Vol 3 Issue 10 Nov 2013 ISSN No : 2230-7850

Monthly Multidisciplinary Research Journal

Indian Streams Research Journal

Welcome to ISRJ

RNI MAHMUL/2011/38595 **ISSN No.2230-7850**

Indian Streams Research Journal is a multidisciplinary research journal, published monthly in English, Hindi & Marathi Language. All research papers submitted to the journal will be double - blind peer reviewed referred by members of the editorial Board readers will include investigator in universities, research institutes government and industry with research interest in the general subjects.

Address:-Ashok Yakkaldevi 258/34, Raviwar Peth, Solapur - 413 005 Maharashtra, India
Cell : 9595 359 435, Ph No: 02172372010 Email: ayisrj@yahoo.in Website: www.isrj.net

Indian Streams Research Journal
Volume-3, Issue-10, Nov-2013
ISSN 2230-7850

Available online at www.isrj.net

Grafting Of *Citrus Reticulate* Microscions Derived From Nucellar Embryos *In Vitro* On Volkamariana Rootstock Growing In Greenhouse

Ebtsam M. Hamza [1] Ahmed A. Nower [1]
Ramadan Aboseree Sayed [2] And Mahdy A. Agwa [2]
[1]Genetic Engineering and Biotechnology Research Institute (GEBRI), Sadat City University, Egypt
[2] Horticulture Research Institute, Agriculture Research Center, Agriculture Ministry, Egypt

Abstract:-Balady mandarin (*Citrus. reticulata*), belongs to family Rutaceae, is one of the ancestral species of citrus, widely grown in the tropical and subtropical areas. Virus and viroids have been recognized as serious problem limiting the vigor, yield and quality of citrus. These diseases are graft transmissible through infected bud sticks. So, obtained of disease-free plants is imperative to provide certified microscions to the growers and to encourage the planting of grafts instead of seedlings. Micrografting is one of virus-free plant production methods, but, it is complex and its successful percent is low especially it is followed by acclimatization stage. Our paper is the first one to examine producing virus-free microscions via *in vitro* propagation of vegetative nucellar embryos and examine in vitro treatments which may affect microshoots propagation and successful of grafting the in vitro producing microscions on volkamariana rootstock in greenhouse. Firstly, the sexual nucellar embryo was determined using RAPD marker. Genetic analysis proved that 5th nucellar embryo (the youngest) is differed than the other four embryos. The similarity between them was 84%. Cytokinin types and concentrations affected multiplication of asexual nucellar embryos of Balady mandarin, the highest significant shoot number/explant through second subculture resulted from the explants cultured on MS medium supplemented with 0.75 and 1.00 mg/l BAP (8.80 and 5.60 shoots/explant, respectively). Concerning grafting of in vitro produced microscions in greenhouse, after 45 days of grafting in greenhouse, Balady mandarin scions (microscions) derived from MS medium supplemented with 1.0mg/l Kin or 0.75 mg/l AS maximized successful grafts percentage (37 and 33% successful grafts, respectively). Also, auxin types and concentrations affected successful grafting, microscions derived from MS supplemented with 0.5 mg/l IAA or 0.5 mg/l NAA gave the highest percentage of successful grafts followed by 1.0 mg/l IBA and control (57, 57, 55 and 30% successful grafts, respectively). Microscions derived from MS medium supplemented with 0.6 mg/l PP333 gave the highest percentage of successful grafts 88.77%. Diameter of rootstock and method of grafting also affected grafting successful percent. Producing of microscions in vitro is good tool for producing virus-free plantlet of Balady mandarin.

Keywords:*Citrus reticulate*, grafting, micropropagation, cytokinins, auxins, growth retardant, rootstock, grafting methods

INTRODUCTION

Balady mandarin (*Citrus reticulata*), belongs to family Rutaceae, is one of the ancestral species of citrus, widely grown in the tropical and subtropical areas. It is a good source of vitamin C and is usually eaten plain or in fruit salads. Among the citrus fruits, mandarin has gained high popularity and is commercially cultivated for its processing quality, fresh consumption and aromatic flavor (Sarma et al., 2011). Citrus orchards and nurseries survey based on the characteristic symptoms expression and serological indexing reported that the major virus, viroid and prokaryotic diseases and its average incidence commonly observed were citrus tristeza closterovirus (CTV) (27%), citrus variegation virus (CVV) (31%), citrus exocortis viroid (CEVd) (16%), citrus cachexia viroid (CCVd) (xyloprosis) (4%), citrus greening (Liberibacter spp.) (4%) and stubborn (Spiroplasma citri) 2%. These diseases are graft transmissible through infected bud sticks. So, augmentation of disease-free foundation plants is imperative to provide certified bud sticks to the growers and to encourage the planting of grafts instead of seedlings (Mukhopadhyay et al., 1997 and Arif et al., 2005)

Pathogen-free citrus selections; including virus-free plants, have been obtained by nucellar embryony culture, nucellar tissue culture, thermotherapy, clonal selection, indexing, and by shoot-tip grafting (STG) (Bitters et al., 1972, Roistacher et al., 1976 and Singh et al., 2008). Micropropagation of shoot tips has been successfully

employed to produce virus-free plants *in citru*s (Navarro et al., 1975, Navarro and Juarez 1977; De-Lange, 1978, Navarro, 1981 and 1984 and Carvalho et al, 2002) On the other hand, Panattoni et al., (2013) reported that tissue culture usually adopted to regenerate plants in biotechnological breeding programs, representes the less used tool for elemenate viruses from plants.

Citrus could be propagated by seeds, grafting, budding, layering or cuttings (Williamson and Jakson, 1994). Apomictic embryos arising from the nucellar tissue give rise to seedlings which are genetically identical to seed parents thus yielding uniform off spring for propagation (Ruiz et al., 2000). But determination of the sexual embryo is required for save production of true to type seedlings if nucellar ones are used as initial explant in tissue culture. In the past, various techniques were used to distinguish nucellar seedlings from sexual zygotic ones viz. colorimetric assays, infrared spectroscopy, enzymatic darkening of polyphenols, chromatography and isozyme pattern analysis, until the discovery of DNA based markers, isozyme analysis remained the widely used technique for sorting out zygotic seedlings, since it is a fast and cheap methodology(Rao et al., 2008). Zygotic embryos of some citrus varities were determined by using various DNA based marker systems such as RAPD, AFLP and SSR (Ruiz et al., 2000, Yaly et al., 2011 and Ahmad et al., 2012). Seedlings classified as zygotic have a different RAPD profile from that of the mother plant or nucellar seedlings. Small embryos located at the micropylar end of the seed do not always produce zygotic seedlings. (Andrade-Rodríguez et. al., 2004).

