Improvement of the Vegetative Propagation of Two Citrus Rootstocks *in vitro*

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Abstract: *Two experiments (in starting stage) were carried out on two citrus rootstocks namely: sour orange (C. aurantium, L.) and volkamer lemon (C. volkameriana, Ten.) to produce healthy, virus- free and non-contaminated explants used in the following stages (multiplication, rooting and acclimatization) .*

Contamination percentage (after 30 days of in vitro culture) was increased in explants of 1 cm length than of 0.5 cm length. The lowest percentage of contamination was found in explants of 0.5 cm and sterilized with 50 % clorox for 15 min with or without ethanol alcohol for 30 sec. in explants taken in active growth period (March) or inactive period (Dec) and this treatment was considered the most proper for both studied rootstocks.

Healthy explants percentage significantly increased in explants of 1 cm length as compared with 0.5 cm length and in explants were taken in March in both propagated rootstocks. Sterilized explants with 10 % clorox (15 min) + 70 % ethanol (30 sec); or with 20 % clorox (5 min) +70 % ethanol (30 sec) and 30 % clorox (5 min) treatment, these 3 treatments were considered the most suitable for sterilizing explants of both rootstocks.

Survival percentage after proliferation (40 days of in vitro culture) was increased in March sampling of 1 cm explant length and in explants culture on MS medium supplemented with 1 mg/ L BA in sour orange rootstock and 3 mg/L in Volkamer lemon rootstock; however, the lowest values of survival were obtained from Ms medium lacking growth in regulators, BA had a clear effect in this concern as compared with NAA and GA_3 at different concentrations. Survival % tended to increase as BA concentration was increased.

Key words: *in vitro, sour orange and Volkamer lemon rootstocks, explant, starting stage, contamination %, healthy explants %, proliferation, growth regulators, MS medium.*

INTRODUCTION

The world market demands for citrus fruits with high quantities and specific quality are annually increased. Producing healthy and standard budded citrus trees, which greatly contribute in increasing yield and improve quality of fruits is considered the first step in citrus industry.

Regardless of the method employed, the production of well grown compatible, disease free , true to type tress require systematic and detailed attention. Plant breeders who searches out genetically assistant or tolerant strains, cultivars and rootstocks and how to use them in improving cultivars and rootstocks through investigation with the traditional and near methods based on biotechnology.

Traditionally, the budded citrus trees require more than two years from planting seed to reach saleable size. Recently, many greenhouse nurseries have been established to produce marketable budded citrus trees in as little as 9-15 months (**Janik and Moore, 1996 and Davies and Albrige, 1998**).

Citrus trees in some countries infested with many serious diseases (tristesa CTV, exocortis viroid CXV and also citrus psorosis) . Choice of the rootstocks should be based on the most important limiting factors to production in a particular region, local climate and soil conditions. For example, sour orange should not be used in areas where CTV is prevalent, while it is used in alkaline and saline soils. However, volkamer lemon is also a lemon hybrid which is rootstock produces large vigorous trees yielding large quantities of moderate to poor quality fruit like rough lemon (**Castle, 1987**). These are not susceptible to CTV, xyloporosis or CEV, but are susceptible to blight and the citrus and burrowing nematodes (**Carpenter *et al.*, 1981**).

Application of new and improved techniques such as plant tissue culture (as a vegetative propagation method) and shoot tip explants are the simplest micropropagation system.

Accordingly, the most promising technique included effect of sampling date, explant size and sterilization agent concentrations on percentages of contamination, healthy and survival explants in both sour orange and Volkamer lemon rootstocks. The effect of some growth regulators such as BA, NAA and GA_3 applied to MS mediums were also studied. Finally, producing healthy, virus free and non- contaminated explants for the following stages are the aim of this study.

MATERIALS AND METHODS

This investigation was carried out in tissue culture laboratory of Horticulture Department, Faculty of Agriculture, Zagazig University on two citrus rootstocks namely : sour orange and volkamer lemon during two successive seasons (2001 & 2002). Tissue culture technique was adopted for micropropagating the above mentioned rootstocks.

Methodology and measurements have been followed in this study could be classificated as follows:

1. Source of plant materials

Sour orange *(Citrus aurantium,* L.*)* and volkamer lemon *(Citrus volkameriana,* Ten.) trees were about 12-years old at the starting of this study and grown in the Experimental Orchard of Agriculture Research Center at El-Kassasin region, Ismaelia Governorate, Egypt were used. Ten healthy trees of each rootstock were selected. Yet, the selected trees are grown in sandy soil at 5m. apart and irrigated by drip irrigation system, they were equal in height, vigorous, cropping. The selected trees were subjected to the normal management used in citrus orchards.

2. Time of explants excision

Shoot tip explants were obtained from spring flush of plant material sources (trees of sour orange and volkamer lemon rootstocks). For this purpose, five branches of 1-year-old or more distributed in different tree locations were selected and labled, then 5 actively new growing shoots of each labled branch were excised from the studied rootstocks. Accordingly, shoot tip explants were collected in two dates : firstly in March (are in active phase) and secondly in December (are in inactive phase). The collected shoot tips directly placed in clean polyethylene bags then transferred to the laboratory.

3. Preparation of shoot tip explants

3.1. Length of shoot tip explants

Shoot tip explants (the terminal portion of shoots) were taken from plant material sources (sour orange and volkamer lemon trees) at length from 3 to 5 cm from active new growing shoots (i.e. March). Yet, in the laboratory, the collected explants were pinched – off by removal older growth, placed in clean polyethylene bags, stripped-off their leaves and spines, divided into nodes (shoot portion at one node) with (0.5 and 1.0 cm length) then directly washed by run water for 10 min. before sterilization treatments.

