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In Vitro Propagation of Citrus Rootstocks

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Abstract

Present investigation was conducted to standardize a protocol for *in-vitro* propagation of citrus rootstocks viz. Rough lemon, Cleopatra mandarin Pectinifera and Troyer citrange. The shoot tip explant was found better for callus induction of these rootstocks than the nodal segment. Maximum callus formation (40.0% and 23.3%) of shoot tip explants was obtained in Cleopatra mandarin, Pectinifera, and Rough lemon and Troyer citrange, respectively in treatment MS basal media + 0.5mg/l Kin, 2.0mg/l NAA, and 2.0mg/l 2, 4-D. Furthermore, the maximum number of shoots per explant was obtained through the callus in Pectinifera, Rough lemon and Cleopatra mandarin in MS basal media + BAP 1mg/l. Maximum rooting of shoots (1.11%) was noted in rootstock Rough lemon followed by Cleopatra mandarin for the ½ MS media supplemented with 10mg/l IBA. Although the callus development and bud proliferation was recorded in rootstock Troyer citrange however, shoot and root formation did not occur. The potting media consisting of soil, sand and FYM in the ratio of 1:1:1 by volume was better with maximum survival rate of hardened plants six weeks after transferring to the pots under greenhouse for Rough lemon followed by Pectinifera and Cleopatra mandarin rootstock.

Keywords: *In-vitro* multiplication, citrus rootstocks, pectinifera, troyer citrange, rough lemon, Cleopatra mandarin

Introduction

Usefulness of citrus rootstocks for the improvement of canopy architecture, fruit production, quality and tolerance to biotic and a-biotic stresses of citrus crops is well known. However these rootstocks, propagated by growing open-pollinated seeds, are highly nucellar and produce true to type plants. Depending upon the rootstocks, generally 1 to 40% zygotic seedlings are produced, which must be culled from seed beds to maintain clonal uniformity. All citrus cultivar selections are usually grafted to selected rootstock seedlings. Some potentially valuable rootstocks produce few or no seed and thus seed shortage of such popular rootstock occurs periodically. Further, the demand of quality planting material of important rootstocks in ample number necessitates for their *in vitro* propagation.

During the past years, micro-propagation techniques have been widely used for several plant species. Also, the plant regeneration in citrus species has been reported by various workers (Singh *et al*., 1994; Parthasarathy *et al*, 2001). Tissue culture technique could be used for propagation of citrus rootstocks and thus, the number of plants produced would not be limited by their seed supply, rather more uniform disease free and quality plant populations might be produced. In spite of micro-propagation of citrus genotypes reported by several workers, a very few reports of *in vitro* multiplication of citrus rootstocks Pectinifera, Troyer citrange, Cleopatra mandarin and Rough lemon are available in the literature (Chaturvedi and Mitra, 1974). In view of this, the present study was undertaken with the objectives to develop protocol for callus induction and regeneration in the above mentioned rootstocks, and to make their utility more effective in orchard establishment of citrus crop.

Materials and methods

The explants viz. shoot tips and nodal segments were collected from Pectinifera (*Citrus depressa* Hayata), Troyer citrange (*Poncirus trifoliata* (L.) Raf. x *Citrus sinensis* Osbeck), Rough lemon (*Citrus jambhiri* Lush.) and Cleopatra mandarin (*Citrus reshni* Hort. ex. Tan.) rootstocks growing at Experimental Orchard of the Department of Horticulture, CCS HAU, Hisar. The surface sterilization of explants was done with 70% aqueous solution of ethanol (v/v) for 30 seconds followed by 0.1% HgCl2 (w/v) for 8-10 minutes and then washed properly with sterile distilled water under aseptic condition.

In order to induct callus as well as for the regeneration of plants, the explants were cultured on MS basal medium (1962) supplemented with different concentrations of phyto-hormones viz. BAP, kinetin, NAA, and 2, 4-D. Surface sterilized and thoroughly washed explants viz. shoot tips and nodal segments were cultured on MS medium containing different concentrations of Kinetin, NAA and 2, 4–D.

