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A Comparative Study of Axillary Shoot Proliferation from the Nodal Explants of Three Varieties Pummelo (*Citrus grandis* [L.] Osb.)

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Abstract: Nodal segments of *in vitro* germinated seedlings of three pummelo varieties [Var.-1 (pulp is pink colour), Var.-2 (pulp is white colour) and Var.-3 (pulp is red colour)] were cultured on half-strength MS medium for axillary shoot proliferation. A large number of shoot buds were produced when such four weeks old culture were subcultured on half-strength MS medium containing 1.0 mg l^{-1} BAP. Roots were induced when the isolated individual shoots were cultured on half-strength MS medium containing 0.1 mg l^{-1} each of NAA, IBA or IAA. Cent percent root were observed on half-strength MS medium having 0.1 mg l^{-1} NAA. These *in vitro* grown plantlets were then successfully transferred to outside natural condition through successive phases of acclimatization. About 93% of the regenerated plantlets survived under *ex vitro* condition.

Key words: Propagation, axillary shoot, analysis of variance, *Citrus grandis*

INTRODUCTION

Pummelo (*Citrus grandis* [L.] Osb.) is a common woody tropical fruits belonging to the family Rutaceae. The fruit is the most important part of the plant and it is the natural source of vitamin C. It has a sour-sweetish taste and is highly valued as a dessert fruit. The fruits are used successfully in the treatment of human scurvy and seeds are also used in the treatment of asthma and bronchitis. Its leaves also contain an essential oil. The bark of pummelo is used as antiseptic. Peel oil of *Citrus grandis* is used for flavouring. The whole tree used as a fuel, or its hard and strong wood may be used for making furniture and agricultural implements[1].

However, Plant regeneration of somatic embryogenesis from tissue culture of *Citrus reticulata* was reported by Altaf *et al.*[2]. Cotyledon tissue culture and shoot buds on roots in *Citrus* were studied by Teo *et al.*[3]. Callus induction, somatic embryogenesis and plant regeneration has been induced from shoot tip and immature ovule of *Citrus junos*[4]. Another review on *Citrus* biotechnology by Gmitter *et al.*[5] could provides information on the depth and dimension of *Citrus* tissue culture, viz. exploitation of somaclonal variation and *in vitro* selection for salt tolerant genotypes by Ben-Hayyim and Goffer[6]. Micropropagation and tissue culture of sweet orange was achieved by Duran *et al.*[7]. Latter, Amin and Akhtar[8] have described *in vitro* protocol for regeneration complete plants using and adventitious shoot proliferation from seedling explants of pummelo. Bowman[9] has reported micropropagation of citrus rootstocks. Subsequently, Carimi *et al.*[10] have also reported somatic embryogenesis and plant regeneration from pistil thin cell layers of *Citrus*. In the present study an efficient and reproducible method of rapid micropropagation protocol has been established of three varieties of pummelo using nodal explants of *in vitro* raised seedlings. One such propagation system is developed that can be used in our commercial horticultural nurseries.

MATERIALS AND METHODS

One-month old *in vitro* germinated seedlings (5-7 cm) of three varieties pummelo were used for axillary shoot proliferation. The shoots of seedlings were cut into two-three segments each having a node. The nodal segments of the three varieties pummelo were then aseptically cultured on half-strength MS media supplemented with various concentrations (0.2, 0.5, 1.0 and 2.0 mg l^{-1}) of BAP or Kn alone or in combination with BAP + Kn for multiple shoot regeneration. For inducing root development 2-4 cm long regenerated shoots were excised and transferred to half-strength of MS medium containing various concentrations either of NAA, IBA or IAA (0.1-0.5 mg l^{-1}). The pH of the medium was adjusted

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Table 1a: Effects of different concentrations and combination of cytokinin in half-strength MS medium for shoot regeneration from nodal segments of three varieties pummelo

