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Influence of Plant Growth Regulators on Micro-Propagation and Somaclonal Variation of Two Strawberry Cultivars.

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ABSTRACT

This study was carried out to investigate the effect of some combinations of two plant growth regulators on the multiplication and stability of the two strawberry genotypes, Sweet Charlie (SC.) and Florida (FL.) cultivars. Shoot tips of the two cultivars were cultured on MS medium supplemented with combinations of BA at 3.0 mg/l with three levels of GA_3at (0.2, 0.4, 0.6 mg/l). Data were collected after 8 weeks of cultured. The results showed that Sweet Charlie produced better multiplication than the Florida. DNA extracted from leaf tissue of *in vitro* derived plantlets were analyzed by RAPD-PCR to detect possible drift in genetic stability of micro-propagated plants.The results showed the different fingerprints of RAPD with concentrations of BA and GA_3 turn for variation within replicates per concentration. The results showed the highest polymorphism appeared in Florida when was compared to Sweet Charlie, the results indicate that the Sweet Charlie is more stable and would be more suitable for *in vitro* micro-propagation.

Keywords*: Strawberry, plant growth regulators, micro-propagation, somaclonal variation.*

INTRODUCTION

Strawberry is cultivated all over the world, not only for its digestive and tonic properties, but also for its nutritional value of the fruits. Conferring to nutrient database for standard reference the strawberry fruits are rich in vitamin C, B1, B2, protein, calcium, potassium, iron, and most of other nutrients essential for human health (Chieng-Ying *et al*., 2009; Kafkas *et al*., 2007).

One of the important goals of the agricultural policy in the world is to increase the acreage of strawberry to meet the increasing demand of local fresh market, processing and export. On a commercial scale, tissue culture-derived strawberry plants are estimated to cost four to five times more than plants produced by conventional propagation (George EF., 1996). However, micro- propagated strawberry has several advantages, such as its ability to rapidly multiply virus-free stock andto improve the capacity of these plants to produce runners for planting in the field (Lopez-Aranda*et al*., 1994, George E., 1996). Regeneration protocols of strawberry are species specific to their regeneration capacity (Passey*et al.*, 2003). Selection of the proper hormone combination, explants, and cultivar are the keys of successful regeneration of strawberry (Barcelo, 1998; Jimenez-Bermudez, 2002). The strawberry cultivars differed in the mass of their shoots in every subculture. Generally, the average number of shoots/jar depended on the number of the subculture and cultivars. This result confirms the findings of (Boxus, 1974). *In vitro* micro-propagation is an important tool for crop improvement in plant breeding and is a prevalent application for induction of somaclonalvariation. Somaclonal variation can be known as variation among plants regenerated from in vitro culture (Larkin and Scowcroft, 1988) and is a common phenomenon in plant tissue culture (Skirvin *et al*., 1993). The regeneration of strawberry depends on the genotype, type of explant and culture conditions (Passey*et al*., 2003) which they would not necessarily the same or even similar for the different genotypes (Folta and Dhingra, 2006).

This study aimed to investigate the influence of some growth hormones on the multiplication and stability of genotype of Sweet Charlie cultivar. Therefore, this study dialed with the influence of hormones concentrations on the efficiency of *in vitro* micro- propagation as well as stability of two strawberry genotypes.

MATERIALS AND METHODS

This study was carried out during 2014 at the Tissue Culture Research Laboratory, Faculty of Agriculture, Mansoura University, Egypt.

Source of plant materials: Two kinds of plant materials of strawberry, Sweet Charlie (SC.) and Florida (FL.) cultivars were used in this investigation. Runner tips of Sweet Charlie and Florida cultivars were collected from greenhouse at Nubaria Research Station, Al Bahira.