Explants were aseptically inoculated on culture medium under white light. Five explants were inoculated per flask and about 150 explants were kept in every set of experiment. All cultures were incubated at 25±20C in culture room under 16/8hrS cycles of light (200 Lux) and darkness. The proliferated shoots (3-4cm) were inoculated individually on MS, half MS media alone or supplemented with various concentration of IBA. The rooted plantlets

were washed thoroughly and transferred into small pots containing potting media prepared of soil, sand and FYM in the ratio of 1:1:1 by volume, and kept at 25±10C All the experiments were carried out following the factorial completely randomized design (Gomez and Gomez, 1984).

Results and discussion

Plant regeneration through indirect organogenesis

Maximum callus formation (40.0% and 23.3%, respectively) in treatment 0.5mg/l Kin, 2.0mg/l NAA and 2.0mg/l 2, 4-D was observed in all the rootstocks, which was significantly better than all other media treatments, followed by 26.67% in treatment 0.5mg/l Kin, 5.0mg/l NAA and 5.0mg/l 2, 4-D in Cleopatra mandarin and Pectinifera, and 16.7% and 23.3% in treatment 0.5mg/l Kin and 5.0mg/l NAA in Rough lemon and Troyer citrange, and Cleopatra mandarin and Pectinifera, respectively (Tab. 1). The maximum per cent callus formation was obtained in shoot tip explants, which was significantly higher than the nodal segment explants for all the rootstocks. The interaction between media and explants was not significant.

This study revealed that among various explants used, the per cent callus formation was found satisfactory only in shoot tip explant in all the rootstocks. The difference in per cent callus formation between rootstocks of citrus might be due to the plant genotype. The results are in conformity with various earlier authors (Chaturvedi and Mitra, 1974; Goyal and Arya, 1981). The maximum per cent callus formation in shoot tip explants was obtained in MS basal + 0.5mg/l Kin, 2.0mg/l NAA and 2.0mg/l 2, 4-D treatment in all the rootstocks. The ratio of auxin and cytokinin concentrations in culture media is known to play a major role in establishment of callus cultures in several citrus species (Chaturvedi and Mitra, 1974).

It can be inferred from the data in Table-2 that shoot tip and nodal explants of rootstocks Pectinifera, Cleopatra mandarin and Rough lemon of citrus produced more (2.61, 2.11 and 1.92, respectively) number of shoots through callus in 1.0mg/l BAP treatment, better than all other treat-

Tab. 1. Effect of phytohormones on per cent callus formation in explants of citrus rootstocks

Treatment (mg/l)			Rough lemon			Cleopatra mandarin			Pectinifera			Troyer citrange		
Kin	NAA	2,4-D	A	B	Mean	A	B	Mean	A	B	Mean	A	B	Mean
-	-	-	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
-	2.0	-	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
-	5.0	-	13.3	6.67	10.0	20.0	13.3	16.6	20.0	13.3	16.7	13.3	6.7	10.0
0.5	2.0	-	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.5	5.0	-	20.0	13.3	16.7	33.3	13.3	23.3	26.7	20.0	23.3	20.0	13.3	16.7
-	-	2.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
-	-	5.0	6.7	6.7	6.7	20.0	13.3	16.7	13.3	6.7	10.0	6.7	6.7	6.7
0.5	-	2.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.5	-	5.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.5	2.0	2.0	26.6	20.0	23.3	46.5	33.3	40.0	46.5	33.3	40.0	26.7	20.0	23.4
0.5	5.0	5.0	20.0	13.3	16.6	33.3	20.0	26.6	26.7	26.7	26.7	13.3	6.7	10.0
C.D (5%) Media: 2.41, Explants: 1.45, Media x Explants: NS														

A – Shoot tip, B – Nodal segment, Kin – Kinetin, NS – Non significant

Tab. 2. Effect of phytohormones on average number of shoots per explant obtained through the callus in shoot tip and nodal segment explants of Citrus rootstocks

