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Micropropagation of sweet orange cv. Mosambi through shoot tips and nodal segments

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ABSTRACT

The present study was undertaken with an objective to develop a protocol for micropropagation of sweet orange cv. Mosambi through shoot tips and nodal segments. Shoot tips of 0.5-1.0 cm, nodal and internodal segments of 1.0-1.5 cm were collected and cultured in Murashige and Skoog (MS) medium. The treatment NaOCl (1%)7 min.+ $HgCl_2$ (0.2%) 3 min. + KCl (1%) 1 min. was found to be the best sterilization treatment. BAP (2.0 mgl^{-1}) + casein hydrolysate (200 mgl^{-1}) was found to be the best treatment for establishment medium with respect to maximum sprouting, minimum days taken for sprouting with highest number of shoots/explant. BAP (2.0 mgl^{-1}) + Kin (1.0 mgl^{-1}) + NAA (0.1 mgl^{-1}) was observed to be the best treatment for multiplication medium with maximum shoot length and highest number of leaves. IBA (2.0 mgl^{-1}) + NAA (0.1 mgl^{-1}) + activated charcoal (500 mgl^{-1}) was found to be significantly superior over all other treatments with respect to maximum root initiation percentage, days to root initiation, highest number of roots and highest root length for shoot tips and nodal segments.

Key words: Mosambi, sweet orange, micropropagation.

INTRODUCTION

Mosambi is one of the important citrus fruit crop and is preferred due to its low acidity and high juice content. Vegetative propagation methods like budding, cutting, layering are practiced during limited period of the year. Moreover, these methods tend to propagate pathogens present in mother explants. By using tissue culture techniques, thousands of genetically identical elite plants can be vegetatively propagated in short time span and limited space with year round availability.

Sweet orange is susceptible to a number of diseases like *Phytophthora* rot (fungal disease), greening (bacterial) disease and psorosis, exocortis (viral diseases). Propagation through nucellar tissue of fertilized or unfertilized ovules is tedious. Moreover, ovules at right stage of development are available only for a very short period during the year. Juvenile characteristics and delayed bearing are disadvantages in production of true to type and virus free plants. The present study was conducted with an objective to develop a protocol for Mosambi micropropagation through shoot tips and nodal segments.

MATERIALS AND METHODS

Explants *viz.* shoot tips (0.5-1.0 cm) and nodal segments (1.0-1.5 cm) were collected from three years old Mosambi plants growing in Horticulture Garden and the experiment was conducted at the Tissue Culture Laboratory, Dept. of Horticulture, Institute of Agricultural Sciences, Banaras Hindu University. The explants were thoroughly washed under running tap water for 15-20 minutes properly. The explants were further treated with 1% cetrimide solution with 0.1% bavistin for 20 minutes on rotator-shaker. This facilitates proper action of sterilant chemicals on the explants taken. The explants were then washed properly with double distilled water (DDW). The explants were than subjected to different surface sterilants i.e. 1% NaOCl and 0.1% $HgCl_2$ and 1% KCl for different durations (Table 1). The explants were dipped in 70% ethyl alcohol for 30 seconds and finally rinsed thrice with double distilled water. Also, one minute dip in 1% KCl was given to different explants to remove excess mercuric (Hg^{++}) ions from explant tissue.

Murashige and Skoog (MS) medium with 3% w/v sucrose and 0.8% w/v agar was taken for inoculation of explants. The pH of the media was adjusted to 5.8 before autoclaving. Culture tubes/flasks after inoculation were incubated in the growth chamber by maintaining 25±2°C temperature with 16/8 hours day/night regime with 300-3200 lux light intensity supplied through white fluorescent tubes.

BAP along with casein hydrolysate / malt extract was used for shoot proliferation in different concentrations in establishment medium (Table 2). Different growth regulators like BAP, NAA and Kin were supplemented

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to MS basal medium for shoot growth in different concentrations and combinations in multiplication medium (Table 3). *In-vitro* proliferated shoots were transferred to ½ MS medium supplemented with different auxins viz. IBA and NAA along with activated charcoal in different combinations (Table 4).

