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Establishment of in Direct Propagation of Mandarin (*Citrus reticulata* L.) Using Tissue Culture

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THE PRESENT investigation deals with development of an efficient micropropagation protocol for mandarin (*Citrus reticulata* L.) via in direct propagation using cotyledons and juice vesicles as explant. The establishment of *in vitro* callus induction from leaf, stem segments and cotyledon system were excised from seedlings has been done. Best results for induction of callus were observed from cotyledons and stem segments 100% on MS medium supplemented 1 or 2mg/l 2,4-D. MS medium supplemented with 300mg/l glutamine or 1.5mg/l casein hydrolysate proved to be the most efficient additive in promoting callus formation from juice vesical with 4.22, 4.23g/callus fresh weight, 100% response and was the most favorable medium. Maximum callus fresh weight (7.96 g) and 100% response were obtained from juice vesicles when cultured on MS medium supplemented with 2mg/l 2,4-D and 10% coconut water. The highest callus regeneration and weight of callus observed from juice vesicles by adding 2mg/l 2,4-D, 300 or 400 mg/l malt extract. Callus raised from juice vesicles showed maximum shoots (96.67%) with 0.5mg/l Kin, 400mg/l malt extract and 2mg/l NAA. Regenerated shoots raised from juice vesicles showed better shoot multiplication and highest length of shoots on MS medium with 2mg/l BA and 0.4mg/l NAA. Regenerated shoots were rooted on MS medium supplemented with different concentrations of NAA and best response (98%) was observed with NAA (2 mg/l) and gave maximum, roots number, roots length and leaves number. Rooted plantlets were successfully acclimatized with survival rate reaching almost 87%. These plants grew normally without showing any morphological variation. Developed protocol can be useful fo*r* application of somatic embryogenesis from juice vesicles to improve mandarin.

Keywords: Casein hydrolyzed, Coconut water, Glutamine, *In vitro*, Juice vesicles, Malt extract, Mandarin, Rooting and adaptation.

Introduction

Citrus belongs to family Rutaceae having 150 genera and 15,000 species and it is distributed mostly in tropical and temperate region of the planet (Ladaniya, 2008). It has high dietary value and is a prosperous source of vitamin C in combination with macromolecules such as amino acids, organic acids and sugar as well as minerals comparable to magnesium and calcium in sufficient quantity (Niaz et al., 2004). Slow growth, long juvenility, insects, pests, diseases, alternate bearing, pre-and post-harvest losses, large number of seeds per fruit, short season of supply and short storage life are the problems facing by citrus species too (Mukhtar et al., 2005). One of the effective methods for citrus propagation is the use of somatic embryogenesis which consequently produces a large number of healthy uniform plants (Gholami et al., 2013). Somatic embryogenesis is an efficient protocol of plant regeneration and rapid propagation of large number of plants within a short time. *In vitro* plant culture has used as a tool for propagation and improvement of many plant species including citrus. In citrus, the production of embryogenic callus lines was reported from excised undeveloped ovules (Starrantino & Russo, 1980), juice vesicles (Nito & Iwamasa, 1990), anthers (Benelli et al., 2010), from immature seeds separated from immature fruits (Gholami, 2013) and from leaves, epicotyls, cotyledons (Kiong et al., 2008). The presence of cytokinin, in concentrations up to 2 mg/l, stimulated the *in vitro* organogenesis when internodal segments-derived explants were used for *Citrus limonia* and *Citrus aurantium*. Although no statistic differences could be detected, culture media supplementation with the combination of BAP and NAA favored the development of adventitious shoots in *Citrus aurantium* (Schinor

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et al., 2011). Micropropagation of citrus offers rapid propagation of such crops in limited space and time under controlled conditions around the year (Usman, 2005). Tissue culture is the best option to produce disease free seedling of the fruit crop rapidly. Micro-propagation and use of the *in-vitro* grafting (micro-grafting) is very helpful for production of virus free planting materials in mandarin (Amgai et al., 2016). Malt extract promoted germination of early cotyledonary stage embryos arising from the *in vitro* rescue of zygotic embryos of sour orange (Carimi et al., 1998). Sugar type and concentration had effects on citrus somatic embryo development. The combination of sorbitol (36.5 mM) with galactose 73 mM was able to augment citrus somatic embryo maturation more effectively than the other concentrations applied (Widoretno et al., 2017). The aim of this research was to determine the best explant type (leaf, stem segments, and cotyledons) and growth regulator and natural additives in order to achieve callus induction. Simple quick response to indirect organogenesis was the main purposes for the use of (juice vesicles and cotyledons) in order to obtained healthy and uniform shoots and rooting of regenerated shoots.

Materials and Methods

This study was carried out during the period from 2014 to 2016 in the Laboratory of Gene Transfer and Germplasm Conservation, Department of Plant Biotechnology and plant material certified immature fruits (90 days after anthesis) were chosen from 10 years old Mandarin (*Citrus reticulata* L.) grafted tree was obtained from the farm at Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City, Egypt.

