Pak. J. Bot., 50(3): 1203-1210, 2018.

ESTABLISHMENT OF AN EFFICIENT PROTOCOL FOR PLANTLETS REGENERATION VIA DIRECT AND INDIRECT ORGANOGENESIS IN *CITRUS RETICULATE* BLANCO (KINNOW MANDARIN)

MUBASHIR HUSSAIN[1], NAVEED IQBAL RAJA[1], HAMID RASHID[2]*, ZIA-UR-REHMAN MASHWANI, ASIM MEHMOOD[2] AND MUHAMMAD IQBAL[1]

[1]*Department of Botany, PMAS Arid Agriculture University Rawalpindi, Pakistan*
[2]*Department of Bio Sciences, COMSATS Institute of Information Technology, Sahiwal, Pakistan*
[*]*Corresponding author's: email: hamidrashid@ciitsahiwal.edu.pk*

Abstract

Studies were initiated to explore the role of plant growth regulators and explant types on efficient plantlets regeneration via direct and indirect organogenesis in Kinnow mandarin [*Citrus reticulata* L. (Blanco)]. Explants were cultured on MS medium containing varying concentrations of phytohormones. The best callus induction response was obtained in MS medium containing 5 mg/l 2,4-D and 1 mg/l BAP where 90% from nucellus tissue, 58% from shoot apical meristem and 56% from nodal segments explants showed callogenic response after 2 weeks of inoculation.Best shoot induction medium via indirect organogenesis was found for nucellus tissue when MS medium was supplemented with 1.5 mg/l Kin and 500 mg/l malt extract which was 46% whereas for SAM and nodal segments, best response was obtained when MS medium was supplemented with BAP and NAA (3.0 + 0.5) mg/l which was 40% and 48% from SAM and nodal segments respectively after 11 weeks of inoculation.The shoot induction as well as multiplication were also studied by using different combinations and different concentrations of phytohormones in growth medium. The best medium for shoot induction percentage via direct organogenesis was [$M_{3=}$ MS + BAP (1.0 mg/l)] whereas 92%, 90% and 82% from nucellus tissue, SAM and nodal segments respectively showed organogenic response after 4 weeks of inoculation, whereas maximum number of shoots per explants was obtained in medium (M_9)containing MS + Kin where 4, 7 and 6 average number of shoots were obtained from nucellus tissue,SAM and nodal segments respectively after 11 weeks of inoculation.Best rooting medium was found when MS medium was supplemented with 2 mg/l NAA where 88% from nucellus tissue and 80% from the nodal segments showed rooting response whereas for SAM best rooting response was obtained when supplemented with 1.5 mg/l NAA i.e 78%. The plantlets were successfully acclimatized in different potting mixtures and highest survival rate (100%) was achieved in potting mixture containing sand and peat moss (2:1).

Key words: Tissue culture, MS medium, Callogenesis, Callus regeneration, Direct regeneration.

Introduction

Citrus is economically one of the most important fruit crop all over the world including Pakistan. The genus citrus belongs to family *Rutaceae* and is the most extensively cultivated fruit crop all over the world. The *citrus* production of the world was relatively invariable during last decade of 20th century. *Citrus* origin is supposed to be southern slope of Himalayan region and northFinally Check Finally Check-eastern area of China and neighboring India (Gmitter*et al.,* 1990). Pakistan is included in the ten major producers of *citrus* all over the world. *Citrus* is grown in Pakistan on area of 193,212 hectares and is increasing 5% annually. Pakistan contributes 95% of the total Kinnow mandarin and supplied to all over the world (Sharif & Waqar, 2005). Punjab province is considered as the main hub of *citrus* fruit crop and provides citrus of high excellence to all over the world. In Pakistan 86% of the *citrus* grown is Kinnow mandarin (*Citrus reticulate* Blanco) followed by Musambi, Feutral and Blood red in proportion of 10%, 4% and 1% respectively.

Kinnow is a hybrid of two varieties, namely *Citrus deliciosa* and *Citrus nobilis*. The export of Kinnow mandarin can be increased by introducing seedless variety. There is a high demand of seedless varieties in the international market and Pakistan's Kinnow mandarin has about 22-26 seeds per fruit. There is a report regarding seedless varieties in Pakistan (Khalil *et al.,* 2011). Propagation of Kinnow mandarin is not very fast through traditional methods of propagation. To cope up with this problem, it is essential that Kinnow mandarin can be propagated through tissue culture methods such as direct and indirect organogenesis so that numerous numbers of plants can be produced in a limited period.

Gene transfer, selection and regeneration of transformantsis now a day & employed by the plant tissue culture techniques (Shah *et al.,* 2009). According to Helal (2011) tissue culture can be employed as substitute to conservative methods *In vitro* propagation with the purpose of increasing the developmental rate of preferred genotypes and commercial micropropagation. Tissue culture of *citrus* has attracted a considerable attention because of its commercial values and due to the important fruit crop of the world. *In vitro* regeneration of *citrus* cultivars has been discussed in various species of *citrus* mainly *Citrus reticulate* (Hassanein & Azooz, 2003); *Citrus sinensis* (Costa *et al*., 2004); *Citrus aurantium* L. (Bordon *et al*., 2000) and *Citrus grandis* (Huang *et al.,* 2002). Micropropagation protocols have been described in various citrus species and explant sources (Usman *et al.,* 2005; Khan *et al.,* 2009; Laskar *et al.,* 2009; Sharma *et al.,* 2009; Pe´rez-Tornero *et al.,* 2010, Hussain *et al.,* 2016).

The research work was carried out to explore the response of explants type and medium composition on callogenesis, frequency of plantlets regeneration and multiple shoot induction in *Citrus reticulata* L.

Materials and Methods

The present research work was carried out at Plant Tissue Culture Laboratory, Department of Botany, PMAS- Arid Agriculture University Rawalpindi, Pakistan during the year 2014-15.

