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ANTHER CULTURE AND PLANT REGENERATION IN CITRUS (*Citrus volkameriana*).

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ABSTRACT

Development of an efficient and cost-effective doubled haploid production system in *Citrus volkameriana* is the prerequisite for the application of doubled haploid technology in a practical breeding program.

An improvement of the protocol for haploid induction through anther culture of *Citrus volkameriana* was achieved following the evaluation of a number of the factors affecting androgenesis. Two types of media were tested for response of anther culture.

In induction cultures, N6 Chu (1978) basal medium was more effective than Murashige and Skoog (1962). The influence of 2,4-dichlorophenoxyacetic acid (2,4-D) and benzyladenine (BA) and three temperature pre-treatments (4ºC, 25ºC, 32ºC) on the floral buds with respect to anther culture of *C. volkameriana* . As well as, the effect of $AgNO_3$ concentration on Volkamer" lemon embryogenesis was investigated. The highest percentages of anthers producing embryoids (47.50) was produced on N6 medium at the concentration of 2.0 mg/l 2,4-D and 0.5 mg/l BA together. Pre-treatment of temperatures at 4ºC and 25ºC were more favorable for embryo production than 32ºC. Silver nitrate concentrations had a strong influence on embryo yields where a 2.0 mg /l significantly gave a higher percentages of anthers (65.00%) producing embryoids compared with 1.0 mg /l or 0.0 mg/l (55.00, 45.83%, respectively). Regeneration of androgenic haploid plantlets from *C. volkameriana* is reported in this study. As embryogenesis proceeded, the normal pattern of development was observed, including heart-shape, torpedo and cotyledonar embryos.

INTRODUCTION

The genus Citrus, which includes few of the most important fruits worldwide, belongs to the family Rutaceae, which comprises 140 genera and 1300 species throughout the world. Citrus is the third most important fruit crop in the world after apple and banana and accounts for the production of about 100 million tons with an area of cultivation spread over a massive 7.2 million hectares (FAO, 2001).

"Volkamer" lemon (*Citrus volkameriana*) is one of the important citrus rootstocks in Egypt especially in sandy soil. It has been introduced annually and popularly used as rootstocks for production of disease-free. In Egypt this rootstock is propagated every year by seed germination.

Not very much attention has been paid to the use of biotechnology as a tool for obtaining new variability. Biotechnological methods can improve the efficiency and increase the speedy of breeding. Anther culture is a widely used method to generate genetic variability. In fact, through this method, it is possible to regenerate gametic or somatic embryos with many applications for plant breeding. Somatic embryos, for example, can be used for protoplast

fusion, genetic transformation or *in vitro* germplasm storage (Sharma *et al.,* 2009).

Pollen embryogenesis through i*n vitro* anther culture is affected by numerous factors and by their interactions, such as: genotype, donor plant growth conditions, pollen developmental stages, pre-treatment applied to the anthers or to the floral buds, culture media and environmental conditions of incubation.

In order to make microspore embryogenesis a reliable tool in *Citrus* breeding, it is necessary to improve the induction response (the frequency of microspores forming embryos) and also to increase the number of responsive genotypes (Germana` *et al.* 2005). On the other hand, techniques for regeneration of haploid plants are important. Haploid plants can not only serve as unique materials for genetic studies, but also through diploidization to become homozygous diploid plants, can be used as crossing parents for the production of hybrid seeds with high growth vigor.

Haploid and spontaneous diploid plant production from anther culture is a well-developed and useful tool in practical plant breeding as well as in basic research. Anther culture in citrus species has been reported to be an effective way to obtain not only haploid plantlets, but also diploid plantlets (Germana` *et al.* 2005). Anther culture is usually used to grow haploid plants, but the regenerates grown from anthers are sometimes polyploids that arise from spontaneous chromosome doubling in the regeneration process (Kiss *et al*., 2001). Accordingly, anther culture can also be used to produce polyploid plants.

There are several factors affecting androgenesis in many species, such as pre-treatments of anthers (Koleva-Gudeva, 2003) and composition of medium (Irikova and Rodeva, 2004) . The response of anthers cultured on different media under certain thermal treatments can go in two directions, callus induction or embryo formation. The mechanism of cold and heat-shock treatment for induction of somatic embryogenesis has been explored and discussed by many authors such as (Matsubara *et al.,* 1998).