Surface sterilization

Mandarin fruits were surface-sterilized by soaking in 10% commercial bleach (containing 5 % active chloride) for 20 min, followed by three rinses with sterile distilled water. Fruits were then dipped in 95% ethanol and flamed for a few seconds. After flamed fruits were cut into two halves for seeds collected and juice vesicles were carefully separated for callus induction (Xiao et al., 2004).

In vitro seed germination

Surface sterilized of seeds were placed individually in 350 culture jar containing 30ml of liquid medium (Murashige & Skoog, 1962) (MS) supplemented with 400 mg/l glutamine, 100mg/l ascorbic acid, 30 g/l sucrose and 7g/l agar. The seeds were maintained at 25 ± 2°C in the dark for three weeks, followed by one week under a 16-h photoperiod 3000 lux. After 5 weeks' explants, (leaf, stem segments and cotyledons) were excised for callus induction.

Callus induction

Effect of dichlorophenoxy acetic acid (2, 4-D) concentrations and different explant parts on callus induction

Different explants like leaf, stem segments and cotyledons (0.5-1.0 cm) were excised from 5 weeks old *in vitro* raised nucellar seedlings for callus induction. Callus was initiated in 350ml culture jars containing 30 ml of MS medium supplemented with 2, 4-D (0.0, 0.5, 1, 2 or 3 mg/l). Explants were incubated in darkness, in a growth room 25±2^{o}C for one month for two times. Data was recorded as percentage of callus induction and callus fresh weight (g/explant).

Effect of different additive on callus induction from juice vesicles and cotyledons

According to the results of previous experiment, 2mg/l 2, 4-D were used to run this experiment. Cotyledons and juice vesicles (1-2cm) excised from fruits were grown on MS medium contained 0, 100, 200 and 300 mg/l glutamine, 0.0, 0.5, 1.0 and 1.5mg/l casein hydrolysate. One explant was cultured in 150 ml a glass jar medium; contained 30ml each treatment had ten replicates. Data were recorded after two months for two times as callus fresh weight (g/explant) and percentage of callus induction.

Effect of different concentrations of coconut water on callus induction

Explants (juice vesicles and cotyledons) which produced with best response from above experiments were used to run this experiment to improvement of callus. Explant were cultured on MS medium containing 2mg/l 2,4-D and 0, 5, 10 or 20 % (v/v) coconut water. One explant (1cm) was cultured in 150 ml a glass jar medium; contained 30ml each treatment had ten replicates. Data were recorded after two months for two times as callus fresh weight (g/explant) and percentage of callus induction.

Effect of malt extract on callus regeneration

The juice vesicles and cotyledons derived callus 1cm were obtained from above experiment and transferred to MS medium supplemented

with 0.3 mg/l α-naphthalene acetic acid (NAA), 0.5 mg/l BA and different concentrations of malt extract (0.0, 100, 200, 300 and 400 mg/l) (Carimi et al., 1998) and 30g/l sucrose. One explant was cultured in 150 ml a glass jar medium; contained 30ml each treatment had ten replicates. The explants were maintained at 25±2°C in dark. Data recorded after two months as callus fresh weight (g/explant) and percentage of regenerated of callus.

Effect of different concentration of NAA on in direct organogenesis from callus derived from juice vesicles and cotyledons

Callus regeneration from malt extract experiment were used as explants for in direct organogenesis. Regenerated callus was cultured on MS supplemented with 400mg/l malt extract, 0.5 mg/l Kin, 30g/l sucrose and different concentrations of NAA (0.0, 0.5, 1.0, 1.5 and 2.0mg/l) and 6g/l agar. The culture explants were kept under dark at 25±2°C. Data were recorded after two months for two times as percentage of shoots induction.

Effect of 2mg/l BA combination with different concentrations of NAA on shoot multiplication

The shoots excised from above experiment were inoculated on MS medium supplemented with 2mg/l BA combination with different concentrations of NAA (0.0, 0.1, 0.2, 0.4 mg/l). The cultures were kept in culture room at 2000 lux and 25±2°C. For each of the treatment, three explants in culture jar 350ml each 50ml of medium and ten replicate were used and the experiment was repeated two times. The number of shoots per explant and length of shoot (cm) were observed after four weeks from culture.

Rooting and acclimatization

Effect of different concentrations of auxins on root formation

Shoots obtained from above experiment were transferred to MS supplemented with 0.5 mg/l Kin for elongation of plantlets for one month and then transferred to different concentrations of Indole-3-butyric acid (IBA) and (NAA) (0, 0.5, 1.0, 1.5 and 2 mg/l) for rooting. Rooting of all the shoots were examined after six weeks as percentage of rooting, roots number, roots length (cm) and plant length (cm).

