International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
IJCS 2019; 7(4): 3106-3111

Received: 19-01-2019
Accepted: 23-06-2019

Amardeep Kour
Department of Fruit Science, Punjab Agricultural University, Ludhiana, Punjab, India

HS Rattan Pal
Department of Fruit Science, Punjab Agricultural University, Ludhiana, Punjab, India

Correspondence
Amardeep Kour
Department of Fruit Science, Punjab Agricultural University, Ludhiana, Punjab, India

In vitro plant organogenesis in sweet orange cv. Mosambi by using epicotyl segments as explants

Amardeep Kour and HS Rattan Pal

Abstract
By genetic transformation techniques, the exogenous genes can be introduced into the plants, but an effective tissue culture protocol with high regeneration percentage is important for genetic transformation. The present study was carried to strandaridzed a plant regeneration protocol for sweet orange (*Citrus sinensis* Osbeck cv. Mosambi) which can be used in plant transformation. Surface sterilization of seeds was carried out by using 0.1% $HgCl_2$ at different time intervals and best results were obtained with 0.1% $HgCl_2$ for 5 minutes exposure time. The seeds were allowed to germinate *in vitro* and were maintaining in the dark for three weeks, followed by one week at 16h photoperiod at 27 ± 2 ºC. For shoot induction the epicotyls segments were cultured on MS medium with different concentrations of BAP and after adventitious bud growth, the shoots were transferred to full strength MS and half strength MS medium with different concentrations of NAA or IBA for rooting. The best results for morphogenetic responses were obtained with 1 mg l^{-1} BAP and for rooting the ½ MS medium supplemented with 1 mg l^{-} IBA was the best. The protocols presented in this work will be suitable for with genetic transformation experiments for this cultivar.

Keywords: Auxins, cytokinins, epicotyl segments, *in vitro* regeneration, Mosambi

Introduction
Sweet orange is highly popular and commercially cultivated for its processing quality, fresh consumption and aromatic flavor. Despite its excessive cultivation, citrus plantations still has some problems such as slow growth and long juvenility, insects-pests, diseases, alternate bearing, pre and post-harvest losses, large number of seeds per fruit, short season of supply and short storage life etc. The recent advances of plant biotechnology open a wide scope of introducing exogenous genes in the plant genome, using gene transfer techniques to overcome the various mentioned biotic, abiotic and physiological stresses. However, for efficient transgenic plant production, defined tissue culture system for better plant regeneration, in association with a genetic transformation system for the gene introduction are necessary (Pérez-Molphe-Balch and Ochoa-Alejo 1997) [21]. During the last decades, it has become possible to regenerate whole plantlets from a wide range of citrus explants, among which epicotyls and internodal segments have been proved to be more satisfying (Tallon *et al*. 2013) [25]. In citrus, some reports have shown the results of experimentation aiming for the efficient regeneration of plants through *in vitro* organogenesis, for use with *Agrobacterium tumefaciens* or particle bombardment for genetic transformation. Pérez-Molphe-Balch and Ochoa-Alejo 1997 [25] studying the effect of BAP and NAA combinations established protocols for plant regeneration of Mexican lime (*Citrus aurantifolia* Christm.) and Monica mandarin (*C. reticulata* Blanco) by direct organogenesis in internodal segments of *in vitro* germinated plantlets. (Ghorbel *et al.* 1998) [7], using combinations of the same growth regulators, defined protocols for grapefruit (*C. paradisi* Macf.), sour orange (*C. aurantium* L.) and alemow (*C. macrophylla* Wester) by organogenesis, from internodal segments of juvenile plantlets grown in the greenhouse. However, such protocol for the important sweet orange cultivar of Punjab i.e. Mosambi is not available. Thus, the present work aimed to establish efficient protocols for plant regeneration by organogenesis of sweet orange (*C. sinensis* Osbeckia). So to undertake citrus rootstocks improvement programme, an effective regeneration system is the prerequisite. In order to develop this base the present study was undertaken with the objectives to develop in vitro regeneration system for citrus rootstock Cleopatra.