Collection of explants: Different explants such as seeds, shoot apical meristem and nodal segments were collected from the healthy trees of Kinnow mandarin from the Bhalwal region of district Sargodha.

Preparation of explants: Immature fruits were washed thoroughly under running tap water to remove all the traces of dust and surface contaminants. Then, they were surface sterilized by immersion in 70% (w/v) ethanol for 5 minutes, then soaked for 10 minutes in 20% commercial Chlorax solution containing 1% NaOCl, and finally rinsed 3 times with sterile distilled water. Fruits were then cut under aseptic condition and immature seeds were separated. For callogenesis, indirect organogenesis and direct organogenesis, immature seeds were cut by scalpel and nucellus halves were separated. Afterwards, nucellus tissues were rinsed in sterile distilled water for 3 times, and then surface sterilized in 20% commercial chlorax solution, containing 1% NaOCl and a drop of tween 20 for 15 minutes. The nucellus tissues were then rinsed three times in sterile distilled water and cultured. Shoot apical meristem and nodal segments were trimmed to approximately size of 2 cm and leaves were detached. They were then brought to laminar air flow cabinet and surface sterilized with 70% ethanol. Afterwards, the explants were washed in 1% NaOCl for 20 minutes. Subsequently, these explants were sterilized in autoclaved distilled water and cultured.

Media preparation and culture conditions: All the *In vitro* work was carried out on MS medium (Murashige & Skoog, 1962). For the preparation of MS medium, exact quantities of all the components of medium were mixed. 30g sucrose was added and final volume of the medium (1 L) was made by the addition of double distilled water. The pH of the medium was adjusted between 5.7-5.8 by adding drops of 1N HCl and 1N NaOH. Medium was solidified with 0.8% agar. The MS medium was heated on hot plate to melt the agar. Finally, the medium was autoclaved at 15 psi for 15 minutes at 121°C. The medium was then poured into petri plates as well as in test tubes and kept in laminar hood. Cultures were then placed in growth chamber at 25°C with a photoperiod of 16 hours light. Light intensity was maintained upto 2500 lux.

Callus induction: All types of explant (Nucellus tissue, SAM and nodal segments) were cultured on MS medium supplemented with different concentrations of PGHs. Four different combinations (16 treatments) as shown in table 1 were tested to find the optimal medium for callus induction in all three explants. All cultures were incubated at 25±1℃ with a 16/8 hours photoperiod. Light intensity was maintained upto 2500 Lux.

Callus regeneration: The smooth compact calli obtained from all three explants were divided and cultured on MS medium containing different concentrations and combinations of phytohormones (Table 2) to check its response for regeneration.

Direct regeneration: MS medium was also supplemented with different concentrations of phytohormones for direct organogenesis. Fifteen different concentrations of three different combinations (Tables 3 & 4) were tested to explore the best medium for shoot induction and the number of shoots per explants respectively in all three types of explants.

Rooting of regenerated shoots: The shoots which were regenerated were separated and cultured on MS medium containing different concentrations (0.5, 1.0, 1.5, 2.0, 2.5 mg/l) of NAA and IBA as shown in table 5 to find the best medium for rooting.

Table 1. Effect of different medium compositions for callus induction in *Citrus reticulata* L. age of cultures: 4 weeks.

Treatment	Media	Conc. (mg/l)	No. of explants cultured	No. of test tubes showing callus induction		
				Nucellus tissue	SAM*	N.S*
C_1	MS + 2,4-D+ malt extract	2.0 + 500	10	6.0 ± 0.264^{bcd}	5.0 ± 0.250^{de}	3.0 ± 0.902^{f}
C_2		3.0 + 500	10	7.0 ± 0.110^{abc}	5.5 ± 0.208^{cd}	3.5 ± 0.513^{ef}
C_3		4.0 + 500	10	7.5 ± 0.310^{ab}	5.8 ± 0.262^{bcd}	4.5 ± 1.322^{def}
C_4		5.0 + 500	10	8.0 ± 0.525^{a}	6.0 ± 1.731^{bcd}	5.0 ± 1.732^{de}
C_5	MS+ 2,4-D + BAP	1.0 + 5.0	10	8.8 ± 0.984^{a}	5.0 ± 0.251^{bc}	4.4 ± 1.969^{cd}
C_6		2.0 + 3.0	10	8.0 ± 0.490^{a}	4.2 ± 1.058^{cd}	3.6 ± 0.692^{de}
C_7		3.0 + 2.0	10	6.2 ± 0.070^{b}	3.6 ± 0.610^{de}	2.7 ± 0.519^{e}
C_8		5.0 + 1.0	10	9.0 ± 0.624^{a}	5.8 ± 0.201^{bc}	5.6 ± 0.171^{bcd}
C_9	MS+ 2,4-D + Kin	1.0 + 0.5	10	6.0 ± 0.941^{bc}	3.8 ± 0.346^{cd}	3.2 ± 0.261^{f}
C_{10}		1.5 + 1.0	10	6.8 ± 0.241^{ab}	4.8 ± 0.721^{de}	3.6 ± 0.529^{f}
C_{11}		2.0 + 1.5	10	7.2 ± 0.722^{a}	5.6 ± 0.872^{cd}	5.5 ± 0.651^{cd}
C_{12}		3.0 + 2.0	10	5.0 ± 0.438^{d}	4.0 ± 0.380^{ef}	3.0 ± 0.556^{f}
C_{13}	MS + BAP + NAA	0.5 + 0.5	10	8.2 ± 0.180^{a}	4.4 ± 0.374^{cd}	3.8 ± 0.191^{d}
C_{14}		0.5 + 1.0	10	6.2 ± 0.330^{b}	3.0 ± 0.296^{e}	2.6 ± 0.377^{ef}
C_{15}		1.0 + 1.5	10	5.8 ± 0.485^{b}	2.8 ± 0.826^{ef}	2.2 ± 0.105^{f}
C_{16}		1.0 + 2.0	10	4.8 ± 0.341^{c}	1.5 ± 0.824^{c}	1.4 ± 0.145^{g}

SAM*: Shoot apical meristem, N.S*: Nodal segments

Mean followed by different letters in the same column differ significantly at p= 0.05 according to Duncan's new multiple range test

Table 2. Effect of different medium compositions on shoot induction percentage via indirect organogenesis in *Citrus reticulata* L. age of cultures: 4 weeks.

