CHAPTER THREE

MATERAILS AND METHODS

3.1. Materials

3.1.1. Source of Genotypes

The sugarcane genotypes used in this study namely G 84-47, Ph8013 and with the commercial genotype GT 54-9. They are under the genus *Saccharum* (Table 1).

Table (1) Sugarcane genotypes parentage and time of release used in the present study.

Genotypes	Parentage	Production location	Time of release
G.84-47	NCo.310 **x** Unknown	Local's fuzz	Promising genotypes (1995)
Ph 8013	CAC.71-312 **x** Phil.642227	Philippines's cutting	Promising genotypes (2002)
G.T.54-9	NCo.310 **x** F.37-925	Taiwan's fuzz	The check variety (1981)

Source: Sugar crop research institute

G.T. Giza-Taiwan (Sugar crop research institute, Giza, Egypt; Taiwan).
Phil. Philippines.
CAC. Collage of Agriculture Cane. Philippines.
NCo. Natal-Coimbatore (Natal, South Africa; Coimbatore, India).
F. Formosa, Taiwan.

The three genotypes were provided from Sugar Crop Research Institute, Genetic and Breeding Department, Cairo, Egypt.

3.1.2. Tissue Culture techniques

3.1.2.1. Murashige and Skoog Media

Murashige and Skoog medium was purchased from Duchefa Biochemical (Duchefa, Postbus 809, Haarlem, Netherlands). The medium composition is shown in Table (2).

Table (2) Murashige and Skoog, (1962) medium composition

Components	Elements	Mg l^{-1}
Macro elements	NH_4NO_3	1650
	KNO_3	1900
	$CaCl_2.2H_2O$	440
	$MgSO_2.7H_2O$	370
	KH_2PO_4	170
Micro elements	KI	0.83
	H_3BO_3	6.20
	$MnSO_4.4H_2O$	22.3
	$ZnSO_4.7H_2O$	8.60
	$Na_2MoO_4.2H_2O$	0.25
	$CuSO_4.5H_2O$	0.025
	$CoCl_2.6H_2O$	0.025
	Na_2.EDTA	37.3
	$FeSO_4.7H_2O$	27.80
Vitamins	Myo-Inositol	100
	Nicotinic acid	0.00
	Pyridoxine HCl	0.50
	Thiamine HCl	0.10
	Glycine	2.00

3.1.2.2. Plant Growth Regulators

The following plant growth regulators were used for sugarcane callus induction and plant regeneration.

1- NAA (1-Nphthylacetic acid): ($C_{12}H_{10}O_6$) LOBA Chemie (LOBA Chemie LTD, P.Box 2042, Mumbai, 400 002, India).

2- 2,4-D (2,4- Dichlorophenoxy Acetic Acid): ($C_8H_6C_{l2}O_3$) Blulux Laboratories Ltd, India.

3- Kinetin (6- Furfurylaminopurine): ($C_{10}H_9N_5O$) Himedia Laboratories Pvt. Ltd. Mumbai, 400 086, India.

3.1.2.3. Other Components

1- Casein hydrolysate (Sigma, USA)	00.5 gm l^{-1}
2- Sucrose (Hullets, SA)	30.0 gm l^{-1}
3- Agar- agar (Biolab)	08.0 gm l^{-1}
4- Coconut water	100 ml l^{-1}

3.1.3. Abiotic Stress Chemicals

- **Mannitol $C_6H_8(OH)_6$:** Mannitol classified as sugar alcohol. (El-Nasr pharmaceutical chemicals Company, Abu Zaabal, Egypt)

3.1.4. Ions Determinations (K^+ & Na^+)

- **Nitric Acid HNO_3 (Sigma, USA) (**El-Nasr pharmaceutical chemicals Company, Abu Zaabal, Egypt).

3.1.5. Molecular Study

RAPD analysis was carried out using five oligonucleotide primers (Table 3) that were selected from the Operon Kit (Operon Technologies Inc., Alabameda, CA).