Cultures were initiated from shoot tip consisting of meristem plus 2 or 3 leaf primordial, which were dissected from stolon of greenhouse-grown plants. The tips were sterilized prior to dissection by washed with tap water for 5 - 10 minutes to remove surface contamination and then sterilized by immersing in 70% ethanol for 2 minute with vigorous shaking followed by 20 minutes in 5% sodium hypochlorite containing one drop of Tween 20. Then the tips rinsed three times with sterile distilled water in laminar flow according to (Moradi *et al.*, 2011). Terminal buds (3 - 4 mm), were dissected and cultured on MS medium supplemented with 30 g/l sucrose and 7 g/l agar for two weeks. Then transferred to regeneration medium (MS) with 0.5 mg/l BA for five weeks and transferred to the same media supplemented with 0.1 mg/l BA for five weeks, then subculture three times on MS media free hormones. After that, small pieces (0.5 g) of leaf tissues of *in vitro* derived plantlets were taken as a random sample of Sweet Charlie and Florida cultivar for molecular analysis. Shoot tips of two cultivars were cultured on MS media supplemented with the following growth regulator combinations:

Level 1: BA+ GA_3 at 3.0 and 0.0 mg/l respectively
Level 2: BA+ GA_3 at 3.0 and 0.2 mg/l respectively
Level 3: BA+ GA_3 at 3.0 and 0.4 mg/l respectively
Level 4: BA+ GA_3 at 3.0 and 0.6 mg/l respectively

After that, small pieces (0.5 g) of leaf tissue of *in vitro* derived plantlets of two cultivars from levels two and four of growth regulator (3.0 mg/l BA + 0.2 mg/l GA_3 and 3.0 mg/l BA + 0.6 mg/l GA_3) were taken for molecular analysis. Also the cultures were kept under 16- hour's illumination (fluorescent light) at 22ºC ± 2ºC. Data were recorded after eight weeks of culturing.

Total DNA was extracted from leaf tissue of *in vitro*-derived plantlets of two cultivars using EZ-10 Spin Column Plant DNA Mini-Preps Kit from Bio Basic Inc. Genomic DNA was used as a template for Polymerase Chain Reaction (PCR) amplification using random RAPD primer. This primer was ISJ-7 (5′TGCAGGTTAGGACCCT3′). Amplification reactions in RAPD technique were performed according to Williams *et al.* (1990). Amplification was carried out in an automated thermal cycle (model Techni TC-3000G). the reaction was subjected to one cycle at 94º C for 4 min followed by 45 cycles at 94º C for 1 min, 37º C for 1 min, and 72º C for 2 min, then a final cycle of 72º C for 10 min. DNA banding patters generated from RAPD techniques were analyzed by GelAnalyzer 3 program. These data scoring amplicons (bands) as present (1) or absent (0) for primer and entered in the form of a binary data matrix.

RESULTS AND DISCUSSION

Influence of BA and GA_3 levels on shoot proliferation of two strawberry cultivars

In this study, shoot tips from the two strawberry cultivars: (Sweet Charlie and Florida) were cultured on MS medium supplemented with combination of BA at 3.0 mg/l with different levels of GA_3 (0.2, 0.4, 0.6 mg/l). The data which were obtained from the four concentrations of growth regulators were set up in a combined analysis and the obtained results are presented in Table 1. The results revealed that the presence of insignificant effects of BA and the different levels of GA_3 on shoot proliferation of both cultivars.