Acclimatization of plants

Rooted plants were transferred into 7cm cup containing a mixture of peat moss and perlite (3:1, v/v). Plants were placed in greenhouses at 27 ± 2°C under a 16 h photoperiod at 4000 lux. Plants were covered with transparent plastic lid cover to prevent water loss. Plants were fertilization with NPK every one week for two months and adapted plants was recorded as survival percentage.

Layout of the experiments

All experiments were designed in factorial completely design and data were compared according to method described by Steel & Torrie (1980).

Results and Discussion

Effect of 2, 4-D concentrations on callus induction from different explant parts

In this experiment, callus induction was done from leaves, cotyledons and stem segments explants were taken from five weeks from germinated seed. High efficiency callus was produced at 2 mg/l 2, 4-D than other concentrations (Table 1 and Fig.1), the color of the callus produced was whitish and yellowish white. The development and maximum of callus induction percentage was observed at cotyledons and stem explants than leaf (97.33). The lower concentration of 2,4-D (0.5mg/l) is not sufficient to induction of callus from leaves (31.66%) but the same concentration sufficient to induce callus from cotyledons and stem (95 and 85%, respectively). This phenomenon suggests that 2,4-D concentration and explant type are more important role in callus formation from explants. The results are in conformity with some of the earlier studies on different *Citrus* spp. which showed good callus induction response under the influence of in different concentrations 2,4-D. Altaf et al. (2009) reported that hormonal combination for good callus induction for seedling leaf of kinnow mandarin is BA+ GA (each at 1 mg/L) + 2,4-D at 0.5 mg/L + proline at 5 mg/L. Amin & Shekafandeh (2015) found that MS medium supplemented with 0.5mg/l 2,4-D induced maximum embryogenic calli of mexican lime. The best response for primary callus induction of kinnow mandarin (90%) was obtained when MS medium was supplemented with 5 mg/l 2,4-D and 500 mg/l malt extract (Hussain et al., 2016). Mahadi et al. (2016) showed that the best a combination of hormones is treatment D2B2 (2 mg/l, 2,4-D, dan 2 mg/l BAP) producing embryogenic callus of Calamansi (*Citrus microcarpa*).

TABLE 1. Effect of MS medium supplemented with different concentrations of 2, 4-D on percent of callus induction from leaf, cotyledons and stem segment excised from 6weeks old seedlings of *Citrus reticulata* L. (Observations recorded after 30 days for two times).

2,4-D con. (mg/l)	Percent callus induction Explant type			
	Leaf	**Cotyledons**	**Stem segment**	**Mean**
0.0	15.00	6.66	5.00	**8.88**
0.5	31.66	95.00	85.00	**75.55**
1.0	88.33	100.00	100.00	**96.11**
2.0	95.00	100.00	100.00	**98.33**
3.0	53.33	95.00	88.00	**78.77**
Mean	65.66	97.33	75.60	
LSD at 5% A		3.96		
B		3.06		
AxB		6.86		

Fig. 1. Callus induction from seed on MS medium supplemented with 2mg/l 2, 4-D.

Effect of different additive on callus induction from Juice vesicles and cotyledons

Micropropagation via embryogeneic callus has become a vital mean for propagating of mandarin. Optimization of the culture medium may require some supplements other than plant growth regulators, which are normally added to the nutrient medium. It is clear from the reported data in Table 2 found that 300mg/l glutamine and 1.5mg/l cazen hydrolyseat has increased callus fresh weight and callus induction % when supplemented to the callus maintenance medium. Juice vesicles exhibited a synergistic positive impact on callus fresh weight and callus induction %, more than seeds explants. Maximum callus fresh weight and callus % observed on 300 mg/l glutamine or 1.5mg/l cazen hydrolyseat at juice vesicles than cotyledons as showed in Fig 2. Glutamine plays an important role in nitrogen assimilation as it is an intermediate in the transfer of ammonia into amino acid. Casein hydrolysate was found to be best medium composition for regeneration protocol developed from suitable explant for callus induction of mandarin. Some studies report mixture of amino acids (like Casein hydrolysate), rather than a single amino acid, as very supportive for shoot multiplication even in prolonged cultures (Nasir et al., 2011). Produced calli from Mexican lime juice vesicles are totipotent for embryogenesis and plant regeneration (Amin & Shekafandeh, 2015).

Effect of different concentrations of coconut water on callus improvement

The effects of different concentrations of

coconut water on embryogenesis fresh weight and improvement of callus % are summarized in Table 3. Maximum callus fresh weight (g) produced on a medium containing 10 % coconut water at juice vesicles explants (Fig.3) than callus derived from cotyledons. Moreover, minimum callus weight and callus induction % were produced at cotyledons than juice vesicles explants. This study shows that the, type of explant and concentrations of coconut water has a very significant effect on callus fresh weight and callus improved %. Coconut water has a very significant effect on callus weight and callus induction %. The results are agreement who those, The use combination of growth hormones and inclusion of other in vitro callus induction and proliferation factors, such as coconut (*Cocos nucifera* L.) extracts (Michael, 2007). Coconut water contains mainly water (94%) and growth promoting substances that can influence *in vitro* cultures including inorganic ions, amino acids, organic acids, vitamins, sugars, sugar alcohols, lipids, nitrogenous compounds and phytohormones (Young et al., 2009). Shoot isolates that proliferated from calli at higher coconut water levels were able to grow to maturity (Michael, 2011).

