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In Vitro Direct Propagation and Genetic Stability of Sugarcanecultivar (G.2003/49)

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Abstract: *Micropropagation of sugarcane cultivar(G.2003/49) through using shoot tips meristem consider and successfully protocol to obtained uniformed diseases- free plants. The obtained results revealed that peak the maximum number of shootlets, leaves and nodes were recorded on MS medium supplement with 2mg/l KIN + 0.25 mg/l NAA. However, the highest shootlets (18.6 cm) wasreported on MS mediumsupplemented with 0.25 mg/l NAA. On the other hand, roots induction indicated that the highest root length (2 cm) resulted from ½MS contained 2mg/l IAA+ 1 mg/l NAA. Whereas, the maximum roots number (7.3) was recorded on ½MS fortifiedwith 1mg/l NAA. Furthermore, stability or somaclonal variation not obtained through genetic analysis of mother leaf tissue (as a control) compared 6 subcultures of in vitro resulted plantlets summarized that all obtained bands were monomorphic.*

Key words: *Micropropagation, shoot tip meristem, sugarcane, kin, NAA, IAA.*

Abbreviations*: KIN, Kinetin; NAA, Naphthalene acetic acid; IAA, indole-3-acetic acid; MS, Murashige and Skoog.*

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I. Introduction

Sugarcane (*Saccharum officinarum L.*) is a vital crop belongs to family Gramineae noticed by**[1].**It is an important photo synthesizer C4plant **[2]**. Sugarcane is planted on 26.1 million ha producing 1.83 billion ton of cane on worldwide as stated by**[3]**.Sugarcane is an importance cash crop and the main source of sugar. It account among 70% of universal sugar production**[4]**. It enters into a large number of industries such as paper, food industry, ethanol production and animal food.Unfortunately, Egypt weather not supported seed production .So that, sugarcane is planted by vegetative reproduction through cultivated contains two or three buds. That traditional way have some disadvantages like, low propagation, take a lot of space and for produced a new variety it takes about (8-10)years for have commercial variety. Therefore, tissue culture is a nessecrey technique for sugarcane propagation and avoiding traditional culturing problems.Tissue culture have various protocols in these studies using micropropagation for sugarcane *invitro* propagation to save space and time and produced uniformed plants like mother plants. Therefor ISSR markers based protocol used were nessecrey for early predicted genetic stability among 15thsubcultures.

II. Materials and methods

2-1 Plant material

In the present study, cultivar (G.2003/49) was used. The germplasm was in form of sugarcane shoot tip meristems which were excised from 5-6 months old field grown sugarcane plants. The plant material was provided by Sugar Crops Research Institute (SCRI), Giza, Cairo, Egypt.

2-2 Explant sterilization and preparation

Healthy young shoot tips were collected by removing the leaves sheath from field grown plants of sugarcane (*Saccharum officinarum L.*). The healthy shoot tips (about 1 – 1.5 cm) were cuted and thoroughly washed under running tap water and soap for 30 min. then washed with sterilized water 5 times. In Laminar Flaw Hood, tips (explants) were treated with 70% ethanol alcohol for 30 seconds for one minute. Subsequently surface sterilized using 30% aqueous solution of commercial Clorox 5.25 g/I "sodium hypochlorite" for 30 min. then washed with sterile distilled water. Apical meristem was used to remove rolled leaves. Then, the shoot tips were submerged in mercuric chloride 0.2% for5 minutes, followed by several washing with sterile distilled water. Finally, the sterilized meristems were cultured on selected media.

2-3 Culture conditions

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). Culturing was done in ~350ml glass jars containing 50 ml of medium for recovery and development of sterilized meristems. However, for shoot induction and plantlets multiplication experiments were carried out using ~150ml glass jars containing 25ml of medium.

2-4 In vitro propagation

For *in vitro* studies, shoot tips were excised from tops of 5-6 months old of field grown plants of sugarcane (G.2003/49) which were prepared as described by **[5].** These explants were cultured on MS basal medium supplemented with 30 g sucrose, 6 g agar and various 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 combination with NAA(0.0 , 0.25 and 0.5 mg/l). 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 were 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:

1. Number ofshootlets. 2. Shootlets length(cm).
3. Number of Leaves. 4. Number of nods.
5. Length of root (cm). 6.Number ofroots.

