In Vitro Propagation of Volkamer Lemon using Nodal Cutting Segments

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ABSTRACT: Citrus is one of the most important commodity worldwide, owing to its tremendous nutritional value, and acceptable as fresh edible food. This study was conducted at the Desert Research Center (DRC) in Cairo, Egypt, during the period 2013-2017. An efficient in vitro propagation system for Volkamer lemon (Citrus volkameriana) was established. The effect of various combination of two plant growth regulators (cytokinin and auxin) was evaluated on the proliferation efficiency of citrus plant via in vitro propagation technique. The sterilized stem nodal segments of the given species were planted vertically on MS culture medium augmented with various combinations of BAP at 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mg/l and NAA at 0.0, 0.1, and 0.2 mg/l, and especially at BAP×NAA at 2.0 ×0.1 mg/l respectively proved to be the best for bud induction. The survival percentage of stem node segment frequency was 100%, especially when MS medium was supplemented with BAP in a range 1.0- 3.0 mg/l. The best shoot initiation was obtained on MS medium augmented with BAP and NAA at 2.0 and 0.1 mg/l, each in turn, which recorded the highest mean value (3.99 shoots/ explant). Higher number of elongated shoots was obtained on MS medium with BAP and NAA at 3.0 and 0.1 mg/l, in order. The higher multiplication rate was recorded on MS medium containing BAP and NAA at either 2.0 or 2.5 mg/l for the former and 0.05 mg/l for the latter. The shoots were then rooted on MS medium containing IBA and NAA at 1.5 and 0.2 mg/l consecutively, with high rooting percentage (100%). The plantlets survival ex vitro swas (80%) when plantlets were transferred to plastic pots containing a mixture of sand and peatmoss (1:1). In conclusion, this study provides reproducible technique for micropropagation of Volkamer Lemon.

Keywords: Citrus volkameriana, plant tissue culture, initiation stage, multiplication stage, rooting and acclimatization stage

INTRODUCTION

Citrus volkameriana, is a member of Rutaceae family and it is commonly known as Volkamer lemon. Citrus is considered as the number one fruit of the world due to its high nutritional value, great production potential and preparation of large number of fruit products from them. Citrus species are cultivated in most tropical and subtropical regions of the world (García-Luis et al., 2006). Volkamer lemon (Citrus volkameriana) is a commonly used as a rootstock in Egypt and it is an excellent rootstock for warm, humid areas with deep sandy soils (García-Luis et al., 2006). Volkamer Lemon being polyembryonic in nature, give rise to several vigorous and virus free nucellar seedling which are difficult to differentiate from zygotic seedling and are ,also, difficult to isolate from zygotic seedling, which necessitate the application of in vitro micropropagation, therefore, very little work has been carried out on the tissue culture of this plant (Ali and Mirza, 2006). Likewise, in vitro propagation is a techno-economically viable and eco-friendly approach to produce disease free planting material on a large scale, utilizing relatively small space and time. Hence, rapid and cost effective in vitro methods of reproducing this rootstock would ensure bulk production of true- to -type and disease- free- planting material. Therefore, the present study was undertaken to standardize the protocol for in vitro propagation of this commercially important Volkamer Lemon.

MATERIALS AND METHODS

Plant materials:

This study was achieved through the period from year 2013 until 2017 in the Tissue Culture Laboratory, Desert Research Center (DRC), and Cairo, Egypt.

The plant materials of citrus rootstock (Volkamer) explants were collected from a private farm located at 70 km Cairo-Alexandria desert road. Actively growing shoots with terminal buds were collected, moistened, wrapped and placed into ice-box container. In the laboratory, the explants were washed under running tap water for 4 hours and the healthy ones were chosen to verify their response to the in vitro propagation procedure. The latter procedure was performed as the following system:

Stage 0 (selection the mother plant and explants sterilization):

Vigours and healthy plants of Volkamer Lemon were selected from the above-mentioned private farm, to collect the explants nodal segments.

Explants sterilization:

Surface sterilization of the given explants was carried out under complete aseptic conditions in the Laminar Air Flow Hood. The explants were subjected to different sterilization treatments using commercial Clorox containing 5.25% sodium hypochlorite (NaOCl) at 1.25% for 20 minutes. After each treatment, the explants were rinsed thoroughly with double distilled sterilized water for 4 times to remove all traces of the disinfectant, with continuous hand shaking agitation in each of the previous steps. Finally, nodal segments were trimmed at both ends to 0.5 - 1cm in length using forceps and scalpel to be ready for culturing.