Materials and Methods
Plant material and explant preparation
The fresh fruits of Mosambi were washed with water containing 1 to 2 drop of Teepol

and then washed thoroughly with running tap water. After squeezing off the juice, the seeds were removed from the fruit and testa of the seeds was peeled off. The surface sterilization of seeds were carried out by using freshly prepared 0.1% mercuric chloride for different time intervals 5 min followed by rinsing with sterile distilled water thrice to remove the toxic effects of the sterility. The de-coated seeds were cultured in MS supplemented with 3% sucrose and 0.8% agar. MS was supplemented with 100 mg/l myo-inositol. The pH of the medium was adjusted to 5.8 before autoclaving. The epicotyl explants with three different cuts (i.e. transverse, oblique and longitudinal, hypocotyls and cotyledon explants from these seedlings were used for regeneration studies.

Seeds of Mosambi were extracted from ripe fruits collected from experimental orchard of Department of Fruit Science, Punjab Agricultural University, Ludhiana. Seed integuments were removed and disinfestation was done in 0.1 % mercuric chloride for 5 minutes. Three washes in distilled and sterilized water were done before the seeds were cultured in test tubes (150 x 25 mm) containing 15 ml of MS medium (Murashige and Skoog, 1962) [16], supplemented with 30 g l^{-1} sucrose. The seeds were maintained at 27 ± 2°C in the dark for three weeks, followed by one week under a 16-h photoperiod. After this period, epicotyls approximately 1.0 cm long were excised.

Shoot regeneration

The epicotyl segments were introduced horizontally in Petri dishes (100 x 15 mm) containing 25 ml of MS culture medium solidified with 0.8% agar and supplemented with 30 g l^{-1} sucrose and different concentrations of benzyl amino purine (BAP): 0.0, 0.25, 0.5, 1.0, 1.5; 2.0 mg l^{-1} and kinetin: 0.0, 0.25, 0.5, 1.0, 1.5; 2.0 mg l^{-1}. Cultures were maintained at 27 ± 2°C, under 16-h photoperiod for 45 days. The adventitious buds were transferred to MS medium with 30 g l^{-1} sucrose and 0.8% agar. A completely randomized factorial was used, with 10 different concentrations of growth regulators, and 10 replications. Each replication consisted of six Petri dishes, with 3 segments each. The observations were recorded for the evaluation of suitable shoot regeneration media combination and explant: time taken for initiation and completion of regeneration; number of adventitious buds produced/ explant; number of shoots produced per explant; shoot length; average leaf number per shoot; leaf size; regeneration percentage.

Rooting of adventitious shoots

Culture media with auxins were used for rooting of the adventitious shoots. Full and half strength MS medium with 30 g l^{-1} sucrose, 0.8% agar supplemented different concentrations was designed with 10 replications and 2 shoots per replication for root induction. The evaluation was done by the determination of time taken for initiation and completion of rooting, rooting percentage, root length and number of roots/ shoot. The data was transformed by (x + 0.5). For both experiments (induction of adventitious buds and rooting of adventitious shoots), data were subjected to ANOVA with 5% significance level.

Results and Discussion

Surface sterilization of seeds: Surface sterilant mercuric chloride (0.1%) was used in this study with exposure time of 2, 3, 4, 5 and 6 minutes for surface sterilization of Mosambi seeds. The data on per cent contamination and seed germination with mercuric chloride are presented in table 1. The surface sterilization showed an inverse correlation between contamination and the period of exposure. In general, the contamination percentage decreased with an increase in the duration of exposure to the $HgCl_2$ (0.1%). The maximum explant survival of 83.33 per cent was achieved at 5 minutes exposure which consequently reduced the contamination to 8.33 per cent. The contamination percentage reduced further with exposure of 6 minutes; however this proved detrimental and reduced the explant survival to 41.67 per cent compared to survival at 5 min exposure. The reduction in explant survival percentage with increase in the duration of exposure might be due to the phytotoxicity caused by mercury (Hg^{2+}) present in mercuric chloride (Kumar and Tiwari 2001). With respect to contamination percentage and mean explant survival with mercuric chloride (0.1%) with five minutes exposure time proved suitable for both the citrus species. The 0.1 per cent of $HgCl_2$ has proved to be the suitable surface sterilant in rough lemon (Saini and Gill 2010) [23] and three citrus cultivars of Kinnow (Usman *et al.* 2005) [26]. In general, the contamination percentage decreased with an increase in the duration of exposure to the $HgCl_2$ (0.1%).