2-5 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 on percentage of plantlets that were successfully acclimatized was recorded. For the multiplication experiment, average number of shoots per explant, average shootlets length (cm) and average number of leaves per shoots were recorded for each treatment.Regarding root induction experiment after30 days.

The following parameters were recorded as follow:

1. Survival rate%. 2. The length of plant (cm).
3. Shoot length (cm). 4. Number of leaves.
5. Number of internodes. 6. Number of roots.
7. Root length (cm).

2-6 DNA isolation

Young leaf tissues from mother plant and *in vitro*obtained plantlets after culturing (1, 3, 6, 9, 11, 15 subcultures) were used for DNA extraction by using CTAB (acetyltrim ethyl ammonium bromide) protocolstated by**[6]** modified by **[7]**. After that, electrophoresis was made and intended DNA concentration in 1.5% agarose gel.

2-7 ISSR analysis

According to**[8]**the ISSR amplification reactions contained 0.6µl of genomic DNA, 1.3 µl 10x buffer, 0.4 µl of each dNTP, 1.0 µl primers and 0.2 µl Taq DNA polymerase, with the final volume adjusted to 12.5 µl with distilled water. The amplification reaction was carried out in Eppendorf Master Cycler. The reaction included an initial denaturation step of 2 min at 94°C, followed by 35 cycles, each consisting of a denaturation step of 30s at 94°C, annealing of 45s at 52°C and 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 photo documentation was taken for each gel. The 100bp DNA Ladder and molecular size marker was used to compare the molecular size of amplified products. Three ISSR primers previously selected from thirteen oligonucleotides were published by the UniversityOf British Columbia (UBC) for application on sugarcane cultivar (G.2003/49).

Table (1): The sequences ofprimers were as follow:

Primers	Sequences
UBC810	5-GAGAGAGAGAGAGAGAT-3
UBC823	5-TCTCTCTCTCTCTCTCTCC-3
UBC812	5-AGAGAGAGAGAGAGAGGT-3

Parameters were carried out as follow:

1. Monomorphic 2. Polymorphic

2-8 Data analysis

The obtained data were exposed to the proper statistical analysis according to **[9].**The least significant differences. Using costat computer program V 6.303(2004). LSD at level as significance was used to differentiate between means.

The data obtained from ISSR markers analysis were recorded as follows:
Monomorphic (+) or polymorphic (-) the polymorphism among different subculture and mother plant were estimated by**[10].**

III. Result and Discussion

Response of different hormones for regeneration stageShoot regenerationData tabulated in Table (2) and Figs (1,2) revealed that the highest shootlet length(18.7 cm)noticed on MS medium supplemented with 0.25mg/l NAA.whereas, the shortest length (4.8 cm) recorded on MS medium fortified with 0.5 mg/l BAp + 0.25 mg/l NAA. The obtained result was in accordance with that obtained by **[11].** Theysummarized that NAA is an important growth regulator for shoot regenerated from sugarcane callus. While for the maximum number of shootlets, leaves and nods was recorded (7, 18.6 and 9.3) repressively, on MS medium supplemented with 2mg/l Kin +0.25 mg/l NAA. On the other side of view, the lowest number of shootlets (1.6) obtained on MS medium with free growth regulators. The data also revealed lowest number of leaves (4.7) and less number of internodes(2) were recorded on MS medium supplemented with 1 mg/l BAp +0.25 mg/l NAA as presented. In this respect,**[12]**found thatsupplementation of MS medium with 1.5mg/l KIN +1mg/l NAA were the best combination for shoot formation. However, **[13]** utilized fall strength of MS medium fortified with 0.5mg/l KIN + 5mg/l NAA was showed the best shoot indication on sugarcane Var. Isd 32.

