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Indirect somatic embryogenesis in sweet orange cv. "Mosambi"

Reena Prusty, O. P. Awasthi*, S.K. Singh and C. Bharadwaj[1]

Division of Fruits & Horticultural Technology, ICAR-Indian Agricultural Research Institute, New Delhi-110012, Delhi, India.

ABSTRACT

This study was carried out to standardize a reliable protocol by using different explants (epicotyl, cotyledon, and root), plant bioregulators (PBRs), and carbon sources on indirect embryogenesis during 2019-2022. Among the treatments tested, T4 (MS + 2,4-D (1.5 mg L-1) + BAP (1.0 mg L-1) + ME (500 mg L-1)) proved best for callusing (90.89 %), callus fresh weight (0.83 g), turgid weight (0.84 g) and dry weight (0.08 g) as compared to others. In terms of callusing (83.72%), callus fresh weight (0.67 g), turgid weight (0.68 g), dry weight (0.05 g) and callus water content (11.28 %), explants epicotyl (E) showed its superiority over others. Similarly, the treatment combination T4 × E surpassed others in callusing potential. Embryogenesis (59.09%) and germination (33.61%) were best in T7 (MS + BAP (2 mg L-1) + NAA (0.1 mg L-1) + ME (500 mg L-1)). Among the different carbon sources, 5% glycerol supplemented with T7 proved best in inducing the highest number of somatic embryos/callus mass (73.26), embryogenesis (65.27%), and plantlet formation (68.77%). Therefore, the standardized protocol can be used for indirect embryogenesis for different genotypes of sweet orange.

Keywords: *Citrus sinensis* L., Epicotyl, Callus, Plantlets.

INTRODUCTION

Citrus, one of the important fruit crops of the world is well known for its high nutritional properties. Among the group of citrus fruits, the area and production of sweet orange (*Citrus sinensis* L.) cv. "Mosambi" has seen an escalating trend. Due to desirable nutritional and medicinal properties, it has been the most preferred fruit among the consumers in the present pandemic. In the recent past, there has been a great demand for the planting materials which, however, are difficult to achieve through the traditional methods of propagation. In this direction, biotechnological tools such as tissue culture can play a vital role in supplying the quality planting material throughout the year. Although, tissue culture has plethora of advantages over the conventional approaches, selection of quality planting materials under *in vitro* necessitate the requirement of a well-developed reliable protocol. In this aspect, somatic embryogenesis is an efficient method of plant regeneration for the production of large numbers of plantlets within a shorter period (Gholami *et al*., 5). The standardization of protocol, however, varies with the species, genotype even the plant parts used, which majorly depends upon the plant bioregulators (PBRs) and additives, keeping other factors in a line (Kayim and Koc, 9). So the present experiment was, therefore, carried out intending to develop a reliable protocol using different explants through somatic embryogenesis following an indirect pathway.

*Corresponding author: awasthiciah@yahoo.com

[1]Division of Genetics, ICAR-IARI, New Delhi-110012, Delhi, India.

MATERIALS AND METHODS

The experiment was carried out at Central Tissue Culture Laboratory, ICAR-National Institute of Plant Biotechnology during 2019-2022. Fully matured ripe fruits were collected from the mother block of sweet orange cv."Mosambi", maintained at the experimental fruit orchard of the Division of Fruits and Horticultural Technology, ICAR-IARI, New Delhi. The collected fruits were kept in an ice box, and immediately brought to the laboratory. The fruits were washed with tap water and treated with 0.1% carbendazim (Bavistin BASF, India) + 0.1% metalaxyl + mancozeb (Ridomil Gold®, Syngenta India) for 1 h. The fruits were taken under the laminar air-flow and surface sterilized with 1% NaOCl solution for 5 min, followed by a quick dip in ethanol (70%, v/v) and subjected to flame sterilization, thereafter. The fruits were cut open and seeds were extracted with the sterilized scalpel and forceps. The seeds were cleaned by rinsing with sterilized autoclaved double distilled water, and cultured in glass jars containing basal MS medium supplied with 30g L^{-1} sucrose for germination in dark. The 15-day-old germinating seedlings were used as an explant source. Nucellar plants were identified based on their development from slow-growing pro-embryos, which were uniform in growth having multiple cotyledons, and were used for *in vitro* culture (Obukosia and Waithaka, 12). The seedlings were trimmed from both ends, and the epicotyls and roots were cut longitudinally into two sections (1-2 cm). The cotyledons were sliced into a thickness of 0.1cm. The explants (epicotyl, cotyledon and root) were excised

and cultured in the Petri-dish (90×15 mm) containing MS medium (Murashige and Skoog, 11) with different plant bioregulators (PBRs) with the cut end, touching the surface of the medium for callusing. For induction of callus, the cultures were maintained in the dark, and the data on callusing were recorded 4 weeks after culture initiation. Callus was sub-cultured fortnightly. The data on fresh weight (FW), turgid weight (TW), dry weight (DW), callus water content (CWC), and relative growth rate (RGR) were recorded 8 weeks after callusing. The callus TW (g) was recorded by keeping the callus clump in the water, and the data were recorded after 24 h. The DW (g) was recorded by drying the callus for 48 h in a hot air oven (70±5°C) until constant weight. The CWC was calculated using the formula of Errabii *et al*. (3). The RGR was calculated using the formula, given by Patade *et al.* (13).