This work has been carried out to improve the induction rate of androgenesis in *Citrus volkameriana*. For this purpose, three steps were investigated: (1) Examined of the effect of growth regulators (2,4-D and BA) and two types of salt media (MS or N6) on induction of embryogenesis from anthers. (2) Examination of pre-treatments of temperature at different degrees (4^oC, 25^oC, 32^oC) and time of exposure (48 or 72h) on embryogenesis from anthers. (3) Examination of the effect of different concentrations (0.0, 1.0, 1.5 and 2.0 mg /l) of silver nitrate ($AgN0_3$) on embryogenesis from anthers.

MATERIALS AND METHODS

This study was carried out at Plant Biotechnology Department, Institute of Genetic Engineering and Biotechnology, Minufiya University, during the period from 2009 to 2011. The experiments were carried out in three consecutive years.

Plant material

Healthy and vigorously growing flower buds were harvested in March from approximately 10 years old trees of Citrus "Volkamer" lemon (*Citrus volkameriana* Ten& Pasq.) grown at Baramon Experimental Farm, Mansoura Horticulture Research Station, Dakahlia Governorate, Egypt.

Pollen developmental stage

The stage of pollen development was tested in one anther per bud size using the acetic-carmine method (Sharma and Sharma 1972). The anthers were collected from flower buds at different stages of development and squashed in 1% acetocarmine in 45% acetic acid for observation under an optic microscope to identify the uninucleate stage of pollen development. For subsequent experiments, only flower buds of the same size (6 – 7 mm in length) as those with anthers with microspores at the uninucleate stage were selected for culture (Fig. 1A).

Experiment of Induction media

The aim of this experiment was to study the effect of salt formulation media and various growth regulators (BA and 2,4-D) at various concentrations on induction of embryogenesis. Two types of salt media, MS (Murashige and Skoog 1962) or N6 Chu (1978) including the two types of growth regulators at various concentrations and their combinations were tested. The tested plant growth regulators included various concentrations of benzyladenine (BA) at 0.0, 0.5 and 1.0 mg/L singly or combined with auxins; 2,4 - dichlorophenoxyacetic acid (2,4-D) at 0.0, 0.5 1.0 and 2.0 mg/L.

The media were gelled with 7.0 g/L agar and supplemented with 40 g/l sucrose, 500 mg/l casein hydrolisate, 200 mg/l L-glutamine, 200 mg/l ascorbic acid, and 1.0 mg/l TDZ (thidiazuron). The media were autoclaved at 121^{o}C and 1.2 kg/cm^{2} air pressure for 20 min. The pH medium was adjusted to 5.8 prior to the addition of agar. The media were distributed in 6-cm-diameter Petri dishes containing 10 -12 ml of solid medium. 40 anthers were placed in each dish.

Anther culture

After cold pre-treatment (floral buds collected and stored in the dark at 4^{o}C for 12-13 h), Unopened flower buds were surface sterilized by immersion for 1 min in 70% (v/v) ethyl alcohol, followed by immersion in commercial disinfectant Clorox (5.25 % NaOCl) solution for 20 min, and finally, the flower buds were washed 4 times in autoclaved distilled water to remove all traces of the disinfectant.

Petals were aseptically removed with small forceps, and the anthers were carefully dissected and placed in 6-cm-diameter Petri dishes containing 10 -12 ml of solid medium. 40 anthers were placed in each dish. The reculture period was 45 days up to 6 months after culture.

Six months after culture, the percentage of anthers producing callus or embryos were calculated. As well as, the percentage of anthers that did not develop (no response) were calculated.

Culture conditions

The Petri dishes with anthers were sealed with Parafilm, incubated initially at 25±2ºC for 21 days in the dark, followed by incubation under a 16/8-h photoperiod with light supplied by cool-white fluorescent lamps.

Influence of temperature pre treatments

To increase callogenesis and embryogenesis, Examination of pre-treatments of temperature at different degrees (4ºC, 25ºC, 32ºC) and time of exposure (48 or 72h) on embryogenesis from anthers were investigated.

