

## Genetic stability detected on micropropagated Egyptian sugarcane cultivar (GT54-C9)

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**Abstract:** Direct regeneration of sugarcane cultivar (GT54-C9) through apical meristem is a sufficient protocol to obtain uniformed diseases-free plants. In this study, regeneration of shoots indicated that the highest number of shootlets, leaves and nodes were obtained from MS medium with 1mg/l BAP +0.25 mg/l NAA. However, the highest length of shootlet (16.9 cm) was noticed on MS medium fortified with 2mg/l KIN + 0.25 mg/l NAA. On the other hand, roots induction revealed that the highest length of roots (4.66cm) was resulted on ½ x MS contained 1mg/l IAA+ 0.5mg/l NAA. Whereas, the maximum number of roots (2.66) were recorded on ½ x MS supplemented with 1mg/l IBA+ 1mg/l NAA. Furthermore, using ISSR technique for mother leaf tissue (as a control) compared 6 subcultures of plantlets summarized that 100% of all obtained bands were monomorphic. Therefore, based on genetic analysis found that genetic stability among control and different subcultures and no soma clonal variations were detected.

**Key words:** Direct regeneration, apical meristem, sugarcane, BAP, KIN, NAA, IBA, IAA, ISSR

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Date of Submission: 30-11-2017

Date of acceptance: 30-12-2017

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### I. Introduction

Sugarcane is an important member in Gramineae [1] which is a distinct crop in the industry and the main source of raw materials for sugar production. It accounts for nearly 70% of row sugar produced globally [2]. In Egypt, sugarcane is cultivated on 139,451 Ha producing 16,055,013 tons of crushable stem [3]. Sugarcane plays an efficient role for sugar production, where about 80% of total sugar productions were obtained from sugarcane, while, the remaining is extracted from sugar beet [4].

The lack of specific weather for flowering of sugarcane in Egypt disturbs the traditional breeding for progressive improvement of traits. Sugarcane is a vegetable crop propagated on commercial level for cultivating by stem cutting. It needs up to 10 years to get an amelioration cultivar for commercial planting. There are also chances of perpetuation of mother plant diseases obtained by [5]. Thus, high rate of sugarcane multiplication can be obtained using tissue culture methods [6].

Tissue culture techniques are useful methods for rapid propagation and produced uniformed plants. In vitro micropropagation technique is a useful tool for rapid regeneration at larger scale and production of disease-free planting material in a number of crops [7&8]. Pathogen-free sugarcane plants and its mass propagation using apical meristem culture provided maximum numbers of germplasm for the farmers throughout the year [9]. Therefore, using modern *in vitro* techniques on a vital crop like sugarcane are given the chances for rapid production to obtain selective commercial sugarcane cultivars to provide the needs of sugarcane growers. In addition, to provide multiplication saving space and time. Micropropagation resulted in sufficient productivity of sugarcane clonal propagation by controlling a lot of problems which are faced during conventional breeding practices and multiplication procedure [10].

According to [11], who reported that molecular markers are necessary tools that can demonstrate complex genetically inherited characters and to directly observe genetic polymorphism. Molecular markers have been quite used to study genetic stability.

The sugarcane cultivar GT54-C9 is a necessary crop in Egypt which was widely spread among farmers [12]. Further, the present study was designed for optimal evaluation of growth hormones required by *in vitro* propagation of sugarcane cultivar (GT54-C9) under controlled conditions, which will further be supplied to farmers for culturing. In this study, a protocol based on a relatively limited number of six subcultures in order to avoid the appearance of somaclonal variation was established as well as to investigate the existence of somaclonal variations in sugarcane cultivar GT54-C9 resulted from micropropagation up until the 15<sup>th</sup> subculture, using ISSR DNA markers.