3.2. Surface sterilization treatments

Two surface sterilization treatments were used in this experiment as follow:

1. Soaking in clorox (commercial bleach which have (5.25% (v/v) sodium hypochloride, Na O Cl) at concentrations of 10, 20, 30, 40 or 50 % for 5, 10 or 15 min.
2. Dipping in ethanol alcohol 70% (v/v) for 30 sec and soaking in clorox at the same concentrations and time used in the above mentioned treatment.

The disinfected plant materials were rinsed 3 times with autoclaved distilled and sterile water as reported by many investigators **(Murashige *et al.*, 1972; Stino *et al.*, 1980; Marin and Duran-Vila, 1991 and Abd El-Zaher, 1998).**

Data concerning contamination and healthy percentages were calculated in every treatment after 30 days of culture for both studied rootstocks in both considered dates and length of explant.

4. Basal nutrient medium

Murashige and Skoog (1962) medium **(MS), was used as** the basal nutrient medium. All culture media used were adjusted at P^H 5.7 ± 0.1 prior to addition of agar.

The prepared culture media were distributed into the culture glass jars (250 ml), and tubes (25 × 15 ml) each contain (20 ml) of culture medium.

The culture tubes were immediately capped with polypropulin closure then were autoclaved at 121 C° and 1.5 b/in^2 for 20 min.

5. *In vitro* plant regeneration

5.1. Establishment of shoot tip explant

Apical meristemes and adjacent tissues with leaf primordia and (1-2) developed leaves excised from source of plant materials were used as shoot tip explants **(Murashige *et al.*, 1972)**. Prepared and sterilized shoot tip explants were cultured on basal MS medium supplemented with BAP (6. Benzyle-amino purine) (commercial name BA or Benzyle-adenine), at concentrations of 0.0, 0.1, 0.3, 0.5, 0.8, 1.0 or 3.0 mg/l, according to the procedure of **(Lukman *et al.*, 1990)**, Gibberellic acid (GA_3) at concentrations of 0.0 or 0.5 mg/l and α-Naphthyle acetic acid (NAA) at concentrations of 0.0 or 0.1 mg/l, according to **(Can *et al.*, 1992)**.

Various combinations between different tested growth regulators were used as follows : 0.1 BA + 0.1 NAA (mg/l); 0.1 BA + 0.5 GA_3 (mg/l) and 0.1 BA + 0.1 NAA + 0.5 GA_3 (mg/l).

All established media were supplemented with 30g/l sucrose and 6 g/l agar and all culture tubes were incubated at (26 ± 1 C°) and (16/8 hrs. day/night) using fluorescent lamps (40 W) approx. induced 2160 Lux.

Data concerning, the survival percentage were calculated after 40 days of culture.

Statistical analysis

Percentages of the obtained results were transformed to angles (Angle = ARC Sin $\sqrt{\text{percentage}}$) before analysis, then the obtained data were subjected to analysis according to the method of **Snedecor and Cochran, (1980)**. The individual comparisons were carried out by using the New Least Significant Difference **(New L.S.D)** at (0.05 level) according to **Waller and Duncan, (1969).**

RESULTS AND DISCUSSION

First experiment (sterilization experiment)
Sour orange rootstock
1.Contamination percentage

As shown in (Table, 1) percentage of contamination of sour orange explants was not affected by date of explanting (March & December) for both explant length of 0.5 or 1.0 cm (51.43, 59.37% against 53.29, 58.68%). In addition, explant length had a clear effect on contamination percentage and the percentage tended to increase as the explant length was increased from 0.5 to 1.0 cm of those taken in March or December.

As for the effect of sterilization treatments (concentration of clorox and ethanol), the obtained data show that, unsterilized explants (control) and those treated with (10% clorox for 5 min) of explant length 0.5 cm were taken either in March or December, in addition to the explant length of 1.0 cm taken in December and treated with (clorox 10% for 5 min + ethanol 70% for 30 sec); (clorox 10% for 10 min) and (clorox 20% for 5 min) gave percentage of contamination reached to (100%).

It could be concluded that, explant length of 0.5 cm treated with 50% clorox for 15 min + ethanol 70% for 30 sec (Tr 31) either taken in March or December was the most proper treatment for sterilizing sour orange rootstock explants.

Data presented in (Table, 1) also show that, the interaction between the studied factors (date of explant removal × concentration of agents × length of explant) were statistically insignificant referred that, the studied factors act independently in this concern.

From another point of view, explants of sour orange of 0.5 cm length taken during active growing period (0.00%) or those explants of 1 cm also taken in the same period and sterilized with clorox 50% for 15 min. + Ethanol 70% for 30 sec were the proper explants as compared with other dates, length or sterilization treatments.

2. Healthy percentage

Data representing in (Table, 2) show that, percentage of healthy explants clearly affected by date of explanting (March and December). As such, the percentage of healthy explants increased with explants taken in March as compared with those explanted taken in December of explant length (0.5 or 1.0 cm) (62.73, 70.55% against 59.87, 68.18%) for 0.5 and 1.0 cm explants in March and December, respectively.

The obtained data also show that, the 1 cm explant length was superior than the short one of March sampling; while, the percentage slightly increased in December for 1.0 cm as compared with 0.5 cm In other words, explant length of 1.0 cm gave the highest percentage of healthy explants, in general, when were taken in March (active growing period) and slightly affected by explant length when was excised in inactive growing period (December).

Concerning the effect of sterilization treatments, untreated explants (control) and those treated with clorox 10% for 5 min of 0.5 or 1.0 cm explant length taken in March or December produced (0.00%) severe browning explants (different dead organs of explant), in addition to, those explants taken in December of both lengthes (0.5 and 1.0 cm) and treated with clorox 10% for 5 min + ethanol 70% for 30 sec.

It is interesting to mention that, healthy explants reached to (100%) in explants of Trs 5, 6, 7, 9 and 14 of the two tested lengthes in March sampling and the same results (100%) were noticed in Trs. 5, 7, 9, 10 and 14 in both lengths of December samples and the other tested sterilization treatments ranged between 0.00 and 100%.