Table 1: Effect of exposure time of Mercuric chloride ($HgCl_2$) (0.1%) on contamination and per cent survival of Mosambi seeds.

Time (min)	Mosambi			
	Contamination (%)		Survival (%)	
Control	100.00	(87.97)	0.00	(0.64)
2	41.67	(40.20)	56.67	(48.87)
3	26.67	(30.95)	63.33	(52.91)
4	15.00	(22.60)	75.00	(60.31)
5	8.33	(16.60)	83.33	(66.26)
6	5.00	(10.67)	41.67	(40.17)
LSD (p=0.05)	(5.05)		(5.16)	

Shoot regeneration: The epicotyl segments cultured on MS + BAP 1.0 mg l^{-1} induced shoot initiation at 9.40 days and was significantly earlier as compared to other media combinations (Table 2). The lower and higher concentrations of the BAP and kinetin significantly reduced the shoot initiation time compared to the basal, however the compared to the optimum dose i.e. BAP 1.0 mg l^{-1} the higher concentrations proved detrimental causing late shoot initiation. The similar trend was observed in completion of shoot regeneration with maximum days required for shoot initiation among the different media combinations were observed in MS + Kin 2.0 mg l^{-1} and this was at par with MS + BAP 2.0 mg l^{-1} and MS + Kin 0.25 and 1.5 mg l^{-1}. However, compared to MS basal all the levels of growth regulators proved significantly better in initiation and completion of regeneration. The supplementation of growth regulators in the media had significant effect on the initiation and completion of regeneration. The BAP 1.0 mg l^{-1} caused initiation 13.3 days early as compared to the MS basal providing an advantage of 19.2 days for the completion of

regeneration. The supplementation of 1.0 mg l^{-1} of BAP was found to be superior as compared to all the other concentrations of the BAP as well as kinetin. Usman *et al.* (2005) [26] observed reduction in the time taken for shoot induction in Kinnow and sweet lime in the BA treated media as compared to the MS basal media. Similar observations have been recorded by Normah *et al.* (1997) [18] and Paudyal and Haq (2000) [20]. The higher doses of BAP were inversely proportional to the time required for shoot induction and completion. These results are in tune with those of Usman *et al.* (2005) [26], Normah *et al.* (1997) [18] and Paudyal and Haq (2000) [20].

The number of adventitious buds obtained per explants showed that the highest adventitious buds per explant (4.04) were found on BAP 1 mg l^{-1} and this was significantly different from other media combinations. The number of adventitious buds was directly proportional to the concentration of different growth regulators in the media. The number of buds increased with the increase in the concentration of BAP or kinetin, however at concentration of >1.0 mg l^{-1} the number of buds per explants decreased. Dejam *et al.* (2002) [6] reported that the maximum number of adventitious buds were produced with 4 mg l^{-1} BAP, but most of these buds were quicken due to higher concentrations of BAP which had an inhibitory effect on shoot elongation. The isolated effect of BAP on the number of shoots per explant showed that BAP @ 1 mg l^{-1} provided the best with 3.52 shoots per explants. This response was significantly different from other concentrations. In addition, BAP concentration above 1 mg l^{-1} caused an antagonistic effect resulting in decrease in the number of shoots per explant. Several reports on these detrimental effects of growth regulators such as kinetin and BAP have been cited in context to citrus regeneration (Maggon and Singh 1995 [14], Perez-Molphe-Balch and Ochoa-Alejo 1997 [21]; Ghorbel *et al.* 1998 [7]; Moreira –Dias *et al.* 2000 [15]). The shoot length also responded to the serial concentrations of the kinetin and BAP with longest shoots of 2.82 cm observed in BAP 1 mg l^{-1} which was significantly higher than Kin 1.0 mg l^{-1} (2.52 cm).. Similarly, Singh *et al.* (1994) [24] obtained shoot length of 1.15-2.60 cm in Khasi mandrain cultured on MS medium, containing BAP (0.05-1.0 mg l^{-1}), kinetin (0.5-1.0 mg l^{-1}) and NAA (0.5 mg l^{-1}). Thus, both genotype and growth regulators might be playing an important role in regulating the shoot length of regenerated tissues. In terms of leaf density also the supplementation of BAP 1 mg l^{-1} proved to be the most suitable concentration resulting in 3.25 leaves per epicotyls segment. Further, it was significantly higher than all other media combinations except MS supplemented with BAP 0.5 mg l^{-1} (2.95 leaves/ explant) and kinetin 1.0 mg l^{-1} (2.80 leaves/ explant). Our studies are in tune with those of Rahaman *et al.* (1996) [22] and Kumar *et al.* (2001) [13] reporting maximum number of leaves (3.93) per explants at BAP mg l^{-1} in Mosambi. The maximum lead width (1.49 cm) was also observed on BAP 1mg l^{-1} which was significantly higher than all other media combinations expect Kin 1.0 mg l^{-1}. The level of BAP or kinetin in the medium was directly proportional to leaf density and size which increased with the increase in the concentration of BAP or Kinetin, compared to the basal medium. Although the increase in either BAP or Kinetin level beyond a certain concentration in the medium caused considerable reduction in the shoot length. The BAP and Kinetin at 1.00 mg l^{-1} in the media proved to be the optimal level for most of the regeneration attributes.

