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FACTORS AFFECTING IN VITRO PROPAGATION AND MICROGRAFTING OF SOUR ORANGE CITRUS ROOTSTOCK

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ABSTRACT

The present investigation was aimed to address two important questions:
1-To what extent is tissue culture can be succeeded in sprouting and rooting the Sour orange?
2-How did the activities of media studied respond to such processes?

The possible application of some commonly in vitro growth regulating compounds is outlined. A number of treatments were applied to determine the best way of inducing in vitro shoot proliferation and rooting on a modified solid MS (1962) medium at full strength. Two explants type (shoot tips and epicotyls) sourced from Sour orange (*Citrus aurantium L.)* Citrus rootstock were cultured for in vitro shoot proliferation and the regenerated shoots for rooting studies. The former process tested the effect of 6-benzylaminopurine (BAP) and 6-furfuryl aminopurine(Kinetin) either alone or in combinations at different concentrations plus a fixed 0.50mg|l NAA. While, the later process tested the effect of combined two auxins, indole-3-butyric acid (IBA) and alpha- naphthaline acetic acid (NAA) at varying concentrations plus a fixed 0.50mg/L Kin.

The obtained results pointed to epicotyls as the better explants to in vitro propagation of the tested rootstock. Medium to best shoot proliferation was MS(1962)basal medium at full strength supplemented with BAP at 1.5mg/L and Kin at 1.00mg/L plus a fixed NAA concentration. Both explants type on this super medium gave a high percentage of shoot multiplication and proliferated shoot of the greatest characteristics except for average shoot length per explant, which the longest shoots were resulted on the same basal medium with the combined two growth regulators at lower concentration (0.25 or 0.50mg/L). Medium to best in vitro rooting was the same basal medium containing IBA and NAA at 2.00mg/L each plus a fixed 0.50 mg Kin/L. The egenerated shoots on such rooting medium succeeded to record the highest rooting percentage, average roots number per explant and the longest proliferated roots as well as relatively the biggest average root diameter per explant.

Micro-grafting study of Washington navel and Valencia scions onto Sour orange rootstock indicated a successful graft union. Micro-graft combination of the former scion onto such rootstock had a degree of graft compatibility greater than Valencia scion onto the same rootstock. This was based on the measurement of three important parameters in this field, grafting success % , survival % and the whole growth vigor of grafted seedlings through the measurement of three characteristics on the grafted seedlings.

INTRODUCTION

Citrus is a highly technical crop. Its successful and profitable cultivation totally depend on the scientific cultivation techniques. The practice of plant tissue culture is part of the recent biotechnology which approaches the field of plant propagation particularly micro-propagation. It has changed the way of most nurserymen, since the applicability of such technology to propagation is of a great benefit not only for Citrus growers, but also for the research workers, students and planners.

Citrus trees are considered the first economic fruit crop in Egypt. According to the statistics of the Ministry of Agriculture (2004), the total area occupied by Citrus trees of the several species and varieties are 143,883 feddan yielded annually 2.5 million metric tons of fruits. Citrus trees are propagated by both seed and vegetative means. The later one are preferred because it ensures true to type plants, uniform quality, regular bearing, etc. Superior trees are likely to arise from certain sources. Tissue culture propagation using explants tissue is now feasible and gaining popularity among nurserymen due to many advantages over conventional methods of vegetative propagation in particular to resolve the major constraint for Citrus rapid adoption by local growers that is the limited availability by elite germplasm pathogen free. Consequently, application of in vitro tissue culture in Citrus is currently expanded worldwide (*Linberger, 2000 and Singh, 2002).*

The intent of the present investigation was to determine the convenient explants tissue (shoot tips and epicotyls) to in vitro propagation of Sour orange Citrus rootstock. The optimum media in complete formation to obtain best shoot multiplication %, shoot proliferation and adventitious roots formation were also studied. In addition, micro-grafting operations in vitro were carried out to estimate the degree of graft compatibility of Washington navel and Valencia sweet oranges(Scions) onto the in vitro cultured rootstock. Three indices effectively used in this respect namely, grafting success %, survival % and the whole growth vigor of grafted seedlings through the measurement of three physical characteristics on them.

MATERIALS AND METHODS

The present research was carried out during three successive years from 2004 to 2007 in two experiments. The first was designed to study the possibility of using tissue culture technique for rapid and economical in vitro micro-propagation of Citrus rootstock Sour orange (*Citrus aurantium L)* and the second one was dealt with this technique in micro-grafting operations aimed to estimate scion-rootstock compatibility of Washington navel and Valencia sweet oranges (*Citrus sinensis Osbeck*) as scions onto this rootstock. Concerning the first experiment factors considered were to determine best explants tissue to be cultured and the optimum medium to obtain a high shoot multiplication percentage as well as maximum shoot and root proliferation. Accordingly, it can be explained as follows:

Explants. Two explants type were tested in this study, the first one was actively shoot tips 2 cm in length each were collected from in vitro newly

growing shoots resulted from 40-day-old seedlings grown from previously in vitro cultured seeds of the rootstocks under study. Seeds culturing was done in glass jars (12 X 6 Cm), 7 seeds per jar at tissue culture laboratory of Horticulture Department, Agriculture College, The University of Mansoura using MS (1962) at full strength hormone-free as culture medium. After leaf removal, shoots were surface disinfested for 10 min in a continuously stirred 10% Clorax solution containing 0.1% Tween 20 (v/v) as spreading agent and the terminal parts were excised 1.0 Cm in length each using scalpel blade and forceps, in sterile Petri dishes under a septic condition. As for the second type, they were epicotyl explants prepared from germinated seeds. The excited explants were surface sterilized in a laminar flow hood by immersing them into 70% ethanol solution for one minute, followed by dipping in 10% sodium hypo-chloride (Na OCL) for 10 min, then rinsed three times in sterile distilled water and transferred to culture glass jars (9X5.5 Cm) containing basal medium in complete formation .

Medium. The culture medium for in vitro shoot proliferation was consisted of Murshigo and Skoog in organic salts and vitamins at full strength as the basal medium (*DeCleene and Ley, 1976*) containing 30 g/L sucrose as carbon and energy source and Bacto agar at the rate of 7mg/L for medium solidification. Such medium also was supplemented with 6-benzylaminopurine (BAP) at 0.00, 0.25,0.50, 0.75, 1.50 and 1.50mg/L and 6-furfurylaminopurine (Kin) at 0.25, 0.50, 0.75 and 1.00mg/L combined either solely or in combinations plus α-naphthaline acetic acid (NAA) at a fixed 0.50mg/L concentration. The culture medium for root proliferation was consisted of the same MS basal medium used for shoot proliferation but combined with 2 auxins, Indole-3-butyric acid (IBA) at 0.50, 1.00, 1.50 and 2.00mg/L and NAA at 0.50, 1.00, 1.50 and 2.00mg/L added either solely or in combinations plus a fixed 0.50mg Kin/L. The pH of shoot proliferation media was adjusted to be 5.8 before the addition of agar using 0.50% potassium hydroxide. Media were dispensed into 9X5.5 Cm glass jars each contained 30ml of nutrient media. The cultured jars with polypropylene sheets that were held in place rubber bands that resistance of higher hot degree through autoclaved at 121C (1.2 kg/Cm^3) for 20 min, then left to cool and harden for 24 hrs before being used.