It could be concluded that, the Trs 5, 7, 9, 10 and 14 were the suitable sterilization treatments for both explant lengthes either taken in March or December from sour orange rootstock. Accordingly, and in this concern treatments (control and clorox 10% for 5 min) were not suitable for sterilization explants of sour orange in most cases.

The obtained results also show that, interaction between the studied factors (date × concentration × explant length) showed insignificant differences and acted independently in this concern (Table, 2).

b. Volkamer lemon rootstock

1- Contamination percentage

Data recorded in (Table, 3) show that, contamination percentage of volkamer lemon explants clearly affected by both explant length and concentration of agents used in sterilization; while, the percentage was not affected by date of explant removal (March or December).

As for the effect of explant removal date, the obtained data show that, both of March (active growing period) and December (inactive growing period) of both lengthes were not affected by date of sampling, in addition, explant length of 0.5 or 1.0 cm slightly differed in contamination percentage in studied periods of sampling (47.57, 51.46% against 49.34, 55.77%) for 0.5 and 1.0 cm length in March or December, respectively. Henceforth, untreated explants (control) and explants treated with clorox 10% for 5 min. were contaminated and the percentage reached to 100% and the same percentages were found also in both explant lengthes of December when treated with 10% clorox for 5 min+ 70% ethanol for 30 sec (Tr 3). Moreover, the lowest percentage of contamination was detected (1.0 and 3.0%) in March and December sampling dates, respectively. In addition, explants of 0.5 cm in length

Table (1) : Effect of sterilization treatments on contamination percentage of sour orange rootstock explants during period (2001- 2002)

Trs.	Treatments (Clorox (%) Ethanol 70 % for 30 sec.)	Contamination (%)					
		March			December		
		0.5 cm	1 cm	Mean	0.5 cm	1 cm	Mean
1	Control (untreated)	100.00	100.00	100.00	100.00	100.00	100.00
2	10 % for 5 min.	100.00	100.00	100.00	100.00	100.00	100.00
3	-- (+).Ethanol (+) 70 % for 30 sec.	90.33	100.00	95.16	97.79	100.00	98.89
4	10 % for 10 min.	90.90	100.00	95.45	91.95	100.00	95.97
5	-- (+).Ethanol (+) 70 % for 30 sec.	70.70	95.50	83.10	81.80	98.00	89.90
6	10 % for 15 min.	70.00	90.00	80.00	90.15	100.00	95.07
7	-- (+).Ethanol (+) 70 % for 30 sec.	70.00	80.55	75.27	80.81	89.89	85.35
8	20 % for 5 min.	95.00	100.00	97.50	92.20	100.00	96.10
9	-- (+).Ethanol (+) 70 % for 30 sec.	87.15	90.75	88.95	80.00	90.92	85.46
10	20 % for 10 min.	80.00	88.50	84.25	80.10	90.00	85.05
11	-- (+).Ethanol (+) 70 % for 30 sec.	67.60	78.70	73.15	69.90	80.00	74.95
12	20 % for 15 min.	60.00	70.05	65.02	60.40	61.43	60.91
13	-- (+).Ethanol (+) 70 % for 30 sec.	20.30	33.35	26.82	30.00	37.70	33.85
14	30 % for 5 min.	70.85	88.88	79.86	90.00	100.00	95.00
15	-- (+).Ethanol (+) 70 % for 30 sec.	70.90	80.12	75.51	70.50	78.70	79.60
16	30 % for 10 min.	40.31	44.45	42.38	50.50	50.79	50.64
17	-- (+).Ethanol (+) 70 % for 30 sec.	28.00	35.13	31.56	30.00	35.05	32.52
18	30 % for 15 min.	29.10	38.18	33.64	25.20	28.80	27.00
19	-- (+).Ethanol (+) 70 % for 30 sec.	18.20	20.25	19.22	16.92	19.17	18.04
20	40 % for 5 min.	60.00	60.60	60.30	70.00	77.77	73.88
21	-- (+).Ethanol (+) 70 % for 30 sec.	50.60	61.00	55.80	30.30	45.14	37.72
22	40 % for 10 min.	50.00	60.67	55.33	60.70	66.60	63.65
23	-- (+).Ethanol (+) 70 % for 30 sec.	18.17	25.50	21.83	20.17	27.27	23.72
24	40 % for 15 min.	40.00	40.48	40.24	10.10	11.16	10.63
25	-- (+).Ethanol (+) 70 % for 30 sec.	10.75	15.13	12.94	7.79	8.21	8.00
26	50 % for 5 min.	40.90	50.19	45.54	49.94	49.99	49.96
27	-- (+).Ethanol (+) 70 % for 30 sec.	37.73	44.44	41.08	40.40	45.00	42.70
28	50 % for 10 min.	12.00	15.15	13.57	10.10	10.17	10.13
29	-- (+).Ethanol (+) 70 % for 30 sec.	8.88	13.18	11.03	10.00	9.90	9.95
30	50 % for 15 min.	6.00	12.13	9.06	3.30	5.50	4.40
31	-- (+).Ethanol (+) 70 % for 30 sec.	0.00	7.77	3.88	1.00	2.10	1.55
Mean		51.43	59.37		53.29	58.68	

New L.S.D :

- **D (Date) (N.S)**
- **C (Concentration) (2.142)**
- **E (Explant size) (2.825)**

Interaction :

- Insignificant

Table(2) : Effect of sterilization treatments on healthy percentage of sour orange rootstock explants during period (2001- 2002)