Treatment	Media	Conc. (mg/l)	No. of explants cultured	No. of test tubes showing shoot induction		
				Nucellus tissue	SAM*	N.S*
N_1	MS + BAP + malt extract	0.5+500	10	4.5 ± 0.231^{a}	2.0 ± 0.103^{e}	1.4 ± 0.590^{fg}
N_2		1.0+500	10	3.8 ± 0.530^{b}	1.8 ± 0.180^{ef}	1.2 ± 0.121^{fg}
N_3		1.5+500	10	3.2 ± 0.295^{c}	1.2 ± 0.305^{g}	1.0 ± 0.153^{g}
N_4		2.0+500	10	2.6 ± 0.550^{d}	1.0 ± 0.153^{g}	1.0 ± 0.230^{g}
N_5	MS + Kin + malt extract	0.5+500	10	4.0 ± 0.780^{a}	1.7 ± 0.710^{cd}	1.4 ± 0.210^{cd}
N_6		1.0+500	10	4.2 ± 0.395^{a}	1.8 ± 0.076^{c}	1.4 ± 0.551^{cd}
N_7		1.5+500	10	4.6 ± 0.290^{a}	2.0 ± 0.180^{c}	1.6 ± 0.480^{cde}
N_8		2.0+500	10	3.2 ± 0.214^{b}	1.2 ± 0.425^{d}	1.0 ± 0.152^{d}
N_9	MS + BAP+ NAA	1.0+0.5	10	3.4 ± 0.895^{bcde}	2.8 ± 0.361^{def}	2.4 ± 0.300^{ef}
N_{10}		2.0+0.5	10	4.0 ± 0.880^{abc}	3.6 ± 0.436^{bcd}	3.2 ± 0.410^{cdef}
N_{11}		3.0+0.5	10	4.4 ± 0.574^{ab}	4.0 ± 0.780^{abc}	4.8 ± 0.571^{a}
N_{12}		4.0+0.5	10	3.0 ± 1.002^{abc}	2.2 ± 0.121^{f}	3.4 ± 0.305^{bcde}

SAM*: Shoot apical meristem, N.S*: Nodal segments

Mean followed by different letters in the same column differ significantly at p= 0.05 according to Duncan's new multiple range test

Table 3. Effect of different medium compositions on shoot induction percentage via direct organogenesis in *Citrus reticulata* L. age of culture: 4 weeks.

Treatment	Media	Conc. (mg/l)	No. of explants cultured	No. of test tubes showing shoot induction		
				Nucellus tissue	SAM*	N.S*
M_1	MS + BAP	0.25	10	7.2 ± 0.229^{ef}	6.5 ± 0.264^{fg}	5.0 ± 0.790^{h}
M_2		0.5	10	8.0 ± 0.305^{cd}	7.8 ± 0.761^{cde}	6.0 ± 0.551^{g}
M_3		1.0	10	9.2 ± 0.173^{a}	9.0 ± 0.551^{ab}	8.2 ± 0.270^{cd}
M_4		1.5	10	8.6 ± 0.360^{abc}	8.4 ± 0.590^{bcd}	7.0 ± 0.712^{f}
M_5		2.0	10	8.0 ± 0.346^{cd}	7.8 ± 0.170^{de}	6.2 ± 0.076^{g}
M_6	MS + Kin	0.5	10	7.0 ± 0.210^{bcd}	6.2 ± 0.430^{def}	4.5 ± 0.390^{g}
M_7		0.75	10	7.2 ± 0.686^{bc}	6.4 ± 0.195^{cdef}	5.6 ± 0.215^{f}
M_8		1.0	10	7.8 ± 0.445^{ab}	7.0 ± 0.315^{bcd}	6.5 ± 0.641^{cde}
M_9		1.5	10	8.4 ± 0.750^{a}	7.8 ± 0.430^{ab}	6.0 ± 0.950^{ef}
M_{10}		2.0	10	7.8 ± 0.515^{ab}	7.4 ± 0.130^{b}	6.0 ± 0.485^{ef}
M_{11}	MS + BAP + NAA + malt extract	0.5+0.25+500	10	6.8 ± 0.361^{b}	5.2 ± 0.566^{cde}	4.0 ± 0.382^{fg}
M_{12}		1.0+0.25+500	10	7.4 ± 0.557^{ab}	5.8 ± 0.617^{c}	5.4 ± 0.220^{cd}
M_{13}		1.0+0.5+500	10	8.0 ± 0.621^{a}	7.2 ± 0.518^{b}	4.8 ± 0.600^{def}
M_{14}		1.5+0.5+500	10	7.0 ± 0.262^{b}	6.6 ± 0.484^{b}	4.4 ± 0.271^{efg}
M_{15}		2.0+0.5+500	10	5.6 ± 0.242^{c}	5.0 ± 0.808^{cde}	3.8 ± 0.121^{g}

SAM*: Shoot apical meristem, N.S*: Nodal segments

Mean followed by different letters in the same column differ significantly at p= 0.05 according to Duncan's new multiple range test

Table 4. Effect of different medium compositions on shoot number per explant via direct organogenesis in *Citrus reticulata* L. age of culture: 11 weeks.