Culture procedures for shoot proliferation. Explants were cultured individually in glass jars (9 x 5.5 Cm) each contained 30 ml of shoot proliferation media and were placed in growth culture room at 26 ± 2C under fluorescent light receiving 16 hrs illumination followed by 8 hrs dark period. Four weeks later culturing date, shoot multiplication percentage, and average shoots number, leaves number along with shoots length per explant were measured on the proliferated shoots on each type of explant used. The former shoot characteristic was calculated according to the following equation:

$$\text{Shoot multiplication\%} = \frac{\text{Number of explants multiple shoots}}{\text{Number of cultured explants}} \times 100$$

The resultant proliferated shoots were used as a mother stock for the subsequent rooting experiments.

Statistical analysis. Shoot proliferation experiments were designed as 42 treatments, 10 replicates each of one cultured jar. The statistical procedure followed those described by Mass, *et al.* ,(1994).

Root proliferation on cuttings. The concerned experiments were designed to examine the efficiency of the tested rooting media in promoting adventitious root formation on cuttings prepared from the regenerated shoots on both explants type and in turn determining best in vitro rooting media. These cuttings were taken from uniformity growing regenerated shoots derived from shoot proliferation experiments. The culture media were MS (1962) basal medium at full strength supplemented with IBA and NAA at the concentrations indicated above combined either solely or in combinations plus a fixed 0.50mgKin/L. Sucrose at 30mg/L and Difco agar at 7mg/L were also added. These rooting media before the addition of agar the PH was adjusted to 5.7 using 0.50% potassium hydroxide and dispensed in glass jars (9 x 4.5 Cm), 30ml each. The cultured jars were closed with plastic covers of high temperature resistant, autoclaved at 121°C for 20 min and left to cool and harden for two days before being used. Regenerated shoot micro-cuttings were carefully excised at 1.0 Cm in length (rooting explants) by removing basal nature leaves and forceps were used, pre-using they were soaked into 70% ethanol and flame treated. Cuttings were individually inserted vertically into culture jars containing 30 ml of rooting media tested and incubated on racks in growth culture room at 25±1°C under 16hrs photoperiod of high intensity (1500 Lux) provided by white fluorescent light followed by 8hrs dark period. Six weeks later culturing date, average roots number, root length and root diameter per cutting wer calculated.

Statistical analysis. Root proliferation experiments were designed as 8 treatments, 10 replicates of one cultured jar each. The statistical procedure were applied according to Pontikis and Sapoutzaki, (1985).

Micro-grafting experiment. Micro-grafting operations of Washington navel and Valencia scions onto Sour orange rootstock were made to determine grafting success % and degree of scion- rootstock compatibility. The plant material used as rootstock was seedlings obtained from previously in vitro cultured seeds of sour orange (cultured in glass tubes containing medium as indicated in explants preparation 2 seeds each) after off-type ones were eliminated basing on visual comparison for uniformity. The sourced of scions were apical portion of shoots (3-5 Cm in length) of new growing shoots on both scions fruiting trees. As for micro-grafting procedure, epicotyls of rootstock seedling were decapitated to be 1-2 Cm each and subjected to make an inverted T incision through the cortex to the cambial region. A thin film from the solid germination medium was put on the incision before insertion of the soaked apical meristems (scions) into incision. The grafted seedlings were individually and carefully transferred to glass tubes (150 x25 Cm) containing 15 ml culture medium of MS basal medium supplemented with 1mg/L thiamine-Hcl, 1mg/L pyridoxine-Hcl, 1mg/L nicotinic acid, 2mg/L glucose, 5mg/L calcium pentathenate and 100mg/L myo-inositol. The medium also contained 30g/L sucrose and 7g/L agar. It was adjusted to pH 5.7 with Naoh-Hcl. The cultured tubes were capped with polypropylene closures, placed on the bridge and kept upright in the

incubator at 27 °C for 4 days and in the dark for 2 weeks then finally transferred to light intensity of 1500 Lux for 4 weeks.

Acclimatization of micro-grafted seedlings was done by carefully taken off them from the glass tubes, thoroughly washed with sterile distilled water at 30 °C to remove any carryout of the medium to eliminate contamination may occurred around the roots and transplanted to Agriculture pots containing a mixture of peat moss, vermiculite, perlite and sand (1:1:1:1 v/v). The agriculture medium before culturing were autoclaved at 121 °C (1.5 kg/Cm) for 2o min. The cultured pots were transferred to glass box (100 x 60 x 25 Cm) covered with white sheet of polyethylene for two weeks in the laboratory in order to keep the grafted seedlings on a high relative humidity. Water plus Hogland nutritional solution was added to the bottom of the box at 0.50 Cm highly. One week later, they were transferred to greenhouse conditions.

Scion – rootstock compatibility determination. This was based on the measurement of three main parameters on the grafted seedlings, average grafting success % at one- month- old grafts in vitro (micro-grafting operation was considered successful when the grafted rootstock in vitro still green, started sprouting and continued growth), graft survival % at 2- month-old grafts in greenhouse, and the whole growth vigor of the grafted seedlings at one-, 2-, 4- and 6 – month- old in 2 seasons of study. The later parameter was presented through the measurement of three physical characteristics on the grafted seedlings, average leaves number, seedling length length of shoot per graft combination under study at the four successive ages.

Statistical analysis. micrografting experiments were designed as 2 treatments, 10 replicates. All the obtaied data for all the experiments were subjected to analysis of variance (ANOVA) by the general linear models (GLMs) procedure using statistical analysis system (2005) (SAS). Mean comparison was performed using the least significant difference (LSD) method at 5% level according to Gomez and Gomez (1984).

RESULTS AND DISCUSSION

The obtained results in this study showed that the effect of tested media in complete formation was differed according to three major factors of a considerable importance including, the type and source of explants used, the concentration of growth regulators and auxins tested , and the procedure of growth regulators added (solely or in combination). Before going further in explaining the effect of these factors, it may be interesting to discuss herein the reason to use the MS (1962) as the basal medium. The importance of such medium was resulted from the suggestion that the development of a truly optimal mineral formulation may be practically impossible. Nevertheless, the MS (1962) formula represents an approximation to this and has proved superior to all other mineral formulations for tissue culturing of several plant species and cultivars. As fore Citrus in vitro propagation *Parthasarathy and Nagaraju, (1995)* reported that the media used for micro-propagation of Citrus tissue have largely involved the basal salts of *Murashigo and Skooge (1962)* for shoot regeneration.