Table (3) The nucleotide sequences of primers used for RAPD analysis.

Primer number	Primer code	Sequence (5`-3`)
1	OPD-02	GGA CCC AAC C
2	OPD-03	GTC GCC GTC A
3	OPH-03	AGA CGT CCA C
4	OPO-01	GGCACGTAAG
5	OPO-02	ACGTAGCGTC

3.2. Methods

The present investigation was carried out during 2011, 2012 and 2013 at the Tissue Culture Laboratory of Agriculture Research Center. El-Sabahya, Alexandria, Central Laboratory at The Faculty of Science, University of Alexandria and Institute of Genetic Engineering & Biotechnology Research. Sadat City University.

3.2.1. Callus Production

Three Sugarcane genotypes ph 8013, GT 54-9 and G 84-47 were selected and tested in the present research. Stem sections containing two lateral buds were planted in plastic pots containing mixed component (v/v) soil: sand: patmos 1: 1: 1 in greenhouse conditions until reaching ~6 months. The explants from 6-8 month old, healthy, disease free were cut the shoot tip which used in our study.

3.2.1.1. Media Content

Murashige and Skoog (MS) basal media was used in the present study for induction embryogenic. The media composition as recommended by **Murashige and Skoog (1962)** was shown in Table (2). Media was supplemented with 3% sucrose and solidified with 8g/L Agar. For callus induction, MS medium supplemented with 3 mg/L 2,4-D, 0.5 gm l^{-1}Casein hydrolysate and 10% coconut water were used as recommended by **Roy *et al.* (2011)**

3.2.1.2. Media Preparation and Sterilization

Into a 1000 ml Erlenmeyer flask, 500 ml of distilled water and eight grams of agar were added and cooked on a hot plate with stirring until they completely melted. Into another 1000 ml Erlenmeyer flask, 400 ml of distilled water was added and the rest of media component were weighted, added to water and mixed well.

After the agar was completely melted, it was mixed with the rest of the media component and the volume was completed to one liter with d-H_2O. The

pH of all media treatments used was adjusted to pH 5.7 - 5.8, using 0.1 M of HCl or 0.1 M of NaOH. The media were poured in 150 ml jars, then, sterilized by autoclaving for 20 min. at 121°C and pressure 1.1 kg/cm^2.