The effect of the length of storage periods at various degrees of heat on subsequent development of embryogenesis of *Citrus volkameriana* was evaluated. Floral buds were harvested, pre-treated at 4ºC, 25ºC, 32ºC in darkness for 48 or 72h and the anthers were then cultured on the best medium from previous experiment (induction medium) as described before.

Then all culture dishes were subsequently transferred to 25±2°C and maintained in darkness for a further 21 days, followed by incubation under a 16/8-h photoperiod with light supplied by cool-white fluorescent lamps. Percentage of anthers producing embryos were calculated after six months.

Silver nitrate ($AgNO_3$) experiment.

The best induction medium (N6 medium supplemented with 2.0 mg /l 2,4-D + 0.5 mg/l BA) was selected from previous experiment to test the effect of silver nitrate ($AgNO_3$) on induction of embryogenesis from anthers **.**

Silver nitrate ($AgNO_3$) was added to the culture media at different concentrations of 0.0, 1.0, 1.5 and 2.0 mg /l. Anther culture was carried out as described above and immediately after plating, Petri dishes were maintained at 25±2ºC. After six months data were collected as, percentage of anthers producing callus or embryos were calculated. As well as, the percentage of anthers that did not develop (no response) was calculated.

Embryo germination

Embryos were transferred to MS medium containing MS vitamins, 30g/l sucrose, 1.0 mg/l gibberelic acid (GA_3), and 0.8% (w/v) agar. As soon as embryos reached 4– 5 mm in size, they were transferred to Magenta boxes contained the germination medium of 1/4 MS and 3.0 g/l Activated charcoal (AC). Embryos were incubated for 4 weeks in the light at 25±2°C with a 16-h photoperiod.

Plant recovery

Plantlets (4–5 cm in length) with normal root development were transplanted ,for the hardening phase, to the greenhouse in pots containing mixture of peatmoss and vermiculite at equal volume.

Data analysis

Experiments were set up in completely randomized block design with five replicates; each replicate consisted of one Petri dish containing 40 anthers. The results were analyzed by analysis of variance (ANOVA) according to Gomez and Gomez (1984).

RESULTS AND DISCUSSION

In order to find an efficient methods for "Volkamer" lemon plant regeneration from anther culture, the optimization of the following parameters was considered: medium composition (type of media and BA and 2,4-D combinations), anther pre-treatment of temperature (Thermal shock pretreatments), and silver nitrate ($AgNO_3$).

Effect of different concentrations of BA and 2,4-D as well as type of media(MS, N6) on percentage of anther produced embryos of *C. volkameriana*.

Results regarding the effect of media type or combination with BA and 2,4-D on the development of anthers after six months of culture are reported in Table (1).

Table (1): Effect of different concentrations of BA , 2,4-D and basal media(MS, N6) on percentage of anther produced embryos of *C. volkameriana after 6 month in vitro.*

		% of anther/embryo			Means of (A x B)	Means of (A)
		BA mg/l Con. (C)				
Type media(A)	2,4-D mg/l (B)	0.0	0.5	1.0		
MS	**0.0**	0.0	0.0	0.0	**0.0**	**9.09**
	0.5	1.66	3.33	3.33	**2.77**	
	1.0	9.16	15.83	13.33	**12.77**	
	2.0	12.50	29.16	20.83	**20.83**	
N6	**0.0**	0.0	0.0	0.0	**0.0**	**15.41**
	0.5	0.83	5.83	3.33	**3.33**	
	1.0	17.50	25.83	24.16	**22.50**	
	2.0	23.33	47.50	36.66	**35.83**	
Means of (C)		**8.12**	**15.93**	**12.70**	**Means of (B)**	
Means of (B x C)	**0.0**	0.0	0.0	0.0	**0.0**	
	0.5	1.25	4.58	3.33	**3.05**	
	1.0	13.33	20.83	18.75	**17.63**	
	2.0	17.91	38.33	28.75	**28.33**	
(A x C)	**MS**	5.83	12.08	9.37		
	N6	10.41	19.79	16.04		

LSD at 5% A = 1.47 B = 2.08 C = 1.80 A x B = 2.95 A x C = 2.55 B x C = 3. 61 A x B x C = 5.11

Data in Table (1 and 2) show the effect of different concentrations of 2,4-D and BA and type of media on the percentage of anther produced embryos. Data of the main effect of media type show that the highest percentage of anther produced embryos (15.41) was obtained with N6 medium as compared with the MS medium (9.09). It was clear that N6 basal medium was more conducive to the induction response than MS when both were supplemented with the same amounts of BA and 2,4-D. This result was in agreement with Zhao *et al.,* (2006) who reported that with coneflower anther culture, N6 medium was more effective than MS medium.