The results concerned the first factor pointed to epicotyls as the better to micro-propagate Sour orange rootstock. They indicated an increasing effect on shoot multiplication percentage higher than shoot tip ones (Table 1). This effect was true with MS basal medium containing the combined two growth regulators at all tested concentrations. In the same line, the results in Tables (2 and 4) and illustrated in Figures (1, 2) concerned the effect of explants type on average leaves and shoots number confirmed the superiority of epicotyls to the shoot tip ones on these characteristics. It was also observed from Table (1) that the applied BAT at the higher concentrations (1.25 and 1.50 mg/L) increased shoot multiplication % at any concentration of Kin used and the minimum values were resulted on explants cultured on MS basal medium BAP-free. The constant and lower effect on both explants type on medium BAP-free combined with Kin at any concentration proved the superiority of BAP and the inferiority of Kin.

Concerning the second factor which was dealt with growth regulator concentrations used, the statistical analysis technique used to indicate the real effect of each growth regulator applied either solely or in different combinations (interactions) on shoot proliferation characteristics on both explants type clearly proved that BAP concentration in complete formation medium for shoot proliferation is considered the limited factor affecting characteristics of proliferated shoot. The superiority was to BAP at 1.50mg/L.

Table (1): Effect of the interactions among explant type, certain concentrations of BAP and Kin on shoot multiplication % during proliferation stage of sour orange explant cultured for 4 weeks on MS solid media.

Explant type (A)	BAP (mg/L) (B)	Kin (mg/L) (C)		
		0.25	0.50	1.0
Shoot tip	0.0	10	10	0
	0.25	20	10	20
	0.50	20	20	30
	0.75	40	50	40
	1.0	70	70	80
	1.25	90	90	90
	1.50	100	100	100
Epicotyl	0.0	50	50	50
	0.25	50	70	60
	0.50	80	90	60
	0.75	80	100	70
	1.0	90	100	70
	1.25	90	100	100
	1.50	100	100	100
L.S.D 5%	5.37			

It was the best to produce significantly the highest leaves and shoots number on Sour orange explants (Tables 2 to 4 and Figures 1&2) if compared with the same explants on medium BAP-free or at the lowest concentration (0.25mg/L). These findings are greatly supported by the results in the same tables which were indicated that culturing explants on MS basal

medium containing any BAP concentration tested had an increasing effect on these shoot characteristics greater than culturing the same explants on basal MS medium BAP-free (Control). The opposite was true in case of average shoot length. A negative association was observed between BAP concentration and average shoot length value measured. The highest value was resulted on Control medium (Table 5 and Figure 3). As for Kin effect, the concerned results in the same tables cleared that Kin at 1.00mg/L counted an average leaves and shoots number per explant higher than the lower concentrations. Comparing the effects of BAP with those of Kin, it could be stated that the response of explants to BAP specially at the high concentration (1.50 mg/L) was superior to Kin concentration used. The effective role of BAP on shoot proliferation was previously confirmed on several studies on either Citrus species and varieties or others. In that respect, Baruah *et al.*, (1996) *studied the response of* Citrus species explants to BAP or Kin at different concentrations. They found variations among the species for shoot proliferation. 6-benzylaminopurine (BAP) was superior to Kin in that respect. Ramsunder *et al.*, (1998) worked on shoots proliferation of acid lime explants.

Table (2): Effect of Explant type and MS medium at full strength supplemented with BAP and Kin at different concentrations on average leaves number, shoots number and shoots length per explant of sour orange rootstock cultured for 4 weeks on MS solid media.

	Treatments	number of leaves	number of shoots	length of shoots (cm)
Explant type (A)	Shoot tip	6.21	0.91	2.01
	Epicotyl	7.30	0.70	3.15
	L.S.D 5%	0.75	0.26	0.51
BAP concentrations (mg/L) (B)	0.0	4.85	1.45	1.30
	0.25	5.13	1.00	1.51
	0.50	5.88	0.93	1.75
	0.75	6.75	0.84	1.95
	1.0	7.11	0.54	2.53
	1.25	7.48	0.53	3.01
	1.50	10.10	0.38	5.98
	L.S.D 5%	0.19	0.07	0.13
Kin conc. (mg/L) (C)	0.25	6.39	0.85	2.26
	0.50	7.00	0.87	2.70
	1.0	6.87	0.71	2.77
	L.S.D 5%	0.28	0.09	0.19

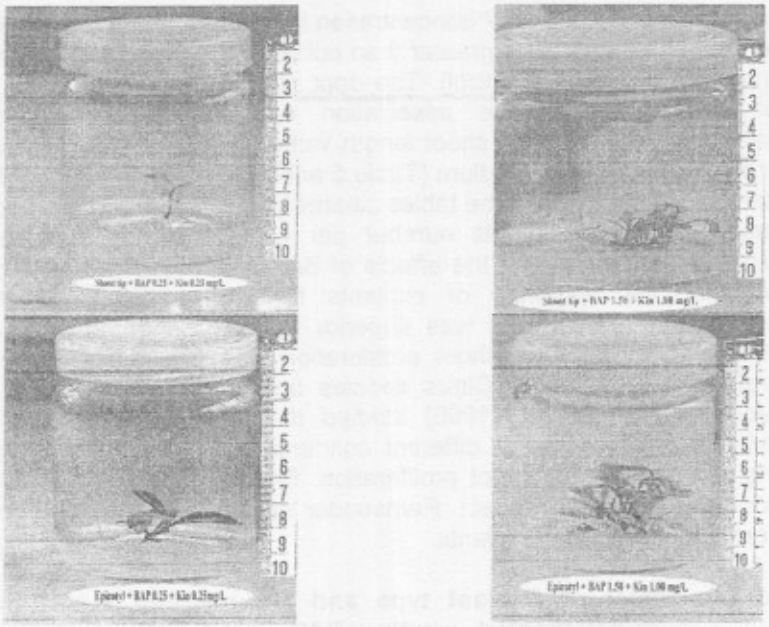


Figure (1): Photo showing the effect of MS (1962) medium supplemented with different BAP and Kin concentrations (mg/L) on average leaves number of the cultured explants of sour orange rootstock for 4 weeks on MS solid media.