Embryogenesis was attempted using epicotyl callus, since it had superior growth as compared to the cotyledon and root explants, when cultured on MS basal medium supplemented with different PBRs. After attaining optimum the size (5-10 mm), the embryos were transferred onto the basal medium supplemented with 1.0 mg L^{-1} GA_3 (gibberellic acid) for enhancing germination. Furthermore, the effect of five different carbon sources (sucrose, glucose, maltose, galactose, and glycerol) were studied using different concentrations (4, 5 and 6%, w/v) to standardize the regeneration medium for indirect somatic embryogenesis. All the media combinations were fortified with 5% sucrose, 500 mg L^{-1} malt extract (ME) and 0.7% agar-agar (Hi-Media, Mumbai). The pH of the culture media was adjusted to 5.7±0.5, and sterilized at 121°C for 20 min, thereafter. Each treatment consisted of 5 explants/ petri-dishes and replicated five times. The cultures were maintained in the laboratory (24±2°C), and programmed to maintain a 16/ 8 hours light/ dark cycle by the use of cool white fluorescent lights (54 µmol $m^{-2}s^{-1}$). The statistical analysis of the regeneration and carbon sources comprising different treatment combinations were analyzed in a completely randomized design (CRD) using statistical analysis system software, SAS package (9.3 SAS Institute, Inc. USA), followed by a t-test (LSD). The first experiment with two factors, *i.e.*, PBRs and explants were analyzed in two factors CRD, where $P \leq 0.05$ were considered significant.

RESULTS AND DISCUSSION

A perusal of data presented in Table 1 showed a significant influence of different treatments and explants used, on the physical parameters of callus. The shortest period for callus induction (13.69 days) and highest callusing (90.89%) were recorded in T_4, while highest CWC (12.01 %) was noted in T_2 having similarity statistically with T_1 and T_3. Treatment T_6 took the longest period (19.34 days) for callus induction, while the least callusing (44.55%) was observed in T_{10}. The lowest CWC (8.22 %) was recorded in T_5. Epicotyl showed promising results on callus induction (83.72%) followed by cotyledon (57.98%), and the lowest callusing

Table 1. Effect of different PGRs and explants on *in vitro* callus induction and its morphological parameters in sweet orange cv. Mosambi.

Treatment (T)	Days taken for callusing	Callusing (%)	CWC (%)
T_1- MS+2,4-D (1 mg L^{-1})+BAP (0.5 mg L^{-1})+ME (500 mg L^{-1})	16.56^{ed}	65.00^{d} (53.98)*	11.51^{a}
T_2- MS+2,4-D (1 mg L^{-1})+BAP (1 mg L^{-1})+ME (500 mg L^{-1})	18.68^{ba}	59.95^{e} (50.93)	12.01^{a}
T_3- MS+2,4-D (1.5 mg L^{-1})+BAP (0.5 mg L^{-1})+ ME (500 mg L^{-1})	16.95^{edc}	87.41^{b} (69.27)	11.85^{a}
T_4- MS+2,4-D (1.5 mg L^{-1})+BAP (1 mg L^{-1})+ME (500 mg L^{-1})	13.69^{f}	90.89^{a} (72.42)	8.96^{de}
T_5- MS+2,4-D (2 mg L^{-1})+BAP (0.5 mg L^{-1})+ME (500 mg L^{-1})	18.32^{bac}	77.07^{c} (61.94)	8.22^{e}
T_6- MS+2,4-D (2 mg L^{-1})+BAP (1 mg L^{-1})+ME(500 mg L^{-1})	19.34^{a}	66.60^{d} (54.99)	9.80^{cb}
T_7- MS+2,4-D (1 mg L^{-1})+KIN (0.5 mg L^{-1})+ ME (500 mg L^{-1})	17.13^{bedc}	78.81^{c} (63.58)	10.47^{b}
T_8- MS+2,4-D (1 mg L^{-1})+KIN (1 mg L^{-1})+ ME (500 mg L^{-1})	18.53^{bac}	55.04^{e} (49.39)	10.09^{cb}
T_9- MS+2,4-D (1.5 mg L^{-1})+KIN (0.5 mg L^{-1})+ ME (500 mg L^{-1})	17.25^{bedc}	58.35^{e} (50.33)	8.88^{e}
T_{10}- MS+2,4-D (1.5 mg L^{-1})+KIN (1 mg L^{-1})+ ME (500 mg L^{-1})	16.43^{ed}	44.55^{g} (42.89)	9.68^{cd}
T_{11}- MS+2,4-D (2 mg L^{-1})+KIN (0.5 mg L^{-1})+ ME (500 mg L^{-1})	15.77^{e}	50.21^{g} (44.46)	9.41^{cde}
T_{12}- MS+2,4-D (2 mg L^{-1})+KIN (1 mg L^{-1})+ ME (500 mg L^{-1})	17.63^{bdc}	53.04^{f} (46.82)	9.47^{cde}
Mean	17.19	65.58 (55.08)	10.03
LSD (P≤0.05)	1.65	1.99	0.75

