results show an intermediary type possessing non-embryogenic tissue covered by embryogenic tissue. To determine the percentage of the embryonic calli, the intermediary type was classified as embryogenic due to the rapid development of the embryogenic tissue in further subcultures comparing with the non-embryogenic tissue. The results in Table 1 and Fig. 1 show the high embryogenic callus percentages ~ 80%. While no significant difference was observed between GT 54-9 and G 84–47, which gave the best response related to the other genotype.

3.2. In vitro drought tolerance

The results show that the maximum RGR was obtained for GT 54-9, while the lowest value was recorded for pH 8013. The results show, on the other side, that control callus gained the highest RGR for GT 54-9 followed by mannitol-treated callus (Table 2). Among the treated samples, callus treated with 100 mM mannitol showed the highest RGR for GT 54-9, while with 300 mM callus recorded the lowest RGR pH 8013. The results indicated that by increasing the mannitol concentration the RGR of callus decreased. Data in Table 2 show a significant effect between three genotypes and treatments. Comparison between means of genotypes, as shown in Fig. 1, indicate that genotypes differ significantly in callus RGR. Genotype GT 54-9 recorded the highest RGR after all treatments. While pH 8013 recorded the lowest value after all treatment.

The obtained results clearly show that by increasing the mannitol concentration, the callus RGR was decreased (Fig. 1). The results show that the highest water content (WC, %) was observed in GT 54-9, while the lowest was observed in pH 8013 (Table 2). On the other hand, under 100 mM mannitol, ph 8013 represented the lowest value of WC (%). Using more than 100 mM mannitol, all genotypes showed decreases in WC (%) (Fig. 1).

3.3. K^+ and Na^+ ion contents

Analysis of variance displayed in Table 3 shows a highly significant difference among the three genotypes for Na^+ and K^+ ion contents. The highest Na^+ value was recorded for G 84-47, while pH 8013 recorded the lowest value. However, the highest K^+ value was obtained with pH 8013 and the lowest value was for GT 54-9. The present experiment proved an inverse relationship between mannitol concentration and Na^+ and K^+ contents. The results show that an increase in mannitol concentrations was accompanied by an increase in the Na^+ content, while a parallel decrease in the K^+ content was detected (Table 4). The results show also that there was no significant variation between the GT 54-9 and G 84-47 for Na^+ content under 300 mM of mannitol. While there is a highly significant variation in genotype pH 8013. The same trend was

Table 1
Callus induction percentage and day to callus initiation of three sugarcane genotypes.

Genotypes	Days to callus initiation	Callus induction (%)	Embryogenic callus (%)	Relative growth rate (RGR)	Water content (WC, %)
GT 54-9	10.0[*a] ± 1.22	86.0[c] ± 3.16	80.0[b] ± 3.16	2.22[a] ± 0.15	77.952[a] ± 2.36
G 84-47	10.0[a] ± 1.58	70.0[a] ± 1.87	78.8[b] ± 2.17	1.57[b] ± 0.13	77.38[a] ± 2.57
ph 8013	14.0[b] ± 2.00	80.0[b] ± 2.00	70.2[a] ± 0.84	0.73[c] ± 0.20	76.12[b] ± 3.38
$LSD_{0.05}$	2.3816	3.8815	3.67	0.0167	1.0306

*Means (±SE) within columns followed by the same letter are not significantly different from each other, LSD test.

Table 2
Means of calli relative growth rate (RGR) and water content (WC, %) derived from leaf explants of sugarcane after treatment with different concentrations of mannitol.

Variety	Mannitol (mM(	RGR	% WC
GT 54-9	Control	2.83[*a] ± 0.011	92.5[a] ± 0.57
	100	2.56[b] ± 0.022	84.3[c] ± 0.13
	200	2.49[c] ± 0.014	71.6[d] ± 0.70
	300	1.01 [g] ± 0.005	63.4[f] ± 0.90
G 84-47	Control	2.49[c] ± 0.011	92.5[a] ± 0.72
	100	1.75[e] ± 0.007	85.8[c] ± 1.14
	200	1.11[f] ± 0.004	68.3[e] ± 0.68
	300	0.93 [h] ± 0.011	63.0[f] ± 1.35
ph 8013	Control	2.39[d] ± 0.014	93.1[a] ± 0.39
	100	0.24[i] ± 0.007	90.2[b] ± 0.63
	200	0.19[j] ± 0.015	66.7[e] ± 0.57
	300	0.09 [k] ± 0.002	54.5 [g] ± 0.80
$LSD_{0.05}$		0.033	2.06

*Means (±SE) within columns followed by the same letter are not significantly different from each other, LSD test.