## **II- Materials and methods**

### **2-1 Plant material**

In this study, one cultivar (GT54-C9) was used. Sugarcane apical meristems were excised from 5-6 months old field grown sugarcane plants, provided by Sugar Crops Research Institute (SCRI) in Giza, Cairo, Egypt. This cultivar was identified by

### **2-2 Explants sterilization and preparation**

Actively growing apical meristems were taken from 5-6 months old healthy mother plants and used as explants. These explants were taken to the laboratory and washed thoroughly under running tap water for 30 minutes and the size reduced to 15 cm length by cutting off at the two ends. Further, sterilized in 30 % (v/v) by Clorox solution for 30 minutes, and then washed four times with sterile distilled water. Apical meristems were used after remove rolled leaves. Finally, they were submerged in mercuric chloride 0.2% for 5 minutes, Followed by several washing with sterile distilled water and sterilized meristems were cultured on selected medium under aseptic condition.

### **2-3 Culture conditions**

After culturing of sterilized meristems in jars containing 50 ml of MS medium. The incubation of jars was done in growth chamber at 25±1°C and exposed to 16 h/day photoperiod controlled automatically at intensity of 3000 lux from white cool light of fluorescent lamps (Phillips, Egypt).

### **2-4 Chemicals**

Agar (Agar- agar, Gum agar), BAP (6-BAP, N6- Benzyl adenine), KIN (6- Furfurylaminopurine, N6- Furfuryladenine), NAA( $\alpha$ - Naphthaleneacetic acid, NAA), IBA (4-(3-Indolyl) butanoic acid, 4-(3-Indolyl) butyric acid and IAA(Heteroauxin, IAA), sigma

### **2-5 In Vitro micropropagation**

For *in vitro* studies, shoot tips were sterilized as described by [13]. These explants were cultured on MS[14] basal medium supplemented with 30 g sucrose, 6 g agar (Agar- agar, Gum agar, sigma) and varying concentrations of BAP (0.0, 0.5, 1, 1.5 and 2 mg/l) or KIN (0.0, 0.5, 1, 1.5 and 2 mg/l) in combinations with NAA(0.0 , 0.25 and 0.5 mg/l). To avoid the carry over effect of multiplication media on *in vitro* rooting, multiplied shoots were maintained on plant growth regulators free MS basal medium for the next two weeks. The rooting response of *in vitro* multiplied shoots was considered on half strength MS basal medium supplemented with 20 g sucrose, 6g agar and different concentrations of IBA (0.0, 0.5 , 1, 1.5 and 2 mg/l ) or IAA ( 0.0 , 0.5 , 1 , 1.5 and 2 mg/l ) with NAA (0.0, 0.5 and 1mg/l ).

The following parameters were recorded as follow:

Number of shootlets per jar, Length of shootlet (cm), Number of leaves per shootlet

Number of nods per shootlet, Length of root (cm) and Number of roots per shootlet

### **2-6 Hardening and acclimatization**

Plantlets with well-developed shoots and roots were transplanted in plastic pot containing a mixture of peat moss, sand and perlite in a 1: 1:1 ratio and transferred to greenhouse for hardening. After 8 weeks, observation the percentage successfully acclimatized were recorded. The following parameters were recorded as follow: Height of plantlet (cm), Length of shoot (cm), Number of leaves per plantlet, Number of nods per plantlet, Number of roots per plantlet and Length of root (cm)

### **2-7 DNA isolation**

Young leaf tissues from mother plant (as a control) and obtained from *in vitro* leaf tissues after culturing (1, 3, 6, 9, 11, 15 subcultures) were used for extracted DNA by Gene Elute™ Plant Genomic DNA Miniprep kit (G2N10- 1KT)

### **2-8 Inter simple sequence repeats polymerase chain reactions (ISSR)**

According to [15] ISSR –PCR for each reaction were prepared the final volume of 25µl containing: 1µl template DNA, 1µl ISSR primer (table 1), 12.5µl of 2X master mix (sigma) , 0.2µl Taq polymerase (sigma) and complete volume to 25 µl of distill H<sub>2</sub>O. Polymerase chain reaction (PCR) for each primer was performed for 35 cycles as following: A) an initial denaturation step of 2 min at 94°C, followed by 35 cycles. B) Each consisting of a denaturation step of 30s at 94°C. C) Annealing of 45s at 52°C. D) An extension of 1 min at 72°C. PCR was terminated with a final extension of 2 min at 72°C. ISSR reaction products were separated on 1.5% agarose gels, in 1 x TBE buffer under ultraviolet light after staining in 2 µl ethidium bromide. Digital photodocumentation was taken for each gel. The 100bp DNA Ladder plus molecular weight marker was used to compare the molecular weight of amplified products.