The basic nutrient medium and culture conditions:

Stem nodal segments were cultured vertically on solidified basal Murashige and Skoog (MS) medium adopted by Murashige and Skoog (1962) supplemented with 100 mg/l myo-inositol and 30 g/l sucrose (3%), which augmented with growth regulators such as benzyl amino purine (BAP), at different concentrations, either independently as 0.0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l or in combinations with β–naphthalene acetic acid (NAA) which was added at 0.0, 0.1and 0.2 mg/l. The pH of the nutrient media was adjusted to 5.7 ± 0.1 with adding few drops of either 0.1N Hydrochloric acids (HCl) or 0.1N Sodium hydroxide (NaOH) prior to addition of 2.7g/l phytagel to solidify the liquid media. Fifteen ml of media were dispensed into culture tubes 25×150 mm long or 30ml volume into 350 ml jars. Then, closed with polypropylene caps and autoclaved at 121℃ under a pressure of 1.1kg/cm² for 20 min, then left to cool, and media were stored at room temperature a day before being used.

Initiation stage

Effect of different growth regulator combinations on the initiation (establishment) stage:

The induction of shoots from the nodal segments was attempted with full strength of solid MS medium supplemented with 100 mg/l myo-inositol, 30 g/l sucrose, 2.7g/l phytagel, 40 mg/l adenine sulphate, 100mg/l glutamine, 1mg/l FeSO4.7H2O and 500mg/l malt extract. Different growth regulators combinations of BAP (0.0, 0.5, 1.0, 1.5, 2.0,2.5 and 3.0 mg/l) individually or in

combination with 0.0, 0.1and 0.2 mg/l NAA were added to the media to obtain the highest percentage of growth induction comparing to MS medium free of growth regulators (as a control). The freshly sterilized explants were cultured into tissue culture tubes or jars and each treatment was represented by 3 replicates.

Multiplication stage:

Established explants (outcomes of initiation stage) were multiplied on MS media for Volkamer Lemon containing different concentrations of BAP (2.0 and 2.5 mg/l) with NAA at 0.05 mg/l for shoots multiplication. As reported above explants were cultured in tissue culture tubes or jars and each treatment was represented by 3 replicates. The explants were subcultured for seven consecutive subcultures on the best-defined multiplication medium using large jars to obtain stock materials to be used for the following experiments.

Rooting stage:

Shoots derived from multiplication stage (ca. 3-5 cm long) were transferred to half strength MS salts with vitamins containing 1g/l activated charcoal (AC), in addition to 100mg/l myo-inositol and 30 g/l sucrose. For rhizogenesis, two types of auxins were tested, viz., IBA at five concentrations (0.0 [nil], 0.5, 1.0, 1.5, and 2.0 mg/l) in combination with NAA at four concentrations (0.0 [nil], 0.2, 0.5 and 1.0 mg/l). The tested media were solidified with 2.7g/l phytagel. Cultures were incubated under the same conditions that used for shoot propagation. The incubation condition was, in general, as 16-hr photoperiod+ 8hr darkness, at 25±2 oC and light intensity of 3000 Lux.

Acclimatization stage:

The obtained plantlets (rooted shoots) were washed, thoroughly, with running tap water to discard media residues, and treated with 0.2 % (w/v) Mon cut 25% (a, a, a-trifluoro-3- isopropoxy-o-Toluanilid) solution as a fungicide for 30 sec., then they were transplanted ex vitro in plastic pots (8 cm in diameter) containing soil potting mix of peat-moss and sand (1:1 v/v). Pots were covered with transparent polyethylene bags and placed in a greenhouse. One week later, the covers were removed, and the plantlets started to acclimatize for one month. The percentage of survived transplants (%) was recorded.

Statistical analysis:

All the experiments carried out during this study were designed as factorial experiments layout in completely randomized design (Gomez and Gomez, 1984). Recorded data were analyzed statistically using analysis of variance technique (ANOVA) Steel et al. (1997). The means significance was compared by applying the least significant difference (L.S.D.) test at 5%level of probability.

