INDIAN JOURNAL OF ARID HORTICULTURE
2011, Vol. 6 (1-2) : 22-27

In-vitro propagation studies of virus tolerant citrus rootstock Cleopatra Mandarin (*Citrus reshnii* Tanaka)

Raj Kumar* [1], M. K. Kaul [2], S. N. Saxena [3], A.K.Singh [4], J. Singh [5] and S.S.Sirrova
Agricultural Research Station (S.K. Rajasthan Agricultural University)
Srikarni Marg, Sriganganagar
[1] SMS (Hort.) KVK, (CIAH), Bikaner, [2]Professor, (Hort.), ARS SKRAU, Bikaner,
[3]Sr. Scientist (Plant Phy.), NRCSS, Ajmer, [4]Sr. Scientist (Hort.), CHES (CIAH), Godhra
[5]Associate Professor (Hort.), CH&F Jhalawar, [6]Scientist (Hort.), CARI, Portbalare

Abstract

Studies on virus tolerant, citrus rootstock Cleopatra (*Citrus reshnii* Tanaka) were carried out by using nodal segments of seedlings as explants. The maximum survival of explants (90 per cent) and minimum time required to bud breaking (19.50 days) was recorde on BAP 0.5mgl^{-1} + kinetin 0.5 mgl^{-1}. However, maximum number of shoot (7.30) was there on BAP 2.0 mgl^{-1} + kinetin 1.0 mgl^{-1} and maximum length shoot (2.40 cm) on BAP 0.5 mgl^{-1} + kinetin 2.0 mgl^{-1}. The maximum (90 per cent) survival of micro shoot for rooting and length of root (6.28 cm) were recorded on M S medium modified with NAA 0.5 mgl^{-1}+ IBA 0.5 mgl^{-1}. The minimum time taken to root induction (22.00 days) was on NAA 0.5mgl^{-1} + IBA 0.1 mgl^{-1} and maximum number of root (5.60) was on NAA 0.5 mgl^{-1} + IBA 1.0 mgl^{-1}. *In-vitro* propagated plantlets were successfully acclimatized by transferring then in pots containing a potting mixture of soil: perlite: vermiculite in equal proportion. Plantlets survival ofv90 per cent was observed in this mixture.

Key words: Cleopatra, *in-vitro*, multiplication, nuceller, BAP, kinetin, NAA and IBA

Introduction

Cleopatra (*Citrus reshnii* Tanaka) is an important citrus species of Rutaceae family and commercially grown for rootstock. It is the important rootstock for mandarin, sweet orange, acid lime etc. because it renders good graft compatibility, productivity, fruit quality, dwarf stature of plant and also tolerance to viruses (Tristiza, Xyloporosis, Exocortis and Gumosis (Chadha, 1970), fungi (*Phytopthora*) and bacterial canker. It is the most salt tolerant citrus rootstock due to ability to exclude sodium and chloride taken up by the foot system (Broembsen, 1984).The production of uniform large number of plant is not possible through seed because of cross pollination nature of citrus. Hence, the maintenance of genetic makeup of genotype in successive generation is not possible through seed propagation. The production of uniform large number of plant is not possible through seed. Tissue culture offers an advantage over conventional methods of propagation (seed) in producing large number of genetically uniform healthy plant within a short period. In tissue culture Plant Growth Regulators (PGRs) play important role on different regeneration parameters. In this perspective studies were carried out to standardize the optimum concentration of PGRs for particular organ regeneration from nodal segments.

Material and Methods

Present investigation was carried out at the Tissue Culture Laboratory, Agricultural Research Station, Sriganganager during 2007-08. Nodal segments of seedlings were used as explants. For this, healthy fruits were collected from citrus repository of the station. They were washed in running tap water for 2-3 hours. The seeds were extracted, washed thoroughly and treated with 0.2 per cent Bavistin. The seed testa was removed under aseptic condition. These decoated seeds were first quick rinsed with 70 per cent ethanol followed by mercuric chloride ($HgCl_2$) @ 0.1 per cent) for five minutes and inoculated in culture tubes (25 x 150 mm) containing 15-20 ml MS basal medium modified with 3 per cent and 0.8 per cent sucrose. All the cultures were incubated in BOD at 25 ± 2° C. After one and half month of inoculation the seedlings were cut into segments, each segment having at least two buds and then inoculated on MS medium (Murashige and Skoog,1962) supplemented with BAP, Kinetin @ 0.0, 0.5, 1.0, 2.0 mg l^{-1} alone or in combinations for axillary shoot proliferation. All the cultures were sub- cultured in fresh medium after 25-30 days. The *in-vitro* generated shoots