Trs.	Treatments (Clorox (%) Ethanol 70 % for 30 sec.)	Contamination (%)					
		March			December		
		0.5 cm	1 cm	Mean	0.5 cm	1 cm	Mean
1	Control (untreated)	0.00	0.00	0.00	0.00	0.00	0.00
2	10 % for 5 min.	0.00	0.00	0.00	0.00	0.00	0.00
3	-- (+).Ethanol (+) 70 % for 30 sec.	0.00	100.00	50.00	0.00	0.00	0.00
4	10 % for 10 min.	0.00	100.00	50.00	0.00	100.00	50.00
5	-- (+).Ethanol (+) 70 % for 30 sec.	100.00	100.00	100.00	100.00	100.00	100.00
6	10 % for 15 min.	100.00	100.00	100.00	0.00	100.00	50.00
7	-- (+).Ethanol (+) 70 % for 30 sec.	100.00	100.00	100.00	100.00	100.00	100.00
8	20 % for 5 min.	0.00	100.00	50.00	0.00	100.00	50.00
9	-- (+).Ethanol (+) 70 % for 30 sec.	100.00	100.00	100.00	100.00	100.00	100.00
10	20 % for 10 min.	94.40	92.90	93.65	100.00	100.00	100.00
11	-- (+).Ethanol (+) 70 % for 30 sec.	94.00	90.11	92.05	93.94	92.90	93.42
12	20 % for 15 min.	90.00	85.90	86.95	92.11	90.90	91.35
13	-- (+).Ethanol (+) 70 % for 30 sec.	100.00	85.00	92.50	90.91	87.80	89.35
14	30 % for 5 min.	100.00	100.00	100.00	100.00	100.00	10.00
15	-- (+).Ethanol (+) 70 % for 30 sec.	92.90	80.80	86.85	90.10	80.91	85.50
16	30 % for 10 min.	90.99	90.91	90.95	90.12	90.92	90.52
17	-- (+).Ethanol (+) 70 % for 30 sec.	90.90	90.00	90.45	86.90	87.97	86.28
18	30 % for 15 min.	90.13	85.80	86.96	88.90	87.80	88.35
19	-- (+).Ethanol (+) 70 % for 30 sec.	87.70	85.13	86.41	88.91	88.00	88.45
20	40 % for 5 min.	90.20	90.14	90.17	80.14	90.00	85.07
21	-- (+).Ethanol (+) 70 % for 30 sec.	90.11	90.00	90.05	90.19	90.11	90.15
22	40 % for 10 min.	90.17	90.00	90.08	92.00	91.90	91.95
23	-- (+).Ethanol (+) 70 % for 30 sec.	70.77	73.90	72.33	80.00	70.72	75.39
24	40 % for 15 min.	70.70	60.17	65.43	85.00	70.00	77.50
25	-- (+).Ethanol (+) 70 % for 30 sec.	40.45	40.40	40.42	55.00	42.42	48.71
26	50 % for 5 min.	40.90	41.10	41.00	49.15	49.05	49.10
27	-- (+).Ethanol (+) 70 % for 30 sec.	42.48	55.50	48.99	30.90	30.13	30.51
28	50 % for 10 min.	30.33	25.20	27.76	30.00	30.17	30.08
29	-- (+).Ethanol (+) 70 % for 30 sec.	27.72	20.20	23.96	12.13	22.20	17.16
30	50 % for 15 min.	10.06	7.10	8.58	20.00	11.10	15.55
31	-- (+).Ethanol (+) 70 % for 30 sec.	10.00	7.00	8.50	10.00	8.79	9.39
Mean		92.73	70.55		59.87	98.18	

New L.S.D :

- **D (Date) (2.124)**
- **C (Concentration) (4.221)**
- **E (Explant size) (2.121)**

Interaction :

- Insignificant

was not contaminated (0.00%) when treated with (50% clorox for 15 min) or (50% clorox for 15 min + 70% ethanol in 30 sec) in March and December for 0.5 cm of explant length only by using sterilization Trs (30 & 31) of (50% clorox for 15 min) and (50% clorox for 15 min. + 70% ethanol for 30 sec).

The obtained data in (Table, 3) also show that, the interaction between the studied factors (data × concentration × explant length) were statistically insignificantly referred that, the studied factors, acts independently in this concern.

It could be concluded that, removal explants of 0.5 cm length in March period and using treatments of 50% clorox for 15 min + 70% ethanol for 30 sec or 50% clorox for 15 min were the most suitable treatments for minimizing the contamination of volkamer lemon explants.

2- Healthy percentage

As noticed in (Table, 4) date of explant removal (March and December) showed insignactnt differences concerning percentage of healthy explants. However, both of explant length and sterilization treatments significantly affected healthy explant percentage. As such, explant length of 1 cm gave the highest values (66.75, 65.20% against 58.23, 57.69%) as compared with 0.5 cm in samples of March and December, respectively. Moreover, sterilization treatments had a clear effect on healthy explant percentage and the ratio ranged between (0.00 - 99.99%) for untreated (control) and explants sterilized with (10% clorox for 5 min) and those treated with (clorox 30% for 5 min), respectively. In addition, percentage of healthy explants reached to (100%) with explants of March sampling either for 0.5 or 1 cm and those sterilized with 10% clorox for 15 min + 70% ethanol for 30 sec; 20% clorox for 5 min + 70% ethanol for 30 sec and 30% clorox for 5 min; while, corresponding values of December samplesd reached to more than (99%) in Trs. of 9, 10, 14 and 15. In other words, the best sterilization treatment, in general, for sterilizing explants of volkamer lemon rootstock of both samples (March and December) or both lengthes (0.5 and 1 cm) was the treatment of 30% clorox for 5 min. while, treatments of control, 10% clorox for 5 min; and clorox 50% for 15 min + ethanol 70% for 30 sec produced severe browning explants and were considered unsuitable for sterilization explants of volkamer lemon rootstock.

Interaction between (date of explant removal × sterilization treatments × explant length) concerning percentage of healthy explant was statistically insignificant (independently factors).