Table 3
The contents of K^+ and Na^+ ions in the three sugarcane genotypes.

Genotypes	Characters	
	Na^+ mg g^{-1} (FW)	K^+ mg g^{-1} (FW)
GT 54-9	0.81[*b] ± 0.05	1.39[c] ± 0.03
G 84-47	0.89[a] ± 0.03	1.55[b] ± 0.07
ph 8013	0.74[c] ± 0.05	1.81[a] ± 0.09
$LSD_{0.05}$	0.03	0.05

[*] Means (±SE) within columns followed by the same letter are not significantly different from each other, LSD test.

detected among the three genotypes for K^+ content under 300 mM mannitol.

3.4. The content of proline

The proline content was increased with the increase in the concentration of mannitol in the three genotypes (Table 4, Fig. 2). Our results also show that the increase or decrease in the proline content was due to the change in mannitol concentration.

3.5. The effect of drought on morphological characters in regeneration

3.5.1. Shoot and root formation

The results in Table 5 indicate no significant differences between GT 54-9 and G 84-47 for the shoot, while pH 8013 had the lowest value (Figs. 3 and 4). However, the pH 8013 genotype showed the largest number of days of root formation comparing with the other two genotypes. The highest number of roots was recorded in GT 54-9 followed by G 84-47 and then pH 8013. Analysis of variance indicates the highest value for shoot formation in GT 54-9 comparing with the other two genotypes, and pH 8013 showed the lowest drought tolerance under different concentrations of mannitol. Analysis of variance indicates the largest number of days for root formation in pH 8013 comparing with the other two genotypes (GT 54-9 and G 84-47) for control, 100, 200, and 300 mM mannitol. The results in Tables 6 indicate the lowest number of days root formation in GT 54-9 under different concentrations of mannitol. Analysis of variance indicates the highest number of root formation in GT 54-9 comparing with the other two genotypes under different concentrations of mannitol.

3.5.2. The RAPD analysis

The RAPD analysis was employed to investigate genetic diversity among the three sugarcane genotypes evaluated under three different mannitol concentrations (100, 200, and 300 mM) using five primers (Fig. 5, Tables 7 and S3). The used primers presented polymorphism ranged from 57 to 94% and gave fragments ranged from 7 to 17%. The OPD-03 primer amplified the maximal polymorphic bands (17), resulting in a 100% polymorphism with a size ranging from 126 to 1619 bp (Fig. 5, Table 7). However, the minimal polymorphic bands (7) were amplified by the OPO-02 primer, resulting in a 57% polymorphism with a size ranging from 156 to 787 bp.

In the current study, five oligonucleotide primers (100%) used in the RAPD analysis gave unique markers. Out of the five primers,

Table 4
The contents of K^+ and Na^+ ions in sugarcane calli after treatment with different concentrations of mannitol.

Genotypes	Mannitol (mM)	K^+ (mg g^{-1} FW)	Na^+ (mg g^{-1} FW)	Proline (μg g^{-1} FW) mean ± standard Error (Y)
GT 54-9	Control	1.61[*d] ± 0.02	0.56[e] ± 0.02	153.3 ± 0.88
	100	1.41 [fg] ± 0.03	0.58[e] ± 0.01	383.3 ± 1.21
	200	1.37 [g] ± 0.02	0.99[b] ± 0.01	723.3 ± 3.18
	300	1.19 [h] ± 0.01	1.09[a] ± 0.03	1120.0 ± 1.73
G 84-47	Control	2.07[b] ± 0.05	0.69[d] ± 0.04	110.0 ± 0.58
	100	1.47[ef] ± 0.04	0.83[c] ± 0.01	363.3 ± 0.67
	200	1.43 [fg] ± 0.05	0.99[b] ± 0.01	656.7 ± 2.33
	300	1.21 [h] ± 0.02	1.07[a] ± 0.03	1043.3 ± 3.18
ph 8013	Control	2.57[a] ± 0.02	0.41[f] ± 0.01	113.3 ± 0.88
	100	1.75[c] ± 0.05	0.56[e] ± 0.02	246.7 ± 1.45
	200	1.57[de] ± 0.04	0.99[b] ± 0.01	603.3 ± 2.73
	300	1.34 [g] ± 0.04	0.99[b] ± 0.01	860.0 ± 3.00
$LSD_{0.05}$		0.10	0.05	