**Table (1):** Three ISSR primers previously selected from thirteen oligonucleotides were published by the University of British Columbia (UBC) for application on sugarcane cultivar (GT. 54-C9)

Primers	Sequences
UBC810	5-GAGAGAGAGAGAGAT-3
UBC823	5-TCTCTCTCTCTCTCTCC-3
UBC812	5-AGAGAGAGAGAGAGGT-3

Parameters were carried out as follow: Monomorphic, Polymorphic

### 2-9 Data analysis

The obtained data were exposed to the proper statistical analysis according to [16], the least significant differences. Using costat computer program V 6.303.LSD test at 5% level was used to differentiate between means. The data obtained from ISSR markers analysis were estimated by [17].

## III Results And Discussion

**In this study, one sugarcane cultivar (GT. 54-C9)** was examined to direct regeneration protocol. Two cytokines, three auxins in different concentrations were used for shoot and root formation in this study. The regenerated plants detected genetic stability through using ISSR markers by the following:

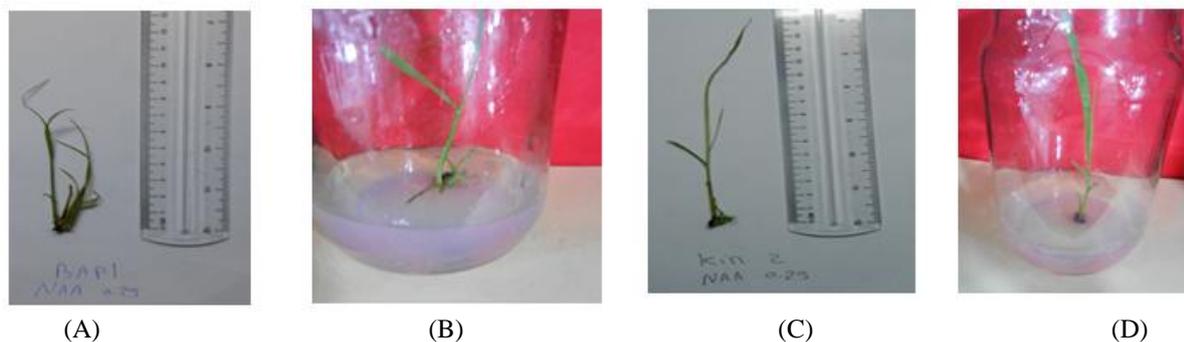
### 3-1 Response of growth regulators for multiplication stage