Cytokinins and auxins are the two most important plant growth regulators used in plant tissue culture. There are relative effects of auxin and cytokinin ratio on morphogenesis of cultured tissues. BAP, at different concentrations, has been the most commonly used cytokinin for multiplication of citrus shoots (Skoog and Miller 1957, Kotsias and Roussos 2001 and Carimi and De Pasquale 2003). It was seen that BAP alone was found to be effective in multiple shoot induction from nucellar embryo explants in C. limonia (Jajoo, 2010). Studies on in vitro shoot regeneration of C. reticulate are rare. A protocoal for shoot regeneration from cotyledonary segments of in vitro grown seedlings was established; BAP had great influence in the shoot proliferation stage (Sarma et al., 2011

Grafting technique is determined as joining and unite pieces of living tissues from different plants. So, they will fuse to form and function as one plant. One of the most important keys to successful budding and grafting is properly positioning scion on the rootstock. Grafting methods include, wedge grafting or cleft grafting, saddle grafting, side veneer or side cleft grafting, epicotyl grafting, splice grafting and whip or tongue grafting (Elam, 1997).

Micrografting is a technique that potentially can combine the advantages of rapid in vitro multiplication with the increased productivity that results from grafting superior rootstock and scion combinations (Gebhardt and Goldbach, 1988). The relationship between grafting success and optimum scion size has been reported by Onay et al., (2004). Micrografting success ranged between 26.7% to 51.88%. in kinnow mandarin.(Naz et al., 2007 and Singh et al., 2008). Production of Gardenia. jasminoides scions in vitro and use it for grafting on G. thunbergia rootstock in greenhouse is established with high successive percent (100%). This method decrease production coast because it does not need to rooting stage and the percent of successive grafting in greenhouse is higher than in vitro micrografting (Nower and Hamza, 2013).

Citrus can be micropropagated via tissue culture techniques, but it has not been a commercial method. The main aim of micropropagation is production of virus-free plantlets through micrografting technique, but, percentage of micrografting success is so low and the successive plantlets need further to be acclimatized with another loss percent. So, the aim of this paper is to determine zygotic embryo genetically and use asexual nucellar embryos which is free of viruses to establish an efficient in vitro propagation protocol for producing free-virus and hard microshoots of Baalady mandarin for using these microshoots as scions to be grafted directly on rootstock cultured and grown in greenhouse as a commercial method. Also, examine the effect of various factors which may affect the grafting successful percent of in vitro produced microscions in greenhouse.

MATERIALS AND METHODS

This study was carried out during the period from 2010 to 2013 in the laboratory of Plant Biotechnology Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), Sadat City University, Egypt.

Plant Materials:

Mother plants is planted in the farm of Ali Moubark Experimental Farm, El bostan, Behera Governorate, Egypt. Seeds of Balady mandarin were used as initial explants for tissue culture and nucellar embryos were used as explant for micropropagation.

Seeds sterilization:

Balady mandarin seeds were divided to two groups; fresh separated seeds and seeds inside fruit. Fresh separated seeds were sterilized by 3.5% NaOCl (v/v) with continuous agitation for 15 min. The sterilizing solution was decanted and explants were washed by distilled sterilized water 3 times (5 min., each), then, dipped in 70% ethanol for 5 sec (chemical method). While, seeds inside fruits group was burned for a few seconds after spray with 75% ethanol in the laminar air flow (flamed method by Xiao et al., 2004 and Nower 2013) and opened with scalpel for separating seeds, then all seeds were cultured in culture jars contained 50ml MS basal medium (Murashige and Skoog, 1962). Each treatment contained 10 replicates (10 jars) and each replicate contained six seed Sterilized seeds number, number of germinated seeds and germinated embryos per seed were recorded after 21, 42, 63, 83, 93 and 103 days.

DNA Finger Print:

In vitro Balady mandarin germinated embryos of ten seeds were arranged from the tallest to the shortest and labeled from 1st, 2nd, 3rd, 4th to 5th according to its tall (Fig. 1). Finger print of the five germinated embryos were done for determine the sexual embryo.

DNA isolation: Total genomic DNA was isolated from each embryo which was germinated in vitro using the CTAB (hexadecyltrimethylammonium-bromide) method (Doyle and Doyle (1990) with few modifications.

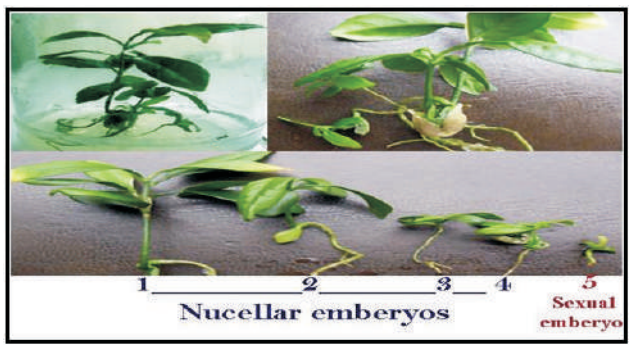

Fig. 1: Germinated embryo of Balady mandarin arranged according to its tall DNA Data analysis.

PCR Amplification and Electrophoresis:

RAPD markers: Nine decamer primers (OP-A12, OP-A17, OP-A18, OP-B12, OP-N16, OP-S147, OP-S238 and OP-S253) were employed on five samples of DNA of germinated embryos. PCR mixture was performed in a total volume of 25 µL, containing 50ng of template DNA, 1 µM of single primer, 1.2 U Taq DNA polymerase (Bangalore Genei, India), 0.40µM of each dNTP, 1.5 mM MgCl2, 10 mM Tris−HCl, and 50 mM KCl. The mixture was illumination.assembled on ice and amplification was performed for 45 cycles, using Biometra thermo cycler, as follow: one cycle at 92Cº for 2 min. and then 44 cycles at 92Cº for 30sec., 35Cº for 60 sec. and 72Cº for 2min.(for denaturation, annealing and extension, respectively). Reactions were finally incubated at 72Cº for 10min and stored at 4 Cº until separation by electrophoresis. All primers used were 10-mer random oligonucleotide sequences (Table, 2). Amplification products were separated by electrophoresis (5V cm-1) in 1.5% agarose gels and stained with ethidium bromide. A photographic record was taken under UV Only clear and repeatable amplification products were scored as 1 for present bands and 0 for absent ones. The specific bands useful for identifying species and cultivars were named with a primer number followed by the approximate size of the amplified fragment in base pairs. Polymorphism was calculated based on the presence or absence bands. The 0 or 1 data matrix was created and used to calculate the genetic distance and similarity using 'Simqual', a subprogram of the NTSYS-PC program (numerical taxonomy and multivariate analysis system program) (Rohlf, 1993). The dendrogram was constructed by using a distance matrix using the unweighed pairgroup method with arithmetic average (UPGMA) sub-program of NTSYS-PC.

Multiplication of nucellar embryos via tissue culture:

Shoot tips of nucellar embryos seedlings (asexual embryos) of Balady mandarin were planted on MS medium supplemented with different cytokinin types [6-benzylamino purine (BAP), adenine sulphat (AS) and kinetin (Kin)] and concentrations (0.0, 0.50, 0.75 and 1 mg/l). Balady mandarin shoots were subculture every 30 days into a fresh medium for two subcultures. Each treatment contained 10 replicates (10 jars) and each replicate contained five explants (about 2cm in length). The cultures were incubated at a temperature of 25±2°C and 16h photoperiod and light intensity 2000lux. Lateral or axilary shoot number/explant and shoot length (cm) were observed and recorded after each subculture.