The rooted plantlets were carefully removed from agar medium and washed in a shallow tray containing sterile water to remove adhering agar. They were then transferred to pot containing sand : soil : compost (1 : 1: 1). The sterilized pots were kept under piant growth chamber with punched polythene covering top of the plants. The plants were kept under such condition for nearly 25-30 days. Then pots were kept under polyhouse conditions for nearly two weeks for hardening and then transferred to the field.

The experiment was subjected to analysis of variance using completely randomized design (CRD). Four replications were taken for each treatment comprising 10 units. The percentage data were subjected to arcsine transformation before statistical analysis.

RESULTS AND DISCUSSION

It is evident from Table 1 that the highest aseptic culture of 88.76% and 92.36% was observed for shoot tips and nodal segments respectively. Similarly, the highest survival percentage of 87.11% and 90.19% was recorded for shoot tips and nodal segments respectively with the treatment NaOCl (1%) for 7 min., $HgCl_2$ (0.2%) for 3 min. and KCl (1%) for 1 min. respectively (Table 1).

The beneficial effects of $HgCl_2$ and NaOCl have been reported earlier by several investigators. In addition to $HgCl_2$ and NaOCl, one min. treatment with 1% KCl improves survival percentage of explant as KCl in known to remove excess Hg^{++} ions. Increase in exposure time of the above surface sterilants drastically affected explant survival. One probable reason for the death of explants when exposed to surface sterilants for longer duration may be due to heavy metal contamination of mercury present in $HgCl_2$, proving detrimental for the survival of explants. The results obtained are in conformity with Rana and Singh (5) and Syamal *et al.* (7) who used NaOCl and $HgCl_2$ as surface sterilants for sterilizing citrus explants.

From Table 2 it is clear that for shoot tips, the treatment BAP (2.0 mgl^{-1}) + malt extract (500 mgl^{-1}) gave maximum (85.20%) sprouting of explants in the establishment medium. Treatment BAP (2.0 mgl^{-1}) + casein hydrolysate (200 mgl^{-1}) was found to be best with respect to maximum sprouting (88.29%) for nodal explants. The same treatment took minimum days for sprouting i.e. 11.98 and 11.14 days and highest no. of 3.95 and 4.57 shoots/explant for shoot tips and nodal segments respectively. Malt extract and casein hydrolysate help in shoot proliferation, multiple shoot induction and early sprouting of explants. Kour *et al.* (2)

Table 1. Effect of exposure time of surface sterilants on per cent aseptic cultures and per cent survival of explants.

Exposure time (in min.)			Percent aseptic cultures		Percent explant survival	
NaOCl (1%)	$HgCl_2$ (0.2%)	KCl (1%)	Shoot tip	Nodal segment	Shoot tip	Nodal segment
0	0	0	0.00 (0.00)*	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
1	1	-	10.00 (18.43)	10.23 (18.65)	12.19 (20.43)	15.26 (22.99)
2	1	-	12.16 (20.40)	15.72 (23.36)	18.19 (25.25)	20.25 (26.74)
3	2	1	40.14 (39.31)	45.68 (42.52)	40.04 (39.25)	45.29 (42.30)
4	3	1	49.15 (44.51)	50.18 (45.10)	48.19 (43.96)	50.19 (45.11)
5	3	1	59.20 (50.30)	61.19 (51.47)	63.18 (52.64)	65.24 (53.87)
5	4	1	65.25 (53.87)	68.27 (55.71)	65.24 (53.87)	67.29 (55.12)
5	5	1	68.19 (55.66)	70.18 (56.90)	68.72 (55.99)	70.18 (56.90)
6	2	1	72.14 (58.14)	75.16 (60.11)	72.18 (58.17)	74.18 (59.46)
6	3	1	79.14 (62.83)	82.29 (65.11)	77.29 (61.54)	80.17 (63.56)
7	2	1	80.16 (63.54)	85.24 (67.41)	80.15 (63.54)	84.25 (66.17)
7	3	1	88.76 (70.41)	92.36 (73.95)	87.11 (68.95)	90.19 (71.75)
8	2	1	76.25 (60.83)	84.16 (66.55)	74.15 (59.44)	82.17 (65.02)
9	2	1	64.25 (53.28)	68.71 (55.99)	62.29 (52.11)	65.81 (54.22)
10	2	1	55.24 (48.58)	56.25 (48.59)	50.08 (45.05)	55.42 (48.11)
CD at 5%			2.84	2.80	3.13	3.38