RESULTS AND DISCUSSIONS

Initiation stage:

Effect of BAP, NAA (mg/l) and their combinations on the given traits of citrus rootstock "Volkamer Lemon" during initiation stage:

Survival percentage of stem explants:

Data presented in (Table 1) declare the effect of both BAP and NAA (mg/l) and their combinations that augmented to MS medium on survival percentage of stem node segment explants during initiation stage. The main effect of BAP showed that BAP exerted highly significant ($P \leq 0.01$) effect on the given trait, whereas, augmenting MS medium with at the range 1-3.0 mg/l and 0.1 mg/l NAA; brought about the highest survival percentage (100%). This finding could be attributed to the mode of action of both BAP and KIN as cytokinins on the stimulation of both cell division and growth promotion of axillary shoots in plant tissue culture as reported previously by Trigiano and Gray (2000) and George et al. (2008). On the other hand, the shoot proliferation depends upon the balance of cytokinins and auxins. The addition of lowest levels of NAA used affected well the initiation of citrus in vitro (Usman et al., 2005).

The mean number of neoformed shoots/explant

Data illustrated in (Table 2) exhibited that tested MS media supplemented with various levels of BAP, NAA, and their combinations on the characteristic. The main effects of BAP, NAA and their combinations, showed a very highly significant effect ($P \leq 0.001$) on the given trait. The highest mean values (3.99) were obtained when MS medium was supplemented with 2.0 mg/l BAP in combination with 0.1mg/l NAA. This finding could be taken place due to the accurate balance between both growth regulators as exogenous application and those of endogenous biosynthesis, which resulted in the best genes expression and subsequently the growth (George et al., 2008).

In this respect the high BAP level compare to NAA level considers as in favor of stimulation cell division, morphogenesis (shoot initiation/bud formation) in tissue culture process, and break of apical dominance and release growth of lateral buds (Raven et al., 1992; Salisbury and Ross, 1992; Davies, 1995) and their combinations exerted highly significant effects on the initiation stages characters of rootstocks, where stem node segment as explants were grown in vitro for 30 days. Further, the obtained results in this study cope with those of Upadhyay et al. (2010) and Marques et al. (2011) who advised to add lower level of NAA to affect the initiation of citrus in vitro and found that the high rates of bud initiation and shoot development were obtained both with BA supplemented medium, in range from 1mg/l to 3 mg/l and with 0.1 mg/l NAA supplemented medium.

Table (1). Effect of different levels of both BAP and NAA (mg/l) and their combinations added to MS culture medium on survival percentage of stem node explants during initiation stage of Volkamer (Citrus volkameriana).

NAA (mg/l)	BAP (mg/l)							Mean NAA	Significance		
	0.0	0.5	1.0	1.5	2.0	2.5	3.0		BAP	NAA	BAP × NAA
	MS medium										
0.0	65	30	80	80	100	100	100	79.28	**	ns	ns
0.1	50.2	50.2	90	90	100	100	100	82.91			
0.2	50.2	30	48	90	100	100	100	74.02			
Mean (BAP)	**55.13**	**36.73**	**72.66**	**86.66**	**100**	**100**	**100**				
L.S.D.									**23.2**	**15.2**	**40.3**

Table (2). Effect of different levels of both BAP and NAA (mg/l) and their combinations added to MS culture medium on mean number of neoformed shoots/explant for stem node segment during initiation stage of Volkamer (Citrus volkameriana).

NAA (mg/l)	BAP (mg/l)							Mean NAA	Significance		
	0.0	0.5	1.0	1.5	2.0	2.5	3.0		BAP	NAA	BAP × NAA
	MS medium										
0.0	0.11	0.11	0.33	0.66	1.88	1.22	0.44	0.67	***	***	***
0.1	0.11	0.11	0.33	1.11	3.99	2.44	0.99	3.02			
0.2	0.11	0.00	0.22	0.44	0.44	0.44	0.33	0.36			
Mean (BAP)	**0.11**	**0.07**	**0.29**	**0.73**	**2.28**	**1.36**	**0.58**				
L.S.D.									**0.27**	**0.18**	**0.48**

L.S.D. = Least significant difference test at 0.05, 0.001 level of probability. **, ***, ns = high significant, very highly significant, not significant, respectively.