Strengthen microshoots of Balady mandarin:

Produced microshoots (Shoot tips about 1.5 to 2cm in tall) of Balady mandarin resulted from multiplication stage were cultured on MS medium supplemented with various cytokinin types (BAP, Kin and AS) at different concentrations (0.0, 0.50, 0.75 and 1mg/l), various auxin types (NAA, IAA and IBA) at different concentrations (0.0, 0.5, 1.0 and 2.0 mg/l) or paclobutrazol (PP333) at different concentrations (0.0, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 mg/l) for one month before used as scions for grafting on volkamariana rootstock grown in greenhouse. The cultures were incubated as mention above. Each treatment contained 10 replicates (10 jars) and each replicate contained five explants (shoots).

Grafting in greenhouse:

Preparation of rootstock: Volkamariana rootstock seeds were cultured in a greenhouse in polyethylene pots (15cm in diameter) three months before grafting.

Preparation of Scions: Microscions which resulted from each treatment in vitro (cytokinin, auxin and PP333 treatments) have been grafted on volkamariana rootstock grown in greenhouse (about 20cm in tall) by cleft grafting (tip pen grafting) method. Also, the effect of different concentration of PP333 in combination with various diameter of volkamariana rootstock (3, 4 and 5mm) and using different grafting methods [cleft grafting (tip pen grafting) or side cleft grafting (side pen grafting)] was examined. Union region between scion and rootstock was rounded by parafilm to fix them and decrease losing water. Scion and union region were covered by polyethylene bags which gradually removed. Each treatment contained 10 replicates and each replicate contained 10 plants. The success of grafting in greenhouse of the in vitro produced microshoots (pre-treated in vitro) was recorded after 45 days of grafts. Also, microscion length and leaves number were recorded.

Data Statistical Analysis:

All trails were designed in factorial completely design. The least significance difference (L.S.D.) was used to compare treatment means at 5% level of significance according to method described by (Steel et al., 1997).

RESULTS AND DISCUSSION

Use asexual nucellar embryos which is free of viruses to establish an efficient in vitro propagation protocol for producing virus-free of Baalady mandarin, reported by Bitters et al. (1972), Roistacher et al. (1976) and Singh et al. (2008) who stated that pathogen-free citrus selections;

including virus-free plants, have been obtained by nucellar embryony culture, nucellar tissue culture, thermotherapy, clonal selection, indexing, and by shoot-tip grafting (STG).

Seeds sterilization:

Results in Table (1) and Fig. (2) showed that flamed method in all periods after cultivation were significantly maximized number and percentage of sterilized seeds (6 seeds and 100%, respectively). Number of germinated seeds increased with increasing time after culture. Method of sterilization affected number of germinated seeds. Flamed sterilization method after 93 and 103 days of cultivation possessed the highest germinated seeds number (5.40 germinated seeds). Also, number of germinated embryos per seed was affected by both method of sterilization and time after culture. The significantly highest number of germinated embryos per seed (4.17 embryos/seed) was possessed by flamed sterilization method after 103 days. These results may be due to the effect of sterilization solution on seed viability in chemical method while seed viability maximized in flamed method as a result of saving it inside fruits. Also, chemical and flamed sterilization methods are surface disinfection and their success depend on if there are not any systemic infection. These came in line with Ali and Mirza (2006) reported that contamination percentage was decreased from 55% to 40% when rough lemon fruits soaked in 0.5% (v/v) solution of sodium hypochlorite for 10 minutes after peeling (40%) compared with soaking in 1% (v/v) sodium hypochlorite solution for 20 minutes before peeling (55%). In another study, Tomaszewska-Sowa and Figas (2011) stated that the sterilization method of yellow everlasting Helichrysum aenarium (L.) in which the seeds were immersed in 96% ethanol and fired over a flame burner allowed 91.67% of sterile samples to be obtained, but further development took place in only 37.5% of all embryos.

Table 1: Effect of sterilization methods and periods after cultivation on seeds sterilization and embryos germination of Balady mandarin in vitro.

Time after culture (day) (B)	Initial seed No	Methods of sterilization (A)								
		No. of sterilized seeds (%)			No. of germinated seed			No. of germinated embryos/ seed		
		Chemical	Flamed	Mean(B)	Chemical	Flamed	Mean (B)	Chemical	Flamed	Mean(B)
21	6	4.0 (67 %)	6.0 (100 %)	5.0 (83 %)	0.6	0.6	0.6	0.0	0.00	0.00
42	6	4.0 (67 %)	6.0 (100 %)	5.0 (83 %)	3.2	3.4	3.3	0.66	0.83	0.75
63	6	4.0 (67 %)	6.0 (100 %)	5.0 (83 %)	3.6	5.0	4.3	1.00	1.33	1.17
83	6	4.0 (67 %)	6.0 (100 %)	5.0 (83 %)	4.0	5.2	4.6	1.83	2.80	2.33
93	6	4.0 (67 %)	6.0 (100 %)	5.0 (83 %)	4.2	5.4	4.8	2.67	3.33	3.00
103	6	4.0 (67 %)	6.0 (100 %)	5.0 (83 %)	4.2	5.4	4.8	2.67	4.17	3.46
Mean (A)		4.0 (67 %)	6.0 (100 %)		3.3	4.17		1.49	2.10	
LSD at level 5%										
A			0.5			0.6			0.48	
B			NS			0.4			0.24	
AxB			0.5			0.8			0.59	

Fig. 2: Effect of sterilization methods on seeds sterilization and embryos germination of Balady mandarin in vitro.

Determination of a sexual embryo by using RAPD technique:

Results in Table (2) and Fig. (3) cleared that number of amplified fragments of Balady mandarin embryos differed according to used primer, i.e., amplified fragments number ranged from 250 to 2000bp. Total amplified fragments of the nine RAPD primers was 85 fragments with total amplified fragments number 18 fragments and polymorphic percent 21%. There were four positive specific fragments (present); were observed with OP-B12, and 14 negative specific fragments (absent), all of them appeared in the 5th embryo.

Phylogenetic relationships among five samples of Balady mandarin nucellar embryos based on RAPD technique:

Similarity tree of germinated Balady mandarin embryos (Fig., 4) indicated that the closest relationship was scored between 1st, 2nd, 3rd and 4th embryos (nearly 100%). While, similarity between all the tallest four embryos and the 5th embryo was about 84%. These result cleared that the 5th embryo (the shortest one) is the sexual embryo in Balady mandarin seeds and the other embryos were vegetative nucellar embryos (asexual embryos). In conclusion, the use of RAPD marker allowed us to compare all germinated embryos of Balady mandarin and determined the sexual one. This result will be benefit in citrus breeding and propagation; ie, determination sexual embryo can be help breeders in find a source of variation in breeding programs, while it can help in propagation by exclusion off type which results from sexual embryo and production true to type seedlings through asexual embryos. Results came in line with Ahmad et al. (2012), EL-Mouei et al. (2011), Golein et al. (2011), Yaly et al. (2011), Rao et al. (2008) and Andrade-Rodríguez et al. (2004) who utilized molecular markers like RAPD, ISSR, expressed sequence tag (EST)-SSR and SSR markers to characterize the zygotic and nucellar seedlings in Citrus, they stated that molecular markers are useful and efficient tools in the identification of some cultivars.