#### 3-1-1 Shoots micropropagation

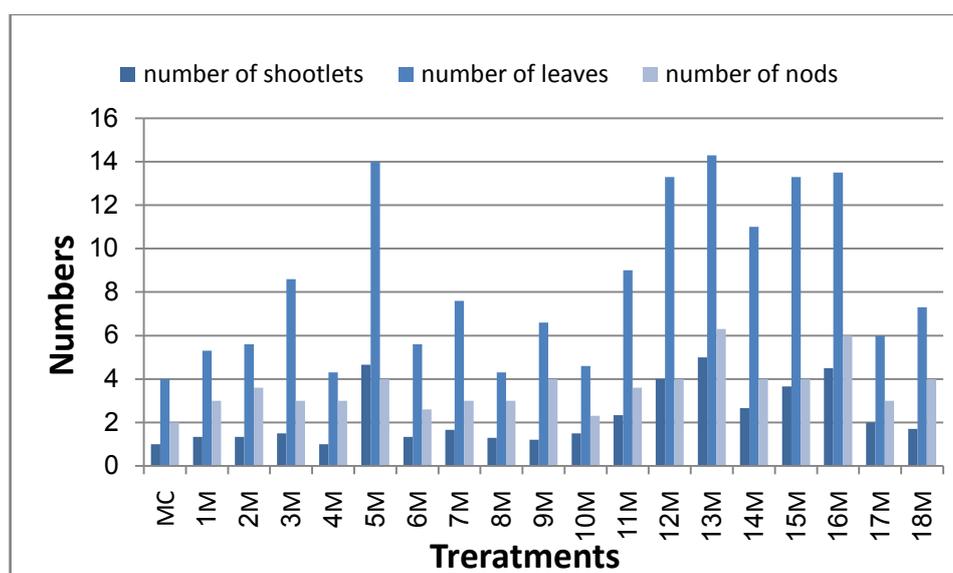
Data tabulated in Table (2) and Figs (1-A, 2) clearly show that the maximum number of shootlet (4.99), leaves (14.3) and nod (6.3) recorded in MS medium supplemented with 1mg/l BAP+0.25 mg/l NAA. Whereas, the minimum number of shootlets (1), leaves (4) and nod (2) observed on free MS medium. In same respect, [18] noticed that MS medium with BAP and NAA was the best for shoot multiplication in sugarcane variety (CO86032). In addition, [19] reported that BAP was essential growth regulator for shoot induction on sugarcane cultivar (HSF-240). Also, [20] described that MS medium supplemented with 1mg/l BAP + 0.5mg/l NAA were essential for shoot regeneration of three sugarcane varieties viz. **Isd16, Isd36 and Isd37**. However, table (2) and fig (1-B) concluded that the highest length of shootlet (16.9cm) observed on MS medium supplemented with 2mg/l KIN +0.5 mg/l NAA. While, the shortest length of shootlet (5cm) achieved on MS medium with 2mg/l BAP+ 0.5mg/l NAA. In agree with [21] reviewed that KIN and NAA were suitable combination for shoot elongation in three sugarcane varieties. Similarly results utilized by [22] on sugarcane variety (Isd32) through studied shoot elongation. Also, In addition, [23] noticed that MS medium with KIN and NAA were favorite mixture for shoot regeneration on sugarcane (*Saccharum spp. hybrid, cv. CoL-54*). [24] Utilized that 1.5 mg/l KIN +1mg/l NAA were favorite combination for shoot elongation on three sugarcane clonal lines, viz., **NIA-98, BL4 and NIA-2004**.

**Table (2):** Effect of various growth regulators added to MS medium on length of shootlet, number of shootlets, leaves and nod of sugarcane cultivar (GT. 54-C9) after 21 days of cultivation under light conditions at 25±1°C

Code	MS medium supplemented with:	Number of shootlets (means)	Length of shootlet (cm) (means)	Number of leaves (means)	Number of nod (means)
MC	Free growth regulators	1.00	14.0	4.0	2.0
M1	0.25mg/l NAA	1.33	15.9	5.3	3.0
M2	0.5 mg/l NAA	1.33	14.2	5.6	3.6
M3	0.5 mg/l Kin +0.25mg/l NAA	1.50	11.9	8.6	3.0
M4	0.5mg/l kin +0.5 mg/l NAA	1.00	12.6	4.3	3.0
M5	1mg/l kin +0.25mg/l NAA	4.66	6.6	14.0	4.0
M6	1mg/l Kin +0.5 mg/l NAA	1.33	14.3	5.6	2.6
M7	1.5 mg/l Kin +0.25mg/l NAA	1.66	12.2	7.6	3.0
M8	1.5 mg/l Kin +0.5mg/l NAA	1.30	6.4	4.3	3.0
M9	2mg/l Kin +0.25mg/l NAA	1.20	16.9	6.6	4.0
M10	2mg/l Kin +0.5mg/l NAA	1.50	13.9	4.6	2.3
M11	0.5mg/l BAP +0.25mg/l NAA	2.33	10.6	9.0	3.6
M12	0.5 mg/l BAP+0.5mg/l NAA	4.00	8.0	13.3	4.0
M13	1mg/l BAP +0.25mg/l NAA	4.99	13.2	14.3	6.3
M14	1mg/l BAP+0.5 mg/l NAA	2.66	6.8	11.0	4.0
M15	1.5 mg/l BAP+0.25 mg/l NAA	3.66	7.8	13.3	4.0
M16	1.5mg/l BAP+0.5mg/l NAA	4.50	8.5	13.5	6.0
M17	2mg/l BAP +0.25mg/l NAA	2.00	8.0	6.0	3.0
M18	2mg/l BAP+0.5 mg/l NAA	1.70	5.0	7.3	4.0
L.S.D 0.05		2.58	11.4	10.76	3.33