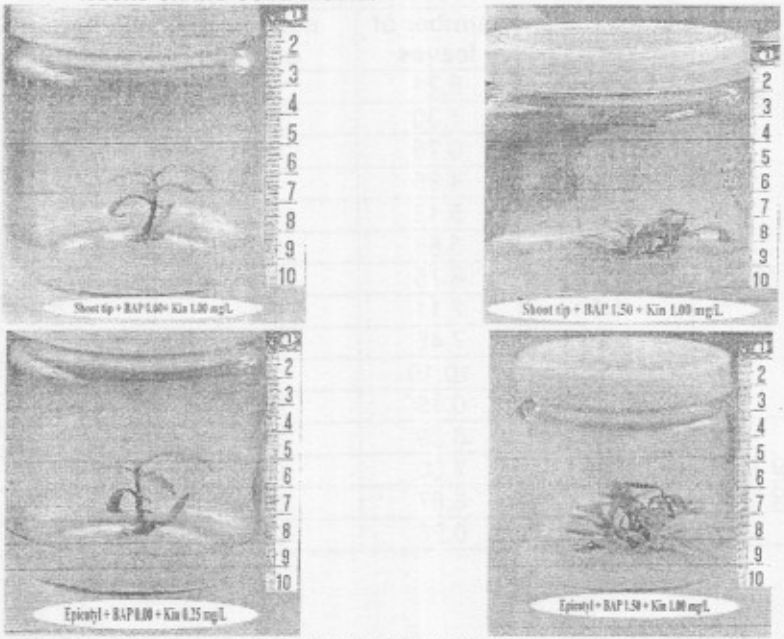


Figure (2): Photo showing the effect of MS (1962) medium supplemented with different BAP and Kin concentrations (mg/L) on average number of shoots of the cultured explants of sour orange rootstock

They found that the greatest number of shoots per explant was achieved on medium containing BAP at 5.00mg/L. A similar study carried out by AL-Khawri and EL- Bahrany, (2001) explained the important role of BAP in stimulating multiple shoot formation. In the same line, Krishan Kumar *et.al.*, (2001) worked with in vitro epicotyls segment of sweet oranges reported that the highest number of explants showing shoot proliferation rate and the highest number of leaves and shoots were obtained on MS medium containing 1.00 mg BAP/L. More recent, Almeida *et al.*, (2002) came to a similar results with *Citrus sinensis* and *C. limonia*. They reported that the best explants bud induction were obtained on medium containing BAP at 1.00mg/L. Similar observation on apples was detected by Sedlak *et al.*, (2006) who reported that the highest shoot proliferation rate was obtained on MS medium with 1.00mg/L BAP.

Table (3): Effect of the interactions among explant type and certain concentrations of BAP and Kin on average leaves number per explant during proliferation stage of sour orange rootstock cultured for 4 weeks on MS solid media.

Explant type (A)	BAP (mg/L) (B)	Kin (mg/L) (C)			Mean of (A ×B)
		0.25	0.50	1.0	
Shoot tip	0.0	6.60	5.30	6.70	6.20
	0.25	2.80	5.10	3.50	3.80
	0.50	4.50	3.30	4.30	4.03
	0.75	5.70	8.90	3.60	6.06
	1.0	5.00	4.30	3.60	4.30
	1.25	7.80	5.10	10.90	7.93
	1.50	9.00	10.70	13.60	11.10
Mean of (A ×C)		5.91	6.10	6.60	
Epicotyl	0.0	10.10	6.30	9.90	8.76
	0.25	5.00	7.30	4.90	5.73
	0.50	4.70	5.00	7.30	5.67
	0.75	5.00	7.50	5.60	6.03
	1.0	8.12	5.50	5.40	6.34
	1.25	6.60	8.40	12.10	9.03
	1.50	6.80	8.60	12.16	9.18
Mean of (A ×C)		6.61	6.94	8.19	
Mean of (B× C)	0.0	8.35	5.80	8.30	L.S.D 5%
	0.25	3.90	4.85	3.70	AB 0.24
	0.50	4.60	4.15	5.80	AC 0.33
	0.75	5.35	7.70	4.60	BC 0.23
	1.0	7.55	8.20	4.50	ABC 0.28
	1.25	7.20	5.80	8.35	
	1.50	7.80	9.55	12.85	

Considering the results of the third factor (applied method of growth regulators), it was cleared that the addition of BAP and Kin solely to MA basal medium had an effect on the cultured explants weaker than in combination. This finding was conducted from the tabulated data of the interactions between explants type and BAP or Kin at the recommended concentration for

leaves number (Table 3 and Figure 1) and shoots number (Table 4 and Figure 2).From these tables and Figures, it was observed in one side a great support to the use of both growth regulators at the highest concentration as a selection shoot proliferating medium. On the other side, they indicated that the effect of BAP and Kin at these concentrations either added solely or in combination had an increasing effect on these characteristics. Therefore, medium consisted of solid MS (1962) basal medium at full strength supplemented with BAP at 1.50mg/ L and Kin at 1.00mg/L plus a fixed 0.50 mg NAA/L is uniquely suited to obtain the highest increasing effect on shoot proliferation characteristics on both explants type used. Otherwise, it is important to state herein that this medium, relatively, caused a decreasing effect on average shoot length. Since a negative association in general, was detected between either BAP or Kin concentration and average shoots length per explant. These association was observed with all used explants. This means that the cultured explants on medium containing the combination of these two growth regulators at lower concentrations (0.25 0r 0.50 mg/L) were more effective to proliferate longer shoots (Table 5 and Figure3).

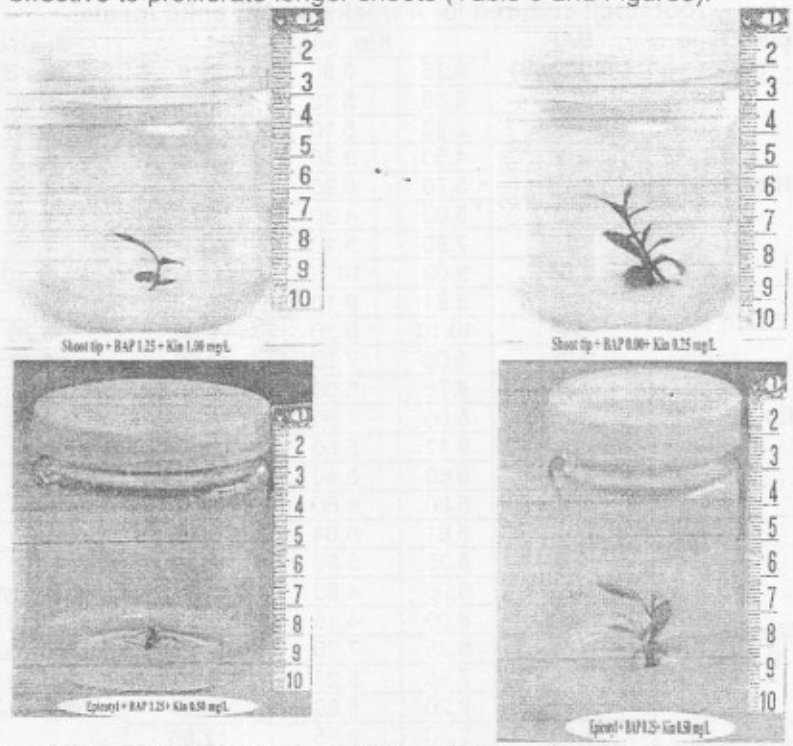


Figure (3): Photo showing the effect of MS (1962) medium supplemented with different BAP and Kin concentrations (mg/L) on average length of shoots of the cultured explants of sour orange rootstock for 4 weeks on MS solid media.