Table (2): Effect of various growth regulators added to MS medium on length ofshootlet , number of shootlets, leaves and nods of sugarcane cultivar (GT. 54-C9) after 21 days of cultivation under light conditions(600-800 lux) and temperature degree (25±1°C)

Code	**MS medium supplemented with:**	Length of shootlets(means)	Number of shootlets (means)	Number of leaves(means)	Number of internodes(means)
MC	Free growth regulators	12.8	1.6	5.6	2.3
M1	0.25mg/I NAA	18.7	2.6	7.0	4.0
M2	0.5 mg/l NAA	13.2	2.4	7.6	3.6
M3	0.5 mg/l Kin +0.25mg/l NAA	10.0	2.0	5.5	2.3
M4	0.5mg/l kin +0.5 mg/INAA	10.9	4.0	11	4.3
M5	1mg/l kin +0.25mg/l NAA	12.6	3.6	11.4	5.7
M6	1mg/l Kin +0.5 mg/I NAA	15.6	3.3	9.0	4.6
M7	1.5 mg/l Kin +0.25mg/l NAA	11.5	2.0	8.0	4.0
M8	1.5 mg/l Kin +0.5mg/l NAA	15.5	5.6	16.3	5.3
M9	2mg/l Kin +0.25mg/l NAA	12.6	7.0	18.6	9.3
M10	2mg/l Kin +0.5mg/l NAA	8.7	3.6	10.9	3.9
M11	0.5mg/l BAP +0.25mg/l NAA	4.8	4.6	10.3	3.4
M12	0.5 mg/l BAP+0.5mg/l NAA	6.0	4.6	14.0	5.6
M13	1mg/l BAP +0.25mg/l NAA	11.6	2.3	4.7	2.0
M14	1mg/l BAP+0.5 mg/l NAA	11.4	3.6	10.6	4.3
M15	1.5 mg/l BAP+0.25 mg/l NAA	9.6	3.6	11.0	3.0
M16	1.5mg/l BAP+0.5mg/l NAA	12.4	4.0	12.0	3.2
M17	2mg/l BAP +0.25mg/l NAA	8.8	3.0	11.3	3.0
M18	2mg/l BAP+0.5 mg/l NAA	14.2	3.3	12.0	3.6
L.S.D(0.05)		4.42	1.48	4.17	2.12

Figure(1): Regeneration of shootlets for sugarcane cultivar G. 2003/49 in MS medium supplemented with 0.25 mg/l NAA.

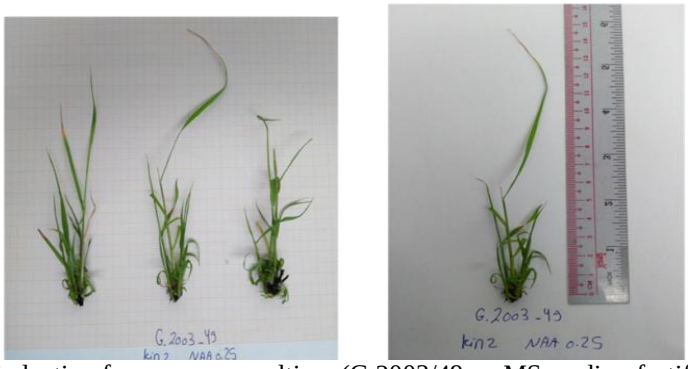


Figure (2): shootlets induction for sugarcane cultivar (G.2003/49 on MS mediumfortified with 2mg/l KIN + 0.25mg/l NAA.

Root induction

The data presented in Table (3) and Figure (4, 5) showed that the highest root length (7.5, 7.2 and 7.0 cm) were recorded on ½ MS medium fortified with 1mg/l NAA, 0.5 mg/l IBA +1 mg/l NAA and 0.5mg/l NAA, respectively. The lowest root length (1.6, 2.6 and 3.3 cm) were recorded with ½ MS supplemented with 0.5 mg/l IAA +1 mg/l NAA , 1mg/l IBA+ 1mg/l NAA and 1 mg/l IAA+ 0.5mg/l NAA, respectively. The data tabulated in Table(3) and Figs (4,5) showed that, the mean of maximum number of roots(2.03) were recorded on half strength MS medium supplemented with 2mg/l IAA + 1 mg/l NAA. Moreover, the mean of lowest number of roots (0.23) was recorded with 1mg/l IBA+ 1 mg/l NAA IN this regard, **[14]** noticed that ½ MS supplemented with IAA+ NAA was confirmed root elongation on 5 sugarcane cultivars. While,**[15]** reported that 5mg/l IAA was an important auxin for root elongation on sugarcane varieties. Also, **[16]** utilized that ½MS with (2.9 -28.5µm) IAA were the best combination for root induction.Also, **[17]** noticed that NAA was suitable growth regulator for root inductionon three sugarcane varieties **viz. Isd16, Isd36 and Isd37.**While, **[18]** reported that half strength MS with 2mg/l NAA was the best medium for root inductionsugarcane variety Co 86032. Also, **[19]**observed that root induction on half strength MS medium with (1-2 mg/l) NAA for two sugarcane varieties CO-6907 and CO-86249.