The obtained data proved that, percentages of contamination and/or healthy of explant significantly affected by explant length. which length of 1.0 cm gave the highest percentages of contaminated explants; while, the youngest explant length (0.5 cm) gave the lowest percentages of both tested rootstocks.

In agreement with the obtained results **Bondok *et al.*, (1985)** on "Hindy" banana proved that, the death of explants during the next 4 weeks of (shoot tip apex plus (6-8) leaf primordia type B) compared to the other (type, A : shoot tip of about 2-3 mm and type C : the apical meristem of about 3-5 mm) types and the large length enhances the occurrence of browning and consequently the culture failed to either survive or proliferate. In the same direction **Sandoval and Miller, (1987)** found that, Musa explants that are too large (10 mm) are also unsatisfactory because they show more blackning and contamination and thus lower resultant survival rates than smaller explants. Also, **Vuylsteke, (1989)** showed that, the most appropriate explant length measuring (2.5 mm) in both height and diameter at the leaf base as compared with the large explant (5 mm) for the establishment of vigorous cultures that multiple profusely in banana.

In addition, **Badr, (1998)** found that, in the starting stage, large explants of banana var. Williams (shoot tips 10 mm in length) indicated higher contamination and browning levels in comparison with medium (5 mm) and small (2.5 mm) length of explants. **Martino *et al.*, (1999)** on olive explants of cv Moraiolo came the above mentioned conclusion.

Concerning the effect of explant removal date, March (active growing period) and December (inactive growing period) on percentage of contamination and / or healthy explants, it is clearly that, date of March was superior than December sampling date of both lengthes (0.5 and 1.0 cm) on sour orange and volkamer lemon rootstocks. The available literature concerning the effect of sampling date on percentage of contamination or healthy explants are vague. While, many reports proved that, the highest percentage and amount of callus formation were noticed on bud explants of sour orange from summer flush of juvenile trees and grown on MS medium and the contamination percentage differed accordingly to the flush and age of explants from mature trees **(Stino *et al.*, 1980)**. In addition, the optimum time to gather *prunus marianna* shoot tips and buds for explants was between May (25) and August (14) to ensure shoot and root formation **(Borred, 1971)**. Furthermore, wood from *Castanea sativa* formed callus, the best in March and worst in December; interestingly another chestunt clone was found to grow best in March but worst in July **(Monsion and Dunez, 1971)**. Shoot tips (0.3 – 1.0 cm) of apple cultivars Fujiand and Red star were removed each month from Oct 1992 to Sep 1993 and cultured on MS medium. Effectiveness of tissue culture was closely related to the period of shoot tip

Table (3) : Effect of sterilization treatments on contamination percentage of volkamer lemon rootstock explants during period (2001- 2002)

Trs.	Treatments (Clorox (%) Ethanol 70 % for 30 sec.)	Contamination (%)					
		March			December		
		0.5 cm	1 cm	Mean	0.5 cm	1 cm	Mean
1	Control (untreated)	100.00	100.00	100.00	100.00	100.00	100.00
2	10 % for 5 min.	100.00	100.00	100.00	100.00	100.00	100.00
3	-- (+).Ethanol (+) 70 % for 30 sec.	87.81	100.00	93.90	100.00	100.00	100.00
4	10 % for 10 min.	90.00	100.00	95.00	98.90	100.00	99.45
5	-- (+).Ethanol (+) 70 % for 30 sec.	88.00	92.90	90.45	90.10	100.00	95.05
6	10 % for 15 min.	82.28	90.92	86.90	80.19	90.00	85.09
7	-- (+).Ethanol (+) 70 % for 30 sec.	80.10	80.95	80.52	81.18	81.90	81.54
8	20 % for 5 min.	98.00	100.00	99.00	99.00	100.00	99.50
9	-- (+).Ethanol (+) 70 % for 30 sec.	80.70	90.90	85.80	82.20	90.92	86.56
10	20 % for 10 min.	60.60	70.70	65.65	60.90	71.17	66.03
11	-- (+).Ethanol (+) 70 % for 30 sec.	40.40	45.90	43.15	45.40	48.28	46.84
12	20 % for 15 min.	35.20	30.30	32.75	28.17	31.13	29.65
13	-- (+).Ethanol (+) 70 % for 30 sec.	20.40	20.94	20.67	25.00	27.72	26.36
14	30 % for 5 min.	80.80	85.80	83.30	70.95	90.10	80.52
15	-- (+).Ethanol (+) 70 % for 30 sec.	70.70	70.65	70.67	72.00	88.10	80.05
16	30 % for 10 min.	20.10	20.40	20.25	30.00	30.15	30.07
17	-- (+).Ethanol (+) 70 % for 30 sec.	20.00	25.00	22.50	18.00	25.10	21.55
18	30 % for 15 min.	18.19	25.00	21.59	10.00	30.00	20.00
19	-- (+).Ethanol (+) 70 % for 30 sec.	6.05	18.00	21.02	10.00	15.00	12.55
20	40 % for 5 min.	60.60	60.90	60.75	99.00	99.10	66.05
21	-- (+).Ethanol (+) 70 % for 30 sec.	60.00	60.00	60.00	60.60	67.76	64.18
22	40 % for 10 min.	20.00	22.00	21.00	22.20	28.00	25.10
23	-- (+).Ethanol (+) 70 % for 30 sec.	12.00	15.00	13.50	20.00	20.15	20.07
24	40 % for 15 min.	4.00	8.40	6.20	8.00	20.00	14.00
25	-- (+).Ethanol (+) 70 % for 30 sec.	4.00	7.00	5.50	5.00	10.90	7.95
26	50 % for 5 min.	60.00	92.01	61.00	67.01	69.18	68.09
27	-- (+).Ethanol (+) 70 % for 30 sec.	60.00	60.21	60.10	55.60	70.00	62.80
28	50 % for 10 min.	8.00	11.10	9.55	11.19	11.18	11.18
29	-- (+).Ethanol (+) 70 % for 30 sec.	7.00	14.10	10.55	10.00	30.00	20.00
30	50 % for 15 min.	0.00	4.40	2.20	2.00	10.11	6.05
31	-- (+).Ethanol (+) 70 % for 30 sec.	0.00	2.00	1.00	0.00	6.00	3.00
	Mean	47.57	51.46		49.34	55.77	