TABLE 2. Effect of modified MS medium supplemented with glutamine and casein hydrolysate at different concentrations and different explant type on callus fresh weight (g) and callus percentage. (Observations recorded after two months for two times).

Different addative conc. mg/l		Callus fresh weight (g)			Callus %		
		Explant type					
		Juice vessels	Cotyledons	Mean (B)	Juice vessels	Cotyledons	Mean (B)
Glutamine	٠,٠٠	2.16	1.33	1.65	10.00	8.00	9.16
	100	4.39	2.49	3.44	90.00	81.66	85.83
	200	3.61	2.86	3.24	95.00	86.66	90.83
	300	4.22	3.00	3.61	100.00	98.33	99.16
Casein hydrolysate	0.5	3.13	1.80	2.46	53.33	76.66	65.00
	1.0	3.60	2.56	3.08	65.00	100.0	82.50
	1.5	4.23	3.10	3.66	100.00	95.00	97.50
Mean (A)		3.62	2.45		78.10	73.33	
LSD at 5% A			0.36			4.30	
B			0.19			2.29	
AxB			0.15			6.08	

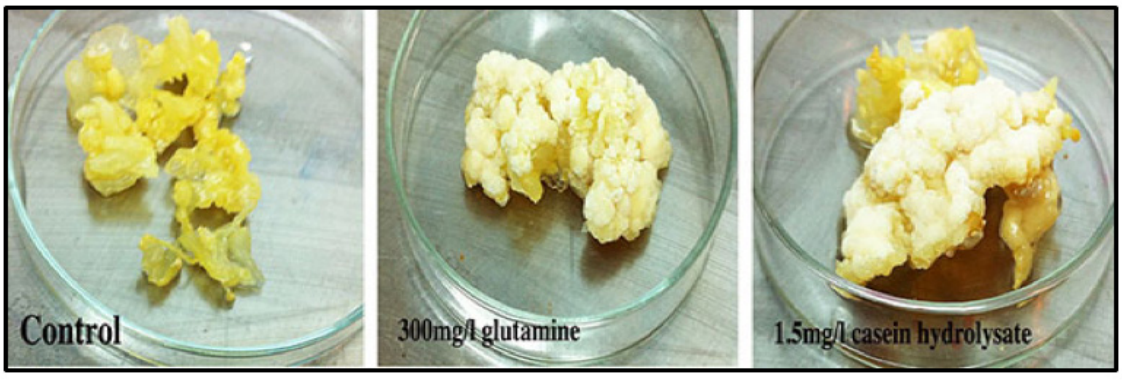

Fig. 2. Callus induction from juice vesicles on MS medium supplemented with 2mg/l 2, 4-D after two months from cultured .

TABLE 3. Effect of different concentrations of coconut water and explant types (juice vesicles and cotyledons) on callus fresh weight (g) and callus percentage. (Observations recorded after two months for two times).

Coconut water (v/v%)	Fresh weight of callus (g)			Callus (%)		
	Explant type					
	Juice vessels	Cotyledons	Mean (B)	Juice vessels	Cotyledons	Mean
0	2.48	1.13	1.80	13.33	11.66	12.50
5	3.06	2.93	2.99	65.00	75.00	70.00
10	7.96	4.23	5.60	100.00	90.00	95.00
20	6.03	3.25	4.64	83.33	93.33	88.33
Mean (A)	4.63	2.88		65.41	67.50	
LSD at 5% A		0.55			6.59	
B		0.38			4.66	
AxB		0.77			9.32	