3.2.1.3. Culture Preparation and Conditioning

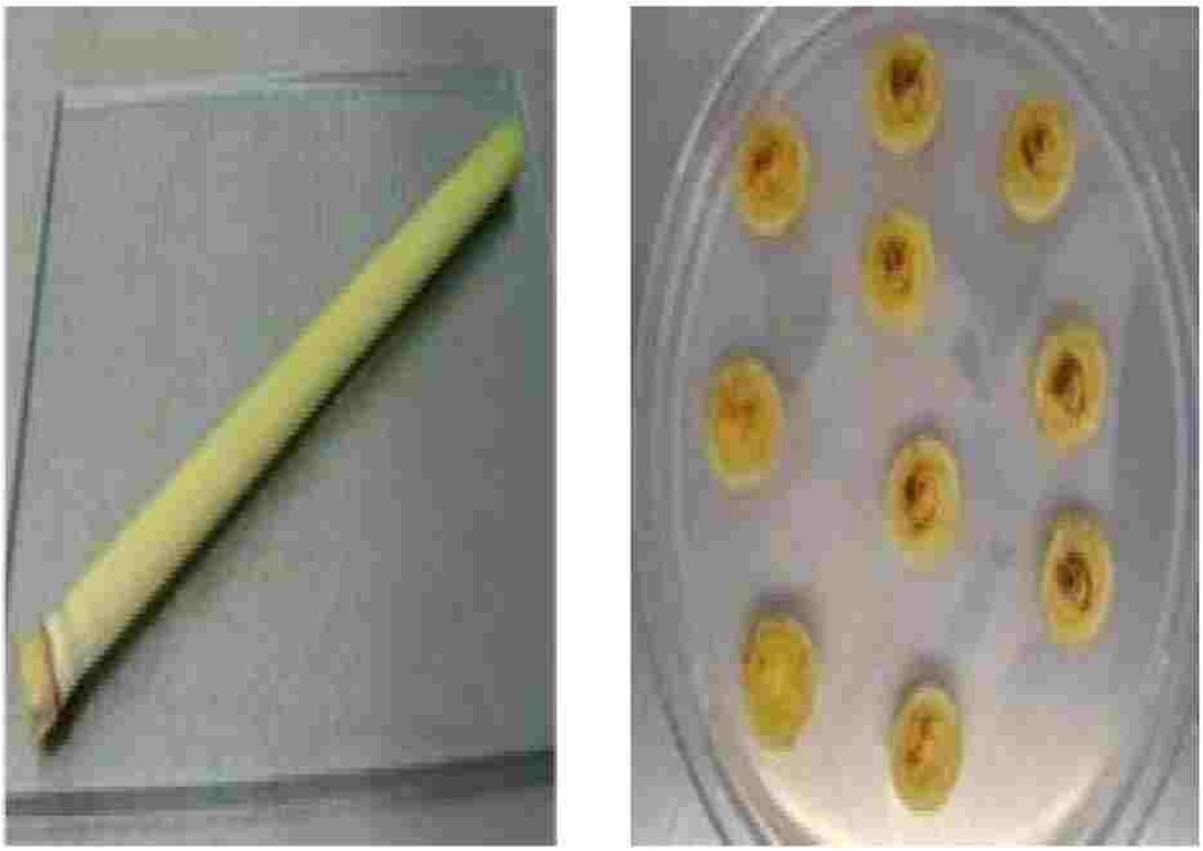
Sugarcane stalks were harvested by cutting the stalk at the base using a pair of secateurs. The immature leaf roll was removed above the natural breaking point of the sugarcane stalk and leaves were pruned from the apical stalk sections. In the laboratory, the leaf roll was cut transversely on the upper end to 100-150 mm (Figure. 1) and surface sterilized by swabbing with 95 % (v/v) ethanol.

Thereafter, in the laminar flow under aseptic conditions, the outer leaf sheaths of the stalk apices were removed using a sterile scalpel until the inner immature leaf roll was exposed. Stem sections were surface sterilized with 75% (v/v) ethanol. Then, immersed in mercuric chloride $HgCl_2$ 0.03% (w/v) for 30 min followed by three rinses with sterile distilled water for 10 min each that recommended by (**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 onto solidified medium in Petri dishes 10 sections per plate (Figure 1) with the ventral surface of each disk in contact with the medium. The experimental were performed in Petri dishes using 25-30 ml of medium. Ten sections were cultured on surface of agar solidified medium in Petri dishes. Each dish was considered as one replication resulted in five replications for each genotype. The plates were sealed with parafilm and incubated in the dark at 25± 2°C, sub culturing on to fresh medium every 2 weeks.

3.2.1.4. Callus Induction Response

The flowing variables were recorded for each Petri dish in the present study: (1) Number of day to callus initiation and (2) Percentage of embryogenic callus derived from the three genotypes.



(a) **(b)**

Figure (1) Material of sugarcane used in the current study (a) Sugarcane leaf roll and (b) leaf sections on solidified medium.

3.2.2. *In Vitro* Drought Tolerance

3.2.2.1. Mannitol ($C_6H_8OH_6$) Treatment

Mannitol was added directly to the culture media before autoclaving at the final concentrations of 0, 100, 200 and 300 mM **(Errabii *et al.* 2007)**. Medium pH was adjusted to 5.7, and then the media was poured in sterilized 150 ml jars, then, sterilized by autoclaving, after ten week from culture 30-35 calli were individually placed on MS medium supplemented or not with the stress factors. Iso-osmotic concentrations mannitol (100, 200 and 300 mM).