The highest shooting percentage (83.89%) was observed on BAP 1 mg l^{-1} which was significantly higher compared to the other media combinations (Fig 1). The increase in the concentration of growth regulators was proportional to increase in the regeneration percentage up to a certain concentration of the growth regulators. However, there was decline in the per cent regeneration at higher levels of growth regulators for both BAP as well as Kinetin and this has been reported by many previous workers (Gloria *et al.* 2000 [10], Beneditu *et al.* 2000 [1], Chandra *et al.* 2003) [3].

Rooting of adventitious shoots

Shoots were considered rooted when a well-developed primary roots and secondary roots were developed. The shoot-lets cultured on ½ MS + IBA 1 mg l^{-1} induced rooting as early as 12.90 days compared to 31.90 days on MS basal. Complete rooting was achieved as early as in 25.60 days on ½ MS+ IBA 1 mg l^{-1} compared to 48.10 days on the MS basal. Thus supplementing with optimum concentration (½ MS+ IBA 1 mg l^{-1}) of growth regulators as well as culture medium concentration provided an advantage of 22.50 days over the basal medium. (Dejam *et al.* 2006) [5]. Also reported better rooting in Bakari micro-shoots on media containing IBA. This has been further supported by the findings of (Kitto and Young 1981) [11] and (Normah *et al.* 1997) [18] reported better rooting with the supplementation of growth regulators such as NAA and IBA in different species of citrus. Among the different levels of IBA and NAA, maximum number of roots (3.70/ planlet) were found on ½ MS+ IBA 1.00 mg l^{-1} and this was significantly superior as compared to all other media combinations expect MS + IBA 1.0 mg l^{-1} (3.15 roots/planlet). For rooting in Bakrai micro-shoots the best result were obtained with IBA (Dejam *et al.* 2006) [6]. The present findings are also in line with those of Dejam *et al.* (2002) [6] and Mooreira-Dias *et al.* (2000) [15] reporting higher number of roots with NAA and IBA supplimentation. The maximum root length (3.89 cm) was also significantly higher on ½ MS+IBA 1.00 mg l^{-1} as compared to all the treatments except MS+NAA 0.50 mg l^{-1} (1.69cm). Similarly Gill *et al.* (1994) [8] obtained 3.1-3.8 cm long roots on MS medium containing NAA (1-2 mg l^{-1}) + IBA (1 mg l^{-1}). Auxins play multiferous role in rhizogenes, which include division of meristematic cells, their elongation and differentiation into primordia (Nanda 1979) [17]. Thus in present findings the number of roots produced per shoot, root length and thickness of roots varied with concentration and combination of auxins used as in the media.