Treatment (mg/l)		Rough lemon			Cleopatra mandarin			Pectinifera		
BAP	Kin	A	B	Mean	A	B	Mean	A	B	Mean
0.0	0.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.5	0.0	1.17	0.67	0.92	1.22	1.17	1.19	1.94	1.17	1.56
1.0	0.0	2.17	1.67	1.92	2.39	1.83	2.11	2.72	2.50	2.61
1.5	0.0	0.83	0.67	0.58	0.72	0.67	0.69	1.50	0.50	1.00
2.0	0.0	0.50	0.33	0.42	0.27	0.33	0.30	0.67	0.33	0.50
0.0	0.5	1.83	1.67	1.75	2.11	1.67	1.89	2.39	2.33	2.36
0.0	1.0	1.00	1.00	1.00	1.06	0.83	0.95	1.17	0.83	1.00
0.0	1.5	0.50	0.33	0.42	0.39	0.50	0.45	0.56	0.33	0.45
0.0	2.0	0.33	0.33	0.33	0.22	0.33	0.28	0.28	0.33	0.30
C.D (5%) Media: 0.24, Explants: , Media : x Explants: NS										

* No shoot formation was observed in rootstock Troyer citrange in all the treatments.

A – Shoot tip, B – Nodal segment, Kin – Kinetin, NS – Non significant

Tab. 3: Effect of phytohormones on per cent bud proliferation in explants of citrus rootstocks

Treatment (mg/l)		Rough lemon			Cleopatra mandarin			Pectinifera			Troyer citrange		
BAP	Kin	A	B	Mean	A	B	Mean	A	B	Mean	A	B	Mean
0.0	0.0	53.3	60.0	56.7	66.7	66.7	66.7	53.3	66.7	60.0	46.7	60.0	53.3
0.5	0.0	60.0	73.3	66.7	80.0	86.7	83.4	73.3	80.0	76.7	66.7	66.7	66.7
1.0	0.0	66.7	86.7	76.7	86.7	93.3	90.0	86.7	93.3	90.0	66.7	73.3	70.0
1.5	0.0	53.3	73.3	63.3	66.7	73.3	70.0	73.3	86.7	80.0	60.0	60.0	60.0
2.0	0.0	53.3	60.0	56.7	60.0	60.0	60.0	66.7	73.3	70.0	40.0	46.7	43.3
0.0	0.5	60.0	80.0	70.0	66.7	66.7	66.7	66.7	66.7	66.7	46.7	53.3	50.0
0.0	1.0	60.0	80.0	70.0	73.3	73.3	73.3	73.3	80.0	76.7	46.7	60.0	53.3
0.0	1.5	40.0	53.3	46.7	53.3	53.3	53.3	53.3	60.0	56.7	26.7	40.0	38.3
0.0	2.0	40.0	40.0	40.0	46.7	46.7	46.7	46.7	53.3	50.0	26.7	33.3	30.0
C.D (5%) Media: 3.59, Explant:2.40, Media x Explant:7.19													

A – Shoot tip, B – Nodal segment, Kin – Kinetin

ments and it was followed by2.36,1.89 and 1.75, respectively in treatment Kin 0.5mg/l. No shoot formation was observed in rootstock Troyer citrange in all the treatments. The minimum average number of shoots (0.28 in Cleopatra mandarin, 0.30 in Pectinifera and 0.33 in Rough lemon) was obtained through callus in treatment 2.0mg/l Kin. There was no culture response in MS basal media treatment without supplementation of growth hormones. Furthermore, significantly higher number of shoots was obtained through callus in rootstock Cleopatra mandarin. Thus it can be inferred that significantly higher number of shoots obtained through callus resulted in Pectinifera was superior to the rootstocks Rough lemon and Cleopatra mandarin. The difference in the number of shoots obtained through callus among the rootstocks might be due to the differences in their genetic makeup. Chaturvedi and Mitra, (1974) reported that isolated stem callus of Citrus *sinensis* also regenerated plantlets.