Table 2: List of the RAPD primers sequences, number of produced amplified fragments, monomorphic, polymorphic, specific fragments and percentage of polymorphism among Balady mandarin nucellar embryos.

Primers	Sequence 5` to 3`	Number of Amplified Fragments	Number of Monomorphic Fragments	Number of Polymorphic Fragments	Number of Spicific Fragments	Polymorphic %
OP-A01	CAGGCCCTTC	9	8	1	1^a	11
OP-A12	TCGGCGATAG	7	4	3	3^a	43
OP-A17	GACCGCTTGT	8	6	2	2^a	25
OP-A18	AGGTGACCG T	9	8	1	1^a	11
OP-B12	CCTTGACGCA	12	8	4	4^p	33
OP-N16	AAGCGACCTG	9	8	1	1^a	11
OP-S147	AGCTGCAGCC	10	8	2	2^a	20
OP-S238	TGGTGGCGTT	8	8	0	0	0
OP-S253	GGCTGGTTCC	13	9	4	4^a	31
Total		85	67	18	14^a+4^p	21

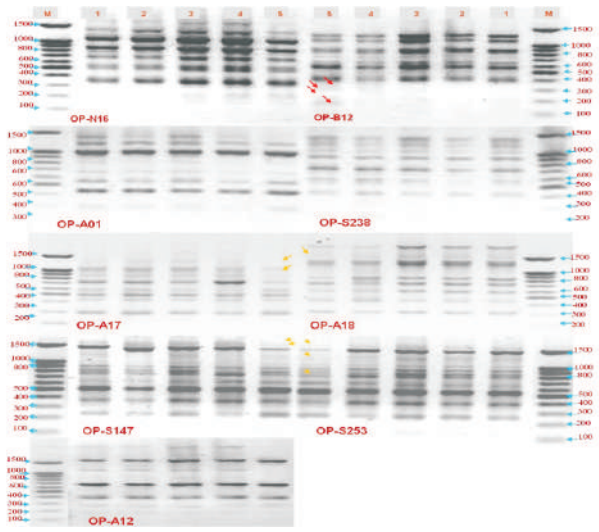

Fig. 3: Amplified fragments obtained from the DNAs of five germinated embryos of Balady mandarin via RAPD-PCR.

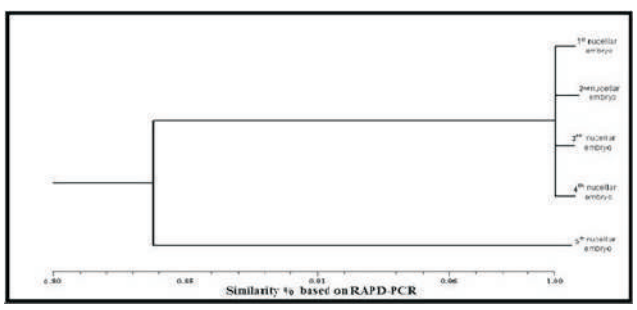

Fig. 4: Similarity tree of germinated Balady mandarin nucellar embryos.

Multiplication of asexual nucellar embryos via tissue culture:

Many factors may be affect propagation of asexual embryos in vitro, i.e., auxins, cytokinins, their concentrations and the combination between them which possess the balance between the two groups which responsible the orientation of the response for shoot formation or root formation or embryos induction and differentiation. So, the effects of different concentrations of auxin and cytokinin and cytokinin types and concentrations on multiplication of asexual embryos (vegetative embryos and true to type) of Balady mandarin derived from germinated seeds in vitro were examined as follow:

Effect of cytokinin types (BAP, AS and kin) and concentrations on shoots multiplication and shoot length of asexual nucellar embryos of Balady mandarin after 2nd subculture:

Table (3) and Fig. (5) indicated that cytokinins types showed the same trend of the first subculture (not presented), BAP was superior followed by AS and kin (4.46, 2.35 and 1.85 shoot/explant, respectively). The highest shoot number per explant was observed on 0.75 and 1.00 mg/l, with no significant difference between them. Interaction between cytokinin types and concentrations showed that, the highest significant shoot number/explant through second subculture resulted from the explant cultured on MS medium supplemented with 0.75 and 1.00 mg/l BAP (8.80 and 5.60 shoots/explant, respectively). Results in Table (3) revealed that shoot length was affected by different cytokinin types. Kin resulted in the tallest shoots followed by AS and BAP (4.40, 3.50 and 3.00 cm, respectively). Also, shoot length was affected by concentrations of ctokinins, 0.50 mg/l maximized shoot length (4.46 cm) while, 1.00 mg/l minimized shoot length (2.26 cm). Interaction between cytokinin types and concentrations conducted that 0.50 and 0.75 mg/l kin and 0.50 mg/l AS resulted in the highest shoot length (5.40, 5.00 and 4.40cm, respectively) with no significant differences between them. These results may be due to the high multiplication rate of BAP which lead to orient all the force of cell for multiplication while elongation may be observed in the low multiplication rate. Also, endogenous content of auxin may be high because Balady mandarin is dicoatyledon plant where an auxin synthesis in lateral and axilary buds, so, it needs exogenous cytokinin to reach the balance which is needed for multiplication. Results came in line with Sarma et al. (2011) and Nower and Hamza (2013) who stated that BAP was superior on other cytokinins in

Table 3: Effect of cytokinin types and concentrations on growth parameters of shoot tips derived from nucellar embryos of Balady mandarin after 2nd subculture.

Cytokinin types (A)	Shoot number/explant					Shoot length (cm)				
	Cytokinin conc. (mg/l) (B)				.	Cytokinin conc. (mg/l) (B)				.
	0.0	0.5	0.75	1.0	Mean(A)	0.0	0.5	0.75	1.0	Mean (A)
BAP	1.0	3.2	8.8	5.6	4.65	4.2	3.6	2.8	1.4	3.0
A.S	1.0	2.2	2.6	3.6	2.35	4.2	4.4	3.2	2.4	3.5
KIN	1.0	1.6	2.2	2.6	1.85	4.2	5.4	5.0	3.0	4.4
Mean (B)	1.0	2.3	4.5	3.93		4.2	4.48	3.67	2.27	
LSD at level 5%	A	0.54				0.57				
	B	0.62				0.66				
	AxB	1.09				1.14				

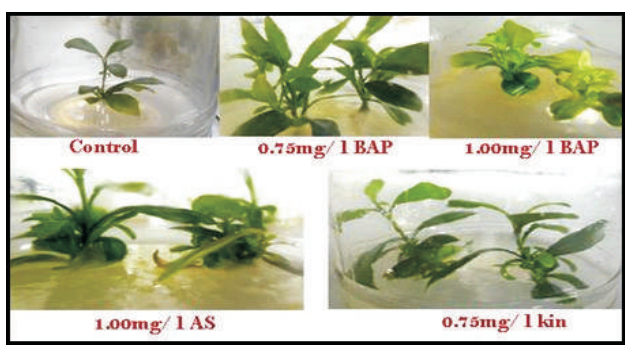

Fig. 5: Effect of cytokinin types and concentrations on growth parameters of shoot tips derived from nucellar embryos of Balady mandarin after 2nd subculture.