New L.S.D :

- **D (Date) (N.S)**
- **C (Concentration) (2.359)**
- **E (Explant size) (1.619)**

Interaction :

- Insignificant

Table (4) : Effect of sterilization treatments on healthy percentage of volkamer lemon rootstock explants during period (2001- 2002)

Trs.	Treatments (Clorox (%) Ethanol 70 % for 30 sec.)	Contamination (%)					
		March			December		
		0.5 cm	1 cm	Mean	0.5 cm	1 cm	Mean
1	Control (untreated)	0.00	0.00	0.00	0.00	0.00	0.00
2	10 % for 5 min.	0.00	0.00	0.00	0.00	0.00	0.00
3	-- (+).Ethanol (+) 70 % for 30 sec.	0.00	100.00	50.00	0.00	0.00	0.00
4	10 % for 10 min.	0.00	100.00	50.00	0.00	100.00	50.00
5	-- (+).Ethanol (+) 70 % for 30 sec.	100.00	100.00	100.00	0.00	100.00	50.00
6	10 % for 15 min.	80.91	80.00	80.45	99.91	88.07	93.99
7	-- (+).Ethanol (+) 70 % for 30 sec.	77.00	75.00	76.00	79.79	77.71	78.75
8	20 % for 5 min.	0.00	100.00	50.00	0.00	100.00	50.00
9	-- (+).Ethanol (+) 70 % for 30 sec.	100.00	100.00	100.00	100.00	98.98	99.49
10	20 % for 10 min.	93.93	92.17	93.05	99.90	98.98	99.44
11	-- (+).Ethanol (+) 70 % for 30 sec.	91.81	90.90	91.35	96.69	92.13	94.41
12	20 % for 15 min.	79.13	77.72	78.42	77.70	77.00	77.35
13	-- (+).Ethanol (+) 70 % for 30 sec.	70.00	70.75	70.37	75.52	70.00	72.76
14	30 % for 5 min.	100.00	100.00	100.00	100.00	99.99	99.99
15	-- (+).Ethanol (+) 70 % for 30 sec.	100.00	99.01	99.50	100.00	99.00	99.50
16	30 % for 10 min.	97.70	99.10	98.40	95.55	97.11	96.33
17	-- (+).Ethanol (+) 70 % for 30 sec.	77.72	80.81	79.26	92.92	88.12	90.52
18	30 % for 15 min.	88.24	88.13	88.18	90.93	88.14	89.53
19	-- (+).Ethanol (+) 70 % for 30 sec.	66.17	67.16	66.66	98.00	69.97	67.68
20	40 % for 5 min.	88.00	80.11	84.05	80.18	80.88	80.53
21	-- (+).Ethanol (+) 70 % for 30 sec.	82.11	80.00	81.05	79.79	80.78	80.28
22	40 % for 10 min.	80.08	75.11	77.59	78.70	77.72	78.21
23	-- (+).Ethanol (+) 70 % for 30 sec.	55.09	55.00	55.04	66.16	60.60	63.38
24	40 % for 15 min.	60.63	60.63	60.63	66.26	61.00	63.63
25	-- (+).Ethanol (+) 70 % for 30 sec.	29.90	35.35	32.62	39.35	36.36	37.85
26	50 % for 5 min.	55.98	55.18	55.58	60.57	56.91	58.74
27	-- (+).Ethanol (+) 70 % for 30 sec.	54.34	55.00	54.67	55.00	56.75	55.87
28	50 % for 10 min.	35.08	30.11	32.59	44.03	40.05	42.04
29	-- (+).Ethanol (+) 70 % for 30 sec.	23.23	22.10	22.66	29.25	25.25	27.25
30	50 % for 15 min.	18.18	0.00	9.09	20.15	0.00	5.07
31	-- (+).Ethanol (+) 70 % for 30 sec.	0.00	0.00	0.00	2.25	0.00	1.12
	Mean	58.23	66.75		56.69	65.20	

New L.S.D :

- **D (Date) (N.S)**
- **C (Concentration) (2.607)**
- **E (Explant size) (2.141)**

Interaction :

- Insignificant

removal, the optimum time being June and during Winter dormancy; while, shoot tips taken in July and August were less successful for tissue culture **(Hb Songlin *et al.*, 1996)**.

From the obtained results, percentage of contamination clearly affected by sterilization treatments; while, length of explant and date of explant removal slightly affected the contamination parcentage. This is clear in both tested rootstocks and using treatment of clorox 50% for 15 min + ethanol 70% for 30 sec, in addition to treatment of 50% clorox for 15 min was the best as compared with other tested treatments for minimizing the contamination of both rootstock explants. However, explant of both length (0.5 and 1.0 cm) were treated with 50% clorox for 15 min or 50% clorox for 15 min + 70% ethanol for 30 sec. In addition, unsterilized (control) explants gave the highest percentage of contamination which reached to (100%) and were not suitable for contamination control in both tested rootstock explants.