*Means (±SE) within columns followed by the same letter are not significantly different from each other, LSD test.

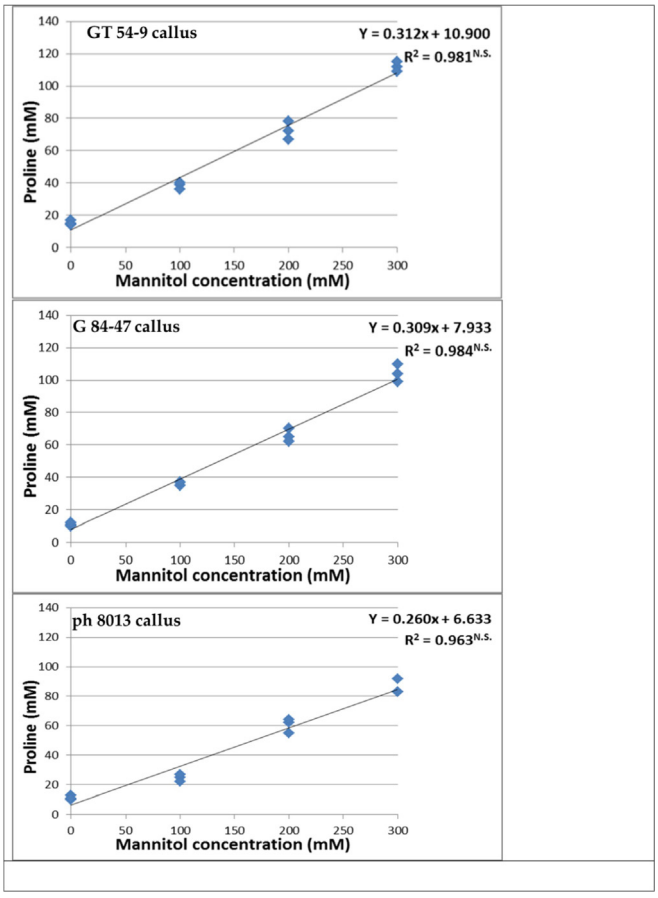

Fig. 2. Regression between mannitol concentrations (X-factor) and the proline (Y-factor) in sugarcane callus.

Table 5
Number of shoots and roots, and number of days for root formation in the three tested genotypes.

Genotypes	Characters		
	Number of shoots	Number of days for root formation	Number of roots
GT 54-9	6.33[*a] ± 0.55	7.58[c] ± 0.51	11.75[a] ± 0.51
G 84-48	6.25[a] ± 0.49	9.25[b] ± 0.49	10.33[b] ± 0.45
ph 8013	4.67[b] ± 0.59	11.25[a] ± 0.46	9.58[c] ± 0.41
$LSD_{0.05}$	0.46	1.29	1.18

*Means (±SE) within columns followed by the same letter are not significantly different from each other, LSD test.

five were detected for GT 54-9 and 8 for G 84-47. Data in Table 7 showed the similarity matrix of five RABD-PCR markers. The three sugarcane genotypes were divided by clustering analysis into two prime groups at 59% similarity. The first group included the genotype G84-47 with three concentrations (100, 200, and 300 mM) of mannitol with 70% similarity, along with control with 68% similarity. The genotypes (ph 8013 and GT 54-9; Table S3) of the second group were divided into two subgroups with 65% similarity. The main consolation for this study indicates a promising drought-tolerant Gt 54-9 genotype comparing with G 84-47 and pH 8013, considering all morphological and molecular studies.