*Figures in parentheses show arc sine transformation value

Table 2. Effect of BAP, casein hydrolysate and malt extract on sprouted explants percentage, days to shoot proliferation and number of shoots per explants in establishment medium.

BAP (mgl^{-1})	Casein hydrolysate (mgl^{-1})	Malt extract (mgl^{-1})	Sprouted explants percentage		Days to shoot proliferation		Number of shoots per explant	
			Shoot tip	Nodal segment	Shoot tip	Nodal segment	Shoot tip	Nodal segment
0.0	0.0	0.0	0.00(0.00)*	0.00 (0.00)	0.0	0.0	0.0	0.0
0.5	100	0.0	49.14 (44.51)	58.16 (49.70)	21.17	20.27	1.25	1.55
1.0	200	0.0	68.29 (55.73)	70.18 (56.90)	15.92	15.17	2.58	2.97
2.0	100	0.0	78.16 (62.14)	80.18 (63.56)	13.17	12.95	3.47	3.85
2.0	200	0.0	85.16 (67.34)	88.29 (69.99)	11.98	11.14	3.95	4.57
2.0	300	0.0	81.12 (64.25)	83.16 (65.77)	15.08	14.75	2.98	3.42
2.5	200	0.0	73.16 (58.80)	75.19 (60.13)	16.17	15.26	1.88	2.10
3.0	200	0.0	62.15 (52.03)	65.14 (53.81)	18.95	18.14	1.56	1.97
0.5	0.0	200	48.19 (43.96)	50.17 (45.10)	22.18	21.17	1.21	1.48
1.0	0.0	300	70.26 (56.95)	75.24 (60.16)	18.16	17.25	2.44	2.67
2.0	0.0	400	80.14 (63.54)	82.39 (65.19)	14.01	12.98	3.19	3.52
2.0	0.0	500	85.20 (67.37)	87.25 (69.08)	12.05	11.58	3.76	4.32
2.0	0.0	600	72.29 (58.24)	75.68 (60.45)	15.79	15.16	2.81	3.33
2.5	0.0	500	68.27 (55.72)	70.17 (56.90)	17.55	16.28	2.01	2.17
3.0	0.0	500	60.76 (51.21)	63.29 (52.71)	19.17	18.92	1.75	1.93
CD at 5%			2.74	2.99	2.90	2.74	0.15	0.24

*Figures in parentheses show arc sine transformation value

Table 3. Effect of BAP, Kin and NAA on shoot length and number of leaves in multiplication medium.

BAP (mgl^{-1})	Kin (mgl^{-1})	NAA (mgl^{-1})	Shoot length (cm)		Number of leaves	
			Shoot tip	Nodal segment	Shoot tip	Nodal segment
0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.5	0.0	0.1	0.67	0.87	1.77	1.92
1.0	0.5	0.2	1.63	1.88	2.67	2.70
2.0	0.5	0.1	2.79	3.01	3.25	3.80
2.0	1.0	0.1	3.85	3.92	3.92	4.15
2.5	0.5	0.1	2.18	2.25	2.63	2.77
3.0	0.5	0.1	1.47	1.52	1.33	1.53
0.0	0.5	0.1	0.61	0.78	0.93	1.14
0.5	1.0	0.1	1.03	1.25	1.62	1.76
0.5	2.0	0.1	2.50	2.90	3.21	3.64
1.0	2.0	0.1	2.98	3.25	3.77	3.97
1.0	2.0	0.5	2.68	2.77	2.97	3.42
1.0	2.5	0.1	1.33	1.50	2.01	2.25
0.5	2.5	0.1	0.72	0.97	1.66	1.75
0.5	3.0	0.1	0.66	0.72	0.77	0.98
CD at 5%			0.17	0.11	0.10	0.09

had also reported best shoot proliferation with BAP (1.5 mgl^{-1}) + malt extract (500 mgl^{-1}) which resulted in the greatest no. of 5.34 shoots with shoot length of 1.96 cm and 8.88 no. of leaves.