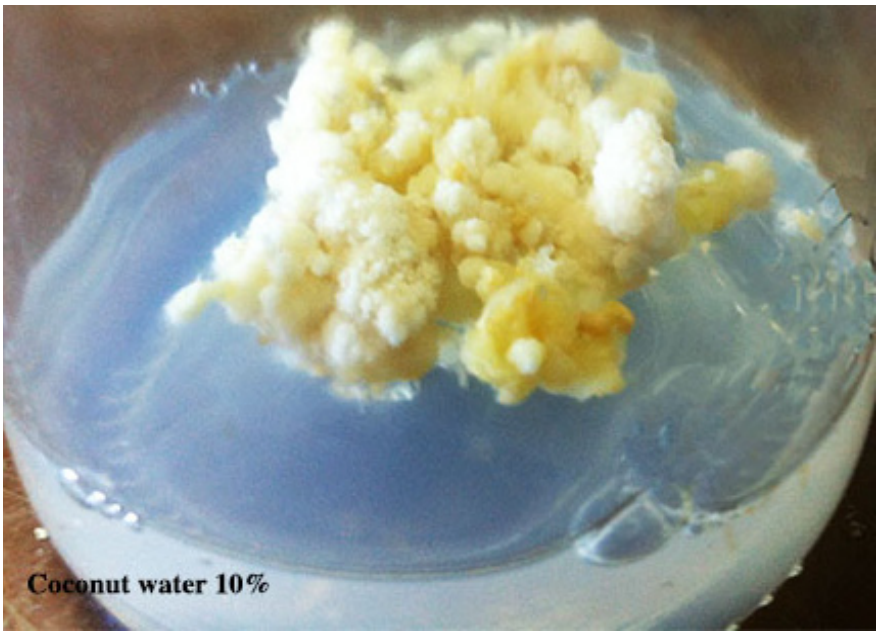

Fig. 3. Improved callus derived juice vesicles on MS supplemented with 2mg/l 2,4-D and 10 %coconut water.

Effect of Malt extract on callus regeneration from callus derived juice vesicles and cotyledons

Malt extract is one of the carbohydrate sources and it has been determined that it stimulate somatic embryogenic callus in citrus. Maximum fresh weight of callus (4.78g/explant) and callus regenerated (97.50 %) derived from juice vesicles was observed on MS media containing 400 mg/l malt extract after ten months. However, the results from the current study produced maximum regenerated callus 93.73% of juice vesicles than derived from cotyledons (82.67%). Interaction between explant type and different concentration of malt extract showed the highest callus weight and percentage of callus regenerated recorded for juice vesicles at 300 or 400 mg/l malt extract than other treatments as shown in Table 4. From the result, it showed that, organic compound such as malt extract enhanced callus regeneration only at high concentration of malt extract for mandarin as shown in Fig. 4. Malt extract seems to play a specific role in cultures of mandarin explants. Malt extract, mainly a source of carbohydrates, was shown to callus regenerated in explants. Sawy et al. (2005) reported that the callus induction and somatic somatic embryogenesis formation from undeveloped ovules in citrus by the use of 500 mg/l malt extract on MS medium. Malt extract or in combination with GA3 enhance somatic embryogenesis formation in different citrus species (Gholami et al., 2013). Somatic embryos of Mexican lime were formed only by the use of MT medium containing 500 to 700mg/l malt extract (Amin & Shekafandeh, 2015).

TABLE 4. Effect of MS medium supplemented with 2mg/l 2,4-D and different concentrations of malt extract on weight of callus and callus regeneration from callus raised from juice vesicles and cotyledons.

Malt extract con. mg/l	Callus weight (g)			Callus regeneration %		
	Explant type					
	Juice vesicles	Cotyledons	Mean	Juice vesicles	Cotyledons	Mean
Control	2.82	1.53	2.17	80.33	61.67	71.00
100	4.43	1.90	3.16	93.33	80.00	86.66
200	4.83	1.98	3.40	95.00	83.33	89.16
300	5.08	3.10	4.09	100.00	93.33	96.66
400	5.66	3.91	4.78	100.00	95.00	97.50
Mean	4.56	2.48		93.73 A	82.67	
LSD at 5% A		0.37			3.27	
B		0.23			2.07	
AxB		0.52			4.62	