For instance, according to the previous studies on citrus and other fruit species, the percentages of contamination or healthy explants vary according to agents of sterilization and the concentration of agents, date of sampling, age and length of plant organ. Many investigations came in line with the above mentioned conclusion **(Abd El-Zaher, 1998)** on citrus species and hybrids **and (Badr, 1998)** on banana . They proved that, shoot tips from juvenile and adult trees of (sour orange, volkamer lemon, troyer citrange, swingle citrumelo, valencia orange and local mandarin), soaked in clorox at 10% for 30 min or mercury chloride at 0.1% for 10 min, were insignificant for contamination control. On the other side, immersing in 70% ethanol then soaking in 20% clorox for 20 min, clorox at 20% for 30 sec or mercury chloride at 0.2% for 10 min. gave good results for controlling contamination in juvenile than 70% alcohol + 30% clorox for 20 min or mercury chloride 0.3% for 10 min for lateral buds of adult plants, it is better to treat them with 30% clorox for 20 min. or mercury chloride 0.3% for 10 min. except that local mandarin where mercury chloride 0.3% for 5 min. was the best treatment. Sodium hypochloride with or without Ethanol was used to sterilize explants of citrus rootstocks **(Abd El-Zaher, 1998)**. In addition, **Badr (1998)** found that, sterilization treatments of various explants of banana (suckers / peepers), all NaOCl concentrations from 0.5 to 2.5% resulted in 100% survival in the starting stage. However, the browning degree was obviously increased with (1.0 – 2.5%) NaOCl. Thus the use of (0.5 – 1.0%) was the best.

Second experiment (establishment experiment)

The second experiment aimed to study the effect of some growth regulators (BA, NAA and GA3) at different concentrations added to MS medium on survival percentage after 40 days of *in vitro* culture in both tested rootstocks.

Survival percentage

a) Sour orange rootstock

Representing data in (Table, 5) concerning March and December samples of (0.5 and 1.0 cm) length showed that, proliferation of buds varied in response to the different tested treatments. As such, explants taken in March were superior than those taken in December and length of 1 cm either taken in March or December was superior than 0.5 cm (51.72, 45.78% against 40.59, 36.77%) for March and December of 1.0 and 0.5 cm length, respectively.

As for the effect of treatments, cultured explants on MS medium supplemented with BA at 1.0 mg/l (Tr 8) significantly increased in survival percentage (68.15%) as compared with other tested treatments. Supplemented medium with 0.5 mg/l BA came after (62.35%), then those supplemented with 3.0 mg/l BA (60.75%) and those supplemented with 0.8 mg/l BA (53.10%) in descending order. Yet, the lowest percentages of survival explants were obtained from control (Tr 1) treatment (23.85%) (explants cultured on basal MS medium only without growth regulators) and the other treatments (MS medium supplemented with 0.1 BA + 0.1 NAA (mg/l) and the treatment supplemented with (0.1 of both BA and NAA + 0.5 GA_3 mg/l) sited between the above mentioned values (Tr 8 and Tr 1).

It could be concluded that, the most successful establishment explants were produced by culturing explants on MS medium supplemented with 1.0 mg/l BA with explant of 1.0 cm length taken in March (active growing period) and the same direction was noticed in December samples (inactive growing period) for 1.0 cm length and also considered the best treatment.

Interaction between (date of sampling × concentration of supplemented agents × explant length) concerning survival percentage was statistically significant and acted independently in this concern (Table, 6).

b) Volkamer lemon rootstock

Data recorded in (Table, 6) show that, in general, percentage of survival explants increased in March samples than in December one. In addition, explant length of 1.0 cm either taken in March or December significantly increased the survival percentage than 0.5 cm (34.88, 31.35% against 47.53, 38.62% for 5.0 or 1.0 cm were taken in March or December, respectively).

As for the effect of treatments (basal MS medium supplemented with different tested regulators) the lowest percentage of survival explants (20, 22.25% in March samples, 19.90, 20% in

Table (5) : Effect of BA, NAA and GA3 (mg/l) added to M.S medium on survival percentage of sour orange rootstock explants during period (2001- 2002)

Trs.	Treatments			Survival (%)					
	BA	NAA	GA_3	March			December		
	Mg/l			0.5 cm	1 cm	Mean	0.5 cm	1 cm	Mean
1	Control (M.S medium only)			20.00	27.70	11.62	19.90	26.50	21.20
2	0.1	--	0.5	25.00	42.25	31.17	22.20	30.30	26.25
3	0.1	0.1	-	22.25	40.10	36.10	20.00	37.75	28.87
4	0.1	0.1	0.5	30.00	42.20	46.35	28.75	40.45	34.60
5	0.3	-	-	40.45	52.25	62.35	37.75	45.00	41.37
6	0.5	-	-	55.90	68.80	53.10	50.50	55.00	52.75
7	0.8	-	-	50.70	55.50	68.15	50.75	56.00	53.37
8	1.0	-	-	65.55	70.75	68.15	50.95	60.60	55.77
9	3.0	-	-	55.50	66.00	60.75	50.15	60.50	55.32
Mean				40.59	51.72		36.77	45.78	

New L.S.D :
- **D (Date) (3.397)**
- **C (Concentration) (3.398)**
- **E (Explant size) (6.421)**

Interaction :
- Significant

Table (6): Effect of BA, NAA and GA3 (mg/l) added to M.S medium on survival percentage of volkamer lemon rootstock explants during period (2001- 2002)

Trs.	Treatments			Survival (%)					
	BA	NAA	GA_3	March			December		
	Mg/l			0.5 cm	1 cm	Mean	0.5 cm	1 cm	Mean
1	Control (M.S medium only)			20.00	30.50	25.25	20.00	28.00	24.00
2	0.1	--	0.5	28.50	40.00	34.25	20.75	25.15	22.95
3	0.1	0.1	-	25.50	25.00	25.25	25.00	22.20	23.60
4	0.1	0.1	0.5	30.00	35.70	32.85	25.15	27.20	26.17
5	0.3	-	-	25.00	45.10	35.05	30.35	40.10	35.22
6	0.5	-	-	40.00	60.50	50.25	32.25	48.45	40.35
7	0.8	-	-	45.00	55.50	50.25	30.90	48.00	39.45
8	1.0	-	-	40.00	65.00	52.50	45.50	50.00	47.75
9	3.0	-	-	60.00	70.55	65.27	52.25	58.50	55.37
Mean				34.88	47.53		31.35	38.62	

New L.S.D :
- **D (Date) (4.347)**
- **C (Concentration) (4.607)**
- **E (Explant size) (5.465)**

Interaction :
-Significant-

December samples) were obtained from MS medium (control) and medium supplemented with 0.1 BA + 0.1 NAA (mg/l) and the same direction were noticed in December for 0.5 and 1.0 cm explant length, respectively. However, the highest survival percentage (60.0, 70.55% for March and 52.25, 58.50% for December) of explants cultured on MS medium supplemented with 3.0 mg/l BA for 0.5 and 1.0 cm length, respectively. Yet, the other tested treatments ranged between the above mentioned values with true differences between the treatments in most cases.