Mean performance of the two strawberry cultivars for the studied traits are presented in Table 2. The results indicated that the highest number of shoots and cluster weight for Sweet Charlie was observed at the level four (3.0 mg/l BA with 0.6 mg/l GA_3) which was 18.2 and 1.48g, respectively. Similarly, the results showed that the highest number of shoots for Florida was observed at the level one (3.0 mg/l BA with 0.0 mg/l GA_3) which was 12.8. While, the highest values of cluster weight was observed at the level two (3.0 mg/l BA with 0.2 mg/l GA_3) which was 1.31g. The results obtained were in agreement with those of Lal *et al.* (2003) who reported that the maximum number of shoots per explant was observed in MS medium supplemented with BAP at 4.0 mg/l. While, Barcelo *et al*. (1998) found that the best results were obtained in the presence of 2.0 mg/l BA and 0.5 mg/l IBA (47% explants formed shoots). Also, Litwinczuk *et al*. (2009) found that the gibberellic acid improved axillary shoot elongation and reduced the growth of callus as well as the formation of roots and the development of adventitious shoots. Kane *et al*., (1994); Folta *et al*., (2006) and Landi and Mezzetti, (2006) found that combinations of auxin and cytokinin promote higher adventitious shoot formation via the shoot organogenesis of strawberry. Rahman *et al* (2015) found that the highest percentage of shoot regeneration (93.33%) and number of shoots (15.00) per leaf disc in strawberry induced on the MS medium supplemented with 3.0 mg L^{-1} BAP and 0.5 mg L^{-1} GA3.

Table 1: Combined analysis of variance and mean squares of genotypes over the four concentration of growth regulators for *in vitro* traits

S.O.V	d.f	Number of shoots per explant	Cluster weight (g)
Levels (L)	3	10.188	0.163
Replicates / (L)	20	40.813	0.267
Genotypes (G)	1	130.021	0.007
G x L	3	27.966	0.399
Error	20	39.629	0.274

Table 2: Effect of various combinations of BA and GA_3 on number of shoots and cluster weight of two cultivars Sweet Charlie and Florida cultivars after 8 weeks of culture *in vitro*.

Genotypes	Number of shoots per explant				Cluster weight (g)			
Growth regulators levels	1	2	3	4	1	2	3	4
Sweet Charlie (SC.)	13.3	16.8	13.5	18.2	0.84	1.18	1.16	1.48
Florida (FL.)	12.8	11.3	12.6	11.8	1.13	1.31	1.19	0.94
LSD 5%	5.999	11.022	7.818	11.432	0.648	0.844	0.708	0.881

Influence of BA and GA_3 levels on genetic stability of strawberry cultivars:

In this study, genomic DNAs of two cultivars (SC. and FL.) were extracted and compared by RAPD-PCR, using random oligonucleotide primer. This primer was ISJ-7(5′TGCAGGTTAGGACCCT 3′). For computer analysis to detect the pair-wise differences between the two cultivars, where intensive bands were considered when present as (1), while weak or absent bands were considered when absent as (0).

Table 3 and Fig. 1 showed the reaction of primer with the cultivar Sweet Charlie at the four replications from control treatment. The primer yielded six different bands with size ranged from 330 to 1060 bp. The results obtained from the RAPD-PCR analysis with the cultivar Sweet Charlie at level two of growth regulators (3.0 mg/l BA + 0.2 mg/l GA_3) from six replications are presented in Table 4 and Fig. 1. The results showed that the fragments obtained from the RAPD-PCR sizes ranged from 330 to 1060 bp. One band was present in

replicate one (S_{R1}) and five (S_{R5}) at size of 454 bp. While this band was absent in other replicates and in control treatment.

As shown from Table 5 and Fig. 1 the reaction of primer with the cultivar Sweet Charlie at level four of growth regulators (3.0 mg/l BA + 0.6 mg/l GA_3) from six replications. The bands showed sizes ranged from 288 to 1060 bp. Two new bands were appeared in this level of hormones with size 316 and 288. The band 316 was presented only in replicate one (S_{R1}) while the band 288 appeared in all replicates.

Table 3: Amplified fragments obtained from the PCR analysis Sweet Charlie cultivar via RAPD-PCR from the control treatment.