4. Discussion

This study aimed at evaluating some sugarcane genotypes (e.g., Gt 54-9, G 84-47, and pH 8013) for their capabilities to induce callus formation, the number of days for callus initiation, and embryogenic calli. The effect of drought stress on tolerance to water scarcity in calli of sugarcane genotypes was examined under different concentration (e.g., 100, 200, and 300 mM) of mannitol. This examination was conducted by assessment of the relative growth

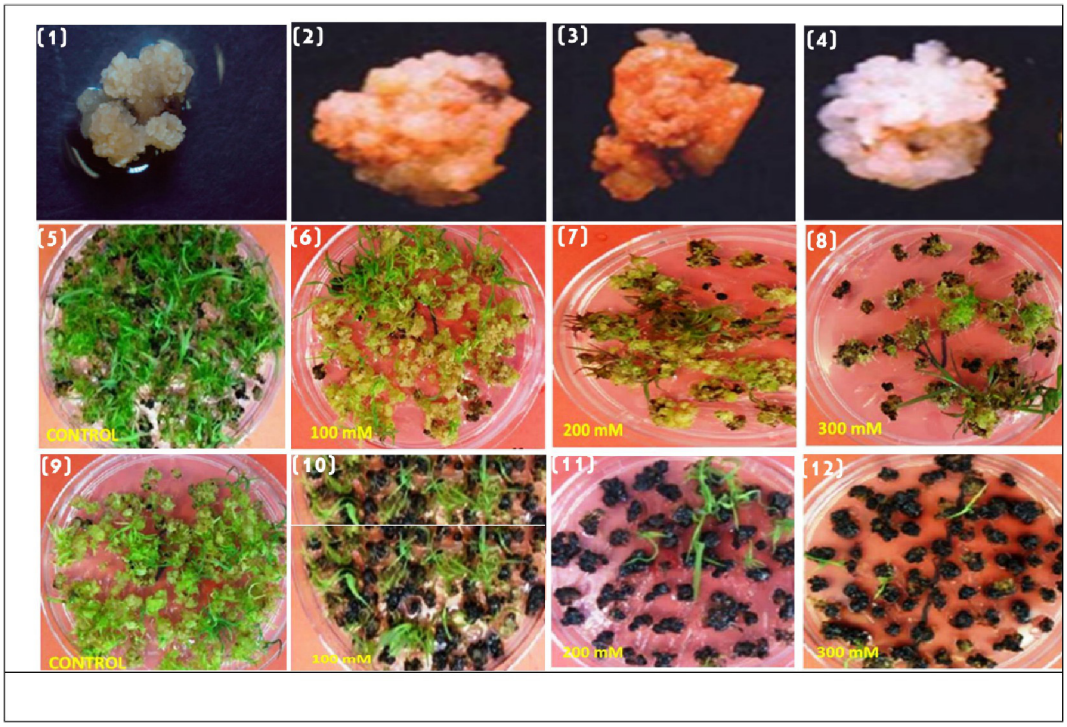

Fig. 3. The effect of different concentrations of mannitol on percentage of callus water content; (1) control, (2) 100 mM, (3) 200 mM, (4) 300 mM, (5–8) Regeneration of GT 54-9 obtained from tolerant callus under different mannitol concentrations, and (9–12) regeneration of pH 8013 under different mannitol concentrations.