Growth regulators (mg l^{-1})	% of shoots formation	No. of total shoots/culture	Average length of shoots (cm)	% of shoots formation	No. of total shoots/culture	Average of length shoots (cm)	% of shoots formation	No. of total shoots/culture	Average length of shoots (cm)
		Var.-1			**Var.-2**			**Var.-3**	
BAP									
0.2	60.0	5.3±0.16	2.5±0.24	40.0	3.3±0.11	1.1±0.14	53.0	3.8±0.46	2.5±0.34
0.5	80.0	6.3±0.35	3.6±0.16	53.0	4.0±0.25	2.6±0.36	73.0	4.3±0.25	3.0±0.26
1.0	93.0	7.8±0.56	4.1±0.18	66.7	4.8±0.36	3.1±0.08	80.0	5.8±0.28	4.0±0.38
2.0	53.0	2.6±0.23	3.0±0.25	33.0	2.0±0.13	2.2±0.45	40.0	2.3±0.13	2.2±0.15
Kn									
0.2	20.0	1.4±0.18	1.2±0.21	6.7	1.1±0.28	1.0±0.11	13.0	1.3±0.08	1.0±0.11
0.5	26.7	2.0±0.25	1.8±0.28	13.0	1.5±0.15	1.0±0.08	20.0	1.4±0.15	1.2±0.18
1.0	40.0	3.6±0.35	2.5±0.15	20.0	2.6±0.05	2.2±0.35	26.7	2.6±0.25	2.2±0.05
2.0	20.0	2.1±0.24	1.5±0.25	6.7	1.1±0.24	1.0±0.15	20.0	2.0±0.14	1.3±0.15
BAP+Kn									
0.2+0.1	33.0	2.2±0.42	1.5±0.18	20.0	2.0±0.22	1.2±0.28	26.7	2.0±0.32	1.4±0.28
0.5+0.1	66.7	4.1±0.35	2.8±0.15	33.0	3.1±0.25	2.0±0.35	46.7	3.2±0.15	2.2±0.35
1.0+0.1	80.0	5.6±0.35	3.5±0.15	46.7	4.0±0.15	3.0±0.45	66.7	4.8±0.25	3.2±0.45
2.0+0.1	53.0	3.7±0.12	2.3±0.13	26.7	2.4±0.02	2.0±0.03	40.0	2.8±0.02	2.2±0.23

15 explants were used in each treatment and data ($\bar{x}$±S.E) were collected after 8 weeks of culture initiation

Table 1b: ANOVA for percentage of shoot formation, number of total shoots per culture and average length of shoots developed from *in vitro* grown nodal segments cultured in half-strength MS medium containing different concentrations and combination of cytokinin

Character→		% of shoot formation		Number of total shoots / culture		Average length of shoots (cm)	
Items	df	MS	F	MS	F	MS	F
Replication (R)	2	956.60	200.12**	208.90	18.78**	146.28	19.40**
Concentration (C)	3	580.00	121.33**	66.45	5.97**	39.14	5.19**
Growth regulator (G)	2	2500.00	523.01**	958.63	86.20**	428.17	56.78**
Variety (V)	2	1100.00	230.12**	227.60	20.46**	106.78	14.16**
C×G	6	180.00	37.65**	46.38	4.17**	33.08	4.38**
C×V	6	185.00	38.70**	33.14	2.98*	23.19	3.07*
G × V	4	68.00	14.22**	22.01	1.97ns	17.81	2.36ns
C×G×V	12	55.00	11.50**	5.32	0.47ns	3.81	0.50ns
Error	70	9.27		11.12		7.54	

** indicates significant at 1% level; * indicates significant at 5% level and ns= not significant

Table 1c: Differences between the pooled means of growth regulators and varieties for three characters

Character→	% of explants showing proliferation	Total number of shoots/culture	Average length of shoots (cm)
Effect of growth regulators			
BAP	60.0a	4.31a	2.42a
Kn	19.4c	1.89c	1.29b
BAP+Kn	51.5b	2.86b	2.14a
Effect of varieties			
Var.-1	46.6a	3.68a	2.45a
Var.-2	28.4c	2.47b	1.78b
Var.-3	37.5b	2.88b	2.16a