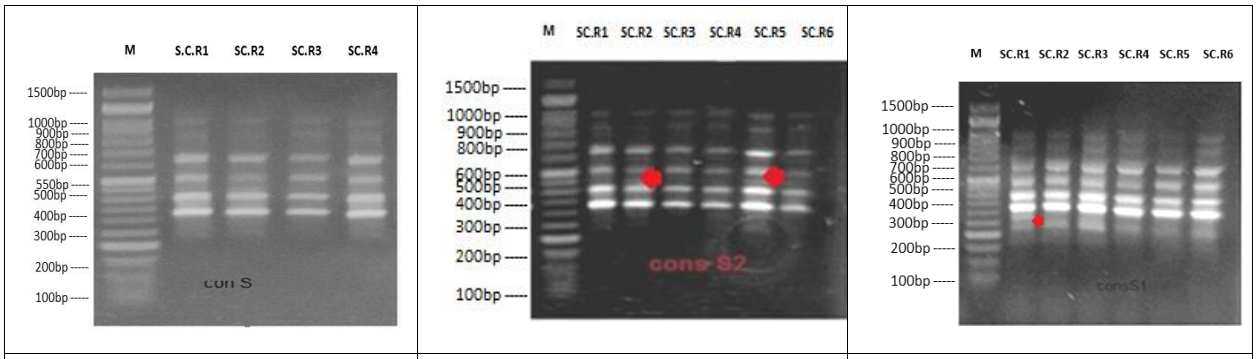
Primer	Size bands (bp)	SC.$_1$	SC.$_2$	SC.$_3$	SC.$_4$
ISJ-7	1060	1	1	1	1
	890	1	1	1	1
	650	1	1	1	1
	520	1	1	1	1
	420	1	1	1	1
	330	1	1	1	1
Total		6	6	6	6

Table 4: Amplified fragments obtained from the PCR analysis of Sweet Charlie at level two of growth regulators (3.0mg/l BA+ 0.2 mg/l GA3) via RAPD-PCR.

Primer	Size bands (bp)	SC.$_{R1}$	SC.$_{R2}$	SC.$_{R3}$	SC.$_{R4}$	SC.$_{R5}$	SC.$_{R6}$
ISJ-7	1060	1	1	1	1	1	1
	890	1	1	1	1	1	1
	650	1	1	1	1	1	1
	520	1	1	1	1	1	1
	420	1	1	1	1	1	1
	454	0	1	0	0	1	0
	330	1	1	1	1	1	1
Total		6	7	6	6	7	6

Table 5: Amplified fragments obtained from the PCR analysis from Sweet Charlie at level four of growth regulators (3.0 mg/l BA + 0.6 mg/l GA3) via RAPD-PCR.

Primer	Size bands (bp)	S_{R1}	S_{R2}	S_{R3}	S_{R4}	S_{R5}	S_{R6}
ISJ-7	1060	1	1	1	1	1	1
	890	1	1	1	1	1	1
	650	1	1	1	1	1	1
	520	1	1	1	1	1	1
	420	1	1	1	1	1	1
	330	1	1	1	1	1	1
	316	1	0	0	0	0	0
	288	1	1	1	1	1	1
Total		8	7	7	7	7	7



Control | **3 mg/l BA + 0.2 mg/l GA_3** | **3 mg/l BA + 0.6 mg/l GA_3**

Fig.1: RAPD-PCR polymorphism of DNA Sweet Charlie cultivar using ISJ-7 primer from leaf tissue of *in vitro*-derived plantlets

Table 6 and Fig .2 showed fragments obtained from the RAPD-PCR analysis with the cultivar Florida at the four replications of control treatment. The primer yielded eight different bands with Size ranged from 250 to 1060 bp. The percentage of polymorphism was 0.00%.

Table 6: Amplified fragments obtained from the PCR analysis of Florida cultivar via RAPD-PCR from the control treatment.