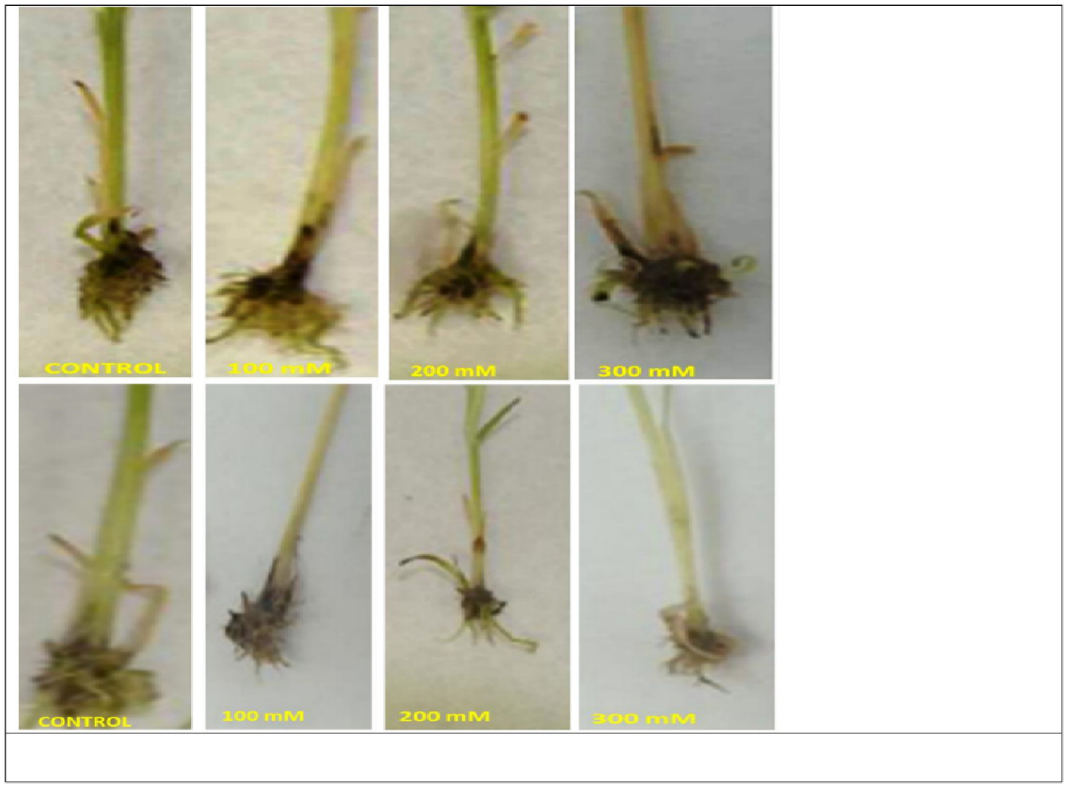

Fig. 4. Difference in number of root formation for GT 54-9 (up) and G 84-47 (down) under different mannitol concentration.

Table 6
Interaction between shoot and root formation, and number day for root formation in the three tested genotypes.

genotypes	Mannitol (mM)	Number of shoot formation	Number of days for root formation	Number of root formation
GT 54-9	Control	9.33^{*a} ± 0.42	07.3^{de} ± 0.9	14.0^{a} ± 1.3
	100	8.00^{c} ± 0.36	07.0^{e} ± 1.0	12.0^{ab} ± 0.7
	200	4.67^{e} ± 0.55	08.0^{cde} ± 1.3	10.7^{cb} ± 0.6
	300	3.33^{f} ± 0.42	08.0^{cde} ± 1.1	10.3^{cb} ± 0.8
G 84-48	Control	9.00^{ab} ± 0.35	09.0^{cde} ± 1.1	11.3^{b} ± 0.9
	100	7.67^{c} ± 0.42	09.0^{cde} ± 1.5	11.3^{b} ± 0.8
	200	5.00^{e} ± 0.36	09.3^{cdeb} ± 0.6	10.3^{cb} ± 0.6
	300	3.33^{f} ± 0.21	09.7^{cdb} ± 0.9	08.3^{c} ± 0.9
ph 8013	Control	8.33^{bc} ± 0.21	11.7^{ab} ± 1.3	10.3^{cb} ± 0.9
	100	6.00^{d} ± 0.36	10.3^{cab} ± 0.6	09.7^{cb} ± 0.5
	200	3.33^{f} ± 0.21	12.7^{a} ± 0.6	10.0^{cb} ± 0.4
	300	1.00 ^{g} ± 0.36	10.3^{cab} ± 0.9	08.3^{c} ± 1.2
$LSD_{0.05}$		0.91	2.59	2.37

*Means (±SE) within columns followed by the same letter are not significantly different from each other, LSD test.