Grafting in greenhouse:

Effect of different concentrations of various cytokinin types (BAP, Kin and AS) on grafting success of microscions of Balady mandarin in greenhouse:

Data in Table (4) and Fig. (6) revealed that after 45 days of grafting, Balady mandarin scions (microscions) derived from MS medium supplemented with 1.0mg/l Kin or 0.75 mg/l AS maximized successful grafts number (3.7 and

3.3, respectively) and percentage (37 and 33%, respectively) compared with control (3 and 30% successful grafts). The highest number and percentage of dead grafts were observed in Balady mandarin scions derived from MS medium supplemented with 1.0mg/l BAP (9.3 and 93%).The highest value of both scion length and leaves number/scion were resulted from scions derived from MS medium supplemented with 1.0 mg/l BAP (8.3cm and 6.3 leaves/scion, respectively). These results may be due to the role of cytokinins in cell division which reflected in grafting success through stimulation formation of grafting unions and enhance scion growth. Results agree with Rafail and Mosleh (2010) who reported that medium supplemented with 2 mg/l BAP produced the highest grafting success percent (90%) for apple, whereas medium supplemented with 1 and 2 mg/l BAP gave the highest grafting success percent (90%) for pear. This confirmed the value of using cytokinins to improve grafting success by promoting and inducing callus growth and the formation of graft union between rootstocks and scions.

Table 4: Effect of cytokinin types (BAP, Kin and AS) and concentrations (0.0, 0.5, 0.75 and 1mg/l) on micrografting success of Balady mandarin in greenhouse after 45 days.

Cytokinintypes (A)	Concentration (mg/l)(B)	Successful grafts		Deadgrafts		Leaves No./ scion	Scion length (cm)
		No.	(%)	No.	(%)		
Control	0.00	3.0	(30%)	7.0	(70%)	6.0	5.3
BAP	0.50	1.7	(17%)	8.3	(83%)	2.3	3.3
	0.75	1.0	(10%)	9.0	(90%)	6.3	8.3
	1.00	0.7	(7%)	9.3	(93%)	5.0	5.0
Kin	0.50	3.3	(33%)	6.7	(67%)	4.3	4.3
	0.75	2.7	(27%)	7.3	(73%)	4.7	3.3
	1.00	3.7	(37%)	6.3	(63%)	5.0	6.0
AS	0.50	2.7	(27%)	7.3	(73%)	4.3	3.7
	0.75	3.3	(33%)	6.7	(67%)	3.3	3.3
	1.00	2.0	(20%)	8.0	(80%)	5.0	8.0
LSD at level 5%		0.82		1.4		1.3	1.2

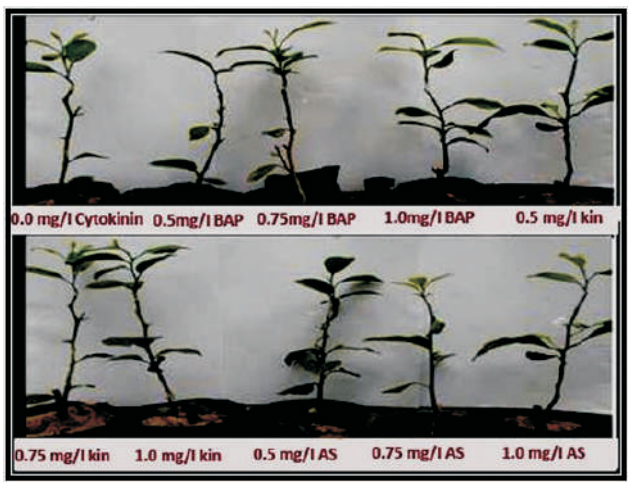

Fig. 6: Effect of different cytokinin types (BAP, Kin and AS) and concentrations (0.0, 0.5, 0.75 and 1.00 mg/l) on micrografting success of Balady mandarin scions derived from tissue culture grafted on volkamariana rootstocks grown in greenhouse after 45 days.

Effect of different concentrations of various auxin types (NAA, IAA and IBA) on grafting success of microscions of Balady mandarin in greenhouse:

Data in Table (5) and Fig. (7) discuss the effect of different auxin types (NAA, IAA and IBA) and concentrations (0.0, 0.5, 1.0 and 2.0 mg/l) on micrografting success of Balady mandarin scions derived from tissue culture in greenhouse after 45 days. Data cleared that Balady mandarin scions derived from MS medium supplemented with 0.5 mg/l IAA or 0.5 mg/l NAA gave the highest percentage of successful grafts (57%) followed by control (30% successful grafts) The highest dead grafts and percentage were observed in the scions derived from control (7.0 and 70%, respectively). Scions derived from MS medium supplemented with 1.00 or 2.00 mg/l IBA were superior in leaves number (15 leaves/scion) followed by 0.5 and 1.00 mg/l NAA (12 and 13 leaves/scion, respectively). The tallest scions resulted from scions derived from MS medium supplemented with 0.5 or 1.00 mg/l IBA (10.00 and 9.00 cm, respectively). It is worth mentioning that scions start to growth after 15 days of grafting process for all scions derived from MS medium supplemented with various concentrations of auxin types. Results may be due to the role of auxin in cell division and elongation which observed in the acceleration of graft union formation between volkamariana rootstock and scions of Balady mandarin and the height of scions which may result from the early beginning of scion growth after formation of grafting union. Results agree with Kotsias and Roussos (2001) and Carimi and De Pasquale (2003) who reported that auxin and cytokinin are the important growth regulators in plant tissue culture and they are the responsible on cell division. Also, Moore (1984); Aloni (1987), Aloni et al. (2010) and Pina and Eraaea (2005) added that the relationships between scion and stock are affected by growth regulators. In grafting, auxin which is released from vascular strands of the stock and scion is an important substance involved in the development of compatible unions, and induces the differentiation of vascular tissues, also, auxin is functioning as morphogenic substances. The same result was obtained by Moghadam et al. (2012) who reported that micrografting in companion with optimal auxin treatment has the strong potential for large scale production of this cactus and might be extended to the propagation of other micrografted Cacti species.

Table 5: Effect of different auxin types and concentrations on growth parameters of Balady mandarin scions derived from tissue culture grafted on volkamariana rootstocks grown in greenhouse after 45 days.