Treatment	Media	Conc. (mg/l)	No. of explants cultured	No. of shoots per explant		
				Nucellus tissue	SAM*	N.S*
M_1	MS + BAP	0.25	10	2.0 ± 1.000^{bc}	3.0 ± 0.925^{b}	3.0 ± 0.091^{b}
M_2		0.5	10	3.0 ± 2.000^{b}	6.0 ± 0.902^{a}	5.0 ± 0.098^{a}
M_3		1.0	10	2.0 ± 0.057^{bc}	5.0 ± 1.000^{a}	3.0 ± 1.000^{b}
M_4		1.5	10	2.0 ± 0.125^{bc}	3.0 ± 2.000^{b}	2.0 ± 0.053^{bc}
M_5		2.0	10	2.0 ± 1.000^{bc}	2.0 ± 0.152^{bc}	1.0 ± 0.005^{c}
M_6	MS + Kin	0.5	10	2.0 ± 0.152^{c}	3.0 ± 0.060^{bc}	3.0 ± 0.925^{bc}
M_7		0.75	10	2.0 ± 0.503^{c}	3.0 ± 0.057^{bc}	4.0 ± 0.060^{b}
M_8		1.0	10	3.0 ± 0.901^{bc}	4.0 ± 0.153^{b}	3.0 ± 1.000^{bc}
M_9		1.5	10	4.0 ± 0.950^{b}	7.0 ± 0.951^{a}	6.0 ± 2.000^{a}
M_{10}		2.0	10	2.0 ± 0.951^{c}	4.0 ± 0.058^{b}	4.0 ± 0.058^{b}
M_{11}	MS + BAP + NAA + malt extract	0.5+0.25+500	10	2.0 ± 0.057^{d}	4.0 ± 0.115^{bcd}	3.0 ± 0.086^{cd}
M_{12}		1.0+0.25+500	10	3.0 ± 1.050^{cd}	5.0 ± 0.854^{abc}	4.0 ± 0.075^{bcd}
M_{13}		1.0+0.5+500	10	4.0 ± 0.058^{bcd}	7.0 ± 2.000^{a}	5.0 ± 1.000^{ab}
M_{14}		1.5+0.5+500	10	2.0 ± 1.000^{d}	5.0 ± 0.155^{abc}	5.0 ± 0.091^{abc}
M_{15}		2.0+0.5+500	10	2.0 ± 0.152^{d}	4.0 ± 0.058^{bc}	3.0 ± 0.163^{cd}

SAM*: Shoot apical meristem, N.S*: Nodal segments

Mean followed by different letters in the same column differ significantly at p= 0.05 according to Duncan's new multiple range test

Table 5. Effect of different medium compositions on root induction percentage in *Citrus reticulata* L. age of culture: 4 weeks.

Treatment	Media	Conc. (mg/l)	No. of explants cultured	No. of test tubes showing root induction		
				Nucellus tissue	SAM*	N.S*
R_1	MS + NAA	0.5	10	6.6 ± 0.458^{de}	5.2 ± 0.215^{g}	5.8 ± 0.401^{fg}
R_2		1.0	10	6.8 ± 0.715^{de}	5.6 ± 0.296^{g}	6.4 ± 0.210^{ef}
R_3		1.5	10	8.2 ± 0.080^{ab}	7.8 ± 0.247^{bc}	7.0 ± 0.952^{de}
R_4		2.0	10	8.8 ± 0.550^{a}	6.4 ± 0.410^{ef}	8.0 ± 0.458^{b}
R_5		2.5	10	8.5 ± 0.371^{ab}	7.2 ± 0.095^{cd}	6.5 ± 0.315^{def}
R_6	MS + IBA	0.5	10	6.0 ± 0.414^{ef}	5.0 ± 0.264^{h}	5.2 ± 0.351^{gh}
R_7		1.0	10	6.2 ± 0.195^{de}	5.4 ± 0.195^{fgh}	5.8 ± 0.180^{efg}
R_8		1.5	10	8.0 ± 0.980^{a}	5.8 ± 0.305^{efg}	7.8 ± 0.095^{ab}
R_9		2.0	10	7.2 ± 0.231^{abc}	6.2 ± 0.200^{de}	6.9 ± 0.390^{cd}
R_{10}		2.5	10	7.0 ± 0.950^{bc}	5.0 ± 0.702^{gh}	5.4 ± 0.451^{efgh}

SAM*: Shoot apical meristem, N.S*: Nodal segments

Table 6. Effect of different potting mixtures on the survival of plantlets of *Citrus reticulata* L.

Sr. No.	Treatments	Frequency of acclimatization of plantlets in pots	Age of plantlets (weeks)
1.	Soil + Sand (1:1)	80	12
2.	Sand + Peat moss (2:1)	100	12
3.	Sand + Soil + Peat moss (1:1:1)	85	12
4.	Cowdung + Soil + Perlite (1:1:1)	96	12

Acclimatization: The plantlets with good root and shoot system were successfully acclimatized in different types of potting mixtures. Plantlets were removed from the culture jars and cleaned with sterile distilled water to remove all the remains of growth medium from the roots. The plantlets were then planted in plastic pots filled with different combinations (Sand + Soil (1:1), Sand + Peat moss (2:1), sand + soil + Peat moss (1:1:1) and cowdung + soil + perlite (1:1:1) hardening mixture. The plantlets were then covered with plastic sheets for 15 days and then cover was removed gradually. The acclimatization process was continued for almost 12 weeks.

Statistical analysis:

All the experiments were laid out in completely randomized design arrangements of treatments. All treatments were replicated three times with ten explants per treatments. To detect the significant difference between means, the collected data was subjected to analysis of variance. Statistical analysis was done using STATISTICS 8.1 and Microsoft excel 2007 software.