Means followed by a common letters within each column are not significantly different by DMRT (P>0.05)

to 5.7±0.1 before autoclaving at 1.1 kg cm^{-2} and 121 °C for 20 min. The medium was gelled with 6 gm l^{-1} agar and 30 gm l^{-1} sucrose (BDH). The cultures were maintained at 25±1 °C under 16 h photoperiod with a light intensity of 2000-3000 lux. All cultures were initiated in 150×25 mm glass tubes containing 15 ml of medium. The cultures were regularly subcultured on fresh half-strength MS medium at four-week intervals in glass tubes or 125 ml conical flask. Observations were recorded over 7 days following inoculation and subculturing. All experiments were repeated thrice at least 15 cultures per treatment. The primary shoots regenerated from explants cultured in half-strength MS basal medium supplemented with 1.0 mg l^{-1} BAP, were isolated and subcultured on the fresh medium of same constituents for four weeks. Thus the regenerated micro-shoots were excised several times and cultured in the same medium.

The plantlets obtained after root initiation were carefully separated from the medium of the culture vessels using forceps to avoid damaging them. The roots of the plantlets were carefully washed with tap water to remove the agar adhering to them. The plantlets were then transplanted into 14 cm diameter nursery pots containing a 2:1 mixture of sand and compost soil. When the plants were established fully under room conditions, they were transferred into 20 cm diameter pots containing compost soil.

The results of analysis of variance (ANOVA) for the data obtained from the shoot and root initiation phase of three varieties pummelo (Table 1c and 2c). The differences

between the means of different varieties and different type of plant growth regulators were compared with the help of Duncan's multiple range test (DMRT).

RESULTS AND DISCUSSION

Shoot multiplication: The nodal segments of the three varieties pummelo were collected from *in vitro* raised seedlings, cultured on half-strength MS media supplemented with various concentrations (0.2, 0.5, 1.0 and 2.0 mg l^{-1}) of BAP or Kn alone or in combination with BAP plus Kn for regenerating of multiple shoots. The data on percentage of shoot formation, total number of shoots per culture and average length of shoots per culture from different treatments were recorded after 8 weeks of culture initiation. The nodal explants showed better performance

Table 2a: Effects of different concentrations of auxin in half-strength MS medium for root induction of *in vitro* regenerated shoots of three varieties pummelo

Type of auxins	Conc. of auxins (mg l^{-1})	% of microcutting rooted	No. of root/ microcutting	Average length of roots (cm)	Days to emergence of roots
			Var.-1		
	0.1	73.0	3.0±0.01	3.0±0.42	26-30
IBA	0.2	53.0	2.0±0.03	2.1±0.31	28-32
	0.5	26.7*	1.0±0.14	2.0±0.16	25-36
	0.1	100.0	5.0±0.06	3.5±0.31	20-25
NAA	0.2	80.0	4.0±0.07	3.1±0.46	24-30
	0.5	53.0	2.0±0.16	2.5±0.06	26-38
	0.1	33.0	2.0±0.11	2.0±0.16	35-48
IAA	0.2	26.7	1.5±0.06	1.5±0.31	28-43
	0.5	20.0*	1.0±0.05	1.2±0.40	25-36
			Var.-2		
	0.1	40.0	2.2±0.11	2.5±0.22	30-35
IBA	0.2	33.0	1.4±0.13	1.2±0.11	32-37
	0.5	13.0*	1.1±0.24	1.1±0.26	28-34
	0.1	66.7	3.7±0.31	3.1±0.21	27-30
NAA	0.2	53.0	3.2±0.17	3.0±0.36	30-35
	0.5	33.0	1.5±0.26	2.1±0.16	35-40
	0.1	20.0	1.4±0.11	2.0±0.26	35-47
IAA	0.2	13.0	1.1±0.46	1.5±0.21	28-43
	0.5	6.7*	1.0±0.35	1.2±0.30	32-40
			Var.-3		
	0.1	66.7	2.5±0.08	2.8±0.22	28-34
IBA	0.2	40.0	1.5±0.23	1.5±0.11	30-35
	0.5	13.0*	1.0±0.44	1.1±0.06	28-37
	0.1	86.7	4.0±0.26	3.3±0.21	25-28
NAA	0.2	73.0	3.4±0.07	3.1±0.26	28-35
	0.5	46.7	1.8±0.46	2.2±0.16	26-38
	0.1	26.7	1.5±0.21	2.1±0.06	30-48
IAA	0.2	20.0	1.2±0.16	1.8±0.11	25-43
	0.5	13.0*	1.0±0.25	1.3±0.20	35-40