Auxin types	Concentration (mg/l)	Successful grafts		Dead grafts		Leaves No./ scion	Scion length (cm)
		No.	(%)	No.	(%)		
Control	0.00	3.0	(30%)	7.0	(70%)	9.00	8.00
NAA	0.50	5.7	(57%)	4.3	(43%)	12.00	7.67
	1.00	4.3	(43%)	5.7	(57%)	13.00	8.00
	2.00	4.3	(43%)	5.7	(57%)	7.00	8.00
IAA	0.50	5.7	(57%)	4.3	(43%)	10.00	8.00
	1.00	4.7	(47%)	5.3	(53%)	10.00	6.00
	2.00	4.3	(43%)	5.7	(57%)	9.00	6.00
IBA	0.50	1.3	(13%)	8.7	(87%)	9.33	10.00
	1.00	5.5	(55%)	4.5	(45%)	15.00	9.00
	2.00	4.3	(43%)	5.7	(57%)	15.00	7.00
LSD at level 5%		1.772		1.538		1.58	1.584

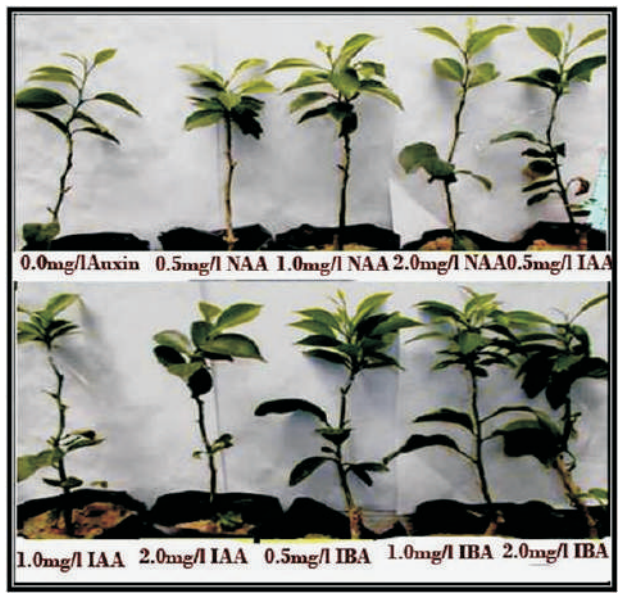

Fig. 7: Effect of different auxin types (NAA, IAA and IBA) and concentrations (0.0, 0.5, 1.0 and 2.0 mg/l) on growth parameters of Balady mandarin scions derived from tissue culture grafted on volkamariana rootstocks grown in greenhouse after 45 days.

Effect of different paclobutrazol (PP333) concentrations on success of Balady mandarin scions derived from tissue culture grafted on volkamariana rootstocks grown in greenhouse after 45 days of grafting:

Data in Table (6) and Fig. (8) concerning the effect of different paclobutrazol (PP333) concentrations on micrografting success of Balady mandarin scions derived from tissue culture on greenhouse after 45 days of grafting. Results cleared that scion derived from MS medium supplemented with 0.6 mg/l PP333 gave the highest number and percentage of successful grafts (8.8 and 88%)followed by scion derived from MS media supplemented with 0.4 mg/l PP333 (7.7 and 77%) and 1.0 mg/l PP333 (6.7and 67%) compared with control (2.7 and 27%). Scion derived from MS media supplemented with 0.0, 0.2, 0.4, and 0.6 mg/l PP333 significantly maximized leaves number (5.0, 5.0, 4.5 and 4.3 leaves/graft, respectively) and scion length (5.1, 5.5, 4.5 and 4.7 cm, respectively).

Table 6: Effect of different paclobutrazol (PP333) concentrations on micrografting success of Balady mandarin scions derived from tissue culture grafted on volkamariana rootstocks grown in greenhouse after 45 days of grafting.

PP_{333} conc. (mg/l)	Successful grafts		Dead grafts		Leaves No./ scion	Scion length (cm)
	No.	(%)	No.	(%)		
Control	2.7	27	7.3	73	5.0	5.1
0.2	4.4	44	5.6	56	5.0	5.5
0.4	7.7	77	2.3	23	4.5	4.5
0.6	8.8	88	1.2	12	4.3	4.7
0.8	6.6	66	3.4	34	2.0	3.2
1.0	6.7	67	3.3	33	2.3	3.0
1.2	3.4	34	6.6	66	3.0	2.7
LSD at level 5%	1.242		0.9589		1.278	1.060

Fig. 8: Effect of different paclobutrazol (PP333) concentrations on micrografting success of Balady mandarin scions derived from tissue culture grafted on volkamariana rootstocks grown in greenhouse after 45 days of grafting.

Further, increase of the PP333 concentration from 0.8 to 1.2 mg/l PP333 decreasing number of leaves and scion length. Results may be due to the ability of PP333, in low concentrations, as antigibrillin to induce storage which may save substances which needed to form grafts union. So, PP333 could be useful in enhancement grafts successful. Also, PP333 at the low concentration may be maximizing leaves number and scion length because it is so diluted and could be degradation quickly and have no negatively effects on growth parameters. Results came in line with Nowello et al. (1992) who stated that the conductivity of water from the paclobutrazol-treated plants of Vitis vinifera was less than that from untreated plantlets growing under 94% relative humidity. It therefore appeared that paclobutrazol treatment would make plantlets be able to withstand acclimatization better. Kamountsis and Chronopoulou-Sereli (1999) reported that Paclobutrazol affects the content of plant growth regulators by inhibiting gibberellin synthesis, reducing ethylene evolution, and increasing cytokinin level.

Effect of PP333 concentrations in vitro and volkamariana rootstock diameter in vivo on success of pen tip and side pen grafting method of scions of Balady mandarin derived from tissue culture in greenhouse:

Tip pen grafting method

Data in Table (7) and Fig. (9) concerning the effect of volkamariana rootstock diameter and PP333 concentrations on success of pen tip grafting method of Balady mandarin microscions derived from tissue culture on greenhouse. Results revealed that the increase the PP333 the maximum successful grafts. The maximum successful grafts percentage (60% successful grafts) was obtained when

microscions separated from shoots which were cultured on MS medium supplemented with 0.80 mg/l PP333. On the other hand, successful grafts number and percentage were negatively affected by volkamariana rootstock diameters, the highest successful percentage (48% successful grafts) was obtained when rootstock diameter was 0.3 cm. considering the effect of PP333 concentrations and rootstock diameter on percentage of successful grafts,. Data cleared that the highest successful grafts percentage (77%) was obtained when microscions were derived from MS medium supplemented with 0.8 mg/l PP333 and diameter of rootstock was 0.3 cm. Results may be due to the more juvenile tissue of rootstock with small diameter (0.3cm) and it may be contained a suitable amount of cambium which enhance grafting union formation. This agree with Elam (1997) who stated that, It is essential to have good contact of cambium or growing layer of the scion and rootstock as the success of graft depends on cambium which is located just below the bark and is a layer of active dividing cells responsible for the production of the conducting vascular system.

Table 7: Effect of in vivo volkamariana rootstock diameter and PP333 concentrations on success of pen tip grafting method of scions of Balady mandarin derived from tissue culture on greenhouse.

Rootstock diameter (cm) (B)	Successful grafts (number and percentage)									
	PP_{333} concentrations (A)									
	0.0 mg/l		0.2 mg/l		0.4 mg/l		0.8 mg/l		Mean (B)	
	No.	(%)	No	(%)	No.	(%)	No.	(%)	No.	(%)
0.3	2.3	23	4.0	40	5.3	53	7.7	77	4.8	48
0.4	1.7	17	3.3	33	5.0	50	6.7	67	4.2	42
0.5	1.0	10	1.7	17	3.0	30	3.7	37	2.3	23
Mean(A)	1.7	17	3.0	30	4.4	44	6.0	60		
LSD at 5% A	1.290									
B	1.490									
AxB	1.490									

Fig. 9: Effect of in vivo volkamariana rootstock diameter and PP333 concentrations on success of pen tip grafting method of scions of Balady mandarin derived from tissue culture on greenhouse.