Table (3): Effect of various growth regulators added to MS medium on length of root and number of roots for sugarcane cultivar (G2003/49) after 21 days of cultivation under light conditions(600-800 lux) and temperature degree (25±1°C)

Code	MS medium supplemented with:	Number of roots(means)	Length of root (cm)(means)
RC	Free growth regulator	0.96	5.0
R1	0.5 mg/I NAA	0.74	7.0
R2	1 mg/I NAA	1.46	7.5
R3	0.5 mg/I IAA+0.5 mg/I NAA	1.42	5.5
R4	0.5 mg/I IAA +1mg/I NAA	0.66	1.6
R5	1mg/I IAA +0.5mg/l NAA	0.43	3.3
R6	1mg/I IAA +1mg/I NAA	1.20	4.0
R7	1.5mg/I IAA +0.5mg/I NAA	1.30	4.1

R8	1.5mg/I IAA+1mg/I NAA	1.44	6.6
R9	2mg/I IAA +0.5 mg/l NAA	1.43	5.9
R10	2mg/I IAA+1 mg/I NAA	2.03	4.5
R11	0.5mg/I IBA+0.5 mg/I NAA	0.37	4.6
R12	0.5 mg/I IBA +1 mg/I NAA	0.94	7.2
R13	1 mg/I IBA +0.5 mg/I NAA	1.26	6.8
R14	1mg/I IBA+1mg/I NAA	0.23	2.6
R15	1.5mg/I IBA + 0.5 mg/I NAA	0.29	6.9
R16	1.5 mg/I IBA +1 mg/I NAA	1.53	4.0
R17	2mg/I IBA +0.5 mg/I NAA	0.70	5.0
R18	2mg/I IBA +1mg/I NAA	1.14	4.3
L.S.D(0.05)		1.94	6.21

Figure3): invitro root elongation for sugarcane cultivar G.2003/49 in½ MS medium with 2mg/l IAA + 1mg/l NAA.

Figure(4): invitro roots multiplication for sugarcane cultivar G.2003/49 in½ MS medium supplemented with 1 mg/l NAA.

Acclimatization stage:

Transferred plantlets to pots with mixture of peat moss, sand and perlite at the ratio of (1:1:1v/v/v) in green house. Showed that, estimated the survival rate after 4 weeks recorded 80%.Such type of result was supported by **[20]** who found that 80% of survival rate on acclimatized plants of sugarcane cultivar US- 633. Also, **[21]** reported that the survival rate of three sugarcane varieties **viz. Isd16, Isd36 and Isd37 was 85%**.

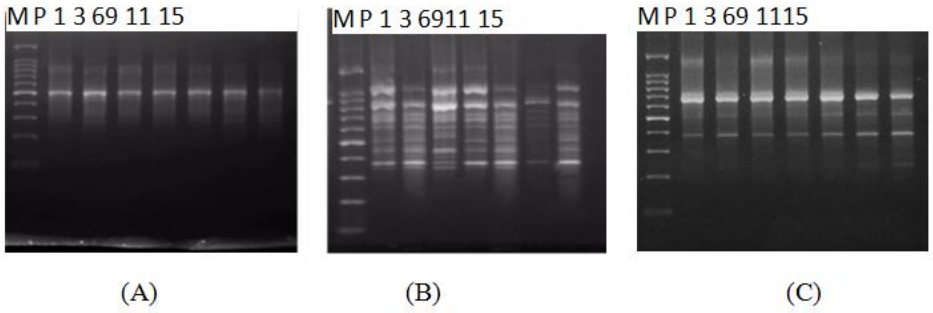
Table (4) Acclimatization of micro propagated plants of sugarcane (G.2003/49) cultivar under greenhouse condition after 8 weeks of cultivation.