Corresponding authors' e-mail: rajhortches@gmail.com

were used as micro cuttings. The dried, under sized leaves and multiple shoots were removed. The micro shoot about 2-3 cm were inoculated in culture tube (25 x 150 mm) containing 15-20 ml MS medium supplemented with various concentrations of IBA and NAA @ 0.0,0.1,0.5,1.0 and 2.0 mg l^{-1} alone or in combination. The rooted plantlets were removed carefully from the culture tubes and their roots were thoroughly washed under running tap water and cleaned with fine brush to remove adhering agar. The plantlets were covered with sterilized cotton wetted with half strength MS medium for 24 hours in culture room. The plantlets were treated with Bavistin @ 0.1 per cent for 10 minutes to prevent fungal contamination. These plantlets were transferred to pots containing sterilized soil, vermiculite, and perlite in equal proportion. These pots were kept in green house at 90 per cent humidity with temperature 26 ± 2°C. The humidity was continuously lowered within 8-10 weeks upto 60 per cent. During this period the plantlets were irrigated with Hoglands solution at three days interval for one month. After that, these were irrigated with Hoglands solution and simple water at an interval of 2-3 days alternately. The observations were recorded for per cent survival of explants, time required to bud break, number of shoot, length of shoot, per cent micro shoot responding to root ,time required to root induction, number of root, length of root and number of plantlets survival after 60 days of planting in pots and incubated in green house. The recoded data was statistically analyzed by C.R.D. (Completely Randomized Design) Cochran and Cox (1957).

Results and Discussion

Per cent survival of explants

The per cent survival of explants was significantly affected by BAP and kinetin levels (Table-1). Among alone levels of BAP and kinetin the maximum 980 per cent) survival of explants were observed when MS culture medium modified with 2.0 mgl^{-1} and 1.0 mgl^{-1} respectively was used. In case of combination of BAP and kinetin the maximum survival of explants (90 per cent) was recorded on BAP 1.0 mgl^{-1} + kinetin 0.5 mgl^{-1}. The results of the studies are concurrent with the findings of Parthasarathy and Nagaraju (1993b) they reported significant interaction between levels of values for plantlet induction. Similar finding were also reported by Baruah *et al.*, 1996a.

Time required to bud break

BAP and Kinetin levels significantly affected the number of days required to bud break. The increasing concentration of BAP and Kinetin significantly decreased time needed to bud break and further, increase in concentration delayed it (Table-1). Among different levels of BAP and kinetin (0.5, 1.0 and 2.0 mgl^{-1}) the minimum (20.30 days) and 22.0 days were recorded on 0.5 mgl^{-1} and 1.0 mgl^{-1} respectively. It is revealed from the data that BAP was better cytokinin than kinetin for reducing time for bud break. The similar findings are also reported by Singh *et al.*(1994) who reported that the mean minimum number of days required to bud beak was directly dependent on citrus species and medium combination. They also reported minimum days to bud beak in *Citrus reticulata* and *Citrus limon* as 17 and 18 days respectively, when explants of both species were cultured in MS medium modified by BAP 1.0 mgl^{-1} + kinetin 0.5 mgl^{-1} + NAA 0.5 mgl^{-1}.