3.2.2.2. Callus Relative Growth Rate and Water Content

After 10 weeks, calli were individually weighed and placed on MS medium supplemented or not with the stress factors. For each treatment (genotype. stress factor concentration), 30–35 Calli were used. After 4 weeks of the exposure to stress conditions, the calli were weighed and then they were characterized for ion and proline concentrations.

Callus relative growth rate (RGR) was determined on a fresh weight (FW) basis according to the formula;

$RGR = [(FW_f - FW_i)/FW_i]$,

Where, FW_f and FW_i are the final and initial FW of the calli.

The % of callus water content was calculated using the formula;

[(FW – DW)/DW],

Where, FW and DW are, respectively, the fresh weight and dry weight of the calli **(Patade *et al.* 2014)**.

3.2.2.3. Determination of K^+ and Na^+ Ions Concentration

For ion measurements, calli were first rinsed for 5 min with cool distilled water in order to remove free ions from the apoplasm without substantial elimination of cytosolic solutes as recommended by **Sacchi *et al.* (1995)**.

Calli were oven-dried at 80 °C for 72 h and they were then grounded. The dry matter obtained was used for mineral analysis. The major contains were extracted after digestion of dry matter with HNO3 acid according to **Lutts *et al.***

(1996). The extract was filtered prior to analysis. Na^+ and K^+ concentrations were determined using a flame spectrophotometer (PHF 90D, France).

3.2.2.4. Proline Determination

Proline was determined according to the method of **Bates *et al.* (1973).** 3% Aqueous Sulfosalicylic Acid, Acid Ninhydrin: 1.25 gm Ninhydrin, 30 ml glacial acetic acid, 20 ml 6M phosphoric acid. The mixture was warmed with agitation until dissolved then kept cool at 4 °C until use.

3.2.2.5. Extraction Procedure

0.5 gm sample of leaf material was homogenized in 10 ml extraction buffer. The homogenate was filtered through Whitman No. 2 filter paper. 2 ml of filtrate were reacted with 2 ml acid ninhydrin and 2 ml glacial acetic acid in a test tube for 1 h at 100 ° C, the reaction was terminated in an ice bath. The reaction mixture was extracted with 4 ml toluene mixed vigorously in a test tube with a stirrer for 15-20 sec. The chromophore containing toluene was aspirated from the aqueous phase and the absorbance was determined with spectrophotometer at 520 nm using toluene as a blank.

3.2.2.6. Proline Standard Curve

Proline standard solution was prepared by dissolving 100 mg proline in 100 ml. of 3% aqueous sulfosalicylic acid. Aliquots of 10 µl to 50 µl of the Proline solution were put into test tubes. Then, the total volume was adjusted to 1 ml using 3% aqueous sulfosalicylic acid. Each tube was treated as previously described.

3.2.3. Shoot Regeneration

The embryogenic calli were selected for plant regeneration and transferred to medium content full strength of MS supplement with 1 mg/l kin. The calli were incubated in 16 hours illumination (2,000 Lux, day light fluorescent tubes) at 20±5 for four weeks. The numbers of calli with green shoot (shoot formation) were recorded.

3.2.4. Root Formation

Shoot plantlet were grown until reaching 5cm in height, then transferred to medium content half strength of MS with other supplement plus 0.2 mg/l NAA. The plantlets were incubated in 16 hours illumination (2,000 Lux, day light fluorescent tubes) at 20±5 to record numbers of days to root indication. Then after four weeks number of roots was recorded.

3.2.5. Molecular Study

3.2.5.1. Total Genomic DNA Extraction

For each sample, genomic-DNA from sugarcane levees were extracted through DNA isolation kit (Gene JET^{TM}, plant genomic DNA purification mini kit. Fermentas). To purify DNA from polyphenol, Lysis Buffer was supplemented with poly vinyl pyrrolidone (PVP) at 2% (W/V) final concentration. DNA was quantified by Gene quant at absorbance of 260/280nm. The quality was further checked on 0.1% agarose gel.