Plant regeneration through direct organogenesis

In citrus rootstocks Rough lemon, Cleopatra mandarin and Pectinifera the maximum bud proliferation (76.7 and 90.0%, respectively) was recorded in treatment BAP 1.0 mg/l and rated best among all the media treatments (Tab. 3). It was followed by rootstock Cleopatra mandarin (86.3%) in treatment BAP 0.5 mg/l, Pectinifera (80.0%) in treatment BAP 1.5 mg/l, and Rough lemon (70.0%) with Kin 0.5mg/l. The highest (86.67 and 93.33%, respectively) bud proliferation was obtained in nodal segment explants in Cleopatra mandarin and Pectinifera followed by Rough lemon (86.7%) and Troyer citrange (73.3%), which was better than explant shoot tip in all the rootstocks. The differences in per cent bud proliferation might be attributed to the genetic behavior of the rootstocks. Shoot regeneration (66-100%) from shoot tip and nodal stem segment of mature plants was reported in C. *mitis* Blanco (Sim *et al*., 1989).

It is clear from the data given in Tab. 4 that maximum number of multiple shoots per explant (2.10, 2.00 and 1.87, respectively) was recorded in rootstocks in the treatment MS +BAP 1.0mg/l in Pectinifera, Cleopatra mandarin and Rough lemon. This treatment was found superior

Tab. 4: Effect of phyto-hormones on number of multiple shoots per explant obtained through callus in shoot tip and nodal segment explants of citrus rootstocks

Treatment (mg/l)		Rough lemon			Cleopatra mandarin			Pectinifera		
BAP	Kin	A	B	Mean	A	B	Mean	A	B	Mean
0.0	0.0	0.00	0.60	0.3	0.00	0.87	0.44	0.00	1.00	0.5
0.5	0.0	0.60	1.60	1.1	0.80	2.00	1.40	0.87	2.00	1.44
1.0	0.0	1.53	2.20	1.87	1.60	2.40	2.00	1.60	2.60	2.10
1.5	0.0	0.87	1.60	1.24	1.00	2.00	1.50	1.20	2.07	1.64
2.0	0.0	0.47	0.87	0.67	0.60	1.00	0.80	0.73	1.40	1.60
0.0	0.5	0.00	1.20	0.60	0.00	1.20	0.60	0.00	1.20	0.60
0.0	1.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.0	1.5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.0	2.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C.D (5%) Media: 0.12, Rootstock: 0.08, Media x Rootstock: 0.23										

* No shoot formation was observed in rootstock Troyer citrange in all the treatments.

A – Shoot tip, B – Nodal segment, Kin – Kinetin

to all other treatments, and it was followed by (1.64, 1.50 and 1.24, respectively) in treatment MS + BAP 1.5mg/l in these rootstocks of citrus. Troyer citrange did not show multiple shoot formation. The maximum number of multiple shoots per explant (2.60, 2.40 and 2.20, respectively) was found in nodal segments as compared to shoot tip explants in above rootstocks. The interaction between media and rootstock was found significant. The difference in the multiple shoots per explant between the two rootstocks might be attributed to the difference in their genetic makeup. Parthasarathy and Nagaraju (1996) recorded the maximum number of shoots when MS basal media supplemented with BAP (0.75mg/l) in C. *limon*.

It is clear that maximum number of roots per shoots per explant (1.34) was obtained in rootstock Rough lemon followed by Cleopatra mandarin and Pectinifera in treatment ½ MS + IBA 10mg/l (Tab. 5). It was rated better than all other rooting media. In rootstock Troyer citrange there was no rooting occurred at all. No rooting was observed in majority of the media treatments. However, the treatments MS + IBA 15mg/l and MS + IBA 10mg/l were also found effective for root formation in these rootstocks. Interaction between media and rootstocks was found to be significant. Rooting percentage relies on the size of the shoot placed for rooting. Large sized shoots show higher rooting percentage (Barua *et al*., 1996).