NAA concentration above 1 mg/l significantly reduced bud initiation and shoot elongation. The highest mean values were obtained on MS medium supplemented with 2.0 and 2.5mg/l BAP in combination with 0.1mg/l NAA for "Volkamer". Furthermore, Upadhyay et al. (2010) mentioned that 2.0 mg/l BAP + 0.1 mg/l NAA was found to be the best treatment for establishment medium of "Sweet orange" , with respect to maximum sprouting, minimum days taken for sprouting with highest number of shoots/ explants. Hence, 2mg/l BAP or 1.0 mg/l KIN + 0.1 mg/l NAA was observed to be the best treatment for multiplication medium with maximum shoot length and highest number of leave.

The mean shoots length (cm)/explant.

Data illustrated in (Table3) exhibited the effect of various levels of BAP, NAA, and their combinations added to the MS medium on the defined characteristic of the mean shoots length of neoformed per culture explants. The main effect of BAP disclosed that it exerted a very highly significant effect (P≤ 0.001) on the given trait, especially when MS culture medium was augmented with BAP at 3.0 mg/l and NAA at 0.1 mg/l, which recorded the highest mean length of shoots (2.01 cm). These results are in agreement with those obtained by Tapati et al. (1995) and Moreira et al. (2001) who found that BAP has been

reported to be the most commonly used cytokinin in citrus tissue culture media, which enhanced culture establishment and minimized the time elapsed to be bud sprouted or outgrowth. This finding may be taken place due to the balance between endogenous and exogenous PGRs (George and Sherrington, 1984; and George et al., 2008) whereas, auxins are capable to control various distinctive processes such as promotion of stem elongation and growth and they are not effective against shoot proliferation (George et al., 2008; Goussard, 1981).

Multiplication stage:

Effect of BAP with NAA and their combinations on the multiplication of Volkamer axillary shoots during 7 successive subcultures

Data in Table (4) and Plate (1) display that the effect of augmenting MS medium with BAP and NAA at best given levels on percentage of explant forming growth, mean number of axillary shoots, and mean length of axillary shoots/ propagule during seven successive subculture of Volkamer rootstock. Whereas, MS medium supplemented with BA at 2.0 or 2.5 mg/l and NAA at 0.05 mg/l; achieved such significant effect on the given traits. The multiplication rate was gradually, increased until the fifth subculture, then declined at the sixth and seven subcultures; especially mean numbers of shoots.

As for the percentage of explant forming out growth, the main effect of BAP and NAA, showed no significant effect on the given trait. For instance, the mean number of shoot formed/ propagule, upon fortifying MS medium with BAP and NAA at either 2.0 or 2.5 and 0.05 mg/l, respectively, showed a significant effect on the given trait, which recorded the highest mean value of the defined character (4.68 or 4.43, each in turn), during the fifth subculture, whereas, after sixth subculture it declined (2.67 or 2.23, serially). On the other hand, augmenting MS medium with BAP and NAA at 2.5 and 0.05 mg/l showed a significant effect on given trait, which recorded the highest mean length of shoots/ propagule (1.75cm).

Table (3). Effect of different levels of both BAP, NAA (mg/l) and their combinations added to MS culture medium on mean shoots length (cm)/explant for citrus rootstocks" Volker" stem node segment during initiation stage.

NAA (mg/l)	BAP (mg/l)							Mean NAA	Significance		
	0.0	0.5	1.0	1.5	2.0	2.5	3.0		BAP	NAA	BAP × NAA
	MS medium										
0.0	0.16	0.33	0.91	1.08	1.23	1.23	1.75	0.95	***	ns	ns
0.1	0.16	0.50	1.83	1.38	1.42	1.42	2.01	1.19			
0.2	0.16	0.00	1.00	1.25	1.66	1.66	1.50	0.96			
Mean (BAP)	**0.16**	**0.27**	**1.24**	**1.23**	**1.17**	**1.37**	**1.75**				
L.S.D.									**0.39**	**0.25**	**0.67**

L.S.D. = Least significant difference test at 0.05, 0.001 level of probability. **, ***, ns = high significant, very highly significant, not significant, respectively.

Table (4). Effect of given levels BAP with NAA on same multiplication traits of Volkamer Lemon axillary shoots during seven successive subcultures.