Among the different levels of IBA and NAA maximum rooting percentage (95.37%) was observed in ½ MS+IBA 1 mg l^{-1}, which was significantly superior over all the media combinations (Fig 2). Pervious studies on supplementation of MS media with NAA alone (Gill *et al.* 1995 [9], Das *et al.* 2000) [4], IBA alone (Oh *et al.* 1991) [19] or in combination has induced roots in the shoots of different species. Thus based on the present findings the supplementation of BAP @ 1.0 mg l^{-1} was found to be the most suitable for shoot regeneration. While the combination of ½ MS supplemented IBA @ 1.00 mg l^{-1} was concluded to be the optimum for rooting in Mosambi. The organogenesis protocol developed will be helpful in tissue culture and genetic transformation of the Mosambi.

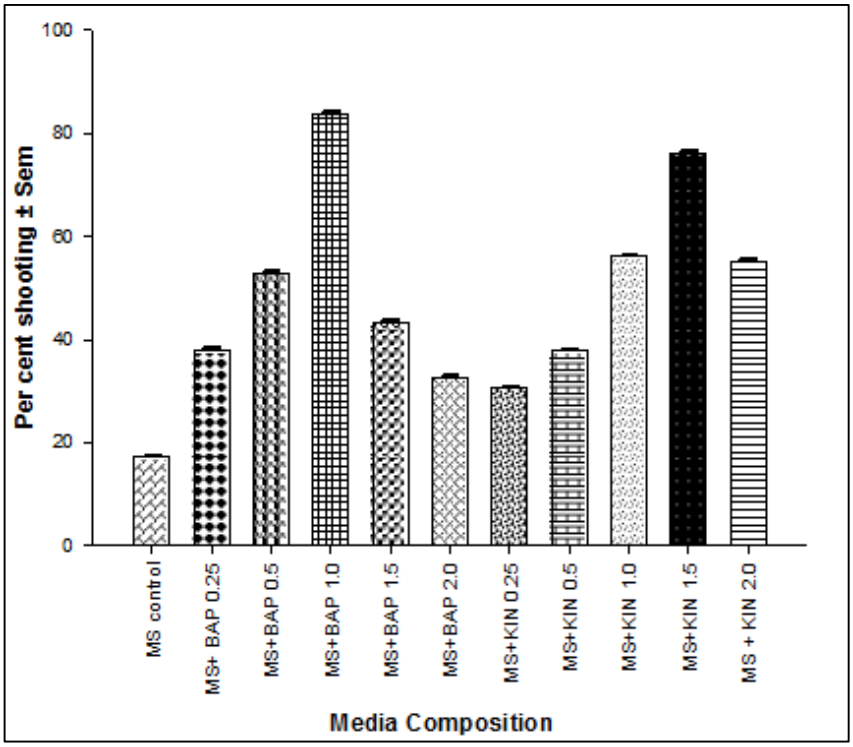

Fig 1: Effect of different concentrations of BAP and Kinetin on per cent shoot regeneration from epicotyl segments