From Table 3 it is evident that the treatment BAP (2.0 mgl^{-1}) + Kin (1.0 mgl^{-1}) + NAA (0.1 mgl^{-1}) gave maximum shoot length of (3.85 cm) and (3.92 cm) with highest number of leaves (3.92) and (4.15) for shoot tips and nodal segments respectively in the multiplication medium (Fig. 1). BAP (1.0 mgl^{-1}) + Kin (2.0 mgl^{-1}) +NAA (0.1 mgl^{-1}) and BAP (2.0 mgl^{-1}) + Kin (0.5 mgl^{-1}) +NAA (0.1 mgl^{-1}) were found to be the next best treatments. The results found in the present study closely resemble with the earlier findings of Rana and Singh (5) and Parthasarathy *et al.* (4).

Cytokinins promote shoot proliferation by inducing cell division, cell and organ enlargement. BAP has been reported to be the best cytokinin for citrus shoot proliferation. The synthetic cytokinins 6-BAP and Kinetin influence inhibition of viruses in certain host systems (Syamal *et al.*,7). 6-benzyladenine is more active than kinetin in inhibiting virus increases and local lesions in various plant materials. The efficacy of BAP was further enhanced when small amount of NAA was added to the medium. The inclusion of auxin to the medium has been found to be beneficial for shoot production in some cases as reported by Bhansali and Arya,1 and Starrantino and Carus, 6. Cytokinins seem to act either by removing free radicals or by preventing their formation. Auxins donot promote shoot proliferation, they are required in culture medium to promote growth of shoots by counteracting suppressive effect of high cytokinin on shoot elongation.

It is evident from Table 4 that the treatment IBA (2.0 mgl^{-1}) + NAA (0.1 mgl^{-1}) + activated charcoal (500 mgl^{-1}) was found to be significantly superior over all other treatments for shoot tips and nodal segments (Fig. 2 and 3). This best treatment gave maximum root initiation of 86.27% and 88.17%, minimum of 15.17 and 14.18 days taken for root initiation, highest number of 3.12 and 3.25 roots with maximum root length of 2.83 cm and 2.92 cm for shoot tips and nodal segments respectively. Treatment combinations IBA (0.5 mgl^{-1}) + NAA (2.0 mgl^{-1}) + activated charcoal (500 mgl^{-1}) and IBA (0.1 mgl^{-1}) + NAA (2.0 mgl^{-1}) + activated charcoal (500 mgl^{-1}) also gave good results. The above results have some resemblance with the earlier findings of Rana and Singh (5) and Kumar *et al.* (3) who has reported good roots initiation results with IBA and NAA combinations.

Auxins promote adventitious root development on intact plants as well as excised stems. Of these, IBA is the most effective one than any other growth regulator

Table 4. Effect of IBA, NAA and activated charcoal on root initiation percentage, days to root initiation, number of roots and root length.