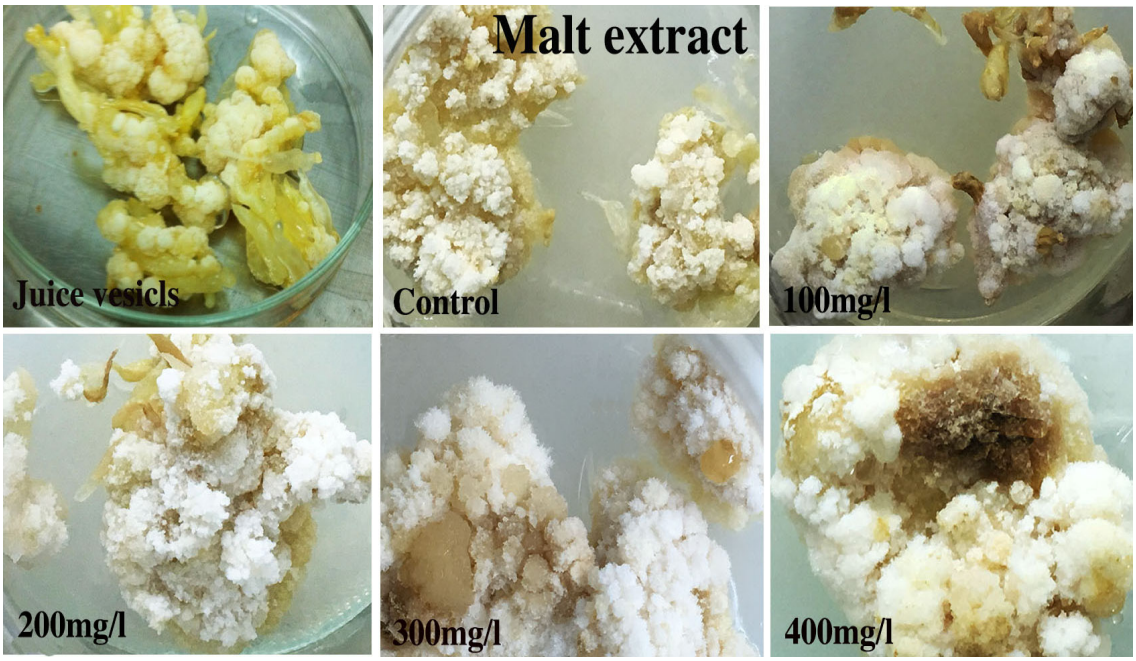

Fig. 4. Callus regeneration from juice vesicles in MS supplemented with 2mg/l 2,4-D and different concentration of malt extract.

Effect of different concentration of NAA on in direct organogenesis from callus derived juice vesicles and cotyledons

The results indicated that good shoots induction was obtained from highest concentration 2.0mg/l NAA in both explants (Table 5). In direct organogenesis increases with the increase of hormonal level (Fig. 5). It was interesting to note that response of both explant type was almost similar at other 2 concentrations level of NAA. According to the results, NAA has maximum shoots formation, due to the presence of auxins at 2mg/l NAA from callus derived from juice vesicles than cotyledons, this depends on its concentration. NAA at low concentration showed a progressive increase in shoots percentage. These results got support from Tomaz et al. (2001) showed that the enhancing effects of auxins (NAA and BAP) on embryos formation percentage in citrus and significant results for embryos. Maximum plantlets were regenerated (92%) from the somatic embryos on half strength MS medium with no hormones (Hussain et al., 2016).

TABLE 5. Effects of MS medium supplemented with 0.5mg/l Kin, 400mg/l malt extract and different concentrations of NAA on percentage of shoots induction percentage from callus (derived from juice vesicles and cotyledons).

Supplement (mg/l) NAA	Shoots induction %		
	Regeneration from juice vesicles callus	Regeneration from cotyledons callus	Mean
Control	23.33	20.00	21,66
0.5	63.33	48.33	54.83
1.0	66.67	53.33	60.00
1.5	76.67	56.67	66.66
2.0	96.67	68.33	82.49
Mean	65.33	48.33	
L.S.D at 5 %A		2.82	
B		1.78	
AxB		4.00	

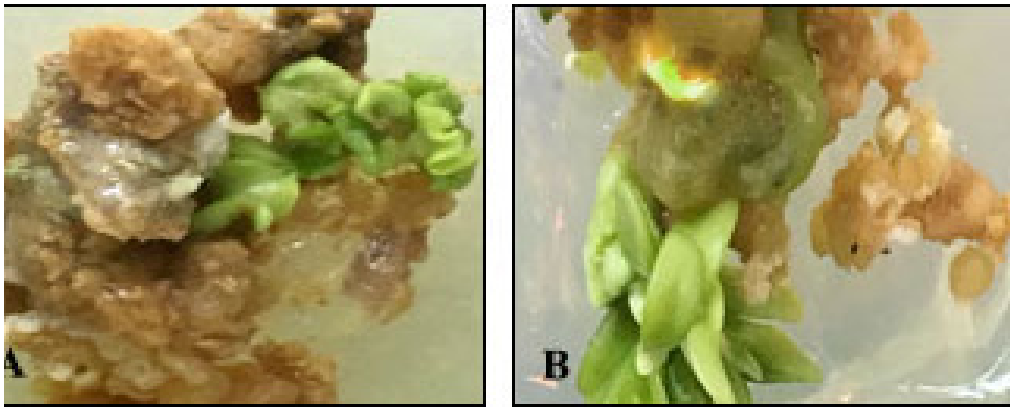

Fig. 5. In direct shoots formation from callus derived juice vesicles on MS medium supplemented with 2mg/l NAA, 0.5mg/l Kin and 400mg/l malt extract. A: initiation of shoots formation. B: Developed shoots.

Effect of 2mg/l BA combination with different NAA concentration on shoot regeneration

The shoots were obtained from above experiment used for shoot regeneration. Amongst various concentration of the four NAA at the concentration of 0.2 and 0.4 mg/l induced maximum number of shoots (49.00) per explant (Fig. 6). The proliferated shoots reached to a maximum plant height (2.90 cm) on MS medium supplemented with 0.2 and 0.4mg/l NAA mg/l (Fig. 7). The shoots obtained from 2mg/l BA supplemented medium were having less number of shoots as compared to shoots obtained on 2mg/l BA and combination with NAA at different concentration. From the result, add 2mg/l BA resulted in increased shoot number while BA combination with 0.2mg/l NAA increased and improved shoots clearly (Fig. 8).