**Figs (1-A,B):** The Best shoot multiplication for sugarcane cultivar (GT. 54-C9) cultured on MS medium with 1mg/l BA + 1mg/l NAA .(C,D) , The Best shoot elongation for sugarcane cultivar (GT. 54-C9) cultured on MS medium with 2mg/L KIN + 0.25 mg/l NAA.



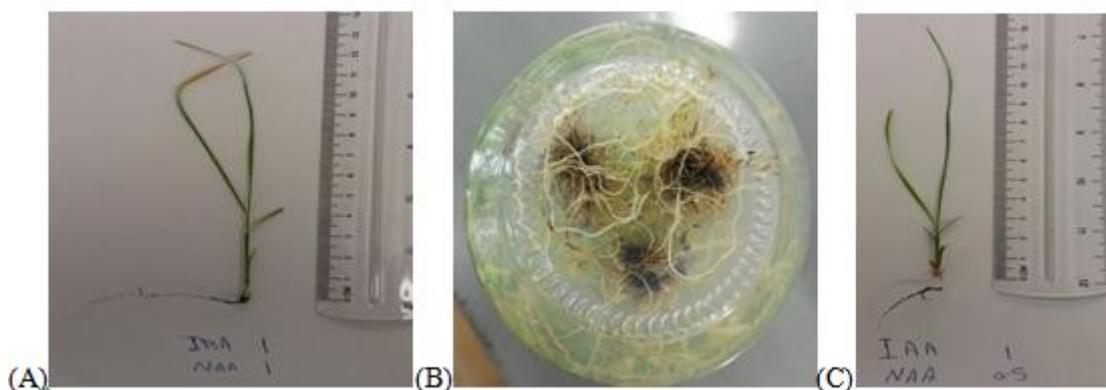
**Fig (2):** Effect of number of shootlets, leaves and nodes *in vitro* sub culturing on shootlets multiplication rate of sugarcane cultivar (GT. 54-C9).

### 3-1-2 Root elongation and formation

Overall results showed non-significant different at the 5% level among different treatments applied. However, data in Table (3) and Figs (2-A, B, C) revealed that the maximum number of roots (3.3) was recorded on ½MS supplemented with 1mg/l IBA+1mg/l NAA. While, the minimum number of roots (1.6) were noticed on 0.5mg/l IAA+ 0.5mg/l NAA. Agreement with obtained results who reported that by [25]. Moreover, [26] stated that supplementation of MS medium with 1mg/l IBA was very important for root induction on *Saccharum officinarum*. In same respect, [27] described that IBA+NAA were the best combination for root formation. Also, [28] indicated that IBA was a suitable growth regulator for root induction *Sorghum bicolor*. Augmented with [29] reported that MS medium augmented with IBA (2.5 and 5.0 µM) for sugarcane varieties (RB855156 and RB72454) respectively, was suitable for rooting. In close with [30] reported that ½ MS medium contained 1mg/l IBA with 0.5mg/l NAA were the best root formation mixture on *Saccharum officinarum* l. Cv. *Us-633*. However, the highest length of root (4.6 cm) was observed on ½MS with 1mg/l IAA + 0.5mg/l NAA. While, the shortest length of root (1.7 cm) noticed on ½MS supplemented with 2mg/l IAA+0.5mg/l NAA. In this regard, [31] concluded that IAA with NAA was effective on root elongation on sugarcane.