These results are in complete agreement with those reported by Singh *et al.*, (1995) who worked with in vitro culture of Citrus species and found that the multiple shoots were obtained on MS medium containing 1.00 mg BAP/L , 0.50 mg Kin/L and 0.50 mg NAA/L. In vitro culture of Pummelo shoot explants, Baruah *et al.*, (1996) indicated that the best shoot proliferation was resulted on medium containing BAP and Kin at 0.75mg/L. A study on epicotyls segment of sweet orange cultivars carried out by Krishan-Kumar *et al.*, (2001) revealed that the highest number of explants showing proliferated shoots of the highest leaves and shoots number were on MS medium combined with BAP at 1.0mg/L. The longest shoots however, were on the same medium containing BAP at 0.25mg/L. In the same line, EL-Bahrany, (2001) working on lime node explants found that in vitro shoots number per node was the highest on MS medium combined with BAP at 0.50mg/L and Kin at 1.00mg/L plus NAA at 0.50mg/L..

Table (4): Effect of the interactions among explant type and certain concentrations of BAP and Kin on average shoots number per explant of sour orange rootstock cultured for 4 weeks on MS solid media.

Explant type (A)	BAP (mg/L) (B)	Kin (mg/L) (C)			Mean of (A ×B)
		0.25	0.50	1.0	
Shoot tip	0.0	1.10	0.90	0.80	0.93
	0.25	1.20	1.10	1.00	1.10
	0.50	1.20	1.20	1.10	1.17
	0.75	1.40	1.40	2.20	1.66
	1.0	2.70	2.90	1.20	2.27
	1.25	2.30	1.80	2.90	2.33
	1.50	3.50	3.50	6.70	4.57
Mean of (A ×C)		1.91	1.82	2.27	
Epicotyl	0.0	1.30	1.30	1.30	1.30
	0.25	1.80	3.20	1.50	2.16
	0.50	2.10	2.50	1.70	1.10
	0.75	1.40	4.30	2.10	2.60
	1.0	2.50	2.70	3.00	2.73
	1.25	4.20	4.10	3.00	3.77
	1.50	4.90	7.00	10.30	7.40
Mean of (A ×C)		1.80	3.20	1.30	
Mean of (B× C)	0.0	1.25	1.25	1.05	
	0.25	1.30	1.95	1.25	L.S.D 5%
	0.50	1.45	2.05	1.40	AB 0.16
	0.75	1.75	2.25	2.10	AC 0.23
	1.0	2.40	2.70	2.15	BC 0.16
	1.25	3.45	3.50	2.95	ABC 0.19
	1.50	4.20	5.25	8.50	

As for rooting experiments, result of preliminary experiments indicated that in vitro culturing cuttings from regenerated shoots of Sour orange rootstock could be rooted. We investigated a number of factors that have been shown affect rooting in vitro. These factors included explants Type,

auxin kind and concentration. Quantity and quality of such effect were estimated through three characteristics measured on the proliferated roots. Cuttings were cultured on solid MA (1962) medium at full strength in complete formation for 6 weeks.The obtained results of the former factor indicated that rooting cuttings of Sour orange epicotyls was accomplished successfully in vitro. These cuttings significantly recorded per cutting higher average roots number, longer main roots and greater average roots diameter (Table 6 and Figures 4&5).Therefore, epicotyls cutting would be desirable for rooting.

Considering the results related to the other factor (Auxin type and concentration), it was observed a linear association between the concentration of combined NAA+IBA and almost the value of measured root characteristics. Therefore, it can be stated that among the used rooting media, the MS (1962) basal medium at full strength supplemented with NAA and IBA in combination at 2.00mg/L each plus a fixed 0.50mgKin/L is the promising rooting medium for in vitro culture Sour orange cuttings.

Table (5): Effect of the interactions among explant type and certain concentrations of BAP and Kin on average shoot length per explant (cm) of sour orange rootstock cultured for 4 weeks on MS solid media.

Explant type (A)	BAP (mg/L) B)	Kin (mg/L) (C)			Mean of (A ×B)
		0.25	0.50	1.0	
Shoot tip	0.0	2.35	1.95	2.18	2.16
	0.25	1.24	1.23	0.96	1.14
	0.50	1.11	0.76	0.53	0.80
	0.75	0.45	1.21	0.68	0.78
	1.0	0.62	0.59	0.44	0.55
	1.25	1.00	0.42	0.22	0.54
	1.50	0.51	0.43	0.26	0.40
Mean of (A ×C)		1.04	0.94	0.75	
Epicotyl	0.0	1.05	1.38	1.29	1.24
	0.25	0.69	1.47	0.58	0.91
	0.50	0.64	1.19	0.75	0.86
	0.75	0.63	0.48	0.66	0.59
	1.0	0.62	0.41	0.48	0.50
	1.25	0.51	0.34	0.55	0.46
	1.50	0.45	0.31	0.33	0.36
Mean of (A ×C)		0.66	0.79	0.66	
Mean of (B× C)	0.0	1.70	1.34	1.42	
	0.25	0.94	1.22	0.91	L.S.D 5%
	0.50	0.81	1.21	0.86	AB 0.08
	0.75	0.81	1.07	0.63	AC 0.12
	1.0	0.65	0.50	0.46	BC 0.08
	1.25	0.53	0.38	0.38	ABC 0.09
	1.50	0.48	0.37	0.29	

Cuttings on this super medium produced significantly the greatest average roots number per cutting , the longest proliferated roots and almost

the biggest average root diameter per shoot (Table 6, Figure 4 & 5). On the other hand, data in the same table and figures showed that MS medium containing the least concentration of such combined auxins (0.50 mg/L each) is not recommended in this respect, since culturing both cutting types recorded the lowest value of root characteristics. These results are strongly confirmed with an increasing number of reports in the field of in vitro root proliferation. In that respect, *Pasqual and Ando,(1990)* working with *Poncirus trifoliata* in vitro rooting; *Can, et al., (1992)* with Sour orange in vitro rooting; *Prez, et al., (1997)* with Mexican lime and mandarin cultivars in vitro rooting. They all found a positive effects on root proliferation in the use of MS medium supplemented with NAA and IBA combined ether solely or in combinations at similar or close to the concentration recommended in this study. More recent, the results of Al-Bahrany, *(2001)* are in complete agreement with our findings. He worked with in vitro lime shoots cultured on rooting media and recommended the MS one containing IBA and NAA at 2.00mg/L as the best rooting medium. Likewise, the present results partially agreed with those obtained in the studies carried out by Moreira-Das, *et al.*, (2000); Al-Khayari and AL-Bahrany,(2001); Kaya and Gubbuc,(2001).

Table (6): Effect of Explant type, IBA and NAA concentrations and their interactions on average roots number, roots length (cm) and roots diameter (mm) per cutting of sour orange explants cultured for six weeks on MS solid media.