Results and Discussions

Effect of different medium compositions on callus induction in Kinnow mandarin: It is evident from the (Fig. 1) that Kinnow mandarin can be *In vitro* propagated via indirect as well as direct organogenesis. Table 1 shows that MS medium containing different concentrations of PGHs to find the best medium for callus induction. MS medium was supplemented with different concentrations of 2,4-D and malt extract and it was found that when MS medium was supplemented with 5 mg/l 2,4-D and 500 mg/l malt extract, the best response for callus induction was obtained which was 80%, 60% and 50% in nucellus tissue, SAM and nodal segments explants respectively. Findings of Kenia *et al*., (2006) reaffirm our results who reported that 2,4-D is indispensable for callus initiation and proliferation. Similarly, Sani & Mustapha (2010) also reported 2,4-D as best phytohormone for callus induction. It was found that Malt extract was an essential substance for callus initiation with asexual embryogenic potential (Tisseret & Murashige, 1977). Similar types of results were reported in "Ponkan" mandarin (Chen *et al.,* 1990) as well as in other citrus cultivars (Pimental & Villegas, 1993). Therefore, it is considered that malt extract is necessary to add in all citruscallus induction media.

Among different concentrations of 2,4-D and BAP, best results were obtained at 5 mg/l 2,4-D and 1 mg/l BAP i.e., 90% in nucellus tissue, 58% in SAM and 56% in nodal segments. Our observations are also in line with Kazmi *et al.,* (2015) who reported similar findings in an attempt to find the effect of various phytohormones for callus induction. For callogenesis, MS medium was also supplemented with varying conc. of 2,4-D and Kinetin and C_{11} medium was found to be best regardless to the type of explants. Similar findings were obtained by Al-Taha *et al.,* (2012) who reported successful embryogeniccalli in *Citrus sinensis*. BAP in combination with NAA was also tried for callus induction and best results for callus induction was obtained in MS medium containing 0.5 mg/l BAP and 0.5 mg/l NAA. Further increase in concentration of either BAP or NAA, the rate of callus induction was decreased in all three types of explants. Singh *et al.,* (2013) also reported that concentration of NAA at 0.5 mg/l and 0.5 mg/l BAP was most effective in callus initiation in *Citrus jambhiri*, thus confirming our results.

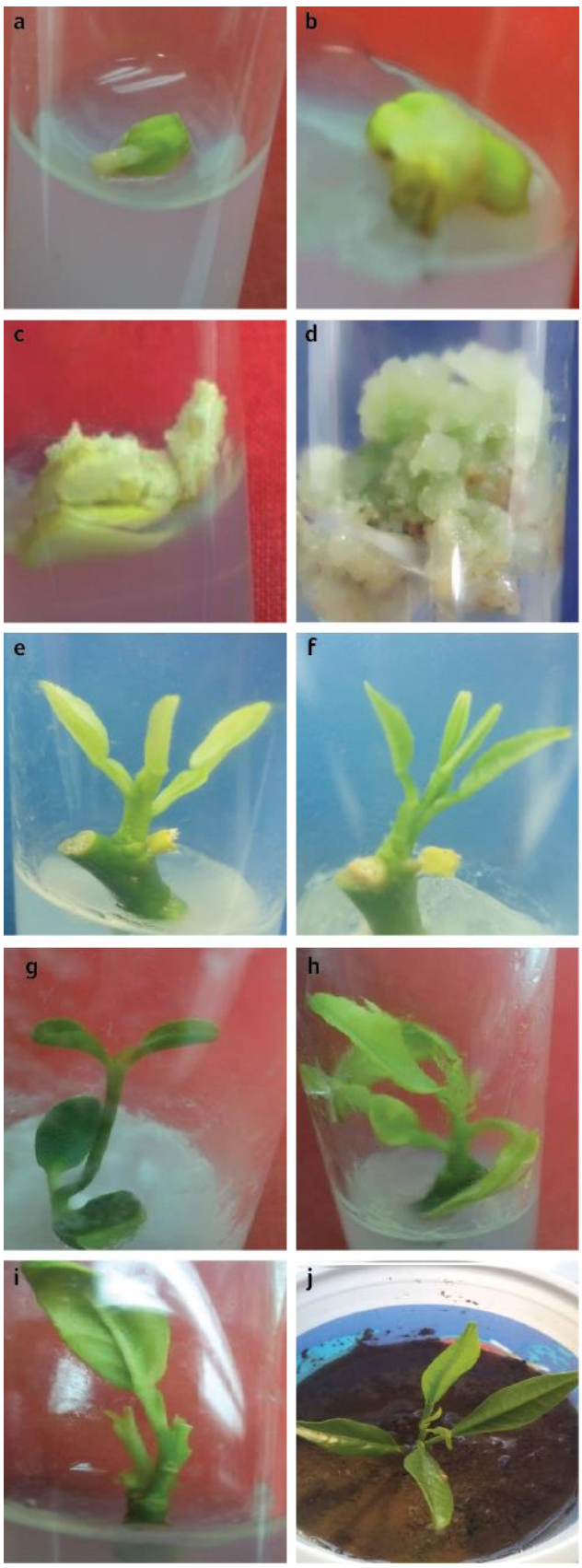

Fig. 1. Time line of *In vitro* regeneration ofKinnow mandarin via direct and indirect organogenesis. **a,** Nodal segment callus; **b,** SAM callus; **c,** Nucellus callus; **d,** Indirect regeneration; **e & f,** Direct regeneration from nodal segments; **g,** Direct regeneration from nucellus tissue; **h & i,** Direct regeneration from SAM; **j,** Plantlets acclimatization.