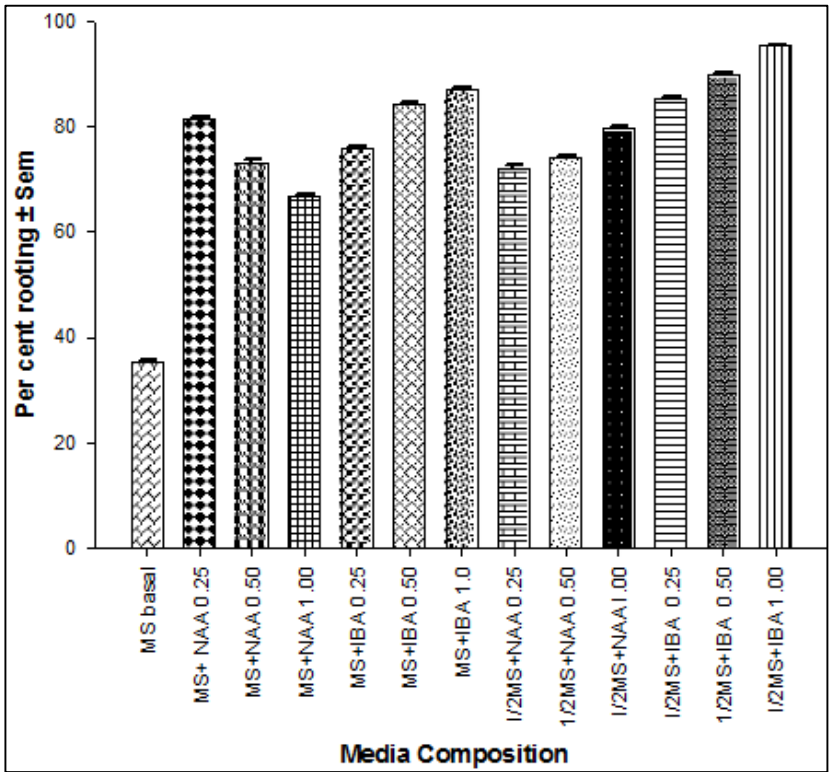

Fig 2: Effect of different concentrations of BAP and Kinetin on per cent rooting

Table 2: Effect of different concentrations of BAP and Kinetin on morphogenetic response in from epicotyl segments in Mosambi

Media	Number of days for initiation of regeneration	Number of Days for completion of regeneration	Number of Adventitious buds/	Number of Shoots/ explant	Shoot length	Leaf density	Leaf length	Leaf breadth
MS control	22.70 ± 0.91	44.30 ± 0.56	0.96 ± 0.08	0.40 ± 0.07	0.31 ± 0.06	1.65 ± 0.15	1.51 ± 0.09	0.74 ± 0.05
MS+ BAP 0.25	12.80 ± 0.57	32.40 ± 0.60	2.04 ± 0.08	1.04 ± 0.10	1.01 ± 0.05	2.55 ± 0.24	1.62 ± 0.08	1.01 ± 0.04
MS+BAP 0.5	11.30 ± 0.50	29.10 ± 0.53	2.84 ± 0.13	1.96 ± 0.11	1.60 ± 0.05	2.95 ± 0.22	1.75 ± 0.03	1.18 ± 0.04
MS+BAP 1.0	9.40 ± 0.45	25.10 ± 0.67	4.04 ± 0.18	3.52 ± 0.14	2.51 ± 0.06	3.25 ± 0.25	2.03 ± 0.04	1.49 ± 0.03
MS+BAP 1.5	13.80 ± 0.66	29.70 ± 0.56	3.20 ± 0.13	1.72 ± 0.12	1.65 ± 0.04	2.50 ± 0.24	1.67 ± 0.04	1.20 ± 0.03
MS+BAP 2.0	15.20 ± 0.80	31.00 ± 0.58	2.94 ± 0.11	1.42 ± 0.13	1.03 ± 0.05	1.90 ± 0.26	1.40 ± 0.03	1.10 ± 0.05
MS+KIN 0.25	15.90 ± 0.64	35.30 ± 0.52	2.72 ± 0.11	0.52 ± 0.10	0.93 ± 0.04	1.60 ± 0.30	1.56 ± 0.04	1.03 ± 0.06

MS+KIN 0.5	13.80 ± 0.66	31.30 ± 0.52	2.40 ± 0.12	0.88 ± 0.09	1.45 ± 0.05	2.00 ± 0.18	1.65 ± 0.03	1.19 ± 0.02
MS+KIN 1.0	12.20 ± 0.65	28.90 ± 0.75	3.12 ± 0.11	1.72 ± 0.11	2.62 ± 0.03	2.80 ± 0.17	1.92 ± 0.05	1.30 ± 0.03
MS+KIN 1.5	14.60 ± 0.65	28.50 ± 0.69	3.00 ± 0.17	2.84 ± 0.18	2.29 ± 0.04	2.05 ± 0.16	1.52 ± 0.07	1.19 ± 0.02
MS + KIN 2.0	16.10 ± 0.64	33.50 ± 0.48	1.96 ± 0.15	1.90 ± 0.12	1.15 ± 0.03	1.70 ± 0.17	1.30 ± 0.04	1.11 ± 0.03
± SEm LSD (p=0.05)	1.83	1.63	0.35	0.33	0.13	0.58	0.15	0.11