The obtained data also show that, MS medium supplemented with high concentrations of BA (especially 3.0 mg/l) was the most suitable for establishment of shoot tip either taken in March or December for 0.5 and 1.0 cm of volkamer lemon explants. In addition, the obtained results proved that, BA had a clear effect in this concern than NAA or GA_3 added to MS medium and the percentage of survival tended to increase by increasing BA concentration.

It could be concluded that, explant length of 1.0 cm taken in March was superior in this concern than 0.5 cm either in March or December when basal medium of MS was supplemented with BA at 3.0 mg/l.

Interaction among different studied factors concerning survival percentage of volkamer lemon rootstock explants was statistically significant, where the high values were obtained from March of 1.0 cm explant length cultured on media consisted of 3.0 mg/l BA and the lowest ones were obtained from MS medium lacking growth regulators (control) for 0.5 cm of March which referred that, the three studied factors were interacted (Table, 6).

The obtained results proved that, explants of both sour orange and volkamer lemon rootstocks gathering in March at (1.0 cm), cultured on MS medium supplemented with (1.0 – 3.0 mg/l) BA increased the establishment of explants as compared with control cultured on only M.S medium, although those treatments received BA at low concentrations or supplemented with BA + NAA + GA_3 (in low concentrations) with true differences in most cases among tested treatments in both studied rootstocks.

In agreement with the obtained herein results **(Lukman *et al.*, 1990; Lin *et al.*, 1992; Omura and Hidaka, 1994 and Singh *et al.*, 1999)** all working on citrus species and hybrids) found that, MS medium supplemented with some growth regulators such as (BA, NAA, GA_3, KIN, IBA, Ads and IAA) increased survival rate, initiation, growth and establishment of shoot tip explants especially M.S medium supplemented with BA, NAA or GA_3 solely or in combination, regarding explant length and explant removal date.

For instance and in this concern **Lukman *et al.*, (1990)** on Troyer citrange apices and **Lin *et al.*, (1992)** on *citrus sinensis,* they decided that, MS medium supplemented with BA, NAA or GA_3 either solely or in combination enhanced initiation and establishment of shoots especially MS medium containing BA with or without NAA or GA_3 application added to M.S medium.

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تحسين الإكثار الخضري لاصلين من الموالح فى المعمل

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** معهد بحوث البساتين بالقصاصين- محافظة الاسماعلية- مصر

- أجريت التجربة في معمل زراعة الأنسجة بقسم البساتين بكلية الزراعة جامعة الزقازيق خلال عامي (2001 ، 2002) حيث اشتملت مرحلة البداية علي تجربتين كان الهدف منهما هو كيفية الحصول علي منفصلات نباتية سليمة خالية من التلوث يمكن استخدامها في المراحل التالية للاكثار (التضاعف والتجذير والأقلمة) وذلك فى أصلى النارنج الواسع الاستخدام في مصر وأصل الليمون الفولكاماريانا .

- أوضحت النتائج أن النسبة المئوية للتلوث زادت في المنفصل النباتي بطول 1 سم أكبر من المنفصل بطول 0.5 سم وكانت أقل نسبة تلوث في المنفصل 0.5 سم والتي تم تعقيمها بالكلوروكس 50 % لمدة 15 دقيقة مع أو بدون كحول الايثانول لمدة 30 ثانية سواء في المنفصلات المأخوذة في مارس أو فى ديسمبر لكل من الأصلين .

- كما أوضحت النتائج أن النسبة المئوية للمنفصلات النباتية السليمة زادت في المنفصلات بطول 1 سم بالمقارنة بالمنفصلات الأقل طولا (0.5 سم) كما زادت في المنفصلات المأخوذة في مارس عن المأخوذة فى ديسمبر وذلك في كلا الأصلين المختبرين . كما اعتبرت الثلاث معاملات التالية أكثر ملاءمة للتعقيم في كلا الأصلين :

عند تعقيم المنفصلات بالكلوروكس 10 % لمدة 15 دقيقة + 70 % كحول إيثانول (30 ثانية) ، معاملة التعقيم بالكلوروكس 20 % لمد ة 5 دقائق + كحول إيثانول 70 % (30 ثانية) ، معاملة التعقيم بالكلوروكس 30 % (5 دقائق)

- كما أظهرت النتائج أيضا أن النسبة المئوية لنجاح المنفصلات النباتية بعد المعاملتين السابقتين وبعد (40 يوم من الزراعة فى المعمل) زادت في عينة المنفصلات المأخوذة في مارس بطول 1 سم والتى أضيف الي بيئة MS BA بتركيز 1 مجم / لتر في أصل النارنج ، BA بتركيز 3 مجم / لتر في أصل الفولكاماريانا ، بينما وجدت أقل نسبة مئوية لنجاح المنفصلات النباتية مع البيئة الأساسية MS الخالية من منظمات النمو (كنترول) كما أتضح أن BA أكثر فاعلية في هذا الصدد (معنوى) مقارنة بكل من GA_3 ، NAA في كل التركيزات . كما زادت النسبة المئوية لنجاح المنفصلات المنزرعة في بيئة MS بزيادة تركيز BA في بيئة النمو .