Number of shoot

Among individual levels of BAP, MS medium modified with 2.0 mgl^{-1} BAP gave maximum number of shoot per explants 5.70 (Table-2). It may be due to more responsiveness of the Cleopatra explants to higher concentration of BAP. The result of present study is in line with the reports of Rahaman *et al.* (1996) who reported 2.80 shoots in Cleopatra when explant was inoculated in MS medium containing 1.0 mgl^{-1} BAP. In case of kinetin a maximum of 5.10 shoots were observed, when explants were inoculated on MS culture medium modified with 2.0 mgl^{-1} kinetin. These findings are similar to those reported by Al-Khayri and Al-Bahrany (2001) who reported that a maximum of 7.0 shoots per explants were observed in MS medium modified by 1.0 mgl^{-1} BAP. This may be due to suppressed apical dominance by cytokinin. It is proved from the study that the rate of shoot multiplication was significantly affected by the concentration of BAP and kinetin. BAP play important role in shoot multiplication, lack of BAP in medium mostly produced single shoot but addition of 0.25 mgl^{-1} BAP significantly increased shoot multiplication. The rate of shoot multiplication on MS media supplemented by kinetin was low. In present study it was observed that BAP was superior cytokinin for shoot multiplication than kinetin. These findings are also supported by Otoni and Teixeira (1991) in Sweet orange. According to them cytokinin promotes shoot proliferation by inducing cell division and enlargement. The similar results was also reported by Bowman (1994) in citrus rootstocks. In interaction effect of BAP and kinetin, the maximum number of shoots (9.30 per explant) were recorded when MS culture media was supplemented by BAP 2.0 mgl^{-1} + kinetin 0.5 mgl^{-1} (Table-2, Fig.-1 and Plate-1A). This may be due to better synergetic effect of the cytokinins. The results of present studies are in line with the report of Karwa (2003) who reported that in Nagpur mandarin the maximum (9.11 ± 0.26) shoots were observed when explants were inoculated on MS medium supplemented with BAP (8.88 µl) + kinetin (2.32 µl). Similar findings have also been reported by Bowman, 1994 in citrus rootstocks, Al-Bahrany (2002) and Al-Khayri and Al-Bahrany (2001) in lime.

Length of root

The length of root was significantly influenced by different concentrations of NAA and IBA. The length of root significantly increased with increasing concentration of IBA up to 1.0 mgl^{-1} level and higher than this had reverse effect. The maximum length of roots (4.20 cm) was observed when MS medium was modified by 1.0 mgl^{-1} IBA, (Table-2). Similar results are also reported by Syamal *et al.* (2007). The increase in level of NAA significantly increased length of root upto 0.5 mgl^{-1} and further increase

in concentration decreased it. The maximum length of roots (2.71cm) was observed when micro shoots were cultured on MS medium modified with 1.0 mgl^{-1} NAA. Kitto and Young (1981) in Carrizo obtained 2.30 cm length of roots when micro shoot inoculated in MS medium fortified by 1.0 mgl^{-1} NAA was usesd. In interaction effect of IBA and NAA, for length of root, maximum length of root (6.28 cm) was observed on MS culture medium supplemented with NAA and IBA each 0.5 mg l^{-1} (Table-2, Fig.-2 and Plate-1D). Syamal *et al.* (2007) reported maximum length of root (2.16 cm) when micro shoots were cultured in MS medium supplemented with IBA 1.0 mgl^{-1} NAA 0.5 mgl^{-1}. The findings of present study are in agreement with Al-Khayri and Al-Bahrany (2001).

Acclimatization

In-vitro regenerated plantlets were successfully acclimatized by transferring them in small pots containing a potting mixture of soil: Perlite: vermiculite in equal proportion. Survival of plantlets upto 75 per cent was observed. It may be due to higher number of roots, high porosity, cation exchange capacity (CEC) and water

Table 1: Effect of BAP & Kinetin, added singly and in combination on basal medium, on different regeneration parameters of Cleopatra

Treatments ($mg\ l^{-1}$)		Per cent survival of explants	No. of days to bud break	No. of shoots	Length of shoot (cm)
BAP	Kinetin				
0.0	0.0	50 (45.00)	22.30	1.60	1.42
0.0	0.5	70 (56.79)	22.10	2.30	1.90
0.0	1.0	80 (63.43)	22.00	3.60	2.10
0.0	2.0	60 (50.77)	23.10	5.10	1.62
0.5	0.0	70 (56.79)	20.30	3.70	1.20
1.0	0.0	70 (56.79)	21.30	4.10	1.50
2.0	0.0	80 (63.43)	21.80	5.70	0.72
0.5	0.5	70 (56.79)	19.50	5.70	1.71
0.5	1.0	70 (56.79)	20.80	4.60	2.00
0.5	2.0	60 (50.77)	20.00	3.60	2.40
1.0	0.5	90 (71.57)	21.20	4.50	1.90
1.0	1.0	60 (50.77)	21.10	6.60	1.71
1.0	2.0	50 (45.00)	21.90	4.30	1.65
2.0	0.5	60 (50.77)	21.10	6.30	1.50
2.0	1.0	60 (50.77)	21.40	7.30	1.82
2.0	2.0	50 (45.00)	23.20	4.30	1.32
S. Em.		0.33	0.26	0.16	0.10
CD (0.05%)		0.99	0.71	0.44	0.27