Table 3: Root development of *in vitro* regenerated shoots of Mosambi on different levels of NAA, IBA and MS media

Media	Number of days for initiation rooting	Number of Days for completion of rooting	Number of roots/shoot	Root length
MS control	31.90 ± 0.82	48.10 ± 0.64	1.05 ± 0.16	1.13 ± 0.03
MS+ NAA 0.25	22.80 ± 0.55	37.80 ± 0.71	1.90 ± 0.18	1.86 ± 0.11
MS+NAA 0.50	21.10 ± 0.71	36.70 ± 0.70	1.70 ± 0.19	1.69 ± 0.04
MS+NAA 1.00	24.70 ± 0.40	40.10 ± 0.38	1.55 ± 0.16	1.48 ± 0.15
MS+IBA 0.25	20.60 ± 0.56	34.00 ± 1.07	2.55 ± 0.25	2.52 ± 0.15
MS+IBA 0.50	18.70 ± 0.60	33.90 ± 0.71	2.70 ± 0.20	2.88 ± 0.09
MS+IBA 1.0	22.10 ± 0.53	37.70 ± 0.45	3.15 ± 0.18	3.54 ± 0.18
I/2MS+NAA 0.25	20.90 ± 0.53	34.90 ± 0.50	2.30 ± 0.13	2.72 ± 0.09
1/2MS+NAA 0.50	21.10 ± 0.72	35.90 ± 0.43	1.95 ± 0.17	2.50 ± 0.05
I/2MS+NAA I.00	21.60 ± 0.50	34.20 ± 0.55	1.90 ± 0.22	2.13 ± 0.07
I/2MS+IBA 0.25	18.70 ± 0.52	33.30 ± 0.76	2.95 ± 0.25	2.97 ± 0.04
1/2MS+IBA 0.50	17.50 ± 0.57	34.70 ± 0.79	3.05 ± 0.29	3.36 ± 0.10
I/2MS+IBA I.00	12.90 ± 0.75	25.60 ± 0.70	3.70 ± 0.27	3.89 ± 0.10
± Sem LSD (p=0.05)	1.69	1.92	0.60	0.29