Higher NAA levels with BA combination showed highest positive effects on shoots number and shoots length. The results are consistent with those of Tallon et al. (2013) and Esmaeilnia & Dehestani (2015). Amgai et al. (2016) found that shooting from explants was significantly higher (71.72%) on medium level of the BAP (0.5 mg/L) and IAA (0.2 mg/L) using *in-vitro* seedling stem as explant of mandarin orange.

Effect of auxin concentration on in vitro rooting

MS medium supplemented with 2mg/l NAA induced roots (Fig. 9). NAA has previously been used for induction of roots from *in vitro* regenerated shoots of citrus. Root induction was observed 15-20 days after transfer of shoots to MS medium. The results indicate that NAA concentration supplied to MS medium, significantly influenced root induction and shoot

growth. The best rooting treatment was 2 mg/l NAA, since it gave the highest root number, root length, leaves number and percentage of root induction. Root number increased fourfold in response to 2mg/l NAA compared to the hormone-free control, but lower levels of IBA reduced root number, as illustrated in Table 6. In contrast, root length was directly related to the concentration of NAA. The longest roots, 5.5 cm, were found in media containing 2 mg/l NAA. To encourage root development, it may be advantageous to transfer shoots from root induction on 2 mg /l NAA, which gave the highest root number, root length, leaves number and percentage of root induction. The plants were successfully transferred to pots in a greenhouse with 98% survival percentage. Similarly, Highest rooting percentage in (*Citrus aurantifolia* and *Citrus sinensis*) was obtained at concentration of 1.5mg/l NAA (Mukhtar et al., 2005). Eight weeks after incubation of embryos on MS medium supplemented with 0.02 mg/l NAA and different levels of GA3 (0, 0.25, 0.5, 0.75 and 1.0 mg/l) plantlets were then transferred into pots containing of 1: 2: 2 volume ratios of soil mixture (sand, peat moss and perlite, respectively) and the rate of survival was 100% of mexican lime (Amin & Shekafandeh, 2015). The highest rooting rates of 76 and 72 % were recorded for media supplemented with 0.5 or 1 mg L-1 NAA, respectively for sweet orange (*Citrus sinensis* L. Osbeck.) (Esmaeilnia & Dehestani, 2015).

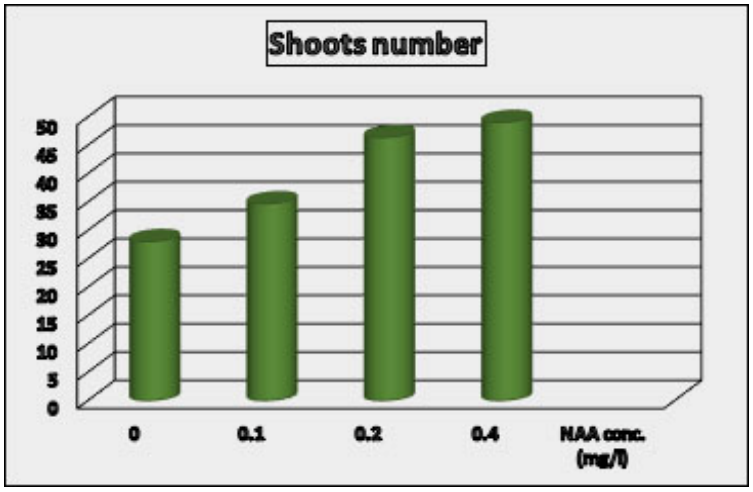

Fig. 6. Effect of different concentrations of NAA and combination with 2mg/l BA on shoots number after four weeks from culture.

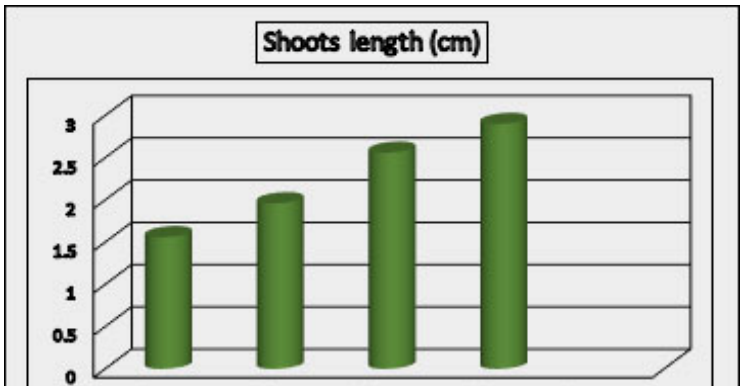

Fig. 7. Effect of different concentrations of NAA and combination with 2mg/l BA on shoots length after four weeks.

Fig. 8. Development of shoots on MS medium supplemented with 2mg/l BA and combination with 0.2mg/l NAA.

Fig. 9. *In vitro* rooting and *ex vitro* acclimatization of mandarin (*Citrus reticulate* L.).

TABLE 6. Effect of MS supplemented with different concentrations of NAA and IBA on roots number, roots length, leaves number and rooting percentage of (*Citrus reticulata* L.).