IBA (mgl^{-1})	NAA (mgl^{-1})	Activated charcoal (mgl^{-1})	Root initiation percentage		Days to root initiation		Number of roots		Root length (cm)	
			Shoot tip	Nodal segment	Shoot tip	Nodal segment	Shoot tip	Nodal segment	Shoot tips	Nodal segment
0.0	0.0	0.0	0.00 (0.00)*	0.00(0.00)	0.0	0.0	0.0	0.0	0.0	0.0
0.1	0.0	0.0	0.00 (0.00)	0.00(0.00)	0.0	0.0	0.0	0.0	0.0	0.0
0.5	0.1	100	20.17 (26.69)	22.16(28.08)	23.16	22.17	0.83	0.98	0.69	0.72
1.0	0.2	200	38.76 (38.50)	42.17(40.56)	21.69	20.68	1.2	1.25	1.48	1.66
1.5	0.3	200	70.25 (56.94)	74.27(59.52)	18.96	18.15	2.09	2.26	1.83	1.97
2.0	0.5	300	79.29 (62.93)	80.19(63.57)	16.92	16.28	2.95	3.12	2.21	2.42
2.0	0.1	500	86.27 (68.25)	88.17(69.88)	15.17	14.18	3.12	3.25	2.83	2.92
2.5	0.1	500	72.1. (58.14)	75.24(60.16)	16.90	16.29	2.39	2.56	2.31	2.38
3.0	0.1	500	60.2. (50.91)	63.22(52.67)	22.14	20.17	1.14	1.21	1.10	1.15
0.0	0.5	100	10.17 (18.60)	12.41(20.63)	24.19	23.14	0.40	0.50	0.40	0.45
0.5	1.0	200	18.19 (25.25)	20.25(26.74)	21.10	20.15	1.17	1.27	1.43	1.52
1.0	1.5	300	50.26 (45.15)	54.26(47.44)	17.96	17.25	2.43	2.52	2.03	2.16
0.1	2.0	500	75.18 (60.12)	78.15(62.13)	16.57	15.92	2.73	2.98	2.32	2.62
0.5	2.0	500	82.16 (65.01)	84.19(66.57)	16.01	15.24	2.97	3.15	2.55	2.84
0.5	2.5	500	58.72 (50.02)	63.77(52.96)	20.01	19.82	1.43	1.57	0.92	1.12
CD at 5%			4.33	3.90	0.87	0.90	0.15	0.13	0.11	0.10

*Figures in parentheses show arc sine transformation value

in most of the cases apparently because it is not destroyed by IAA oxidase or other enzymes and therefore persists longer. Auxins stimulate cell expansion by cell wall loosening after a short time following exposure, in addition, auxins promote protein synthesis by controlling gene expression as a response after a long time interval.

Activated charcoal may also induce the formation of adventitious roots in some species. Its presence in the medium reduces the light supply to *in vitro* regenerated shoots and helps in improvement of absorption of growth regulators in cultures.

The complete plantlets with well developed roots were transferred to glass jars filled with mixture of sand : soil : compost (1:1:1) before transferring to field directly (Fig. 4).

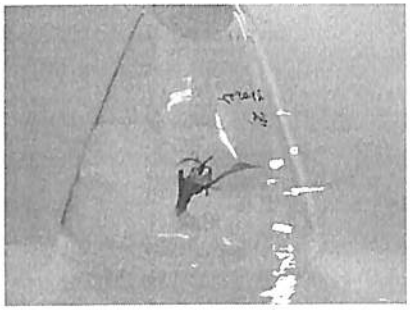

Fig.1. Effect of BAP (2.0 mgl^{-1}) + Kin (1.0 mgl^{-1}) + NAA (0.1 mgl^{-1}) on growth of shoots in multiplication medium.

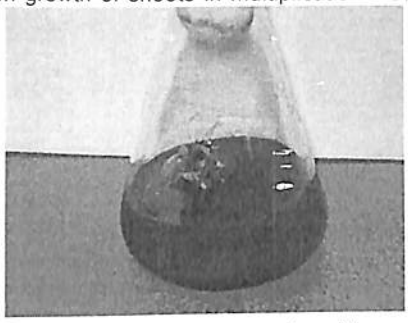

Fig.2. Grown shoots of explant cultured in root initiation medium IBA (2.0 mgl^{-1}) + NAA (0.1 mgl^{-1}) + activated charcoal (500 mgl^{-1}).

Fig. 3. Subculturing of grown roots by application of treatment IBA (2.0 mgl^{-1}) + NAA (0.1 mgl^{-1}) + activated charcoal (500 mgl^{-1}) in ½ MS medium.

Fig. 4. Hardening of tissue culture grown Mosambi plant in soil : sand : compost (1:1:1) medium.

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