Effect of different medium compositions on indirect regeneration: Table 2 shows that MS medium was supplemented with different concentrations of three different combinations of phytohormones to find the optimal medium for regeneration from the calli obtained from all three types of explants. MS medium was supplemented with BAP and malt extract and it was found that 0.5 mg/l BAP and 500 mg/l malt extract was most suitable for indirect regeneration. Comparable results were obtained by other scientists in their research work on pummelo and mandarin (Begum *et al.,* 2003; Sarma *et al.,* 2011). Kinetin in combination with malt extract was also tested and it was found that N_7 medium (1.5 mg/l Kin + 500 mg/l malt extract) showed best response. At this concentration the rate of shoot induction was 46%, 20% and 16% from the calli obtained from nucellus tissue, SAM and nodal segments respectively. Some studies have shown that use of BAP alone is the better treatment for shoot regeneration in different *Citrus* spp. (Raman *et al.,* 1992; Costa *etal.,* 2002). There are some reports which indicate that Kin is better PGH for shoot regeneration (Rahaman *et al.,* 1996). For indirect organogenesis, MS medium was also supplemented with BAP and NAA and best results were obtained at 3 mg/l BAP and 0.5 mg/l NAA i.e., 44%, 40% and 48% in calli obtained from nucellus tissue, SAM and nodal segments explants respectively. Inclusion of auxin in the medium has been found to be beneficial for shoot induction in many cases (Chaturvedi & Mitra, 1974; Bhansali & Arya, 1978). The findings of Negpal *et al.,* (2010) reaffirm our finding who also report best callus regeneration response (71%) with nodal segments derived calli cultured on MS medium containing 3 mg/l BAP and 0.5 mg/l NAA.

Effect of different medium compositions on shoot induction percentage and number of shoots per explant via direct organogenesis in Kinnow mandarin: It was shown in table 3 that MS medium was supplemented with varying concentrations of different combinations to explore the best medium for shoot induction via direct organogenesis. Data presented in table 3 showed that by increasing or decreasing the concentration of BAP beyond 1 mg/l, declined in the shoot induction percentage. At this concentration, shoot induction percentage was 92% in nucellus tissue, 90% in SAM and 82% in nodal segments explants. Similar results were obtained by Mukhtar *et al.,* (2005) who reported that 1 mg/l BAP was most effective in shoot induction percentage by using shoot apical meristem and nodal segments. The results are also in confirmation with the results of Kim *et al.,* (2002) and Vestri *et al.,* (2003) that varying the concentrations of different phytohormones appreciably influences the shoot formation percentage in citrus species. BAP at different concentration has been the most commonly used for shoot induction percentage and multiplication of citrus cultivars (Carimi *et al.,* 2003). BAP is more effective for regeneration percentage as compared to Kinetin described by various scientists (Moreira-Dias *et al.,* 2001; Almeida *et al.,* 2002; Silva *et al.,* 2005 and Germana *et al.,* 2011).

Different concentrations of Kinetin were also supplemented in MS medium for shoot induction and better results for shoot induction was obtained at the concentration of 1.5 mg/l. Mukhtar *et al.,* (2005) also reported that 1.5 mg/l was most efficient for regeneration percentage. Hassanein & Azooz (2003) demonstrated that Kinetin was effective for regeneration of *Citrus reticulata* via *In vitro* seed germination as well as for shoot cuttings. Shoot formation percentage was less in presence of Kinetin as compared to BAP (Molina *et al.,* 2007). When MS medium with various concentrations of BAP + NAA+ Malt extract was used, the concentration 1 mg/l BAP + 0.5 mg/l NAA and 500 mg/l Malt extract showed 80%, 72% and 48% shoot induction from nucellus tissue, SAM and nodal segment explants used. Supplementation of the MS medium with a cytokinins and auxins combination has produced the contrasting result regarding the organogenesis of citrus. While some scientists reported that NAA in combination with BAP was fundamental for callus initiation and bud formation (Bordon *et al.,* 2000; Ghorbel *et al.,* 2000; Almeida *et al.,* 2003).

Data presented in (Table 4) showed that MS medium supplemented with varying concentrations of different combinations were used to check the response for number of shoots per explant. Data found in table 4 showed that further increase or decrease in concentration of BAP beyond 0.5 mg/l did not show good results for shoots number per explants. Our findings were in agreement with that of Mukhtar *et al.,* (2005) who obtained highest number of shoots per explants by using shoot apical meristem followed by nodal segments. Al-Khayri & Al-Bahray (2001) also obtained similar types of results. MS medium containing different concentrations of Kinetin were also tested and better response for shoots number per explants was obtained at 1.5 mg/l. At this conc., shoots number per explants was 4, 7 and 6 in nucellus tissue, SAM and nodal segments explants. The findings of Mukhtar *et al.,* (2005) reaffirmed the present study who successfully obtained maximum shoots number per explants using shoot apical meristem and nodal segments at 1.5 mg/l concentration of Kinetin. Different concentrations of BAP, NAA and malt extract were also tested for shoot number per explants. Best result in this combination was obtained in M_{13} medium. At this shoot number per explants was 4, 7 and 5 in nucellus tissue, SAM and nodal segment explants respectively. Our results did agree with Haripyaree *et al.,* (2011) who reported the maximum number of shoots per explant when MS medium was supplemented with BAP at 0.25 mg/l and NAA at 0.5 mg/l.

Effect of different medium compositions on root induction percentage in Kinnow mandarin: MS medium was also supplemented with different concentrations of auxins such as NAA and IBA to explore the best medium for root induction (Table 5). Data presented in (Table 5) showed that by increasing or decreasing the concentration of NAA beyond 2 mg/l declined in the root induction. At this concentration, rate of root induction was 88% in nucellus tissue, 64% in SAM and 80% in nodal segments explants. While for SAM, best results for root induction (78%) was obtained when MS medium was supplemented with 1.5 mg/l NAA. These results are in conformity with the Kim (2002), who noted that basal media containing 1.5 mg/l NAA was found to be most suitable for *In vitro* root initiation in mandarin. Similarly, Cheong *et al.,* (2003) also got the same result that by increasing level of various auxins such as NAA and IBA in the media. For rooting MS medium was also supplemented with varying concentrations of IBA and it was found that 1.5 mg/l concentration of IBA showed best results for root induction i.e., 80%, 58% and 78% in nucellus tissue, SAM and nodal segments respectively. Haripyaree *et al.,* (2011) reaffirmed the present study who successfully achieved root induction when MS medium was supplemented with IBA at 1.5 mg/l. Ling *et al.,* (2002) and Chandra *et al.,* (2003) observed good root formation percentage when supplemented with auxins.