Average roots number

Explant type (A)	NAA + IBA(mg/L) (B)				Mean of (A)
	0.50*	1.00*	1.50*	2.00*	
Shoot tip	1.20	2.10	2.30	3.70	2.33
Epicotyl	1.10	2.40	3.20	5.00	2.93
Mean of (B)	1.15	2.25	2.75	4.35	
L.S.D 5%	(A) NS		(B) 0.27		(AB) 0.33

Average root length

Explant type (A)	NAA + IBA(mg/L) (B)				Mean of (A)
	0.50*	1.00*	1.50*	2.00*	
Shoot tip	1.20	2.30	2.47	3.30	2.32
Epicotyl	1.98	3.12	3.50	4.78	3.35
Mean of (B)	1.59	2.71	2.99	4.04	
L.S.D 5%	(A) S		(B) 0.28		(AB) 0.33

Average root diameter

Explant type (A)	NAA + IBA(mg/L) (B)				Mean of (A)
	0.50*	1.00*	1.50*	2.00*	
Shoot tip	0.22	0.26	0.34	0.44	0.32
Epicotyl	1.45	0.43	0.53	0.62	0.76
Mean of (B)	0.84	0.35	0.44	0.53	
L.S.D 5%	(A) S		(B) 0.12		(AB) 0.15

*** These values were for each of the used auxins.**

They tested the effect of certain auxins at various concentrations used either alone or in combinations to induce adventitious roots formation on Citrus regenerated shoots. On the other hand, these findings are contradicted with the results obtained by *Lukman, et al., (1990)* who worked with in vitro culturing of Troyer citrange shoot apices and found no appear effect to NAA on promote rooting.

Considering the results obtained in the first experiments, it could be recommended MS (1962) basal medium at full strength supplemented with BAP at 1.50 mg/L and Kin at 1.00mg/L plus a fixed 0.50 mg NAA/L as the uniquely suited to induce best proliferated shoots on both shoot tip and epicotyl explants sourced from Sour orange Citrus rootstock. Culturing epicotyls explants were the super to in vitro proliferate shoots of a higher qualities. As for root proliferation medium, such results pointed to the same basal medium combined with NAA and IBA at 2.00mg/L each plus a fixed 0.50 mgKin/L as the best rooting medium to form adventitious roots of the greatest root characteristics on cuttings from both explants type sourced from Citrus rootstock studied. Once again in vitro epicotyl cuttings were more convenient than those of shoot tip ones in this respect.

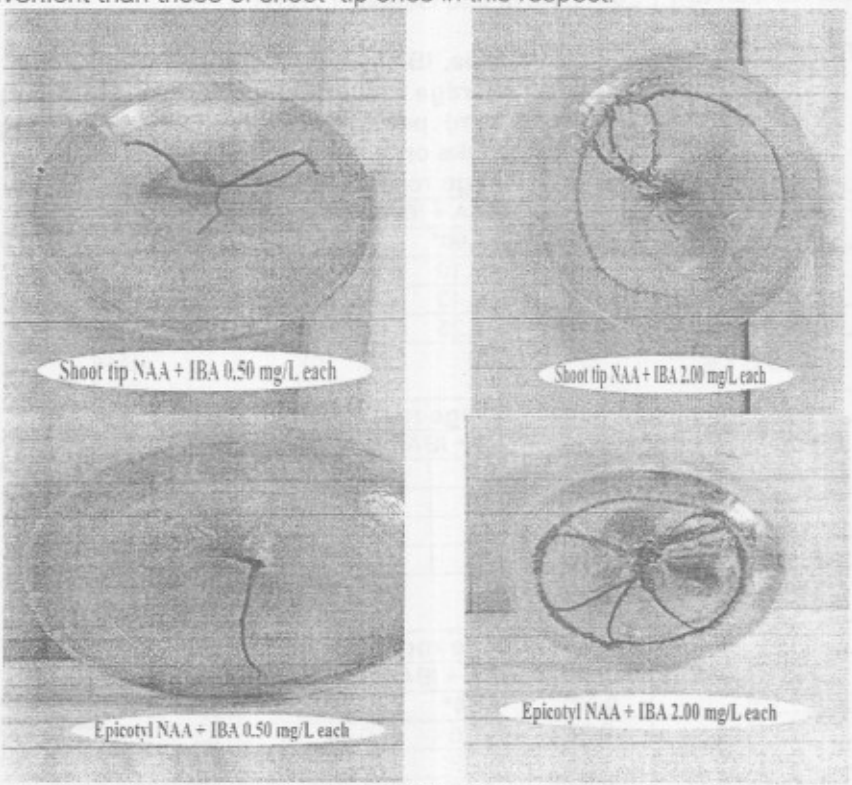


Figure (4): photo showing the effect of explant type cultured on MS (1962) medium supplemented with NAA + IBA at 0.5 and 2.0 mg/L for 6 weeks on average roots length per cutting of sour orange explants.

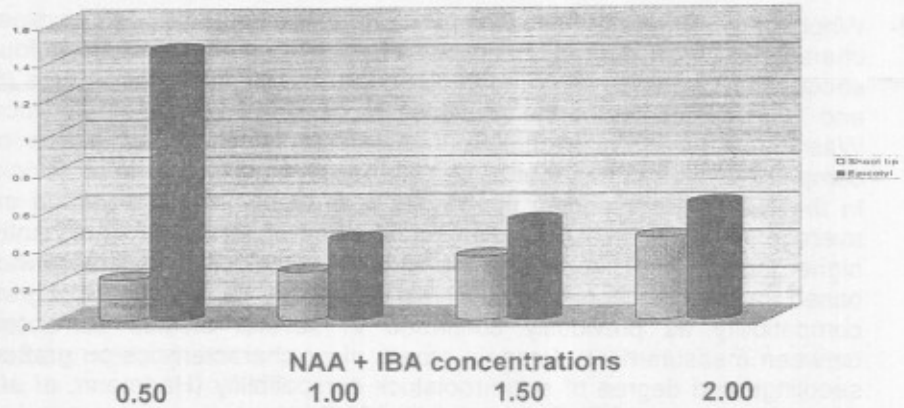


Figure (5): Diagram showing the effect of explant type cultured on MS (1962) medium supplemented with NAA + IBA at 0.5 and 2.0 mg/L for 6 weeks on roots diameter per cutting of sour orange explants.