*Figures given in parentheses are angular transformed values

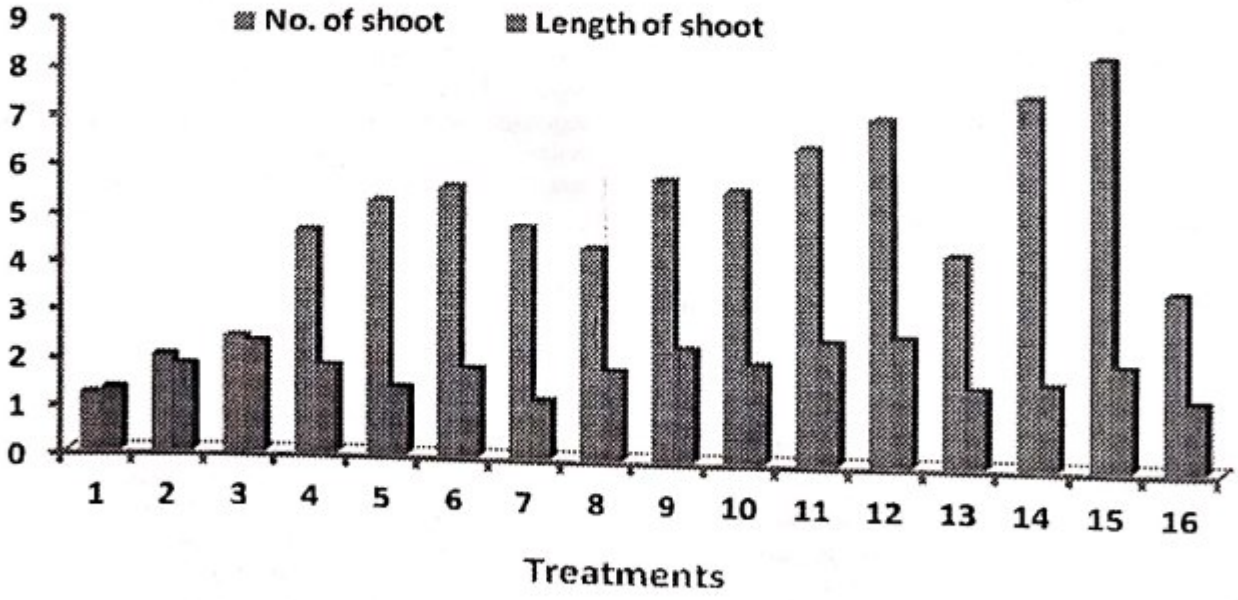

Fig. 1 Effect of cytokinin (BAP and Kinetin) added singly and in combination in MS basal medium on no. of shoot and length of shoot (cm) in Cleopatra

Table 2: Effect of NAA and IBA added singly and in combination in basal medium on different root parameters of root in Cleopatra

Treatments (mg l^{-1})		Treatments (mg l^{-1})	No. of days taken to root induction	No. of roots	Length of root (cm)
NAA	IBA				
0.0	0.0	0.0 (0.00)	0.0	0.00	0.00
0.1	0.0	60 (50.77)	25.10	1.20	1.80
0.5	0.0	70 (56.79)	22.60	2.20	2.29
1.0	0.0	80 (63.43)	26.20	3.60	2.71
2.0	0.0	70 (56.79)	27.90	2.20	2.21
0.0	0.1	60 (50.77)	26.20	1.40	1.26
0.0	0.5	70 (56.79)	24.30	1.90	2.16
0.0	1.0	80 (63.43)	23.50	2.50	4.20
0.0	2.0	50 (45.00)	26.20	2.30	3.69
0.1	0.1	70 (56.79)	23.50	3.00	1.85
0.5	0.1	70 (56.79)	22.00	3.30	3.12
1.0	0.1	80 (63.43)	28.10	3.30	2.38
2.0	0.1	70 (56.79)	27.10	3.00	1.91
0.1	0.5	80 (63.43)	23.70	4.00	4.13
0.5	0.5	90 (71.57)	23.80	4.40	6.28
1.0	0.5	70 (56.79)	26.70	3.70	3.30
2.0	0.5	60 (50.77)	28.60	2.90	1.82
0.1	1.0	70 (56.79)	23.30	3.90	3.30
0.5	1.0	70 (56.79)	25.80	5.60	2.81
1.0	1.0	60 (50.77)	26.10	4.10	2.38
2.0	1.0	60 (50.77)	27.50	4.10	1.90
0.1	2.0	60 (50.77)	25.40	3.80	2.61
0.5	2.0	50 (45.00)	25.70	4.20	2.79
1.0	2.0	50 (45.00)	27.40	3.20	2.42
2.0	2.0	40 (39.23)	28.20	2.50	1.25
S. Em.		0.45	0.40	0.45	0.16
CD (0.05%)		1.27	1.13	1.27	0.44