Side pen grafting method:

Table (8) and Fig. (10) clear the effect of PP333 concentrations and in vivo volkamariana rootstocks diameters on success of side pen grafting method of microscions of Balady mandarin derived from tissue culture on greenhouse. Data showed that adding PP333 to MS medium at concentrations 0.0, 0.20, 0.40 mg/l increased number of successful grafts, the maximum of successful grafts percentage (70%) was obtained from 0.4 mg/l PP333 compared with control (4%). On the other hand, the highest successful grafts percentage (20%) were obtained when diameter of in vivo volkamariana rootstocks were 0.4 cm. The highest successful grafts percentage (60%) were obtained when concentration of PP333 was 0.8 mg/l PP333 and in vivo volkamariana rootstock diameters were 0.3 or 0.4 cm. Finally, tip pen grafting method was superior on side pen grafting (77 and 70% grafting successful). This may be due to the good vascular system formation in the case of tip pen grafting.

Table 8: Effect of PP333 concentrations and in vivo volkamariana rootstocks diameters on success of side pen grafting method of microscions of Balady mandarin derived from tissue culture on greenhouse.

Rootstock diameter (cm) (B)	Successful grafts (number and percentage)									
	PP_{333} concentrations (A)									
	0.0 mg/l		0.2 mg/l		0.4 mg/l		0.8 mg/l		Mean (B)	
	No.	(%)	No	(%)	No.	(%)	No	(%)	No	(%)
0.3	0.0	00	0.0	00	0.0	00	6.0	60	1.6	15
0.4	1.0	10	1.0	10	0.0	00	6.0	60	2.0	20
0.5	0.0	00	0.0	00	2.0	20	4.0	40	1.6	15
Mean(A)	0.4	40	0.4	40	0.7	7	5.3	53		
LSD at level 5% A	0.7									
B	0.8									
AxB	0.8									

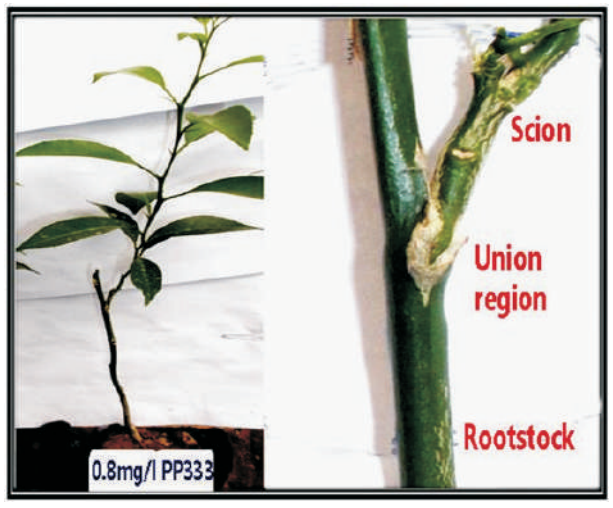

Fig. 10: Effect of PP333 concenrations in vitro and volkamariana rootstock diameters in vivo on success of side pen grafting method of microscions of Balady mandarin derived from tissue culture on greenhouse.

CONCLUSION

Determination of sexual embryo of Balady mandarin using genetic markers is very benefits for propagation and breeding programs. Using RAPD-PCR cleared different genetic distance between 5th nucellar embryo and the other embryos. Use nucellar vegetative embryos as initial explants of Balady mandarin propagation is effective method for producing virus-free plants commercialy. Cytokinins, auxins, PP333, grafting methods and rootstock diameters are factors affected grafting of in vitro produced microscions of Balady mandarin in

greenhouse. Microscions derived from MS medium supplemented with 0.6 mg/l PP333 gave the highest number and percentage of successful grafts (8.8 and 88%) in greenhouse when it was grafted on 3mm diameter of volkamariana rootstock. This paper is a step on the road to commercially produce virus-free citrus plantlets and eliminate viruses spread via grafting manipulation. But good treatments in citrus orchard are very important to save it from viruses infections.

REFERENCES

Ahmad, M., A. Javaid, Hafeez ur-Rahman, S. Hussain, A. Ramzan and Abdul Ghafoor, 2012. Identification of mandarin x orange hybrids Identification of mandarin x orange hybrids using simple sequence repeat markers. J. Agric. Res., 50(2):225-232

Ali, S. and Mirza B. 2006. Micropropagation of rough lemon (Citrus jambhiri Lush.): Effect of explants type and hormone concentration. Acta Bot. Croat. 65 (2), 137–146

Aloni R (1987). Differentiation of vascular tissues. Ann. Rev. Plant Physiol. 38: 179-204.

Aloni, B, Cohen R, Karni L, Aktas H and Edelstein M 2010. Hormonal signaling in rootstock-scion interactions. Sci. Hortic. 127: 119-126.

Andrade-Rodríguez M., A. Villegas-Monter, G. Carrillo-Castañeda, and A. García-Velázquez, 2004. Polyembryony and identification of Volkamerian lemon zygotic and nucellar seedlings using RAPD. Pesq. agropec. bras., Brasília, 39.(6):.551-559

Arif, M., A. Ahmad, M. Ibrahim and S. Hassan, 2005. Occurrence and distribution of virus and virus-like diseases of citrus in North-West Frontier Province of Pakistan. Pak. J. Botany, 37: 407-421.

Bitters W.P., T. Murashige, T.S. Rangan and E.Nauer, 1972. Investigations on eistablishing virus-free citrus plants through tissue culture. In: W.C. Price, (ed.), Proc. 2nd Conf. Inter. Organization Citrus Virol. Univ. Florida Press Gainesville, pp: 267-271

Carimi F. and F. De Pasquale, 2003. In: Jain SM, Ishii K (eds). Micropropagation of woody trees and fruits. Kluwer, Netherlands, pp: 589-619

Carvalho S. A., F.A Santos., M. A. Machado, 2002. Psorosis virus complex elimination from citrus by shoot-tip grafting associated to thermotherapy. Fitopatol Bras, 27: 306-308

De Lange, J.H., 1978. Shoot-tip grafting, A modified procedure. Citrus and Subtropical Fruits Journal, 539: 13-15.

Doyle J.J. and J.L. Doyle, 1990. Isolation of plant DNA from fresh tissue. Focus 12:13–15

Elam P., 1997. Budding and grafting citrus and avocados in the home garden. Publication 8001 by the Regents of University of California, Division of Agriculture and Natural Resources. Available on the DANR Communication Services website at http://danrcs.ucdavis.edu.

El-Mouei, R., Choumane W. and Dway F., 2011. Molecular characterization and genetic diversity in genus Citrus from Syria. Int. J. Agric. Biol., Vol. 13, No. 2: 351–356

Gebhardt, K. and H. Goldbach, 1988. Establishment, graft union characteristics and growth of Prunus micrografts. Physiol. Plant. 72:153-159.