15 micro-cuttings were cultured of each treatment and data ($\bar{x}$ ± S.E) were collected after 6 weeks of culture
* indicates slight callusing from the cutting base of microshoots

Table 2b: Analysis of variance for number of total roots per shoot and average length of roots developed from micro-shoots cultured in different concentrations of auxin for root induction

Character→		% of micro shoots rooted		Number of total roots/shoot		Average length of roots (cm)	
Items	df	MS	F	MS	F	MS	F
Replication (R)	2	1200.0	72.55**	208.12	15.76**	194.36	24.14**
Concentration (C)	2	840.0	50.78**	84.78	6.42**	57.71	7.16**
Growth regulator (G)	2	2780.0	168.07**	567.44	42.98**	432.00	53.66**
Variety (V)	2	1426.0	86.21**	226.32	17.14**	165.69	20.58**
C×G	4	214.0	12.93**	27.02	2.04ns	15.86	1.97ns
C×V	4	195.0	11.78**	42.31	3.20*	18.78	2.63*
G × V	4	218.0	13.18**	41.11	3.11*	10.78	1.3ns
C×G×V	8	68.0	4.11**	10.11	0.76ns	5.19	0.6ns
Error	50	16.5		13.20		8.05	

** indicate significant at 1% level; * indicate significant at 5% level and ns= not significant

Table 2c: Differences between the pooled means of different auxin and varieties for three characters

Character→	% of micro shoots rooted	No. of root/microshoot	Average length of roots (cm)
Effect of growth regulators			
IBA	35.5b	1.74b	1.92b
NAA	65.8a	3.17a	2.87a
IAA	20.0c	1.31b	1.62b
Effect of variety			
Var.-1	51.7a	2.38a	2.32a
Var.-2	30.9c	1.84a	1.83a
Var.-3	42.8b	1.98a	2.13a

Means followed by a common letters within each column are not significantly different by DMRT (P>0.05)