In addition, the main effect of 2,4-D on the percentage of anther produced embryos indicated that the highest percentage of anther produced embryos (28.33) was observed with 0.2 mg/l 2,4-D, while the lowest

percentage (3.05) was obtained with 0.5 mg/l 2,4-D as compared with the control.

The interaction effect of the two growth regulators (2,4-D and BA) on the percentage of anther produced embryos indicated that percentage was significantly increased with increasing the concentration of both growth regulators as compared with the control. The highest record (38.33) was produced at the concentration of 2.0 mg/l 2,4-D and 0.5 mg/l BA combination (Table,1 and 2). This result is in accordance with the findings of anthers cultured on the medium supplemented with 0.5 mg/l 2,4-D and 2.0 mg/l BA resulted in the highest callus formation rate (Tang *et al.,* 2009).

The presence of an appropriate concentration of growth regulators in the medium plays an important role in callus formation from anther culture. Especially 2,4-D was indispensable and critical for the dedifferentiation of anthers (Li and Zhuang 2006).

After 2 weeks of culture, most of the anthers were swollen. one month later, most of the anthers produced callus which appeared compact and green, or friable and white.

Sometimes direct embryo formation without callus production was observed. Well structured embryos showed normal dicot developmental patterns: globular, heart, torpedo and cotyledonary stages (Fig., 1 D). Embryo could be converted into vigorous plantlets (Fig. 2).

Callus induction rates of the cultured anthers were determined on day 30 and are shown in Table (2). It was obvious that N6 basal medium was more conducive to the induction of callus than MS when both were supplemented with the same amounts of BA and 2,4-D, and the combination of BA with 2,4-D at the given levels had better effects than 2,4-D at the three tested levels. As to the texture of the calluses, no clear differences were observed between those induced on MS and N6 media. However, on media containing 2,4-D alone, the calluses were more friable, especially when 2,4-D was used at the higher concentrations (2.0 mg/l). These results are in accordance with other findings such as (Jian *et al.,* 1986) where the importance of 2,4-D in combination with BA was emphasized for a significant increase of the androgenic callus frequency.

Percentage of anther produced embryos was determined after 6 months as shown in Table (1). As mentioned in other previous report (Germana and Chiancone 2003) who reported that on anther culture of *Citrus clementina* results were recorded after 11 months from culturing. This reducing of the culturing time might help in controlling variation in plantlets. This agreement with Barry-Etienne *et al*. (2002) who reported that, reducing the culturing time might decrease the somaclonal variation frequency; another major commonly encountered difficulty and it will probably ensure the formation of more homogenous plantlets. In this study, the new medium including (N6 medium supplemented with 2.0 mg /l 2,4-D + 0.5 mg/l BA, 40 g/l sucrose, 500 mg/l casein hydrolisate, 200 mg/l L-glutamine, 200 mg/l ascorbic acid, and 1.0 mg/l TDZ) improved the yield and reduced the culture time, which in tarn reduced the variation and the cost. That result will be particularly interesting and helpful for clonal mass propagation and/or genetic engineering in *volkameriana* plants.

Table *2.* Effect of different concentrations of BA , 2,4-D and basal media(MS, N6) on Percentages of anthers that did not develop, Percentages of anthers producing callus and Percentages of anthers producing Embryoids in *Citrus volkameriana*