Genotypes	Volkamer Lemon					
Growth regulators	2.00 mg/l BAP+ 0.05 mg/l NAA			2.50 mg/l BAP + 0.05 mg/l NAA		
No. of subculture	Survival %	Mean number of shoots	Mean length of shoots (cm)	Survival%	Mean number Of shoots	Mean length of shoots(cm)
1_{st} subculture	100^{a}	0.63^{d}	1.52^{a}	100^{a}	0.55^{d}	1.75^{a}
2_{nd} subculture	100^{a}	0.88^{cd}	1.49^{a}	100^{a}	1.12^{d}	1.47^{abc}
3_{rd} subculture	100^{a}	1.12^{cd}	1.39^{a}	100^{a}	2.12^{c}	1.26^{bc}
4_{th} subculture	100^{a}	1.83^{bc}	1.37^{a}	100^{a}	3.22^{b}	1.19^{bc}
5_{th} subculture	100^{a}	4.86^{a}	1.24^{a}	100^{a}	4.43^{a}	1.07^{c}
6_{th} subculture	100^{a}	2.67^{b}	1.38^{a}	100^{a}	2.23^{c}	1.34^{abc}
7_{th} subculture	100^{a}	0.86^{cd}	1.62^{a}	100^{a}	0.96^{d}	1.58^{ab}

Plate (1). Multiplication stage of Volkamer Lemon, when cultured on MS media and the best combination 2.0 mg/l BA with 0.05 mg/l NAA.

In this respect, cytokinins, together with auxin, take part in regulation of the cell cycle in plant cells (i.e. stimulation of cell division, break apical dominance, enhance axillary shoot proliferation, and adventitious, inhibition root formation). Also, the interaction between auxin and cytokinis or their ratio between other represents an important signal in the formation of cell phenotype and in the onset and maintenance of the process of cell division (Stickens et al., 1996). In the same line, Kumar et al. (2001) mentioned that the plantlets were regenerated by direct organogenesis from epicotyls segments of in vitro germinated nucellar seedlings of sweet orange cultivars. When epicotyls segments (1.0-1.5 cm long) were cultured on MS medium supplemented with BA and NAA, in Mosambi; the highest number of explants showed shoot proliferation (14.33) and the highest number of shoot (2.06) and leaves (4.56) were obtained upon using 1.0mg/l BA. In Jaffa, 2.0mg/l of BA; gave the highest number of explants showing shoot proliferation. Likewise, Upadhyay et al. (2010) mentioned that 2.0 mg/l of BAP + 200 mg/l of casein hydrolysate was found to be the best treatment for establishment medium for sweet orange , with respect to maximum sprouting, minimum days taken for sprouting with highest number of shoots/ explants. It was observed that augmenting the culture medium with 2.0mg/l BAP + 1.0 mg/l KIN + 0.1 mg/l NAA; was recorded to be the best treatment for multiplication medium with maximum shoot length and highest number of leave. In vitro organogenesis of citrus was studied by Schinor et al. (2011) for the micropropagation of genotype Citrus sinensis cv. Natal, C. limonia, C. volkameriana and C. aurantium, with the use of epicotyls segments –derived explants, and cultured in MT medium supplemented with different concentration of BAP and NAA. For the recalcitrant genotypes C. limonia and C. aurantium the in vitro organogenesis was, also, studied with intermodal segments, cultured in MT medium supplemented with BA ad NAA. In the same year, the factors affecting in vitro adventitious shoot formation on internode explants of Citrus aurantium L. was recorded by Marques et al. (2011) and found that the high rates of bud initiation and shoot development were obtained due to supplementing the culture medium with BA, in range from 1.0 mg/l to 3.0 mg/l and with 0.1 mg/l NAA. Notably, NAA concentration above 1.0 mg/l; significantly reduced bud initiation and shoot elongation.

Rooting stage (Rhizogenesis)

Effect of IBA, NAA and their combinations (mg/l) on percentage of rooted shoots /propagule during rooting stage of citrus rootstock " Volkamer".

Data tabulated in Table (5) display the effect of various level of both applied growth regulators viz, IBA, NAA and their interactions on percentage of rooted shoots/ propagule of the tested rootstocks "Volkamer Lemon" during rooting stage. As for, the main effects of IBA, NAA in addition to their combinations, generally, they exerted very highly significant effect ($P \leq 0.001$) on the given trait. For instance, augmenting MS medium with IBA, NAA and IBA× NAA at 1.5, 0.2 and 1.5×0.2 mg/l; resulted in the highest percentage values as 49.75, 46.60 and 100%, consecutively.

Effect of NAA, IBA and their combinations (mg/l) on mean number of roots formed/explant during rooting stage of citrus rootstocks'' Volkamer ''.