rate (RGR), Na^+, and K^+ ion accumulations, proline content, and shoot and root formation. Identifying the genetic variation among sugarcane genotypes was also examined via the RAPD-PCR analysis. Our results confirm previous findings on sugarcane (Badawy et al., 2008) and rice (Van Sanford et al., 2001), as they found that callus induction capacity is genotype-dependent. Additionally, Errabii et al. (2007) tested the sugarcane callus with MS medium with some stress factors and reported that these stresses reduced the RGR for all the tested genotypes. Patade et al. (2011) reported a progressive decrease in RGR of sugarcane callus with an increase in the level of stress. Our results confirm the findings of Patade et al. (2011), where they indicated a sharp decrease in growth of sugarcane callus on salt-stressed media at equal osmotic levels. This indicates a stress sensitivity of undifferentiated tissues and their growth. The current research was also in the line with that of Errabii et al. (2007) who observed a decrease in the water content and a radical effect on ion concentrations in sugarcane callus under both mannitol and NaCl. They added that the maximum RGR and water content are reversed under mannitol-induced osmotic stress. This finding has also been reported with other species; *Tagetes minuta* (Mohamed et al., 2000) and *Populus euphratica* (Watanabe et al., 2000). Like our findings, Patade et al. (2008) reported Na^+ ion accumulation, along with K^+ ion and osmolyte (glycine betaine and proline) accumulations due to their pivotal roles in osmotic adjustment in the stressed cells of the sugarcane callus. However, ions tend to accumulate in excess in the cytoplasm as cells continue to be able to compartmentalize ions in their vacuoles until their capacity to contain ions is completed. This leads to acute ion imbalances and conformational alterations in the electrical potential of the plasma membranes (Chinnusamy et al., 2006). However, Most of the K^+ ions would primarily affect bulk solution properties (as opposed to the μmolar amounts

required to meet tissue nutritional requirements) such as the electrical charge of the system (Niedz and Evens, 2008). Besides, the increase in K^+ ion helps to remove ionic confounding in the callus for well growth and regenration. K^+ ion played an effective role in drought tolerance through increasing its content in the callus tissues as an osmoprotectant to maintain sufficient water for growth and regeneration processes (Alzahrani et al., 2018; Rehman et al., 2018; Obaid and Reddy, 2019; Rady et al., 2019b).

Under stress, the elevated Na^+ concentration was previously observed along with the low K^+ concentration in *Cynara cardunculus* (Benlloch-González et al., 2005). Some species can replace Na^+ ions with K^+ ions to positively modify the osmotic modification. Similar results were obtained concerning proline accumulation, which is an effective strategy for stressed plant tissues and cells (Errabii et al., 2007). Balibrea et al. (1999) also obtained variation in proline content associated with plant tolerance and ability to adapt to saline conditions. Besides, the high accumulation of proline is often associated with sugarcane, which is more salt-tolerant than unselected (Gandonou et al., 2005; Elrys et al., 2018; Sitohy et al., 2020). The trend of our results confirms the findings of Cano et al. (1996) who reported high proline accumulation in stress-resistant genotypes as compared to stress-sensitive ones. Besides, proline has been widely adopted as a marker for stress resistance (Alvarez et al., 2003). Additionally, proline can be employed as an organic N reserve, which is ready to use after attenuating the stress to keep the synthesis of both protein and amino acids (Sairam and Tyagi, 2004; Mahajan and Tuteja, 2005; Ashraf, 2010; Elrys et al., 2019). In general, an increase in the con-