The per cent survival of *in vitro* derived plants in potting media was recorded six weeks after transferring to the pots under greenhouse condition. The survival of plants varied with potting media and rootstocks (Tab. 6). Maximum survival was obtained in soil + sand + FYM (1:1:1v/v) media, which was significantly better than all other potting media and was followed by soil + river sand (1:1v/v) and soil + sand (1:1v/v). The survival was significantly higher (80.00 and 71.43%, respectively) in soil + sand + FYM (1:1:1) media in Rough lemon and Cleopatra mandarin. The higher plant survival in the soil + sand + FYM (1:1:1) potting mixture can be attributed to the retention of optimum moisture with adequate aeration. These results are in conformity with the observations made by Goyal and Arya (1987).

The per cent survival of in vitro derived plants in potting media was recorded six weeks after transferring to pots under greenhouse condition. The survival of plants varied with potting media and rootstocks (Tab. 6). Maximum survival was obtained in soil + sand + FYM (1:1:1 v/v) media, which was significantly better than all other potting media and was followed by soil + river sand (1:1 v/v) and soil + sand (1:1 v/v). The survival was signifi-

Tab. 5: Effect of media and IBA on number of roots/ shoot in citrus rootstocks

Media (MS or ½ MS)	Treatments IBA (mg/l)	Rough lemon	Cleopatra mandarin	Pectinifera	Mean
MS	0.0	0.00	0.00	0.00	0.00
MS	1.0	0.00	0.00	0.00	0.00
MS	2.0	0.00	0.00	0.00	0.00
MS	4.0	0.00	0.00	0.00	0.00
MS	5.0	0.00	0.00	0.00	0.00
MS	10.0	0.64	0.53	0.60	0.59
MS	15.0	0.78	0.74	1.08	0.86
MS	20.0	0.00	0.00	0.00	0.00
½ MS	0.0	0.00	0.00	0.00	0.00
½ MS	1.0	0.00	0.00	0.00	0.00
½ MS	2.0	0.00	0.00	0.00	0.00
½ MS	4.0	0.00	0.00	0.00	0.00
½ MS	5.0	0.00	0.00	0.00	0.00
½ MS	10.0	1.34	1.27	1.11	1.24
½ MS	15.0	0.63	0.65	0.71	0.66
½ MS	20.0	0.00	0.00	0.00	0.00
C.D (5%) Media: 0.22, Rootstock: 0.07, Media x Rootstock: 0.32					

IBA – Indole butyric acid

Tab. 6: Effect of potting media on survival % of citrus rootstocks under greenhouse

Potting media mixture	Rough lemon	Cleopatra mandarin	Pectinifera	Mean
Soil + Sand (1:1)	50.00	45.45	61.54	52.33
Soil + River sand (1:1)	71.43	69.23	66.67	69.11
Soil+ Sand + FYM (1:1:1)	80.00	71.43	73.33	74.92
Mean	67.14	63.04	67.18	
C.D (5%) Media: 3.41, Rootstock: 1.73, Media x Rootstock: 4.62				

cantly higher (73.33%) in soil + sand + FYM (1:1:1 v/v) media in Pectinifera followed by Rough lemon and Cleopatra mandarin. There was no shoot formation in Troyer citrange, hence, plants were not available for planting in greenhouse. The higher plant survival in the soil + sand + FYM (1:1:1 v/v) potting mixture can be attributed to the retention of optimum moisture with adequate aeration. These results are in conformity with the observations made by Goyal and Arya (1987).

In the present investigation efforts have been made to standardize the protocol for in vitro multiplication of Citrus rootstocks. It may be possible to propagate some Citrus rootstocks viz. Rough lemon, Cleopatra mandarin and Pectinifera in vitro if they are desirable enough to justify the labour and expense that would be involved. However, further studies are needed to be carried out to optimize the procedures for the establishment of tissue culture derived plants in soil, to examine genetic uniformity and evaluate their performance in field in comparison with sexual propagated plants.

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