Results outlined in Table (6) and Plate (2) disclosed the effect of IBA, NAA (mg/l) and their combinations on mean number of roots formed/ shoot of Volkamer Lemon during rooting stage. Whereas, IBA at 1.5, NAA at 0.2 mg/l and their interaction at 1.5×0.2 mg/l, in series, have very significant effects ($P \leq 0.001$) of the given trait, which recorded the highest mean values as 1.27 for IBA, 1.15 for NAA and 2.44 for interaction.

The obtained results could be explained on the bases that auxin induced number of responses which involved cell division, cell enlargement, protein and nucleic acids syntheses which are concomitant of auxin-induced growth and changes in wall plasticity of plant cell and increase the apical dominance as there are essential and rapid processes involved in growth and elongation (Wilkins, 1989). The use of auxins and many other factors and changes in the rooting environment have been described in order to enhance the rooting of microcuttings (Brand and Lineberger, 1986). Similarly, in a previous study on in vitro rooting of cv. "Pinot noir" microshoots (Heloir et al., 1997), where it was shown that IBA is a suitable auxin, while other types of auxins (e.g., NAA) may lead to callus formation. Jaskani et al. (2008) reported that media having 10µM (2.0mg/l) IBA proved to be the best for root formation in microshoots while its absence shoots of vitis showed complete failure in root formation. Hicks and Dorey (1998) also reported that roots at high frequency was achieved on MS medium plus IBA but level of IBA was different than the treatments in the present study which may be due to different varietal response. These results are close to those of HuXinXI et al. (2007) who indicated that about 97.7% of the adventitious shoot from Citrus sinensis was rooted on 1/2 MS medium + 3% sucrose + 0.7% agar + 2.0 mg/l IBA, pH 5.8. Also, Upadhyay et al.(2010) reported that IBA (2.0 mg/l) + NAA (0.1 mg/l) + activated charcoal (500mg/l) was found to be significantly superior over all other treatments with respect to maximum root initiation percentage, days spanned to root initiation , highest number of roots and length for nodal and intermodal segments of C. sinensis. Alemow and "Cleopatra" mandarin shoot were rooted well using these plant growth regulators.

Table (5). Effect of IBA, NAA and their combinations (mg/l) on percentage of roots /propagule during rooting stage of citrus rootstocks" Volkamer ".

NAA(mg/l)	IBA (mg/l)					Mean NAA	Significance		
	0.0	0.5	1.0	1.5	2.0		IBA	NAA	IBA×NAA
0.0	0.00	22.0	44.0	44.0	0.00	22.00	***	***	***
0.2	0.00	0.00	100	100	33.0	46.60			
0.5	0.00	0.00	0.00	55.0	33.0	17.60			
1.0	11.0	0.00	0.00	0.00	44.0	11.00			
Mean (IBA)	2.75	5.5	36.0	49.75	27.5				
L.S.D.							8.0	7.0	17.0

L.S.D. = Least significant difference test at 0.05,0.001 level of probability.
*, ***, ns significant, very highly significant, not significant, respectively.

Table (6). Effect of IBA, NAA and their combinations (mg/l) on mean number of roots formed/explant during rooting stage of citrus rootstocks "Volkamer ".

NAA (mg/l)	IBA (mg/l)					Mean NAA	Significance		
	0.0	0.5	1.0	1.5	2.0		IBA	NAA	IBA×NAA
0.0	0.00	1.66	1.16	1.17	0.00	0.79	***	ns	***
0.2	0.00	0.00	2.33	2.44	1.00	1.15			
0.5	0.00	0.00	0.0	1.50	1.00	0.50			
1.0	0.66	0.00	0.00	0.00	2.00	0.53			
Mean (IBA)	0.16	0.41	0.87	1.27	1.00				
L.S.D.							0.40	0.36	0.80

L.S.D. = Least significant difference test at 0.05,0.001 level of probability.
*, ***, ns = significant, very highly significant, not significant, respectively.

Likewise, NAA/IBA combinations; produced higher rooting percentages than did the IBA/ IAA combinations, and in sour orange nearly 100%of explants developed roots. Regenerated shoots of Citrus limon L. showed root induction on MS medium containing 1.0 mg/l IBA which was recorded by Goswamiet al. (2013). Root initiation commenced within 12 days, and after three weeks, vigorous roots could be seen on each plantlet they were, successfully, acclimatized and transferred to the glasshouse.