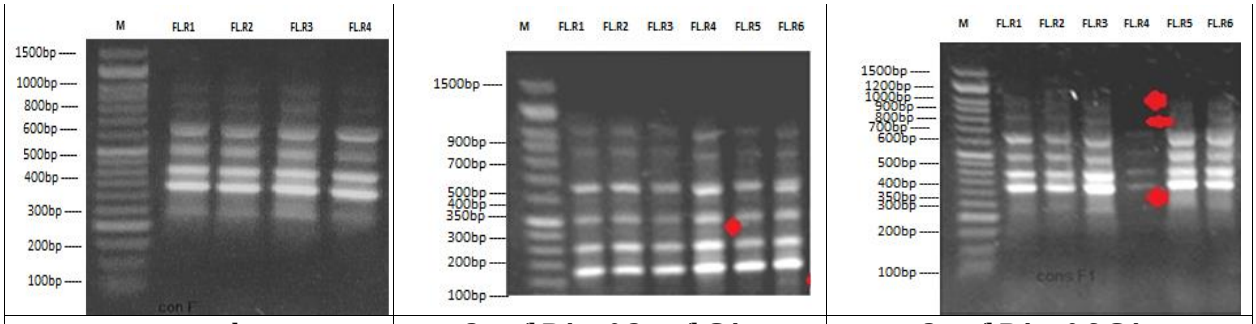
Primer	Size bands (bp)	F_1	F_2	F_3	F_4
ISJ-7	1060	1	1	1	1
	890	1	1	1	1
	650	1	1	1	1
	520	1	1	1	1
	420	1	1	1	1
	330	1	1	1	1
	250	1	1	1	1
Total		7	7	7	7

Table 7 and Fig. 2 showed fragments obtained from the RAPD-PCR analysis from cultivar Florida at level two of hormones (3.0 mg/l BA with 0.2 mg/l GA3). The obtained bands sizes ranged from 250 two 1060 bp. One band was appeared in replicate four (FR_4) with size at 395 bp while this band absent in other replicates and in control treatment. The band with size 250 bp was absent in all replicates except for replicate six (FR_6).

Table 7: Amplified fragments obtained from the PCR analysis Florida at level two of growth regulators (3.0mg/l BA + 0.2 mg/l GA_3) via RAPD-PCR.

Primer	Size bands (bp)	F_{R1}	F_{R2}	F_{R3}	F_{R4}	F_{R5}	F_{R6}
ISJ-7	1060	1	1	1	1	1	1
	890	1	1	1	1	1	1
	650	1	1	1	1	1	1
	520	1	1	1	1	1	1
	420	1	1	1	1	1	1
	395	0	0	0	1	0	0
	330	1	1	1	1	1	1
	250	0	0	0	0	0	1
Total		6	6	6	7	6	7

Table 8 and Fig. 2 showed fragments obtained from the RAPD-PCR analysis for Florida cultivar at level four of growth regulators (3.0 mg/l BA with 0.6 mg/l GA_3) from six replications. The primer yielded seven different bands with sizes ranged from 250 to 1060 bp. Three bands were absent at replicate four (F_{R4}). Their sizes were 250 bp, 890 bp and 1060 bp



control | **3 mg/l BA + 0.2 mg/l GA_3** | **3 mg/l BA + 0.6 GA_3**

Fig. 2: RAPD-PCR polymorphism of DNA Florida cultivar using ISJ-7 primer from leaf tissue of *in vitro*-derived plantlets

Table 8: Amplified fragments obtained from Florida at level four of growth regulators (3.0mg/l BA + 0.6 mg/l GA_3) via RAPD-PCR.

Primer	Size bands (bp)	F_{R1}	F_{R2}	F_{R3}	F_{R4}	F_{R5}	F_{R6}
	1060	1	1	1	0	1	1
	890	1	1	1	0	1	1
	650	1	1	1	1	1	1
	520	1	1	1	1	1	1
	420	1	1	1	1	1	1
ISJ-7	330	1	1	1	1	1	1
	250	1	1	1	0	1	1
Total		7	7	7	4	7	7