	2,4-D	BA	Percentages of anthers that did not develop	Percentages of anthers producing callus	Percentages of anthers producing embryoids
MS	0.0	0.0	100	0.0	0.0
		0.5	94.16	5.83	0.0
		1.0	٩١,٦٦	8.33	0.0
	0.5	0.0	٧٠,٨٣	27.50	1.66
		0.5	٦٨,٣٣	28.33	3.33
		1.0	٦٤,١٦	32.50	3.33
	1.0	0.0	٤٠,٨٣	50.00	9.16
		0.5	٣٨,٣٣	54.83	15.83
		1.0	٢٨,٣٣	50.83	13.33
	2.0	0.0	٢٩,١٦	58.33	12.50
		0.5	٢٩,١٦	58.33	29.16
		1.0	٣٥,٨٣	41.66	20.83
N6	0.0	0.0	١٠٠,٠	0.0	0.0
		0.5	٩٠,٨٣	0.0	0.0
		1.0	٨٩,١٦	9.16	0.0
	0.5	0.0	٥٠,٠٠	10.83	0.83
		0.5	٤٥,٠٠	19.16	5.83
		1.0	49.16	48.33	3.33
	1.0	0.0	٤٢,٥٠	47.50	17.50
		0.5	٣٠,٠٠	40.00	25.83
		1.0	٢١,٦٦	44.16	24.16
	2.0	0.0	٣٠,٠٠	51.66	23.33
		0.5	٢١,٦٦	30.83	47.50
		1.0	٣٣,٣٣	30.00	36.66

LSD at 5% Percentages of anthers producing callus= 11.74
Percentages of anthers producing embryoids= 5.11
Percentages of anthers that did not develop = 11.52

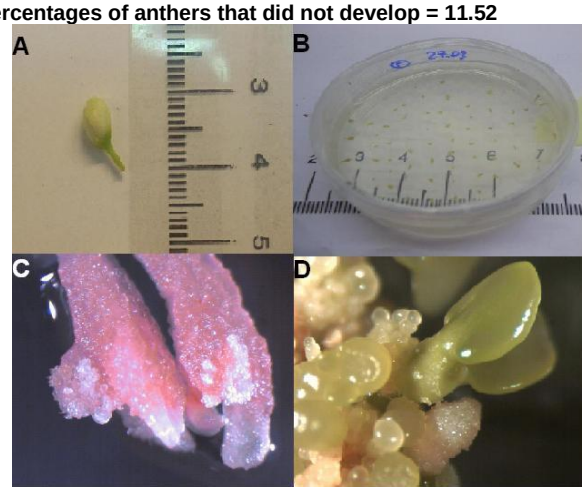

Fig. 1. *Citrus volkameriana* anther culture. A-Flower bud of *Citrus volkameriana* (6 -7 mm), B- Anther culture in Petri dish. C- Callus emerging after 2 weeks of anther culture. D-Groups of embryoids at different stages of development.

Thermal shock and chilling treatments

Present experiment focused on the effect of thermal shock and chilling treatments of anthers on the embryo yields in citrus (*C. volkameriana*) (Table 3).

The duration and temperature at which the anthers were exposed appeared to be very important and critical factors in deciding the embryo yields.

Data in Table (3), on the main effect of the duration which the anthers were exposed show that 72h was significantly more effective than 48h in percentage of anther produced embryos after 6 weeks of anther culture *in vitro*. Data on the main effect of pre treatments indicate that 4^{o}C was more effective than other pre treatments (25, 32^{o}C). In that concern, Deng *et al*. (1992) reported that, in Citrus anther culture, a low temperature pretreatment (at 4–5^{o}C for either 2 h, overnight, or for 3–6 days) has been previously used. In the same line, Qi *et al.,* (2011) reported that, cold or heat shock pretreatment has been applied to a variety of plant species.

Concerning the interaction, results in Table (3) show that, the highest percentage of anther produced embryos (16.33) was found with the anther exposed to 4^{o} C for 72h as pretreatments followed by the 4^{o}C for 48h (13.66) when both compared with all tested periods and other pre treatments (32 and 25^{o}C). However, Data in Table (3) reveled that, minimum percentage of anther produced embryos (3.00) in the present study was obtained with the treatment of 48 or72h at 32^{o}C. As indicated in previous work by German and Chiancone (2003) who reported that, the highest temperature (32^{o}C) applied to the floral buds before culture is not recommended for androgenesis in Citrus.

Table 3. **Effect of pre-treatment and their time of exposure on percentage of anther produced embryos of *C. volkameriana* after 6 months in vitro.**

Pre-tretments (A)	Percentage of anthers producing embryos		Means of (A)
	Time (B)		
	48h	72h	
4^{o}C	13.66	16.33	**15.00**
25^{o}C	13.33	12.00	**12.66**
32^{o}C	3.33	2.66	**3.00**
Means of (B)	**10.11**	**10.33**	

LSD at 5% A = 2.94 B = 2.40 A x B = 4.16

The mechanism of cold and heat-shock treatment for induction of somatic embryogenesis has been explored and discussed by many authors such as (Matsubara *et al.,* 1998).