Measurements	Survival rate	Height of plant (cm)	Length of shoot	Number of leaves	Number of nodes	Number of roots	Length of root (cm)
Mean	80%	36.4	23	4	2.33	6.33	3.5±

Figure(5): refer to sugarcane **(G.2003/49)** cultivar after 8 weeks of acclimatization.

Genetic stability through ISSR:

Initially, samples from six subcultures of sugarcane cultivar (G.2003/49) were used in order to select the most informative oligonucleotides. Thirteen oligonucleotides were tested and published by the University of British Columbia (UBC). From these, three were selected and the amplification products provided clear and good repeatability in G.2003/49 cultivar. On this cultivar using the primer UBC 810, eight polymorphic band, band with a molecular mass range from(1281 to 783)bp. With the primer UBC 823, ten polymorphic bands with molecular mass (1528to 762 bp). Also, for UBC 812 primer observed five polymorphic bands from (1513 to 809 bp). ISSR technique in the study of DNA polymorphism in revealed high degree of consistency. This was in accordance with**[22]** they revealed that ISSR marker based on molecular analysis as an efficient technique for early predicated of soma clonal variations. Also,**[23&24]** reported that somaclonal variations was absent through 15th subcultures of sugarcane plantlets. In this respect,**[25]** described that uniformed plants like mother plant were recognized after 6-30 months of in vitro culturing of ZingiberrubensRoxb.



Figs.(6): electrophoretic pattern of G.2003/49 cultivar with UBC- type ISSR. Electrophoretic pattern obtained from amplification of DNA from sugarcane cultivar G.2003/49 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.

Table (4):DNA polymorphism obtained from G.2003/49 with UBC810 Primer

MS(bp)	P	1	3	6	9	11	15	Polymorphism
1281	+	+	+	+	+	+	+	**Monomorphic**
1207	+	+	+	+	+	+	+	**Monomorphic**
1019	+	+	+	+	+	+	+	**Monomorphic**
998	+	+	+	+	+	+	+	**Monomorphic**
938	+	+	+	+	+	+	+	**Monomorphic**

864	+	+	+	+	+	+	+	**Monomorphic**
819	+	+	+	+	+	+	+	**Monomorphic**
783	+	+	+	+	+	+	+	**Monomorphic**

Where M.S (bp) = molecular size M= molecular marker
P = Mather plant (as control) 1, 3, 6,9,11 and 15 are subcultures

Table (5): DNA polymorphism obtained from G.2003/49 with UBC823 Primer

MS(bp)	P	1	3	6	9	11	15	Polymorphism
1528	+	+	+	+	+	+	+	**Monomorphic**
1324	+	+	+	+	+	+	+	**Monomorphic**
1203	+	+	+	+	+	+	+	**Monomorphic**
1105	+	+	+	+	+	+	+	**Monomorphic**
1054	+	+	+	+	+	+	+	**Monomorphic**
1020	+	+	+	+	+	+	+	**Monomorphic**
922	+	+	+	+	+	+	+	**Monomorphic**
858	+	+	+	+	+	+	+	**Monomorphic**
793	+	+	+	+	+	+	+	**Monomorphic**
762	+	+	+	+	+	+	+	**Monomorphic**

Where M.S (bp) = molecular size M= molecular markerP = Mather plant (as control) 1, 3, 6,9,11 and 15 are subcultures

Table (6): DNA polymorphism obtained from G.2003/49 with UBC812 Primer

MS(bp)	P	1	3	6	9	11	15	Polymorphism
1513	+	+	+	+	+	+	+	**Monomorphic**
1140	+	+	+	+	+	+	+	**Monomorphic**
1085	+	+	+	+	+	+	+	**Monomorphic**
948	+	+	+	+	+	+	+	**Monomorphic**
809	+	+	+	+	+	+	+	**Monomorphic**

Where M.S (bp) = molecular size M= molecular marker
P = Mather plant (as control) 1, 3, 6,9,11 and 15 are subcultures

IV. Conclusion

These studypresented that direct regeneration was effective method for obtained disease- free and uniformed plants on sugarcane cultivar (G.2003/49).

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