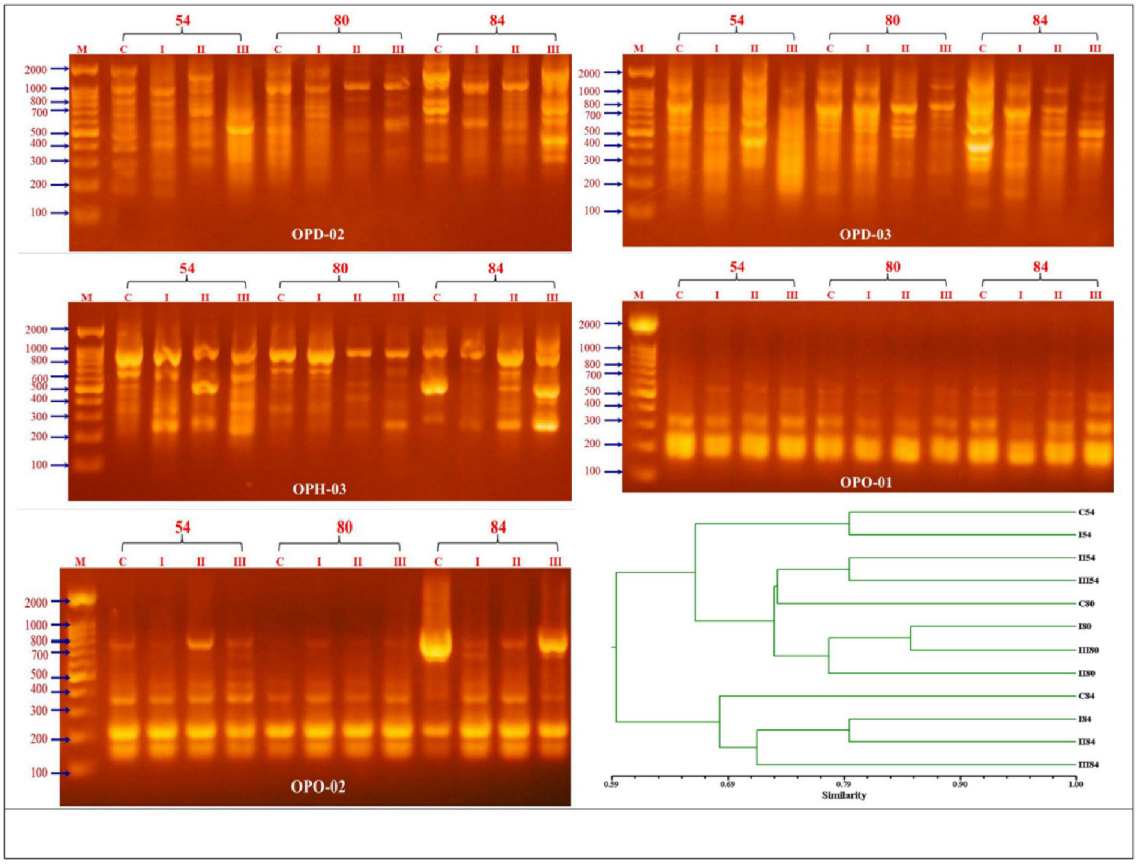

Fig. 5. The DNA polymorphism of sugarcane (*Saccharum officinarum* L.) treated with mannitol using randomly amplified polymorphic DNA with primers OPD-02, OPD-03, OPH-03, OPO-01 and OPO-02 (54: GT 54-9, 80: ph 8013, 84: G 84-47, M: marker, C: control, I: 100 mM, II: 200 mM, and III: 300 mM mannitol) and dendrogram of the three genotypes using five random amplified (RAPD) primers.

Table 7
The DNA polymorphism using randomly amplified polymorphic DNA with primers OPD-02, OPD-03, OPH-03, OPO-01, and OP0-02 under different mannitol concentrations.

Parameters and primers name	Primer Polymorphism				
	OPD-02	OPD-03	OPH-03	OPO-01	OPO-02
Monomorphic fragments	1	0	1	2	3
Polymorphic (without Unique)	14	11	7	5	3
Unique fragments	2	6	4	2	1
Polymorphic (with Unique)	16	17	11	7	4
Total number of loci	17	17	12	9	7
Polymorphism (%)	94%	100%	92%	78%	57%
Mean of fragments frequency	0.377	0.323	0.417	0.509	0.726

centration of mannitol as *iso*-osmotic stress in sugarcane leads to an increased accumulation of proline. In another study, María et al. (1997) reported that somatic embryogenesis is renowned as a crucial pathway for plant regeneration and a model system helpful for investigating the events of molecular biology, biochemistry, and morphology that all occur during early plant development.