**Table (3):**Effect of various growth regulators added to MS medium on length of root and number of roots for sugarcane cultivar (GT. 54-C9) after 21 days of cultivation under light conditions at 25±1°C

Code	MS medium supplemented with:	Number of roots(means)	Lengthof Root (cm)(means)
RC	Free growth regulator	2	4.1
R1	0.5 mg/l NAA	2	2.7
R2	1 mg/l NAA	2	3,6
R3	0.5 mg/l IAA+0.5 mg/l NAA	1.6	2.4
R4	0.5 mg/l IAA +1mg/l NAA	2.3	2.9
R5	1mg/l IAA +0.5mg/l NAA	2.3	4.6
R6	1mg/l IAA +1mg/l NAA	2	3.16
R7	1.5mg/l IAA +0.5mg/l NAA	2	3
R8	1.5mg/l IAA+1mg/l NAA	2	3
R9	2mg/l IAA +0.5 mg/l NAA	1.6	1.7
R10	2mg/l IAA+1 mg/l NAA	2	3.1
R11	0.5mg/l IBA+0.5 mg/l NAA	1.6	1.9
R12	0.5 mg/l IBA +1 mg/l NAA	1.6	1.3
R13	1 mg/l IBA +0.5 mg/l NAA	2	2.8
R14	1mg/l IBA+1mg/l NAA	3.3	3
R15	1.5mg/l IBA + 0.5 mg/l NAA	2	4.1
R16	1.5 mg/l IBA +1 mg/l NAA	2.6	4.4
R17	2mg/l IBA +0.5 mg/l NAA	2	2
R18	2mg/l IBA +1mg/l NAA	2.3	2.3
L.S.D0.05		0.84	2.28



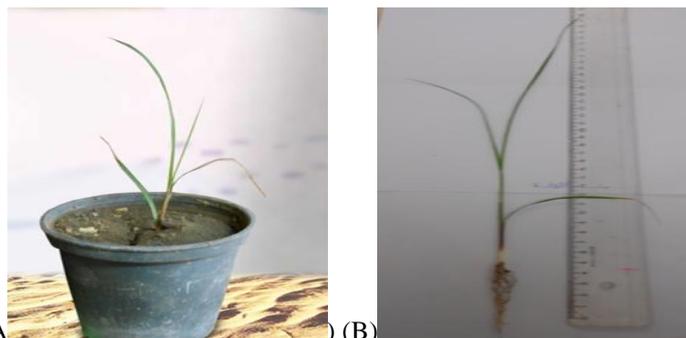
**Figures (3):** (A,B) The Best roots multiplication for sugarcane cultivar GT. 54-C9 observed on MS medium supplemented with 1mg/l IBA + 1 mg/l NAA. (C) The Highest root elongation for sugarcane cultivar GT. 54-C9 obtained on MS medium with 1mg/l IAA + 0.5mg/l NAA.

### 3-1-3 Acclimatization stage

The estimated survival rate after 4 weeks were recorded 90% tabulated in table(4) and figs (4).In this concern, [32]noticed that highest survival rate on mixed medium sand, soil and peat (1:1:1). In close with [33]who reported that 80% survival rate among acclimatized of sugarcane plants. In this concern, [34] concluded that 85% survival rate for acclimatized plantlets on sugarcane varieties.