*Figures given in parentheses are angular transformed values

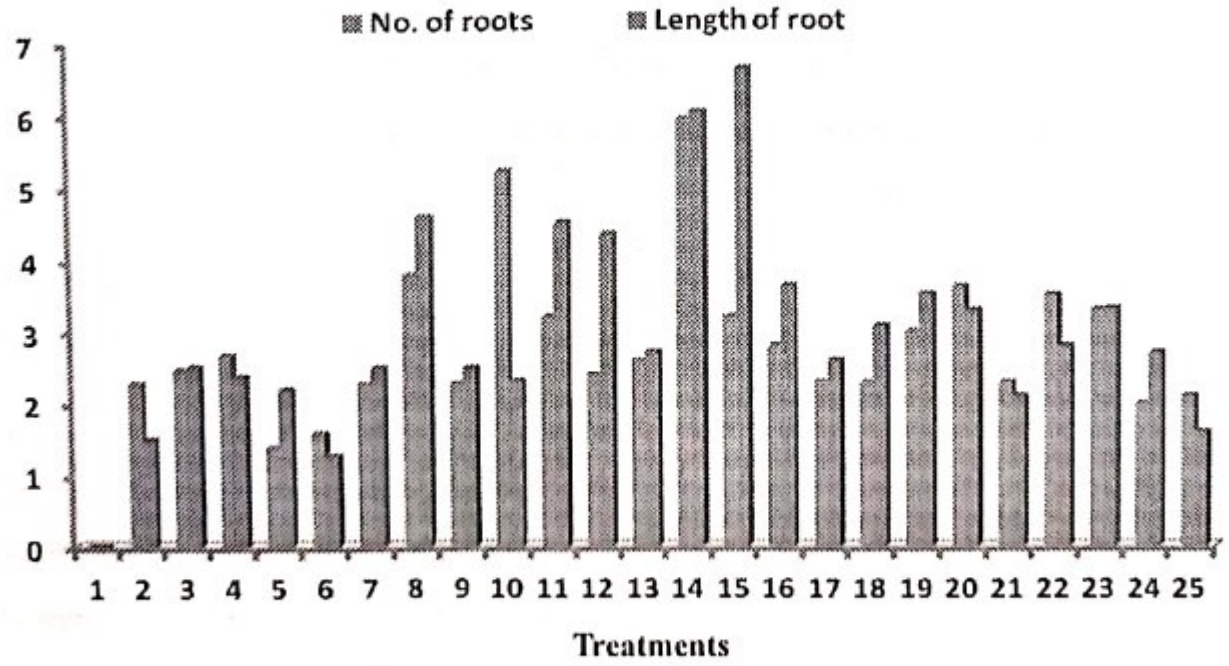

Fig. 2. Effect of auxin (NAA and IBA) added singly and in combination in MS basal medium on no. of roots and length of roots (cm) in Cleopatra