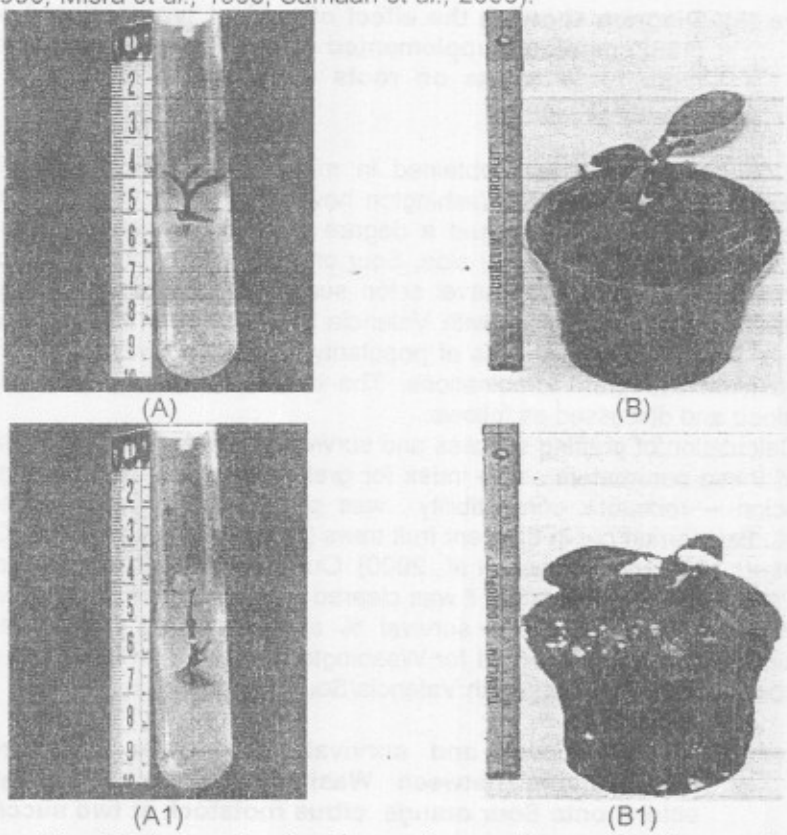
Concerning the results obtained in micro-grafting experiment, it was found in one side that both Washington navel and Valencia scions made a successful graft unions that had a degree of graft compatibility with Sour orange rootstock. On the other side, Sour orange is the promising stock that can make with Washington navel scion successful compatibility of greater degree than the same stock with Valencia scion. To find these statements three graft compatibility indices of popularity in this field were measured on the resulted two graft combinations. The obtained measurements can be explained and discussed as follows:

1- Calculation of grafting success and survival percentages. The application of these parameters as an index for grafting success and the degree of scion – rootstock compatibility was previously confirmed in several studies carried out in different fruit trees (Williamson *et al.*, 1992; Guindy *et al.*, 1995 and Samaan *et al.*, 2000). Our results herein are presented in Table (7). From this table, it was cleared that the higher grafting success % at one-month-old and survival % at 2-month-old grafted seedling significantly were recorded for Washington navel onto Sour orange graft combination comparing with Valencia/Sour orange one.

Table (7): Graft success and survival percentages of micrograft combinations between Washington navel and Valencia scions onto Sour orange citrus rootstock at two successive ages.

Graft participators		Graft success % at One-month-old			Graft survival % 2-month-old		
Rootstock	Scion	1st culture	re-culture	x̄	1st season	2st season	x̄
Sour orange	W. navel	86.67	85.49	86.08	84.00	82.00	83.00
	Valencia	46.67	47.12	46.90	57.10	56.20	56.65
LSD 5%		6.53	6.50		5.16	5.17	

2- Whole growth of grafted seedlings. The measurements of three physical characteristics on grafted seedlings of each graft combination at four successive stages as recorded in Table (8) and illustrated in Figures (6 and 7) greatly confirmed the results of the above two parameters. Since Washington navel scion onto Sour orange rootstock had a degree of compatibility higher than that of Valencia scion onto the same rootstock. In the two tested seasons, the former graft combination produced an average leaves number, seedling length and shoot length significantly higher than Valencia scion onto the same rootstock. This statement was based on the acceptance of whole growth as a criterion for graft compatibility as previously confirmed in several studies correlated between measurement of certain growth vigor characteristics on grafted seedlings and degree of scion-rootstock compatibility (Hartmann, *et al., 1990;* Misra *et al.*, 1995; Samaan *et al.*, 2000).



(A) (B)

(A1) (B1)

Figure (6): Photograph showing micrograft combinations of Sour orange rootstock with Washington navel and Valencia scions.

A- Washington navel onto sour orange rootstock at one-month-old.
B- Washington navel onto sour orange rootstock at 2-month-old.
A1- Valencia onto sour orange rootstock at one-month-old.
B1- Valencia onto sour orange rootstock at 2-month-old.

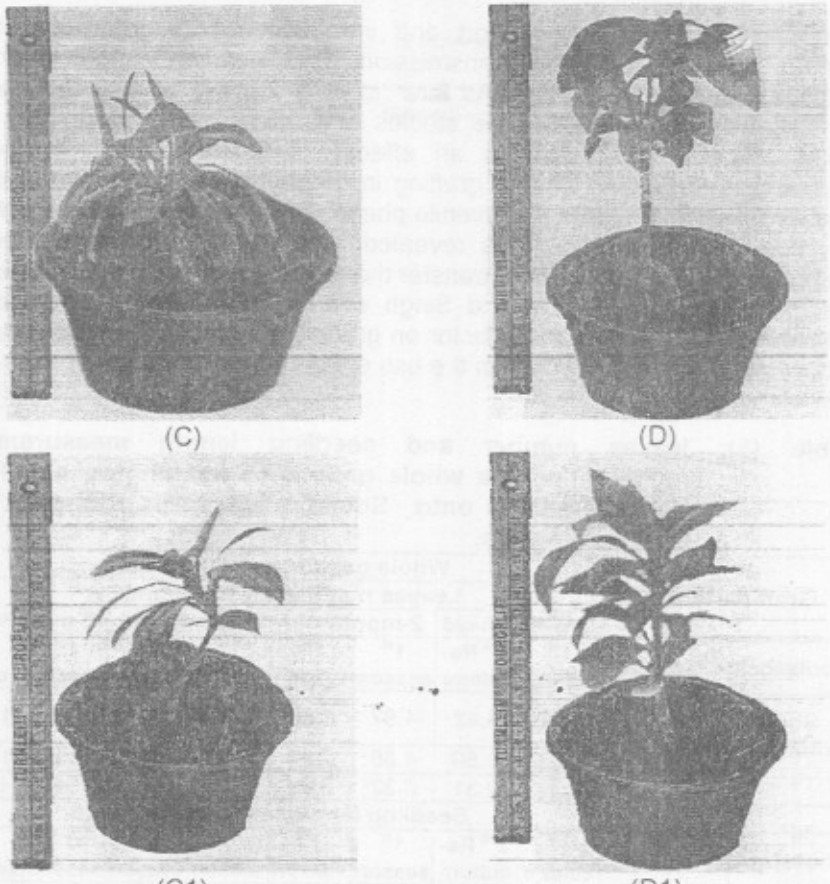


Figure (7): Photograph showing micrograft combination of Sour orange rootstock with Washington navel and Valencia scions.
C- Washington navel onto sour orange rootstock at 4-month-old.
D- Washington navel onto sour orange rootstock at 6-month-old.
C1- Valencia onto sour orange rootstock at 4-month-old.
D1- Valencia onto sour orange rootstock at 6-month-old.