**Table (4):**Acclimatization of micro propagated plantlets of sugarcane (GT54-C9) cultivar under greenhouse condition after 8 weeks of cultivation

Measurements	Survival rate	Height of plant (cm)	Length of shoot	Number of leaves	Number of nodes	Number of roots	Length of root (cm)
Mean	90%	26.90±2.4	23.00±2.3	3.66±0.57	2.66±0.57	6.33±2.3	4.66±2.3



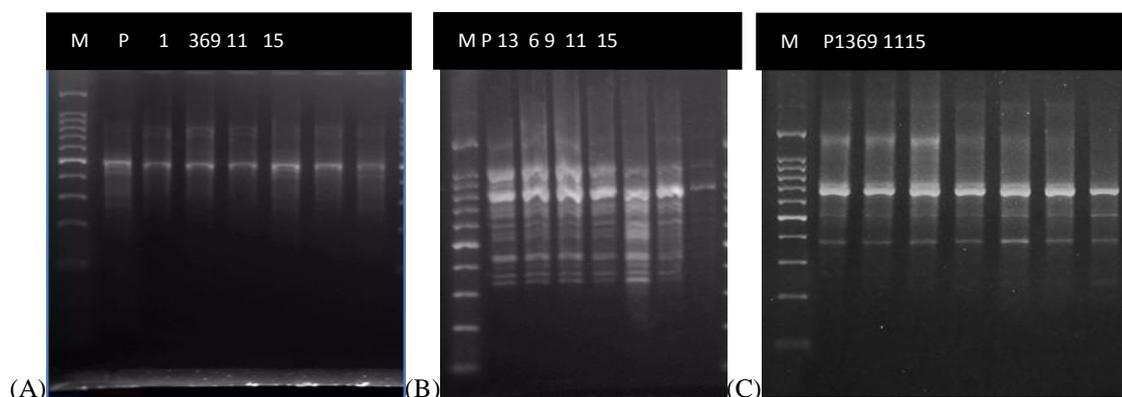
Figures (4-A, B):refer to sugarcane (GT54-C9) cultivar after 8 weeks of acclimatization.

### 3-2 Genetic stability through ISSR

The most informative oligonucleotides bands for sugarcane cultivar (GT.54-C9) tabulated in Table (5) and Figs (8- A, B and C).Indicating that none of the three oligonucleotides used detected somaclonal variation when compared with the standard array plant (control plant). Similarly results were obtained by [35] in sorghum. In contrast, [36] they found that 100% monomorphic bands were observed in subcultures of the same sugarcane varieties **RB943365** and **RB92579**. Moreover, [38] indicated that direct regeneration less risk of somaclonalvariations.

**Table (5):**ISSR oligonucleotides profiles of mother leaf tissue (as control) and in vitro propagated sugarcane (GT.54-C9)

ISSR primers	Rang of molecular size (BP)	Number of total bands
UBC 810	1282.9 - 613	7
UBC 823	1339.5 – 767.3	12
UBC 812	1495.8 – 790.6	6



**Figures (5):** electrophoretic pattern of GT.54-C9 cultivar with UBC- type ISSR. Electrophoretic pattern obtained from amplification of DNA from sugarcane cultivar GT.54-C9 in each of the subcultures in the order listed (M, P, 1, 3, 6 , 9 ,11 ,15). Where P is the mother plant (as a control). M is the molecular marker weight (1.5 kb ladder).by respective primers A.UBC 810 B. UBC 823 C.UBC 812. Primer set. University British Columbia. Vancouver. Canada.

### IV. Conclusion

This present study indicated that direct regeneration was effective method for obtained uniformed plants on Egyptian sugarcane cultivar (GT.54-C9).

### Acknowledgment

I would like to extend my deepest gratitude to Dr.Khaled Adly Khaled, senior Research of Genetics, Department of Breeding and Genetics, Sugar Crops Research Institute, Agricultural Research Center for his effort to obtain the items under study. Also, Great thanks would be expresses to Mr. Mohammed Fathy (Tissue culture laboratory, Agricultural center for genetic engineering and biotechnology “ACGEB” (Faculty of Agriculture, Ain Shams University), for his effort on tissue culture experiment of sugarcane.

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H.A.M. Abd-Alla "Genetic stability detected on micropropagated Egyptian sugarcane cultivar (GT54-C9)." *IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB)* 3.6 (2017): 56-63.