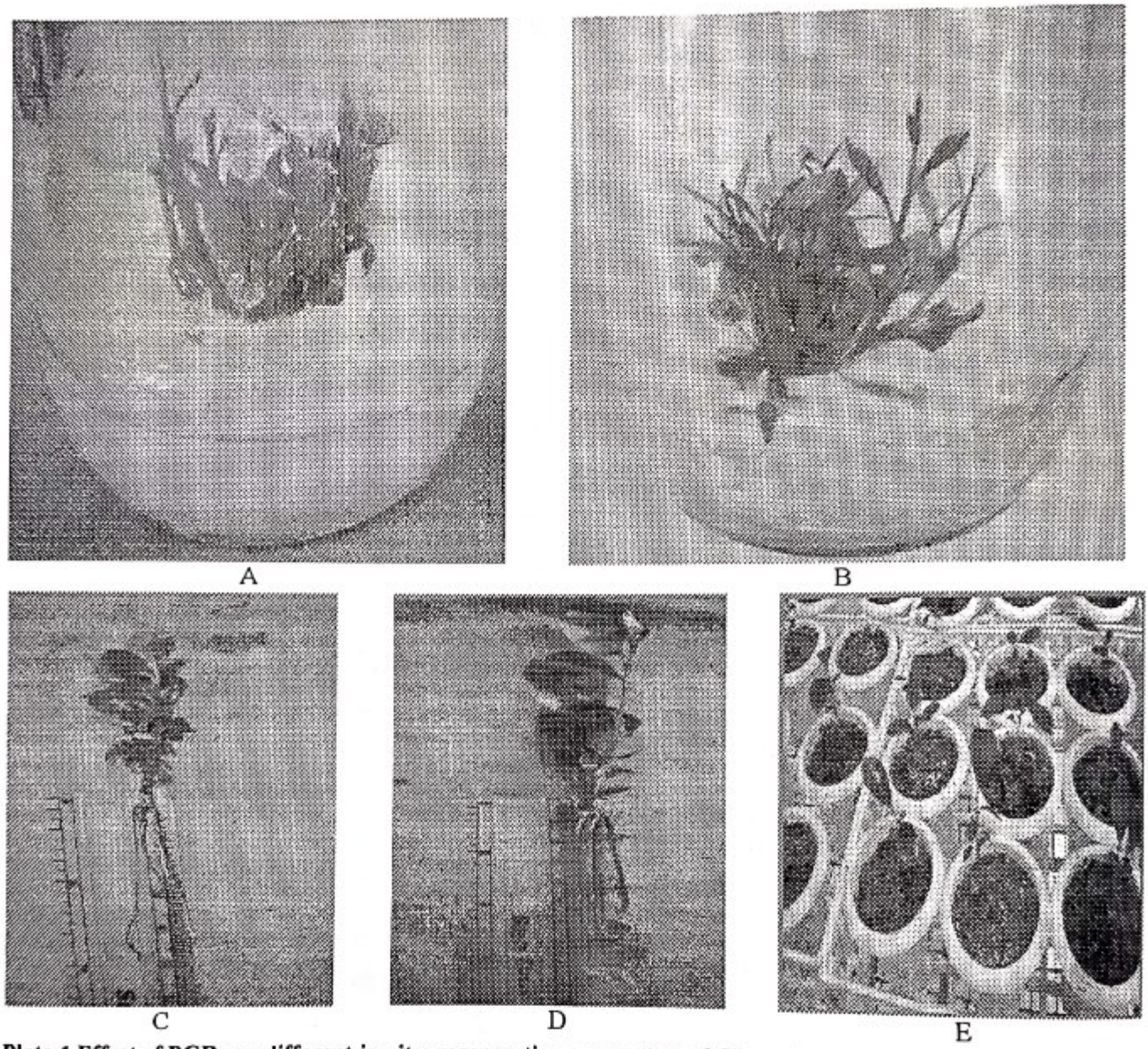

Plate-1 Effect of PGRs on different *in-vitro* regeneration parameters of Cleopatra
A- No. of shoot (BAP 2.0 mgl^{-1} + kinetin 1.0 mgl^{-1}), **B-**Length of shoot (BAP 0.5 mgl^{-1} + kinetin 2.0 mgl^{-1}) **C-** No. of root (NAA 0.5 mgl^{-1} +IBA 1.0 mgl^{-1}), **D-** Length of root (NAA 0.5 mgl^{-1} +IBA 0.5 mgl^{-1}) **E-** Hardening of *in-vitro* regenerated plantlets.

holding capacity of potting medium (Plate-1E). According to Gill *et al.* (1995 and Baruah, *et al.*, 1996b) survival per cent of *in-vitro* plantlets is directly related to number of roots. The results of the present study are in line with earlier reporters. Singh *et al.* (2007) reported 92.00 per cent success of *in-vitro* plantlets *in vivo* condition when vermiculite and coco peat @ 3:1 ratio were used as potting medium. Singh *et al.* (1994) reported 60 per cent establishment of plants when planted in soil in case of khasi mandarin. In case of Rough lemon Singh *et al.* (1999a) reported 80 per cent survival of plantlets in soil upon planting..

Refrences

Al-Bahrany, A.M. 2002. Effect of photohormones on *in-vitro* shoot multiplication and rooting of lime (*Citrus aurentifolia* Christm.) Swing. *Scientia Horticulture*, 95 : 285-295.