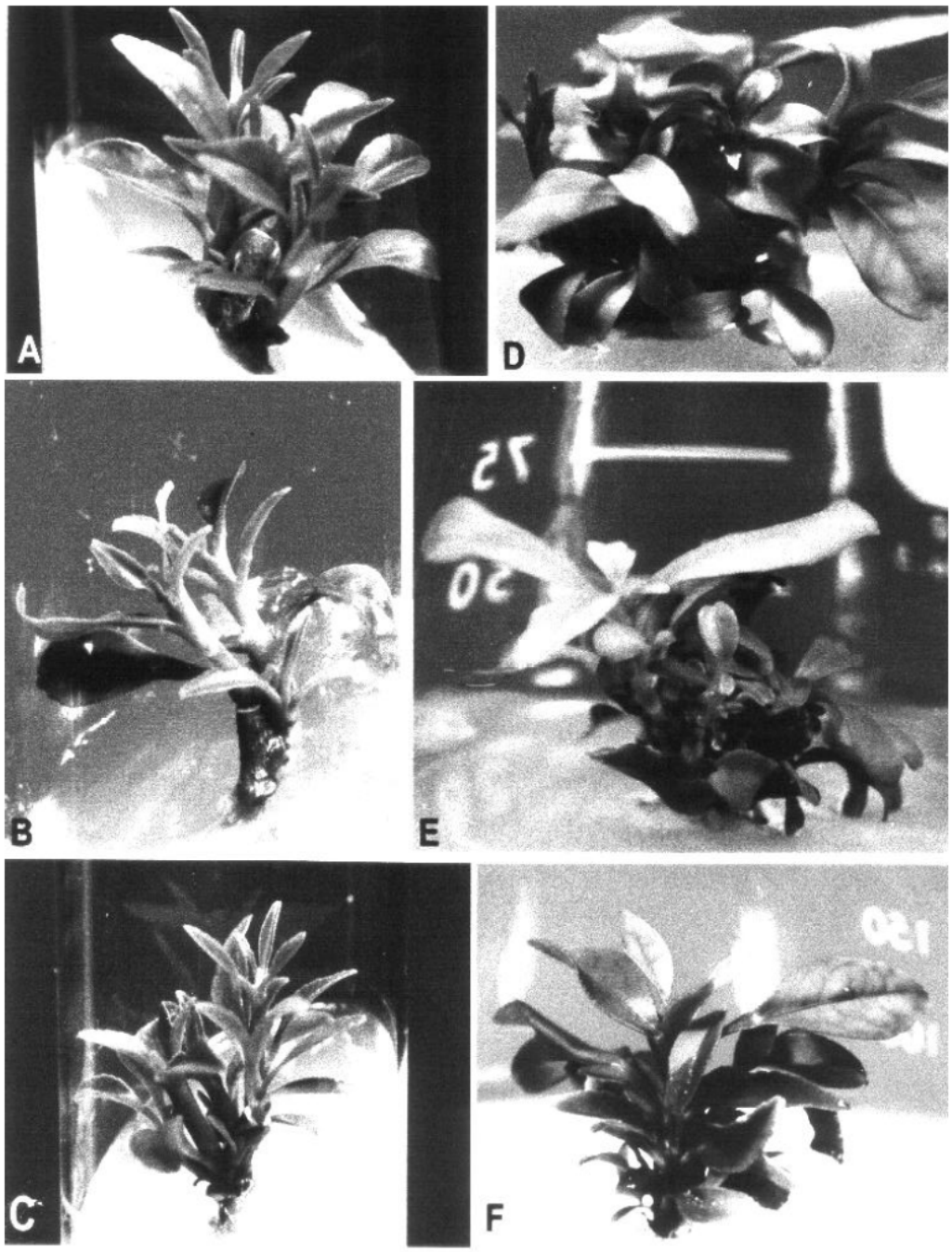

Fig. 1A-F: Growth and development of shoots in vitro from the nodal explants of three varieties pummelo (*Citrus grandis* [L.] Osb.)

A-C: Initial development of axillary shoots from the nodal explants on half-strength of MS medium with 1.0 mg l^{-1} BAP of Var.-1, Var.-2 and Var.-3, respectively after 5 weeks of culture incubation

D-F: Proliferation and elongation of the axillary shoots from the nodal explants on the same medium of Var.-1, Var.-2 and Var.-3, respectively after 8 weeks of culture incubation