Auxin con. Mg/l		Roots number	Roots length (cm)	Leaves number	Rooting %
NAA	0.0	1.00	2.50	10.33	35.55
	0.5	2.00	3.16	11.00	67.78
	1.0	2.66	4.50	12.00	83.66
	1.5	3.33	4.16	12.67	88.33
	2.0	4.66	5.50	14.33	98.00
IBA	0.5	1.33	3.00	8.00	66.67
	1.0	2.33	3.33	9.33	68.89
	1.5	2.66	3.56	10.00	85.55
	2.0	3.33	3.36	11.00	88.66
L.S.D at 5 %		0.64	0.31	0.88	3.46

Conclusion

In the present investigation, it has induction of multiple shoot buds from callus mass of juice vesicles explants provided a novel protocol for in direct propagation of *Citrus mandarin* in tissue culture. It resulted in the regeneration of a large number of plantlets from single explants. This technique, therefore, is an efficient system for germplasm conservation and mass multiplication of this important fruit plant, as compared to propagation by seed.

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تأسيس نظام الاكثار الغير مباشر لليوسفي (*Citrus reticulate* L.) باستخدام زراعة الانسجة

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تهدف هذه الدراسة إلى تطوير بروتكول الاكثار الغير مباشر لليوسفي من خلال تحفيز انتاج الكالس باستخدام الفلقات والاكياس العصيرية كمنفصل نباتي. وكذلك تأسيس نظام لتحفيز انتاج الكالس باستخدام المنفصل النباتي الأوراق والفلقات وكذلك العقل الساقية الناتج من انبات البذرة معمليا. وتم تحفيز عالي للكالس بزراعة المنفصل النباتي للأوراق والفلقات وكذلك العقل الساقية على بيئة مواراشيج وسكوج المحتوية على ٢ او ٣ ملجم/لتر ٢و٤- ثنائي فينوكسس حامض الخليك. واوضحت الدراسة اعلي نسبة مئوية في تكوين الكالس تم الحصول عليها عندما زرعت الأجزاء النباتية من الفلقات وكذلك العقل الساقية على بيئة مواراشيج وسكوج المحتوية علي ٢ ملجم/لتر ٢و٤- ثنائي فينوكسس حامض الخليك. اعطت بيئة مواراشيج وسكوج المحتوية على الاضافات الفعالة ٣٠٠ ملجم/لتر جلوتامين او ١.٥ ملجم/لتر كازين هيدروليزد لتشجيع تكوين الكالس من الكياس العصيرية ٤.٢٢ و٤.٢٣/ جم لوزن الكالس الطازج وكانت البيئة مناسبة وعالية الاستجابة ١٠٠ ٪. تم الحصول على أكبر معدل للوزن الطازج للكالس ٧.٩٦ جم واستجابة عالية ١٠٠ ٪ من الاكياس العصيرية المنزرعة على بيئة مواراشيج وسكوج المحتوية علي٢ ملجم/لتر٢و٤ ثنائي فينوكسس حامض الخلييك و١٠ ٪ ماء جوز الهند. وتم تشجيع زيادة تضاعف الكالس من زراعة الأكياس العصيرية على بيئة مواراشيج وسكوج المحتوية على ٢ملجم/لتر ٢و٤ ثنائي فينوكس حامض الخلييك و٣٠٠ او٤٠٠ ملجم/لتر مستخلص الشعير. ولقد تم الحصول اعلي نسبة ٩٦.٦٧ ٪ لإنتاج النبيتات بالطريق الغير مباشر من الكالس الناتج من الأكياس العصيرية مع ٠.٥ ملجم/لتر كاينتين و ٤٠٠ ملجم/لتر مستخلص الشعير و٢ملجم/لتر نفثالين حامض الخلييك. سجلت أفضل النتائج للكالس الناتج من الأكياس العصيرية اعلي عدد للنبيتات وأعلي طول للنبيتات عندما زرعت على بيئة موارشيج وسكوج المحتوية علي ٢ملجم/لتر بينزيل ادينين و٠.٤ ملجم/لتر نفثالين حامض الخلييك. وتم تجذير اللنبيتات المتكونة على بيئة مواراشيج وسكوج المضاف اليها تركيزات مختلفة من نفثالين حامض الخلييك واعطت اعلي استجابة ٩٨ ٪ باستخدام ٢ملجم/لتر نفثالين حامض الخلييك و زيادة عدد للجذور وطول الجذور وكذلك عدد الاوراق. وتمت اقلمة النباتات الناتجة من مرحلة التجذير بنسبة نجاح٨٧ ٪. ونمت هذه النباتات طبيعيا دون ملاحظة أي تغير او اختلافات في الشكل الخارجي لهذه النباتات. ويمكن استخدام هذا البرتوكول في انتاج الأجنة الجسدية باعداد كبيرة من الأكياس العصيرية لليوسفي.