Effect of different potting mixtures on the survival of plantlets in Kinnow mandarin: *In vitro* rooted explants of *Citrus reticulate* were removed from the rooting medium and detached from the adhering gel (Table 6). Then they were shifted into plastic pots in 4 different types of potting mixtures. The plants were then covered with polypropylene sheets. The polypropylene sheets were slowly removed after a period of almost 2 weeks. The process of acclimatization was continued for almost 3 months. The highest survival rate was achieved (100%) in potting mixture containing sand and peat moss (2:1) followed by the soil, perlite and cowdung (1:1:1). The percentage of success during acclimatization was 85% by using sand, peat moss and soil (1:1:1).The findings of Al-Taha *et al.,* (2012) also supported our results who reported that plantlets which were obtained and transferred to potting mixture containing sand and peat moss and maximum survival rate (100%) was achieved.

Conclusion

The results of present investigation standardize the protocols for callogenesis, direct and indirect organogenesis ultimately leading to efficient plantlets regeneration from nucellus tissue, SAM and nodal segments in *Citrus reticulata* L. In the present study, we established regeneration protocols for *Citrus reticulata* that could be used in future to transgene and enhance the commercial values of *Citrus reticulata* L. The techniques established in present study can also be tried for other fruit crops which are difficult to propagate through conventional methods.

References

Al-Khayri, J.M. and A.M. Al-Bahray. 2001. *In vitro* micropropagation of *Citrus aurantifolia* (Lime). *Curr. Sci.*, 81: 1242-5.

Almeida, W.A.B., F.A.A. Mourao Filho, L.E. Pino, R.L. Boscariol, A.P.M. Rodriguez and B.M.J. Mendes. 2003.

Genetic transformation and plant recovery from mature tissues of *Citrus sinensis* L. Osbeck. *Plant Sci.,* 164: 203-211.

Al-Taha, H.A.K., M.J. Abbas and F.A. Muayed. 2012. Somatic embryogenesis and plantlet regeneration from nucleus tissues of Local orange (*Citrus sinensis* (L.) Osbeck). *Actaagricult. Sloven.,* 99(2): 185-189.

Begum, F., M.N. Amin, S. Islam, M.A.K. Azadand and M.M. Rehman. 2003. *In vitro* plant regeneration from cotyledon-derived callus of three varieties pummelo (*Citrus grandis* (L.) Osbeck). *Onl. J. Bio. Sci.,* 3(8): 751-759.

Bhansali, R.R. and H.C. Arya. 1978. Tissue culture propagation of citrus trees. International Citrus Congress (3rd: Sydney, Australia) *Int. Soc. Citri.,* 1: 135-140.

Bordon, Y., J.L. Guardiola and A. Garcia-Luis. 2000. Genotype affects the morphogenic response *In vitro* of epicotyls segments of *Citrus* rootstocks. *Ann. Bot.,* 86: 159-166.

Chandra, A., V. Gupta, P. Burma and D. Pental. 2003. Patterns of morphogenesis from cotyledon explants of Citron (*C. medica* L.). *In vitro Cell. Dev. Biol.*, 39: 514-519.

Chaturvedi, H.C. and G.C. Mitra. 1974. Clonal propagation of Citrus from somatic callus cultures. *Horti. Cult. Sci.,* 9: 118-120.

Chen, R.Z. and L.Y. Zhang. 1990. A preliminary study of the factors affecting embryogenesis and plantlets regeneration from nucellus tissues of *Citrus reticulata* cv. Ponkan. *Acta-Botanica Aust.,* 6: 75-80.

Cheong, E. and M.R. Pooler. 2003. Micropropagation of lemon (*C. limonia* L.) through axillary bud and induction of adventitious shoots from leaf pieces. *In vitro Cell.and Dev. Biol.*, 39: 455-8.

Costa, M.G.C., V.S. Alvesm, E.R.G. Lani, P.R. Mosquim, C.R. Carvalho and W.C. Otoni. 2004. Morphogenic gradients of adventitious bud and shoot regeneration in epicotyls explants of *Citrus*. *Sci. Hortic.,* 100: 63-74.

Costa, M.G.C., W.C. Otoni and G.A. Moor. 2002. An elevation of factors affecting the efficiency of *Agrobacterium*-mediated transformation of *Citrus paradise* (macf.) and production of transgenic plants containing carotenoid biosyntheticgenes. *Plant Cell Repro.,* 21: 365-373.

Germana, M.A., M. Micheli, B. Chiacone, L. Macaluso and A. Standardi. 2011. Organogenesis and encapsulation of *In vitro*-derived propagules of Carrizo citrange [*Citrus sinensis* (L.) Osb. *Ponciriustrifoliata* (L.) Raf]. *Plant Cell Tiss. Org. Cult.,* 106: 299-307.

Ghorbel, R., A. Dominguez, L. Navarro and L. Pena. 2000. High efficiency genetic transformation of sour orange (*Citrus aurantium*) and production of transgenic trees containing the coat protein gene of citrus tristeza virus. *Tree Physiol.,* 20: 1183-1189.

Gmitter, F.G. and X. Hu. 1990. The possible role of Yunnan, China, in the origin of contemporary citrus species (*Rutaceae*). *Econo. Bot.,* 44(2): 267-277.

Haripyaree, A., G. Kshetrimayum, S. Huidrom and D. Maibam. 2011. *In vitro* propagation of *Citrus megaloxycarpa. Env. and Exp. Bio.,* 9: 129-132.

Hassanein, A.M. and M.M. Azooz. 2003. Propagation of *Citrus reticulata* via *In vitro* seed germination and shoot cuttings. *Biol. Plant.,* 47(2): 173-177.