Acclimatization stage:

Effect of acclimatization mixture on plantlets survival, during plantlets acclimatization stage of citrus rootstocks.

Data presented in Table (7) and Plate (3) demonstrated the ex vitro successful acclimatization of neoformed plantlets of citrus rootstocks" Volkamer ". A high percentage of "Volkmer" plant survival (80%) was achieved by transplanting of plantlets in pots containing peatmoss and sand at ratio of 1:1(v/v). Generally, the well- defined mixture declared mixture the best mixture of growing "Volkamer" plantlets. As well as, no significant difference was dectected regarding plant survival observed between all mixtures. The number of newly formed leaves (true leaves) of "Volkamer", especially the highest mean number value were achieved due to the mixture ratio above-mentioned of

peatmoss: sand (1:1) and the lowest mean value was recorded with the ratio of peatmoss and sand (1:2). While, the plantlets length of 'Volkamer" was, significantly promoted by the effect of physical and chemical properties of acclimatization mixture which resulted in (9.5cm), but the lowest height was 3.4 cm which was recorded owing to the mixture from the sand: peatmoss (1:2).

Table (7). Effect of acclimatization mixture on plantlets survival, during plantlets acclimatization stage of Citrus Volkameriana.

Acclimatization mixture	Volkamer		
	Plant survival %	Number of newly leaflets	Shoot length (cm)
Sand+ Peatmoss 1:2	40	3.4	5.6
Sand + Peatmoss 2:2	40	3.9	7.5
Sand + Peatmoss 1:1	80	5.8	9.5
Sand + Peatmoss 2:1	60	3.7	5.6
L.S.D.	40.561*	0.868***	1.545***

L.S.D. = Least significant difference test at 0.05,0.01,0.001 level of probability.
*, **,***, ns= significant, high significant, very highly significant, not significant, respectively.

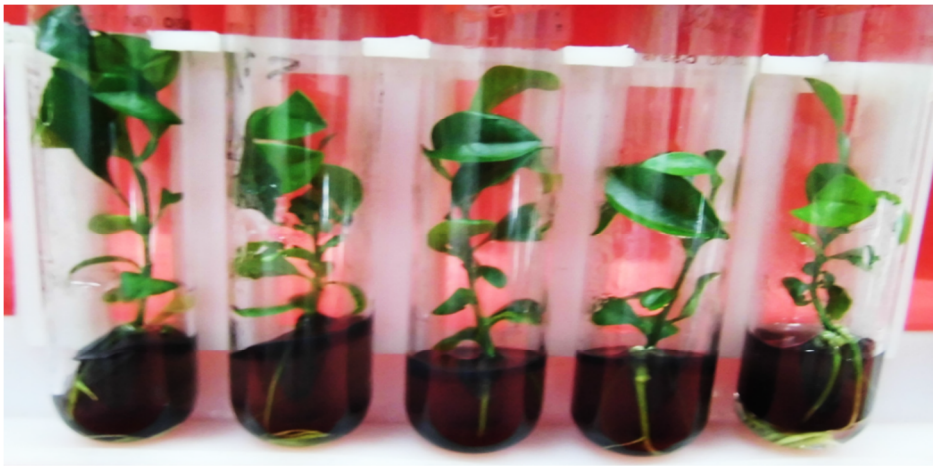

Plate (2). Rooting stage of Volkamer using MS medium containing 2.0 mg/l IBA and 0.2 mg/l NAA.

Plate (3). Acclimatization stage of Volkamer.

In this context, Kumar et al. (2001) found that the complete plantlets survival rate of regenerated were obtained with the highest survival rate of 68.8% due to transfer the neoformed plantlets to pots containing sand and soil (2:1) was 66.02% in Mosambi and 67.5% in Jaffa. Usman et al. (2005) found that Kinnow explants registered the highest rooting percentage (91%)and recorded the highest number of root per shoot (1.3). the plantlets were grown in the greenhouse on mould, sand, peat moss, and loam, then transplanted into the field after 2-3 month. Prez-Tornero et al. (2010) reported that the success during the acclimatization was close to 100% and the plantlets exhibited normal growth in soil under greenhouse condition. In addition, Roussos et al. (2011) claimed that the rooted explants were successfully acclimatized under mist (85%). Also, Khalil et al. (2011) noticed that the regenerated plantlets of C. senensis were successfully acclimatized when planted in jiffy pots containing sterilized soil mixture of sand, silt and clay in 1:1:1 ratio to study their response to in vivo condition.