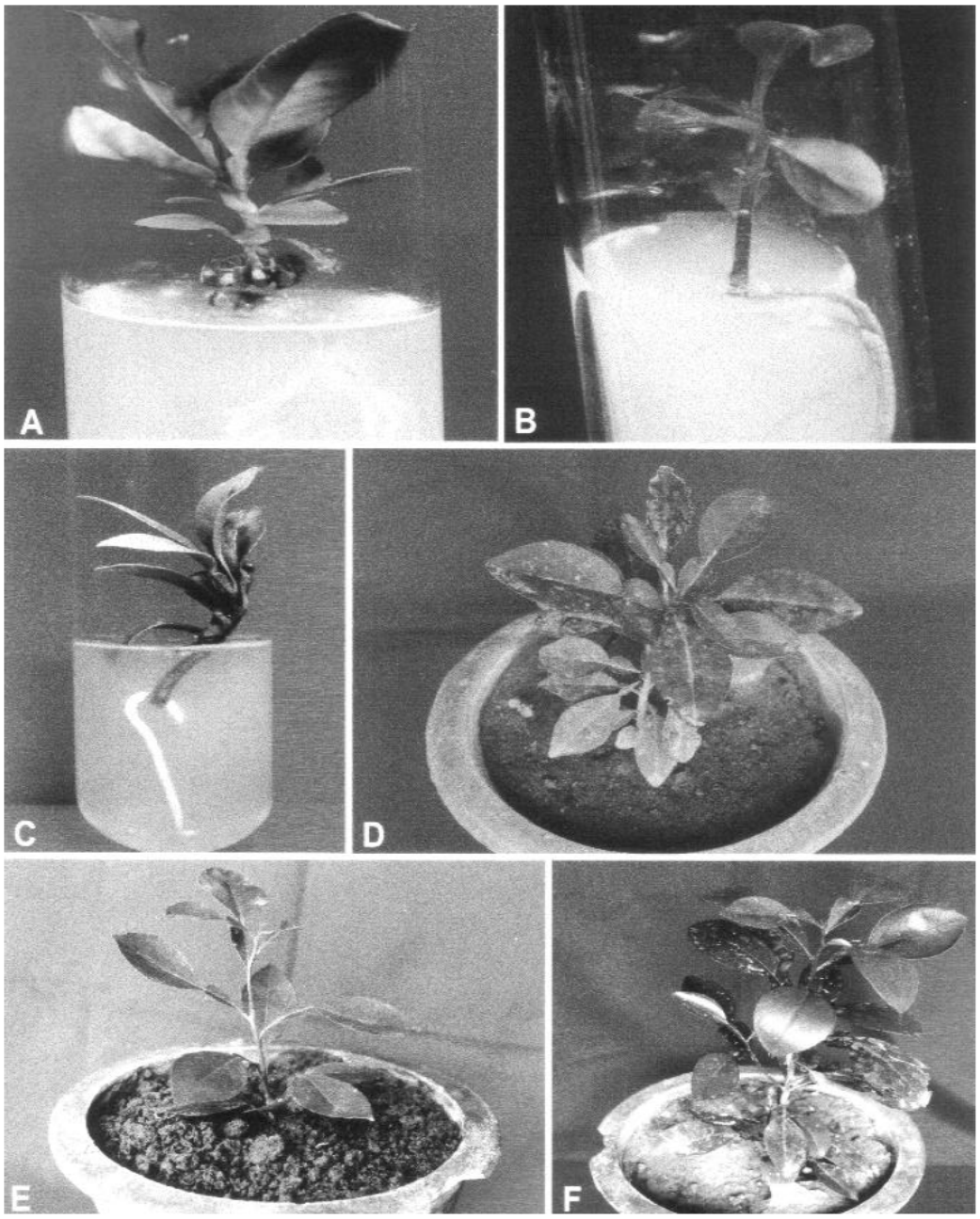

Fig. 2A-F: Plant regeneration from in vitro grown shoots of three varieties pummelo (*Citrus grandis* [L.] Osb.)

A-C: Development of roots from the bases of microcutting on half-strength of MS medium with 0.1 mg l^{-1} NAA of Var.-1, Var.-2 and Var.-3, respectively after 3 weeks of culture incubation

D-F: Growth and development of *in vitro* grown plantlets on the soil after 8 weeks of transfer under *ex vitro* condition of Var.-1, Var.-2 and Var.-3, respectively

on the medium with 1.0 mg l^{-1} BAP of three pummelo varieties (Fig. 1A-C). The highest frequency of 93 and 80% of the cultures were found to regenerate shoots in this medium and the number of regenerated shoots per culture was 7.8±0.56 for Var.-1, 4.8±0.36 for Var.-2 and 5.8±0.28 for Var.-3, respectively (Fig. 1D-F). Medium containing Kn alone showed less performance than BAP and this concentration produced 40, 20 and 26.7% shoots and total number of shoots per culture were 3.6±0.35, 2.6±0.05 and 2.6±0.25 for Var.-1, Var.-2 and Var.-3, respectively. The medium in combinations with BAP and Kn induced considerable frequency of multiple shoots (Table 1a). On medium containing 1.0 mg l^{-1} BAP, all the three varieties responded well and produced more shoots than the medium containing 1.0 mg l^{-1} Kn and 1.0 mg l^{-1} BAP with 0.1 mg l^{-1} Kn. This result is due to the synergistic effect of BAP as reported for *Citrus grandis*[8,11]. Using 1.0 mg l^{-1} BAP of shoot regeneration from nodal and shoot tip explants has also been achieved in a wide range of pummelo[12].

In the present investigation, nodal segments of three varieties pummelo were regenerated successfully and the most suitable medium was half-strength of MS+1.0 mg l^{-1} BAP. The technique described here appears to be readily adaptable for large scale clonal propagation.