Golein B. , R. Fifaei and M.Ghasemi, 2011. Identification of zygotic and nucellar seedlings in citrus inter specific crosses by inter simple sequence repeats (ISSR) markers. Afr. J. Biotechnol., Vol. 10(82):18965-18970.

Haripyaree A.; Guneshwor K.; Sunitibala H. and Damayanti M., 2011. In vitro propagation of Citrus megaloxycarpa . Environmental and Experimental Biology, 9: 129–132

Jajoo A., 2010. In vitro propagation of Citrus limonia Osbeck through nucellar embryo culture. Current Research Journal of Biological Sciences, 2 (1): 6-8

Kamountsis A.P. and A.G. Chronopoulou-Sereli, 1999. Paclobutrazol affects growth and flower bud production in gardenia under different light regimes. HORT. SCI. 34-4: 674-675.

Kotsias D.and P.A. Roussos, 2001. An investigation on the effect of different plant growth regulating compounds in in vitro shoot tip and node culture of lemon seedlings Sci Hortic., 89: 115-128.

Moghadam A. R. L.;Ardebili Z. O. and Rezaie L., 2012. Effect of indole butyric acid on micrografting of cactus . African Journal of Biotechnology Vol. 11(24): 6484-6493.

Moore R. (1984). A model for graft compatibility-incompatibility in higher plants. Am. J. Bot. 71(5): 751-758

Mukhopadhyay, S., J. Raj, B.C. Sharma, A. Gurung, R.K. Sengupta and P.S. Nath, 1997. Micropropagation of Darjeeling orange (Citrus reticulata Blanco) by shoot-tip grafting. J. Hortic. Sci., 72 (3): 493-499.

Mukhtar R., M.M Khan., R. Rafiq, A. Shahid and F.A Khan. (2005). In vitro regeneration and somatic embryogenesis in (Citrus aurantifolia and Citrus sinensis). International Journal of Agriculture & Biology Vol 7(3): 518-520

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassay with tobacco cultures. Physiol. Plant., 15: 473- 497.

Navarro L. and J. Juarez, 1977. Tissue culture technique used in Spain to recover virus-free citrus plants. Acta Hortic. 78:425-435

Navarro L., 1984. Citrus tissue culture. In: Micropropagation of selected root crops, plants, citrus and ornamental plants (plant protection and production, paper 59). FAO Rome, PP: 113-154

Navarro L., C.N. Roistcher and T.Murashige, 1975. Improvement of shoot-tip grafting in vitro for virus-free citrus. J. Am Soc Hortic Sci, 100: 471-479

Navarro, L., 1981. Shoot-tip grafting in vitro (STG) and its application: A review. Proc. Int. Soc. Citriculture, 1: 452-456.

Naz A.A., M.J. Jaskani, H.Abbas and M. Qasim, 2007. In vitro studies on micrografting technique in two cultivars of Citrus to produce virus free plants. Pak. J. Bot., 39(5): 1773-1778.

Nowello V., I. Grimaudo and A.V.,Robers 1992. Effects of paclobutrazol and reduced humidity on stomatal conductance of micropropagated grapevines. Acta Hort. 319: 65-70.

Nower A.A. (2013): In vitro production of somatic embryos from nucellus of mango (Mangifera indica L.) Life Science Journal;10 (2) 1164-1174

Nower A. A.and E. M. Hamza, 2013. Production of Gardenia jasminoides scions via tissue culture for grafting on Gardenia thunbergia under greenhouse.conditions. Journal of Applied

Sciences Research, 9(4): 3118-3128.
Onay, A., V. Pirinc, H. Yildirim and D. Basaran, 2004. In vitro micrografting of mature pistachio (Pistacia vera Siirt). Plant Cell Tissue and Organ Culture, 77:215-219.
Panattoni A., A. Luvisi and E. Triolo, 2013. Review. Elimination of viruses in plants: twenty years of progress. Spanish Journal of Agricultural Research, vol. 11, No. 1:173-188
Pina A and P Errea, 2005. A review of new advances in mechanism of graft compatibility-incompatibility. Sci. Hortic. 106: 1-11.
Rafail S. T. and M. S. Mosleh, 2010. Factors involved in micropropagation and shoot-tip grafting of apple (Malus domestica Borkh.) and Pear (Pyrus sp. L.) "World Food System A Contribution from Europe" Tropentag, September14-16, 2010 in Zurich.
Rao, M.N., J.R. Soneji, C.X. Chen, S. Huang and F.G. Gmitter, 2008. Characterization of zygotic and nucellar seedlings from sour orange-like citrus rootstock candidates using RAPD and EST-SSR markers. Tree Genetics Genomes. 4(1): 113-124
Rohlf, F.J., 1993. NTSYS-PC Numerical Taxonomy and Multivariate Analysis System Version 1-80. Exeter Software, Setauket, New York
Roistacher C.N., L. Navarro and T. Murashige, 1976. Recovery of Citrus selection free of several viruses, Excortis viroid, and Spirolasma citrus by Shoot-Tip grafting in vitro. Surveys, Citrus improvement , and other subjects, pp: 186-193
Ruiz, C., M.P. Breto and and M.J. Asins, 2000. A quick methodology to identify sexual seedlings in citrus breeding programs using SSR markers. Euphytica. 112(1): 89-94
Sarma; C, A. Borthakur, S Singh, M K Modi and P Sen, 2011. Efficient In Vitro Plant Regeneration From Cotyledonary Explants Of Citrus reticulata .L. Blanco Annals of Biological Research, 2 (6):341-348
Singh B., S. Sharma, G. Rani, V. Hallan, A.A. Zaidi, G.S. Virk and A. Nagpal, 2008. In vitro micrografting for production of Indian citrus ringspot virus (ICRV)-free plants of kinnow mandarin (Citrus nobilis Lour x C. deliciosa Tenora). Plant Biotechnology Report, 2: 137-143
Skoog, F. and C.O. Miller, 1957. Chmical regulation of growth and organ formation in plant tissue cultured in vitro.Symp Soc Exp Biol 11:118-131
Steel, R.G.D., J.H. Torrie and M.A. Boston, 1997. Principles and procedures of statistics. 2nd edition, McGraw-Hill Book Co. Inc., USA. 633p.
Tomaszewska-Sowa M. and A Figas, 2011. Optimization of the processes of sterilization and micropropagation of Cup plant (Silphium perfoliatum L.) from apical explants of seedlings in in vitro cultures. Acta Agrobotanica Vol 64 (4):3-10
Williamson, J.G. and L.K. Jakson, 1994. Citrus propagation. In Fact Sheet (HS-86) of Florida Cooperative Extensio Service, Univ. of Florida
Xiao J.N., X.L. Huang, Y.J. Wu, X.J. Li, M.D. Zhou and F.Engelmann, 2004. Direct somatic embryogenesis induced from cotyledons of Mango immature zygotic embryos, In Vitro Cell Dep. Biol. Plant, 40: 196-199.
Yaly, M. C., V. M. Novelli, M. Bastianel and M. A. Machado, 2011. Transferability and level of heterozygosity of microsatellite markers in citrus species. Plant Molecular Biology Reporter. 29(2): 418-423.