A number of hypotheses exist to explain the beneficial effect of a cold pre-treatment with respect to embryo induction. It seems that a cold pre-treatment increases the number of pollen grains with two equal nuclei and stimulates embryoid formation by delaying and modifying pollen mitosis or by blocking starch production, thereby dissolving microtubules (Nitsch, 1977) or maintaining viability of the cultured P-grains (Heberle-Bors, 1985). Cold pre-treatments are routinely used in the anther culture of many crops, and their

effects are genotype-dependent (Osolnik *et al.*, 1993). In additional, Tenhola-Roininen *et al.* (2005) reported that a cold pretreatment (4° C) was beneficial for the induction of the anther culture response in rye.

Application of cold pretreatment has become an essential measure to increase the efficiency of androgenesis in many species (Wang, 2009).

Effect of silver nitrate ($AgNO_3$) concentrations

In general the response of cultured anthers to addition of silver nitrate ($AgNO_3$) was better than the controls (those not receiving additional silver nitrate) (Table 4). As indicated in previous work by Kumar *et al*., (2009) who emphasis that silver nitrate has proved to be a very potent inhibitor of ethylene action and is widely used in plant tissue culture.

The response of anthers varied with silver nitrate concentration. Overall, an increase in the concentration of silver nitrate resulted in an increase in the production of embryo yields per anthers (%). Silver nitrate concentrations had a strong influence on embryo yields where a 2.0 mg /l gave a significantly higher percentage (65.00) of anthers producing embryoids than a 1.0 mg /l or 0.0mg/l (55.00, 45.83 respectively).

Table 4. **Effect of silver nitrate ($AgNO_3$) concentrations on the embryo yields in *Citrus volkameriana***

Conc. Of $AgNO_3$ (mg/l)	Percentages of anthers producing embryoids	Percentages of anthers producing callus	Percentages of anthers that did not develop
0.0	45.83	30.00	25.00
1.0	55.00	34.16	11.66
1.5	52.50	37.50	13.33
2.0	65.00	34.16	5.83

LSD at 5% Percentages of anthers producing embryoids= 6.35
Percentages of anthers producing callus= 8.43
Percentages of anthers that did not develop = 10.35

It was clear from the present study that the increase in the concentration of silver nitrate in the culture, gradually increased the percentages of anthers producing embryoids . However, since embryos were also formed in the absence of silver nitrate, in the control, the overall results suggests that silver nitrate may not be essential for induction but for increasing yields of embryos from anther culture and decreasing anthers that not developed of *C. volkameriana*. Similar results were described by Achar (2002) who reported that, the responsiveness of anthers to addition of increasing concentration of silver nitrate ($AgN0_3$) (the ethylene inhibitor) to the culture medium, showed a progressive increase in the embryo yields in all the genotypes. In the same line, Lashermes (1992) reported that, numbers of anthers responding and, consequently, of embryoids and the produced plants were enhanced when anthers were cultivated on media containing 5 mg/l silver nitrate.

$AgN0_3$ known to be a potent inhibitor of ethylene action in plants (Beyer, 1976), and may promote wheat pollen embryogenesis and direct generation by blocking the inhibitory effect of endogenous ethylene on the embryo (Purnhauser *et al.,* 1987). An optimum level of ethylene production is

important for pollen embryogenesis as reported in barley (Cho and Kasha, 1989) and *Solanum carolinense* (Reynolds, 1987).
Silver nitrate concentrations had a strong influence on percentages of anthers that did not develop where a 2.0 mg/l gave a significantly higher percentages of anthers that develop than a 0.0 mg/l.