The analysis of variance indicated that statistically significant variations were present in case of concentration (C), growth regulator (G), variety (V), concentration × growth regulator (C×G) and concentration × variety (C×V) for the characters like percentage of shoot formation, number of total shoots per culture and average length of shoots (cm). On the other hand, the interaction items growth regulator × variety (G×V) and concentration × growth regulator × variety (C×G×V) for number of total shoots per culture and average length of shoots (cm) were not statistically significant, but percentage of shoot formation was statistically significant (Table 1b). The differences between the means of different varieties and different type of plant growth regulators were compared with the help of Duncan's multiple range test (DMRT). Among three types of plant growth regulators for the character like highest percentage of explants produced shoots and total number of shoots per culture were obtained in BAP followed by BAP plus Kn and Kn and the differences among them were significant. On the other hand, average length of shoots per culture was obtained in BAP followed by BAP plus Kn and Kn where the differences between BAP and BAP plus Kn were statistically not significant. Besides, highest percentage of explants produced shoots was obtained in Var.-1 followed by Var.-3 and Var.-2 and the differences among them were significant. However, other two characters in Var.-1 and Var.-2 were not found any significant variation but Var.-3 was showed statistically significant (Table 1c).

***In vitro* rooting:** The microcuttings (2-4 cm long) were prepared from the *in vitro* proliferated usable shoots and cultured on half-strength MS medium supplemented with 0.1-0.5 mg l^{-1} either of IBA, NAA or IAA for adventitious rooting. The percentage of root formation, number of roots per shoot and average length of the longest root were recorded after 20-25 days of culture incubation for Var.-1, 27-30 days for Var.-2 and 25-28 days for Var.-3. Among the three type of auxins, NAA was found to be comparatively more effective than IBA and IAA at different concentrations tested for producing roots on the shoot cuttings. The microcuttings of Var.-1 showed better rooting performance than that of Var.-2 and Var.-3 (Fig. 2A-C). Among various concentrations tested 0.1 mg l^{-1} of all the three auxin produced highest frequency of root formation. The frequencies of root formation for the three varieties were 100, 66.7 and 86.7 for NAA, 73.0, 40 and 66.7% for IBA and 33.0, 20 and 26.7% for IAA for Var.-1, Var.-2 and Var.-3, respectively (Table 2a). The findings are in agreement with those observed in *Citrus jambhiri*[13], *Punica granatum*[14], *Citrus grandis*[15].

ANOVA showed that the items concentration (C), growth regulator (G), variety (V) and concentration × variety (C×V) for the characters like percentage of micro shoots rooted, number of total roots per shoot and average length of roots (cm) were statistically significant. The interaction item concentration × growth regulator (C×V) and concentration × growth regulator × variety (C×G×V) for number of total roots per shoot and average length of roots (cm) were not significant, but percentage of micro shoots rooted was significant. Besides, the growth regulator × variety (G×V) for percentage of micro shoots rooted and number of total roots per shoot were significant, whereas average length of roots showed statistically non significant (Table 2b). The differences between the pooled means of different auxin and varieties were observed to be statistically significant by DMRT. The percentage of root formed that was obtained in NAA followed by IBA and IAA and the differences among them were significant. Besides, other two characters in all the three tested auxins did not found any significant variation. On the other hand highest percentage of explants produced root that was obtained in Var.-1, followed by Var.-3 and Var.-2 and the differences among them were significant. However, other two characters in all the three tested varieties did not found any significant variation (Table 2c).

Transplantation: Rooted plantlets were successfully transferred into 14 cm diameter nursery pots containing a 2:1 mixture of sand and compost soil. During initial period of acclimatization transferred plantlets were kept in high relative humidity maintained by covering the plantlets with polythene bags. At the time of rearing, shoots elongated and the plants were very healthy. When the plants were established fully under room conditions, they were transferred into 20 cm diameter pots containing compost soil (Fig. 2D-F). About 93% of the transplanted plants survived under field condition. Mass propagation of plant varieties through *in vitro* culture is one of the best and most successful examples of commercial application of plant tissue culture technology. Recently, there has been much progress in this technology for horticultural plants.

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