The successful application of tissue culture technique in micro-propagation of Sour orange rootstock in this study offers certain advantages not possible with conventional propagation techniques. It allows the production of a great number of genetically identical plants from small pieces of stock plants in relatively short period of time which accelerated asexual propagation (Matsumoto, *et al.*,2000). In addition, the original tissue explants, with most species, does not destroy the parent plant. Once it is established actively divided to be a continuous source of micro-cuttings which can result in plant production under greenhouse conditions without seasonal interruption. In the same line, Mas Camacho, *et al.*, (1991) reported that in vitro propagation of Citrus permits the production of disease-free planting

materials which allows transport and introduction of vegetative material without the risk of disease transmission. This is advantageous over the traditional quarantine system. As fore in vitro micro-grafting technique, its benefits were explained in the studies of Obeidy and Smith (1991) who reported that micro-grafting is an effective technique for elimination of viruses, the early diagnosis of grafting incompatibilities, the rejuvenation of mature tissue and bypass the juvenile phase in fruit trees. Furthermore, Ke *et al.*, (1993) working on fruit trees revealed that micro-grafting was succeeded to shorten the time required for transfer the grafted plants to field. In the same line, Navarro *et al.*, (1975) and Singh *et al.*, (2008) were pointed to the rootstock age as an important factor on grafting success. The highest rate of successful grafts was resulted in the use of younger rootstock seedlings (1 or 2-week- old).

Table (8): leaves number and seedling length measurements representing the whole growth of washington navel and Valencia scions onto Sour orange citrus rootstock at 4 successive ages.

Graft parteners		Whole growth characteristices							
		Leaves number per micrograft at							
		One-month-old		2-month-old		4-month-old		6-month-old	
Rootstock	Scion	1st culture	2nd Re-culture	1st season	2nd season	1st season	2nd season	1st season	2nd season
Sour orange	Washington navel	3.47	3.42	4.87	4.89	10.40	10.21	15.87	15.50
	Valencia	2.53	2.50	4.80	4.84	8.73	8.10	12.80	12.92
LSD 5%		0.32	0.31	0.32	0.32	0.39	0.42	0.44	0.44
		Seedling length/micrograft (cm) at							
Rootstock	Scion	1st culture	2nd Re-culture	1st season	2nd season	1st season	2nd season	1st season	2nd season
Sour orange	Washington navel	3.79	3.60	8.27	8.30	14.15	14.11	25.45	25.53
	Valencia	2.75	2.81	6.80	6.85	12.22	12.33	21.54	21.60
LSD 5%		0.30	0.30	0.33	0.33	0.38	0.38	0.46	0.38
		shoot length micrografting at							
Rootstock	Scion	1st culture	2nd Re-culture	1st season	2nd season	1st season	2nd season	1st season	2nd season
Sour orange	Washington navel	0.51	0.49	3.53	3.45	4.93	4.99	10.40	9.97
	Valencia	0.25	0.52	1.70	1.80	3.79	3.60	8.68	8.78
LSD 5%		0.10	0.12	0.38	0.39	0.39	0.32	0.44	0.34

They attributed this increasing effect with younger seedlings to their capability to cover most shoot tips grafted onto them with precocious callus formation. In addition, they reported that most shoot tips grafted on older rootstocks were dried, turned brown and died. Vijayakumari and Singh, (2003) working on commercial Citrus propagation, they reported that micro-budding propagation technique is applicable for fast getting the marketable budded seedlings earlier with reduced cost. On the other hand, the conventional budding is season dependent, limited and requires longer time for field release bud-grafts.

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العوامل المؤثرة على الإكثار المعملى والتطعيم الدقيق لأصل الموالح النارنج

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أجرى هذا البحث خلال الفترة من ٢٠٠٤ - ٢٠٠٧ فى معمل زراعة الأنسجة والصوبة الزجاجية لقسم البساتين بكلية الزراعة جامعة المنصورة. وقد اشتملت الدراسة على نوعين من التجارب الأول منها أهدف الى دراسة امكانية استخدام بعض المركبات المنظمة للنمو والشائعة الإستعمال فى عدد من المعاملات للوصول لأفضل بيئة زراعية معدلة (موراشيج وسكوج ١٩٦٢ كاملة القوة) لإنتاج أقوى أفرخ خضرية وتجزير معملى على نوعين من الأجزاء المفصولة (Explant) هما القمم النامية والسويقات الجنينية العليا (Epicotyl shoot tips) لأصل الموالح النارنج.

وقد تم اختبار منظمى النمو بنزيل أمينو بيورين (BAP) والكينيتين بتركيزات مختلفة أضيفت للبيئة بتركيزات منفردة أو متجمعة وفى كل المعاملات زودت بتركيز ثابت من نفثالين حامض الخليك (NAA) وذلك فى حالة دراسة بيئات تفريع الأفرخ shoot proliferation أما فى حالة الوصول لأفضل بيئات التجزير تم اختبار نوعين من الأكسيين هما أندول حامض البيوتريك (IBA) ونفثالين حامض الخليك (NAA) اضيفت لنفس البيئة إما بصورة فردية أو مجتمعة وايضا فى كل الحالات زودت البيئة بتركيز ثابت من الكينتين.

وقد أشارت النتائج المتحصل عليها إلى أن السويقة الجنينية العليا كانت الأفضل فى الإكثار المعملى لأصل النارنج بالمقارنة مع النوع الأخر. أما بالنسبة لأفضل البيئات للوصول لأفضل تفريع أفرخ فقد كانت بيئة الاستزراع السابق ذكرها بعد تدعيمها بمنظمى النمو بنزيل أمينو بيورين عند تركيز ١,٥ ملليجم فى اللتر والكينتين بتركيز ١ ملليجرام فى اللتر الى جانب اضافة نفثالين حامض الخليك بتركيز ٠,٥ ملليجرام فى اللتر.

حيث أن كلا النوعين من الأجزاء المفصولة والمزروعة على البيئة المنتخبة سجل أعلى نسبة من تفريع الأفرخ وصفات الأفرخ الناتجة فيما عدا صفة متوسط طول الافرخ حيث كانت أطول الافرخ نتجت على نفس البيئة ولكن مع التركيزات المنخفضة من منظمات النمو المختبرة.

كانت افضل البيئات لإنتاج تجذير معملى هى ما تكونت من البيئة الاساسية السابق ذكرها محتوية على أكسين أندول حامض البيوتريك ونفثالين حامض الخليك عند تركيز ٢ ملليجرام فى اللتر لكلا منهما مع اضافة الكينتين بالتركيز الثابت ٠,٥ ملليجرام فى اللتر حيث ان الأجزاء المزروعة على هذه البيئة نجحت فى تسجيل اعلى عدد من الجذور لكل جزء مزروع وأطول جذور ناتجة هذا الى جانب أن الجذور كانت نسبيا أكبر فى القطر.

أما النوع الثانى أجرى فيه دراسة نوعين من التركيبات التطعيمية المعملية بين طعوم لأشجار مثمرة من صنفى البرتقال بسرة والفلنشيا مع أصل النارنج المزروع معمليا وقد أشارت النتائج ان استخدام طعم (Washington navel) على أصل النارنج (Sour orange) قد اعطى أعلى النتائج لجميع الصفات المدروسة لتقدير درجة التوافق بينهما بالمقارنة بالتركيب الأخر.