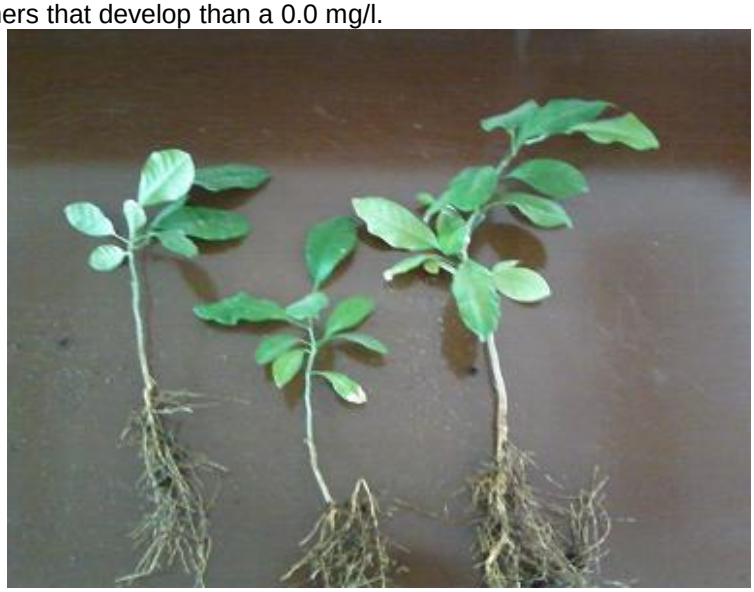

Fig. 2. A normal complete plant differentiation from anther-derived calluses of *Citrus volkameriana*

Anther culture is often the method of choice for doubled haploid production in many crops because the simplicity of the approach allows large scale anther culture establishment and application to a wide range of genotypes (Sopory and Munshi 1996).

Chromosome doubling can occur spontaneously during *in vitro* anther culture, and the genotype, developmental stage of the microspores, type of pretreatment and pathway of development affect the percentage of doubling (Castillo *et al*., 2009).

Conclusion

2,4-D in combination with BA supplemented to the basal N6 nutrient medium has a better effect on induction of androgenesis in *citrus volkameriana* anther cultures. Treatments with 4°C (48 h) and 4°C (72h) stimulate callus induction, and regeneration in *citrus volkameriana* anther cultures. Addition of 2.0 mg/l $AgNO_3$ to the culture media anthers proved to be most effective. The results of these experiments show that the ability to produce haploids of *C. volkameriana* can be improved investigating the factors affecting gametic embryogenesis. For this reason, further and more basic studies on the sporophytic pathway of pollen grains should be carried out in the future.

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زراعه متوك الموالح وتكوين نباتات لأصل الفولكا

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تعتبر زراعه المتوك لإنتاج نباتات كامله من أهم الطرق التى تساعد مربى النبات فى إنتاج أصناف جديده وكذلك التحسين الوراثى فى الانواع الموجوده.
تم فى هذا البحث وضع تقنيه لإنتاج نباتات من أصل الفولكا عند زراعه المتوك المفصوله. أختبر لذلك نوعين من بيئات الزراعه N6 , MS ونوعين من منظمات النمو وهى بنزيل أدنين و ٤,٢ داى كلورو فونكسى حامض الخليك وحده أو مندمجين معا بتركيزات مختلفه للوصول لافضل توليفه لدفع المتوك المنزرعه لانتاج أجنه.
أوضحت النتائج أن إستخدام بيئه N6 أفضل من بيئه MS لهذا الغرض وكان أفضل تركيز عباره عن توليفه من ٢ ملليجرام / لتر ٤,٢ داى كلورو فونكسى حامض الخليك و 0.5 ملليجرام / لتر بنزيل أدنين.
درست بعض العوامل المؤثره على إنتاج الاحنه من زراعه المتوك ومن أهمها معاملات ماقبل الزراعه , عرضت البراعم الزهريه لدرجات حراره مختلفه (٤, ٢٥, ٣٢ درجه مئويه) لاوقات مختلفه (٤٨ و ٧٢ ساعه) أوضحت النتائج أن أفضل معامله تجرى على البراعم الزهريه هى تعرضها لدرجه حراره منخفضه ٤ يليها ٢٥ لمده ٧٢ ساعه بدون فرق معنوى عن ٤٨ ساعه. كذلك تم دراسه تاثير نترات الفضه بتركيزات مختلفه على تكوين الاجنه من المتوك أصل الفولكا وكان أفضل تركيز ٢ ملليجرام/لتر نترات فضه حيث أدى الى حث ٦٥% من المتوك المنزرعه لتكوين أجنه. لوحظ جميع مراحل تكون الاجنه حتى ظهور الطور الفلقى بشكل واضح.

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