The used primers were selected from the Operon kits (Operon Technologies Inc., Alabameda CA, USA). RAPD-PCR analysis was performed according to the method of **Williames *et al.* (1990).**

3.2.5.2. Random Amplified Polymorphic (RAPD- PCR) Analysis

The polymerase chain reaction mixture (25 µl) consisted of 12.5µl of Maximo *Taq* DNA Polymerase 2X-preMix (Gene ON, Germany) contains (0.1U/ul Taq DNA Polymerase, 0.4mM of each dATP, 4mM MgSO4, 20mM KCl, 16mM $(NH_4)_2SO_4$, and 20mM Tris-HCl, pH8.8), 0.5µM of primer and 50 ng of genomic DNA.

PCR amplification was performed in a Biometra *T1* gradient thermalcycler for 35 cycles after initial denaturation for 5 min at 94°C. Each cycle consisted of denaturation at 94°C for 1 min; annealing at 35°C for 1 min; extension at 72°C for 2 min and final extension at 72°C for 10 min. Amplification products were separated on 1% agarose gels at 100 volts for 1.30 hrs with 1 x TBE buffer **(Williames *et al.*, 1990)**. To detect ethidium bromide/DNA complex, agarose gels were examined on ultraviolet transilluminator (302 nm wavelength) and photographed. Using 100 pb DNA ladder (Fermentas), the lengths of the different DNA fragments were determined.

3.2.5.3. Data Analysis

Phoretix electrophoresis gel image analysis, ID software was used for scanogram tracing of protein bands of the tissues. The molecular weights of protein bands were determined against protein calibration kits (Spectra™ Multicolor Broa Range Protein Ladder; fermentas life sciences under the license for Strep-tag® technology).

Data matrices were entered into the NTSYS (Numerical Taxonomic and Multivariate Analysis System) program, version 2.1, Applied Biostatistics Inc. **(Rohlf, 2000)**. Similarity coefficients were used to construct dendrograms using the UPGMA (Un-weighted Pair Group Method with Arithmetic average) and the SAHN (Sequential Agglomerative Hierarchical Nested clustering) routing in the NTSYS software.

3.2.6. Statistical Analysis

One Way ANOVA in completely randomized design was used to reveal the significant differences among the samples. The L.S.D. (least significant differences) test was conducted to identify the significant differences among the means at 5% level of probability. Comparison of the mean values is usually calculated after an ANOVA **(Waller and Duncan, 1969)**. Each analysis done by Comparing Means procedure which indicated at the least significant difference (L.S.D.) for the means at the chosen level of significance. The L.S.D. is often used for doing just of few planned comparisons of means. The L.S.D. should be used for comparing all pairs of means only if an ANOVA indicates that significant differences exist. (**Waston, 1952).**

The statistical analysis was conducted using SAS® (1990) software program. GLM procedure of SAS was used. Mean differences were tasted by Duncan's New Multiple Range Test **(Duncan, 1955)**. When significant P value was obtained. 6-2- For the all traits analysis of variance was based on a factorial experimental of 3 x 4 according to the following model.

$$Y_{ijkl} = \mu + R_i + V_j + T_k + (VT)_{jk} + e_{ijk}$$

Where:

Y_{ijkl}	is the observation on the ijklth individual,
.μ	is the overall mean common to all observations,
R_i	is the effect of ith replicates (.i = 1,2,3),
V_j	is the effect of jth varieties level (.j = 1,2,3),
T_k	is the effect of kth treatments (k = 1, 2).
$(VT)_{jk}$	is the interaction effect of jth varieties by kth trearments
.e_{ijkl}	is the random error

Regression coefficient (r) was calculated between these two variables. proline which was concerned as the dependent one (Y) while the mannitol concentration was determined as the independent variable (X).

$$b = \frac{\sum xy - \frac{(\sum x)(\sum y)}{n}}{\sum x^2 - \frac{(\sum x)^2}{n}}$$

Where, b: is the slope of the regression line.