References

1. Beneditu VA, Filho AM, Mendes MJ. Callus induction, somatic embryogenesis and protoplast isolation from sweet orange varities. Sci Agric J. 2000; 57:132–38.
2. Bond JE, Rose ML. *Agrobactrium*-mediated transformation of the commercially important citrus cultivar Washington navel orange. Pl Cell Rep. 1998; 18:229-234.
3. Chandra A, Gupta V, Burma P, Pental D. Patterns of morphogenesis from cotyledon explants of Citron (*C. medica* L.). *In Vitro* Cell and Dev Biol. 2003; 39:514-19.
4. Das JM, Moreira Molina RV, Bordon Y, Guardiola JL Luis A, Garcia. Direct and Indirect Shoot Organogenic Pathways in Epicotyl cuttings of *Troyer Citrange* differ in hormone requirements and in their Response to light. Ann Bot. 2000; 85:103-10.
5. Dejam M, Khosh-khui M, Shekafandeh A. Adventitious Bud Induction and Plant Regeneration in Epicotyl Segments of Bakrai (*Citrus reticulata* Blanco C. Umetta Swing). Intl J Agric Res. 2006; 1:14-19.
6. Dejam M, Khoush-Khui M, Shekafandeh A. *In vitro* propagation of sweet orange (*Citrus sinensis* L. Osbeck) by direct organogenesis in epicotyls segments. Iran J Hortic Sci Technol. 2002; 2:85-94.
7. Ghorbel R, Navarro L, Durán-Vila N. Morphogenesis and regeneration of whole plants of grapefruit (*Citrus paradisi*), sour orange (*C. aurantium*) and alemow (*C. macrophylla*). J Hort Sci Biotech.1998; 73:323–27.
8. Gill MIS, Singh Z, Dhillon BS, Gosal SS. Somatic embryogenesis and plantlet regeneration on calluses derived from seedling explants of 'Kinnow' manadrin (*Citrus nobilis* Lour X *Citrus deliciosa* Tenora). J Hort Sci. 1994; 69:231-36.
9. Gill MIS, Singh Z, Dhillon BS, Gosal SS. Somatic embryogenesis and plantlet regeneration in mandarin (*Citrus reticulata* Blanco). Scientia Hort. 1995; 63:167-74.
10. Gloria FJM, Filho FAAM, Camargo LEA, Mendes BMJ. Caipira sweet orange + Rangpur lime: a somatic hybrid with potential for use as rootstock in the Brazilian Citrus Industry. Gen Mol Biol. 2000; 23:661-65.
11. Kitto SL, Young MJ. *In vitro* propagation of Carrizo citrange. Hort Sci. 1981; 16:305-306.
12. Kumar K, Dhatt AS, Gill MIS. In vitro plant regeneration in sweet orange (*Citrus sinensis* L. Osbeck.) cv. Mosambi and Jaffa. Indian J Hort. 2001; 58:208-11.
13. Kumar R, Tiwari JP. Effect of chemicals, light intensity and its duration on *in vitro* establishment of Chinese guava (*Psidium friedrichsthalianum*). Prog Hort. 2001; 33:1-6.
14. Maggon R, Singh B. Promotion of Adventitious bud regeneration by ABA in combination with BAP in epicotyl and hypocotyls explant of sweet orange (*Citrus sinensis* L. Osb.). Scientia Hort. 1995; 63:123-28.
15. Moreira Dias JM, Molina RV, Bordon YLJ, Guardiola JL, Garcia Luis A. Direct and indirect shoot organogenic pathways in epicotyl cuttings of Troyer Citrange differ in hormone requirements and in their response to light. Ann Bot. 2000; 85:103-10.
16. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant. 1962; 15:473-97.
17. Nanda KK. Adventitious root formation in stem cuttings in relation to hormone and nutrition. In: S S Bir (ed.) Recent researches in plant sciences. Kalyani Publishers, New Delhi. 2000, 461-541.1979.
18. Normah MN, Hamida S, Ghani FD. Micropropagation of *Citrus halimii*–an endangered species of South-east Asia. Pl Cell Tiss Org Cult. 1997; 50:225–27.
19. Oh SD, Song, WS, Cho HM. Plant regeneration in dangyooza (*C. grandis*) through embryogenesis. Four effects of plant growth regulators on direct somatic embryogenesis and plant regeneration from immature ovule. Research reports of the Rural Development Administration. Biotechnol. 1991; 33:38-43.
20. Paudyal KP, Haq N. In vitro propagation of pummelo (*Citrus grandis* l. osbeck). In Vitro Cell Dev Biol-Pl. 2000; 36:511-16.
21. Perez-Molphe-Balch E, Ochao-Alejo, N. *In vitro* plant regeneration of Mexican lime and mandarin by direct organogenesis. Hort Sci. 1997; 32:931-34.

22. Rahman AAS, Nagaraju V, Parthasarthy VA. Response of embryoids of certain citrus species to two cytokinins. Ann Pl Physiol. 1996; 10:45-49.
23. Saini HK, Gill MIS. Factors affecting shoot regeneration in rough lemon (*Citrus jambhiri* lush) rootstock. Indian J Plant Physiol. 2010; 15:94-98.
24. Singh S, Ray BK, Bhattacharya, Deka PC. *In vitro* propagation of *Citrus reticulata* Blanco and *Citrus Limon* Burm. Hort Sci. 1994; 29:214-16.
25. Tallon CI, Porras I, Perez-Tornero O. High efficiency in vitro organogenesis from mature tissue explants of Citrus macrophylla and C. aurantium. In Vitro Cellular and Developmental Biology – Plant. 2013; 49:145-155.
26. Tallon CI, Porras I, Perez-Tornero O. High efficiency in vitro organogenesis from mature tissue explants of Citrus macrophylla and C. aurantium. In Vitro Cellular and Developmental Biology – Plant. 2013; 49:145-155.
27. Usman M, Muhammad S, Fatima B. *In vitro* multiple shoot induction from nodal explants of citrus cultivars. J Central European Agri. 2005; 6:435-42.