Table 9 summarizes the obtained results from the two cultivars including the present bands at different levels of growth regulators (BA and GA_3) are confirmed to be present in the fingerprints and the percentage of polymorphism. The percentage of polymorphism was 14.3 and 12.5% at level two and four of growth regulators from six replications in Sweet Charlie, respectively. While, the percentage of polymorphism in Florida was 25% at level two of growth regulators. The highest polymorphism appeared in cultivar Florida at level four of growth regulators (3.0 mg/l BA with 0.6 mg/l GA_3) the percentage of polymorphism was 42.9 %. It means in all cases, that the RAPD fingerprints produced with different levels of BA and GA_3 turn for variation within replicates per levels were almost identical. In this study different of hormones in tissue culture techniques were applied to induce somaclonal variation among different strawberry cultivars. Random amplification of polymorphic DNA (RAPD) is an important tool which has been extensively used to identify polymorphism among the genotypes.

These results agreement with Gaafar and Saker, (2006). They reported that the concentrations of auxin in culture media and number of subcultures are important factors for induction of somaclonal variation in an *in vitro* system. Larkin and Scowcroft, (1988) and Kuksova *et al*., (1997) decided that *in vitro* tissue culture techniques have been known to be an important tool in the induction of variation leading to the development of new plant genotype . Simpson and Bell, (1989) they found that the use of low BA concentrations on strawberry has been recommended by several authors, since it decreases the risk of phenotypic abnormalities after the field establishment of micro-propagated plants. While, Adel (2007), studied the somaclonal variation in micro-propagated strawberry detected at the molecular level from Chandler, Sweet Charlie and Gaviota. He found that most of the obtained bands from *in vitro*-derived plantlets in all primers used with the three cultivars were found to be present in the fingerprints of standard propagated plants, demonstrating no variation in the pattern obtained with DNAs from the two sources of strawberry plants.

Table 9: Primer with arbitrary sequence tested for two effectiveness in the RAPD-PCR analysis that produced polymorphic bands of strawberry.

cv.	Levels of growth regulators (BA + GA_3)	M.W bp	Bands total	Monomorphic	N.	Polymorphic bp.	Polymorphism %.
SC.	control	1060-330	6	6	---	------	------
	3 + 0.2 mg/l	1060-330	7	6	1	454 (S_{R1}- S_{R5})	14.3
	3 + 0.6 mg/1	1060-288	8	7	1	316 (S_{R1})	12.5
FL.	control	1060-250	7	7	---	----	------
	3 + 0.2 mg/l	1060-250	8	6	2	250 (F_{R6}) 395 (F_{R4})	25
	3 + 0.6 mg/l	1060-250	7	4	3	250 890 (F_{R4}) 1060	42.9

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تأثير منظمات النمو النباتية على الإكثار الدقيق والتباين الجسدي لصنفين من الفراولة.

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أجريت هذه الدراسة لمعرفة تأثير بعض منظمات النمو علي التضاعف والاستقرار في التركيب الوراثي لصنفين من اصناف الفراولة هى :سويت شارلي و فلوريدا .زرعت القمم النامية لكلا الصنفين علي بيئة موراشيج و سكوج مضافا اليها البنزيل ادنين) 3 ملجم / للتر + (حمض الجبريلك -0,2 - 0,0) 0,6 -0,4ملجم / لتر (تم تسجيل النتائج بعد 8 اسابيع من الزراعة . أظهرت النتائج تفوق صنف سويت شارلي على صنف الفلوريدا فى عدد النباتات الناتجة بعد مرور 8 اسابيع من الزراعة .وقد تم تحليل الحمض النووي المستخرج من قطع صغيرة من اوراق النباتات الناتجة من زراعة الانسجة معمليا عن طريق RAPD-PCRللكشف عن مدى الاستقرار الجيني لهذه النباتات .أظهرت النتائج وجود اختلافات وراثية بين المعاملات والمكررات عن المقارنه وسجلت اعلى نسبه للاختلافات مع الصنف فلوريدا. مما يشير الى ان الصنف سويت شارلى هو الأكثر ثباتا وعدم التأثر بالتكاثر المعملى.