In conclusion, the stem node segment was the best of the citrus " Volkamer Lemon" explant showed the maximum shoot initiation on MS medium supplemented with BAP at 2.0 and NAA 0.1 mg/l each in turn. While, MS medium containing BAP at 2.0 or 2.5 and NAA at 0.05 mg/l; brought about the highest multiplication rate of the given traits. Whereas, the maximum rooting was obtained on MS medium augmented with IBA 2.0 mg/l + NAA at 0.2 mg/l. Generally, the mixture ratio of sand: peat moss (1:1) was the best mixture of growing "Volkamer Lemon" plant on the given trait.

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الملخص العربي

الإكثار المعملي الدقيق لليمون الفولكامر بإستخدام الأجزاء الساقية البرعمية

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تعتبر الموالح من أهم محاصيل الفاكهة حول العالم لما لها من قيمة غذائية عالية وايضا مقبولة كغذاء طازج. تم تنفيذ هذه الدراسة في مركز بحوث الصحراء–القاهرة–مصر خلال الفترة من ٢٠١٣ الى ٢٠١٧. لقد تم إيجاد بروتوكول كفء ويعتمد عليه للإكثار المعملي الدقيق لأصل ليمون الفولكامر. لقد تم تقييم تأثير توليفات مختلفة من أثنين من منظمات النمو (سيتوكينين وأوكسين) على كفاءة تكاثر هذا النوع من الموالح من خلال تقنية الإكثار المعملي الدقيق. تمت زراعة البراعم رأسياً على بيئة موراشيج وسكوج MS المزودة بتوليفات مختلفة من السيتوكينين بنزيل أمينو بيورين بتركيزات صفر، ٠,٥، ١,٠، ١,٥، ٢,٠، ٢,٥، ٣,٠ ملجم/ لتر) وكذلك الأوكسين نفثالين حمض الخليك بتركيزات صفر، ٠,١ و ٠,٢ ملجم/لتر، وأثبت التفاعل بين البنزيل أمينو بيورين× نفثالين حمض الخليك عند ٢,٠×٠,١ ملجم/لتر على التوالي هي التوليفه الأفضل لأستحثاث نمو البراعم المنزرعة. كانت النسبة المئوية لحياة الأجزاء الساقية البرعمية ١٠٠% خاصة عندما زودت بيئة موراشيج وسكوج بمدى من البنزيل أمينو

بيورين يتراوح بين ١,٠ الى ٣,٠ ملجم. كان أفضل تنشئة للمجاميع الخضرية قد تم الحصول عليه عندما زرعت الأجزاء النباتية على بيئة موراشيج وسكوج المزودة بنزيل أمينو بيورين ونفثالين حمض الخليك عند ٢,٠ كذلك ٠,١ ملجم / لترعلى التوالي، والتي سجلت أعلى قيمة متوسطية (٣,٩٩) مجموع خضرى لكل جزء نباتى. ولقد تم الحصول على مجاميع خضرية طويلة عندما زرعت تلك المجاميع الخضرية على بيئة موراشيج وسكوج المزودة بنزيل أمينو بيورين ونفثالين حمض الخليك عند٣,٠ كذلك ٠,١ ملجم على التوالي. أما اعلى معدل تضاعف لتلك المجاميع الخضرية كانت قد لوحظت عندما زرعت تلك المجاميع الخضرية على بيئة موراشيج وسكوج المزودة بنزيل أمينو بيورين ونفثالين حمض الخليك عند أما٢,٠ أو ٢,٥ ملجم/لتر بالنسبة للأول وكذلك ٠,٠٥ ملجم/لتر للأخير. تم تجذير المجاميع الخضريةعلى بيئة موراشيج وسكوج المزودة بالأوكسين أندول بيوترك حمض الخليك ونفثالين حمض الخليك عند ١,٥ كذلك ٠,٢ ملجم/ لتر على التوالي مع أعلى نسبة مئوية للتجذير والتي كانت ١٠٠%. كان معدل حياة النبيتات ٨٠% عندما تم نقلها الى أصص بلاستيكية تحتوى على خليط من الرمل والبيتموس (١:١). ولهذا فالدراسة الحالية تقدم تقنية يمكن الأعتماد عليها للإكثار المعملى الدقيق.