Al-Khayri, J.M. and Al-Bahrany, A.M. 2001. *In-vitro* micro-propagation of *Citrus aurantifolia*. *Current Science*, 81(9-10): 1242-46.

Baruah, A. Nagaraju, V. and Parthasarathy, V. A. 1996b. Micro-propagation of three endangenered citrus species-2, rooting ex vitro, *Ann Plant Physiol* 10: 129-32.

Broembsen, L.V.1984. *Citrus, in SubTrop. Fruit J.*, 610 : 5-12.

Cochran, W.C. and Cox, G. 1957. Experimental Designs 2nd (Ed). John Wiley and Sons. New York.

Baruah, A., Nagaraju, V., and Parthasarathy, V. 1995. Cytokinin Mediated Response in Shoots of *Citrus grandis* Osbeck, *Ann. Plant Physiol.*, 9 (1) : 13-16.

Baruah, A. Nagaraju, V. and Parthasarathy, V.A. 1996a. Micro-propagation of three endangered citrus species-1. Shoot proliferation *in vitro*. *Ann. Plant Physiol.*, 10 : 124-28.

Bowman, K.D. 1994. Micro-propagation of smooth flat Seville and Yuma citrus rootstocks. *Fla State Hort. Soc.*, 107 : 15-18.

Chadha, K.L. 1970. Rootstocks. Pp. 9-25 *in* Citrus Decline in India - Causes and Control (K.L. Chadha, N.S. Randhawa, G.S. Bindra, J.S. Chohan, and L.C. Knoor, eds.). A joint publication of Punjab Agril. University, Ohio State University and USAID.

Gill, M.I.S., Singh, Z., Dhillon, B. S. and Gosal, S. S. 1995. Somatic embryogenesis and plantlet regeneration in mandarin (*Citrus reticulata* Blanco). *Scientia Hort.*, 63 : 167-74.

Karwa, A. 2003. *In vitro* propagation of *Citrus reticulata* Blanco (Nagpur mandarin). *Ind. J. Genet.*, 63 (2) : 187 - 188.

Kitto, S.L. and Young, M. J. 1981. *In vitro* propagation of Carrizo citrange. *Hort. Science*, 16 (3) : 305 306.

Kour, K., Bakshi, P. and Kher, R. 2007. Studies on micro-propagation of rough lemon. *Indian J. Hort* , 64 (4) : 454-455.

Kumar Krishan, Dhatt, A.S. and Gill, M.T.S. 2001. *In vitro* plant regeneration in sweet orange (*Citrus sinensis*, L. Osbeck cv. Musambi and Jaffa). *Indian J. Horti*, 58 : 11-13.

Murashige,T.L. and Skoog,F.1962. A revised medium for rapid growth and biomass with tobacco tissue cultures. *Physiol Plant*, 15:473-97

Otoni, W.C. and Teixeira, S. L. 1991. Influence of the length and position of juvenile nodal segments obtained from *Citrus sinensis* (L.) Osb. Cv. Pera mother plants on axillary bud multiplication. *Revista Ceres*, 38 : 474-84.

Parthasarathy, V.A. and Nagaraju, V. 1993. Morphogenetic response of embryoids of three citrus species to benzylamin purine. *Ann. Plant Physiol.*, 7 : 169-74.

Parthasarathy, V.A. and Nagaraju, V. 1994. Effect of NAA and malt extract on *in vitro* growth of excised embryos of citrus. *Int. J. Trop.Agric.*, 12 : 107-11.

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

Singh, D., More, T.A., Awasthi, O.P., Niwas, R. and Srivastava, S. 2007. *In vitro* morphogenesis in Mulberry. *Nat. Seminar on "Opportunities and Challenges of Arid Horticulture for Nutrition and Live hood"* March 8-9, 2008.

Singh, S., Ray, B. K. and Deka, P. C. 1999a. Micropropagation of *Citrus jambhiri* cultivar Rough lemon. *J. Interacad*, 3 : 140-45.

Singh, S., Roy, B. K., Bhattacharya, S. and Deka, P. C. 1994. *In vitro* propagation of *Citrus reticuleta* Blanco and *Citrus limon* Burm. *Hort Sci.*, 29 (3) : 214 216.

Syamal, M.M., Upadhyay, S., and Biswas, B. 2007. *in vitro* clonal propagation of kagzi lime (*Citrus aurantifolia* Swingle). *Indian J Hort.*, 64 (1) 84 : 86.