Helal, N.A.S. 2011. The green revolution via synthetic (artificial) seeds: *A Rev. Res. J. Agri. Biol. Sci.* 7(6): 464-477.

Huang, W.L. and L.F. Liu. 2002. Carbohydrate metabolism in rice during callus induction and shoot regeneration induced by osmotic stress. *Bot. Bull. Acad. Sin.,* 43: 107-113.

Kazmi, S.K., S. Khan, N. Kabir, A.A. Mirbahar, M. Raziq and N. Kauser. 2015. Embryogenic callus induction, somatic embryogenesis, regeneration and histological studies of Kinnow mandarin (*Citrus reticulate* Blanco L.) from nucellar embryo and epicotyl region. *Pak. J. Bot.*, 47(1): 305-310.

Kenia, T., G.A. Enriquez, C. Yanaysi, N. Soto and D.F. Alejandro. 2006. Development of a system for rapid plant regeneration from In vitro sugarcane (*Saccharum officinarum* L.) meristematic tissue. *Biotecnologia Aplicada*, 23: 22-24.

Khalil, S.A., A. Sattar and R. Zamir. 2011. Development of sparse-seeded mutant Kinnow (*Citrus reticulate* Blanco) through bud wood irradiation. *Afr. J. Biotechnol.,* 10(65): 14562-14565.

Kim, M.H., H. Lee, M.S. Chung and J. Joo. 2002. Factor affecting efficiency of shoot induction in *Citrus junos*. *M.Sc. Thesis*, Department of Animal Sciences and Biotechnology, Kyungpook National University, Daegu, Korea.

Laskar, M.A., M. Hynniewta and C.S. Rao. 2009. *In vitro* propagation of *Citrus indica* Tanaka- An endangered progenitor species. *Indian J. Biotech.,* 8: 311-316.

Ling, Y.X., A. Kitajima, K. Hasegawa and X.L. Yang. 2002. Callus induction and embryoid regeneration from the endosperm culture of 'Tosa–Buntan' pummelo [*C. grandis* (L.) Osb.]. *Env. Control Biol.,* 38: 241-6.

Molina, R.V., S. Castello, A. Garcia-Luis and J.L. Guardiola. 2007. Light cytokinin interactions in shoot formation in epicotyl cuttings of Troyer citrange cultured *In vitro*. *Plant Cell Tiss. Org. Cult.,* 89: 131-140.

Moreira-Dias, J., M.R.V. Molina, J.L. Guardiola and A. Garcia-Luis. 2001. Day length and photon flux density influence the growth regulator effects on morphogenesis in epicotyl segments of Troyer citrange. *Sci. Hortic.,* 87: 275-290.

Mukhtar, R., M.M. Khan, B. Fatima, M. Abbasi and A. Shahid. 2005. *In vitro* Regeneration and Multiple Shoots Induction in *Citrus reticulata* (Blanco).*Int. J. agri. and Biol.,* 7: 414-416.

Pe´rez-Tornero, O., O.C. Tallo´n and I. Porras. 2010. An efficient protocol for micropropagation of lemon (*Citrus limon*) from mature nodal segments. *Plant Cell Tiss. Org. Cult.,* 100: 263-271.

Pimental, R.B. and V.N. Villegas. 1993.Induction of somatic embryogenesis in selected *Citrus* species. *Philip. J. Crop Sci.,* 18.

Rahaman, A.A.S., V. Nagaraju and V.S. Parthasarthy. 1996. Response of embryoids of certain *Citrus* species to two cytokinins. *Ann. of Plant Physiol.,* 10: 45-49.

Raman, H., S.S. Gosal and D.S. Brar. 1992. Plant regeneration from callus cultures of *Citrus limon* and *C. jambhiri*. *Crop Improve*, 19: 100-103.

Sani, L.A. and Y. Mustapha. 2010. Effect of genotype and 2,4-D concentration on callogenesis in sugarcane (*Saccharum* spp. Hybrids). *Bayero J. Pure Appl. Sci.,* 3(1): 238-240.

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 Bio. Res.,* 2(6): 341-348.

Shah, A.H., N. Rashid, M.S. Haider, F. Saleem, M. Tahir and J. Iqbal. 2009. An efficient, short and cost-effective regeneration system for transformation studies of sugarcane (*Saccharum officinarum* L.). *Pak. J. Bot.,* 41(2): 609-614.

Sharif, M. and Akhtar. 2005. "Fruit Exports Prospects in Pakistan" DOC, *www.parc.gov.pk/data/SSD-Database/.2005*.

Sharma, S., A. Prakash and A. Tele. 2009. *In vitro* propagation of *Citrus* rootstocks. Notulae Botanicae Horti. *Agrobotanici.*, 37: 84-88.

Silva, R.P., A.J. Souza, B.M.J. Mendes and F.A.A. Mourao-Filho. 2005. Sour orange bud regeneration and *In vitro* plant development related to culture medium composition and explant type. *Rev. Bras. Frutic.*, 32: 1-8.

Singh, B., S. Sharma, G. Rani, G.S. Virk, A.A. Zaidi and A. Nagpal. 2013. *In vitro* flowering in embryogenic cultures of Kinnow mandarin (*Citrus nobilis* Lourx *C. deliciosa* Tenora). *Glob. J. Agri. Res.*, 1(1): 012-015.

Tisseret, B. and T. Murashige. 1977. Probable identity of substances in Citrus that repress asexual embryogenesis. *In vitro*, 13: 785-789.

Usman, M., S. Muhammad and B. Fatima. 2005. *In vitro* multiple shoot induction from nodal explants of *Citrus* cultivars. *J. Central Europ. Agri.*, 6: 435-442.

Vestri, F., S. Schiff and A. Bennici. 2003. *In vitro* shoot regeneration in rough lemon (*C. jambheri* Lush). *In vitro Cell. and Dev. Biol.*, 39: 586-94.

(Received for publication 12 July 2017)