Our results confirm the findings of Watt et al. (2009), who studied *in vitro* sugarcane regeneration and reported various outcomes of the genetic stability of *in vitro*-derived plantlets. The variation ranged from little or no evidence of somaclonal variation to high levels regardless of the morphogenesis path. Moreover, when phenotypic differences are discovered, they are in transit as the variants are due to the original parent phenotype in the first ratoon crop. Our results also confirm the findings of Singh (2003), who stated that after induction of somatic embryos, calli were subcultured on a medium for plant regeneration. The results indicate the maximum plant regeneration in control cultures to regenerate the plant for each genotype. The author also mentioned that the efficiency of plant regeneration decreases under the increased stress of NaCl. He also mentioned that no regeneration is observed on MS4c and MS4b cultures of the Thatta-10 and SPHS-19 genotypes, while the CPF-237 sugarcane genotype regenerated 2.21 ± 0.17 plantlets per callus. The same trend was noticed by Rao and Ftz (2013), who developed an *in vitro* selection system for drought-tolerant callus in sugarcane lines. The number of shoots per callus was higher (98 ± 0.15) in the non-selected callus comparing with the selected callus (36 ± 0.18), and the plantlets generated from the selected callus displayed 86% rooting (10–12 roots per plantlet) within 10 days of transferring (Rao and Ftz, 2013, Elrys et al., 2020). In another study, Koch et al. (2012) reported a considerable decrease in sugarcane plantlet production associated with untreated samples. An elevated proline level due to drought stress indicates that the species will tolerate the stress. Thus, an increased proline level in GT 54-9 indicates that it is more tolerant to drought stress than pH 8013, which resulted in a lowered proline level. The disturbance in plant metabolism resulting from abiotic treatments generally affects the different metabolic pools of stressed plants (Abdel Latef et al., 2021a, b). These differences in the contents of the metabolites under drought stress may indicate improvement or delay in metabolite synthesis and accumulations. Since it is often accumulated in stressed cells compared to other amino acids, proline is always related to the form of stress experienced by plant cells, and proline levels differ depending on the degree of stress.

The present work used a review (Koebner and Summers, 2003), in which it has been stated that genetic differences between individual species or organisms are represented by genetic markers. Usually, all genetic markers occupy specific genomic positions within chromosomes. Using DNA markers can help breeders improve the quickness and accuracy of the process. Genetic labeling and DNA fingerprinting are particularly suitable for building a hierarchy of wanted characteristics. Ten elite sugarcane clones were tested for genetic diversity utilizing RAPD analysis. The activity of sucrose synthase was assayed *via* TRAP and tolerance to drought was assessed using STS techniques/field experiment. The RAPD examination displayed that genetically most similar genotypes were Thatta-10 and AEC82-223 (80.4%) and the most dissimilar genotypes were AEC71- 2011 and NIA-2004 (49.8%). Based on the dendrogram, the varieties could be divided into four clusters (A to D). The AEC 82-223 variety generated a specific allele of 311 bp with the B-02 primer.

Finally, it has been mentioned that RAPD results in amplification of few random segments of DNA, allowing for variation in length and number of amplified segments when the sequence of the segments is altered (Shinohara et al., 1997; Desoky et al., 2019). RAPDs have been used widely for the analysis of genetic variation. The results obtained by Ngezahayo et al. (2007) and Cuesta et al. (2010) recommend the use of RAPDs for the analysis of somaclonal variation.

5. Conclusions

A high degree of callus proliferation was obtained and ranged from 70 to 86% and the highest frequency was recorded for the GT 54-9 genotype comparing with the other genotypes (G 84-47 and pH 8013). Besides, the highest percentage of embryogenic callus was 80%. The results showed a maximum RGR value for the GT 54-9 genotype, while the analysis of variance showed a highly significant difference among the three genotypes for Na^+ and K^+ ion accumulations. As the concentration of mannitol increased, the proline content increased. Analysis of variance indicated the highest values for shoot formation in the GT 54-9 genotype compared with the G 84-47 and pH 8013 genotypes. The main result of this study was that the GT-54-9 genotype is considered the most promising genotype, which showed more tolerance to drought stress based on all the studied traits. Besides, the five oligonucleotide primers (100%) used in the RAPD analysis gave unique markers. Out of the five primers, five were detected for the GT 54-9 genotype and eight for the G 84-47 genotype. The results showed the similarity matrix of five RABD-PCR markers. Cluster analysis divided the three sugarcane genotypes into two main groups in 59% similarity.

6. Ethical disclosures**a

The authors announce that no experiments were performed on animals and no data were collected from patient in this research.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2021.08.047.

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