Data presented in parenthesis are Arc Sin transformed values; Mean values in a column are separated by different alphabetical letter(s) in superscript are significant as per Tukey's test. 2,4-D (2,4- Dichlorophenoxy acetic acid); BAP (6- Benzylaminopurine); CWC (Callus water content)

(53.22%) was observed in root (Fig. 2A). Amongst explants the highest CWC (11.29 %) and the shortest period for callusing (15.01 days) were observed in epicotyl segments, while the lowest CWC (9.33 %) was noted in the cotyledon. The longest period for callus induction (18.51 days) was recorded in roots (Fig. 2B).

The highest FW (0.83g), TW (0.84g) and DW (0.08g) was documented in T_4havingsimilarity statistically with T_1, while the lowest FW (0.28g) and TW (0.29g) was recorded in T_{10} and DW (0.027g) in T_8 (Fig. 1). RGR was witnessed to be maximum (3.82%) in the treatment T_8. Similarly, among tested explants, epicotyl exhibited maximum FW (0.67g) and TW (0.68g) with minimum RGR (2.31%). The DW was recorded highest in the epicotyl (0.054g) which maintained the statistical parity with cotyledon (0.057g) (Fig. 2C). Contrary to the higher values, obtained concerning the parameters mentioned above in the epicotyl segments, FW (0.42g), TW (0.43g) and DW (0.039g) exhibited lower values except for RGR (2.82%) in roots. The interaction between the treatments and explants showed the significant variations for different callus parameters (Table 2). The highest callusing (99.70%), FW (1.20g), TW (1.23g) and DW (0.11g) with a minimum period for callusing (10.48 days) was noted in T_4×E. The CWC (21.80 %) and RGR (5.15%) were highest in T_3×E and T_3×C, respectively.

The superior effect of treatment T_4 on callus induction was possibly due to the addition of cytokinin in lower concentration that stimulated cell division, and resulted in a rapid upsurge of the callus mass to the auxin-rich medium. The findings of the study agree with the earlier reports of Hidaka and Omura (8) and Grosser *et al.* (7) in citrus. Similar to our result, Krishan *et al.* (10) also reported that the callusing and embryogenic potential of epicotyl was best among the explants undertaken (leaf and epicotyl) in *C. jambhiri*.

The callus derived from epicotyl exhibited the profuse callusing with nodular structure of embryonic origin. Conversely, the callus produced from the cotyledons and roots gave rise to yellowish-white compact and whitish friable callus, and were, therefore discarded because of its splitting and non-embryonic nature (Fig.4. A-C). Thus, the epicotyl segment derived callus clump was used for regeneration through embryogenesis, supplemented with different PBRs to the MS medium. Embryogenesis capacity of the callus was characterized by the changes in the colour of the callus from yellowish white to greenish globular structure. For embryogenesis, different cytokinins (BAP and kinetin) and auxins (2,4-D and NAA) were supplied at different concentrations (0.1-3.0 mg L^{-1}) alone or in combinations (Fig. 3). Of the different treatments tested, promising results on embryogenesis (59.09%) and germination (33.61%) were noted in T_7, while lowest embryogenesis (20.34%) and germination (11.84%) were noticed in T_3. Supplementation of BAP (T_4) alone resulted in poor embryogenesis (26.64%) and maturation of embryo (19.17%), while BAP and auxin when fortified at equal concentrations (T_3), drastically suppressed the regeneration. The different stages of embryo regeneration (globular, heart, torpedo and cotyledon) were observed as non-uniform in nature (Fig. 4 D-E). However, the primary embryos, when sub-cultured in the same media fortnightly produced the secondary embryos (Fig 4 F). The embryos were then germinated, and developed into plantlets (Fig. 4G- I).

The positive role of BAP on somatic embryogenesis has also been reported by Sajeva *et al.* (15). MS medium containing malt extract along with cytokinins (BAP and Kinetin) and/or NAA have been reported to give significantly higher mean plant regeneration as compared to medium containing cytokinins and auxins

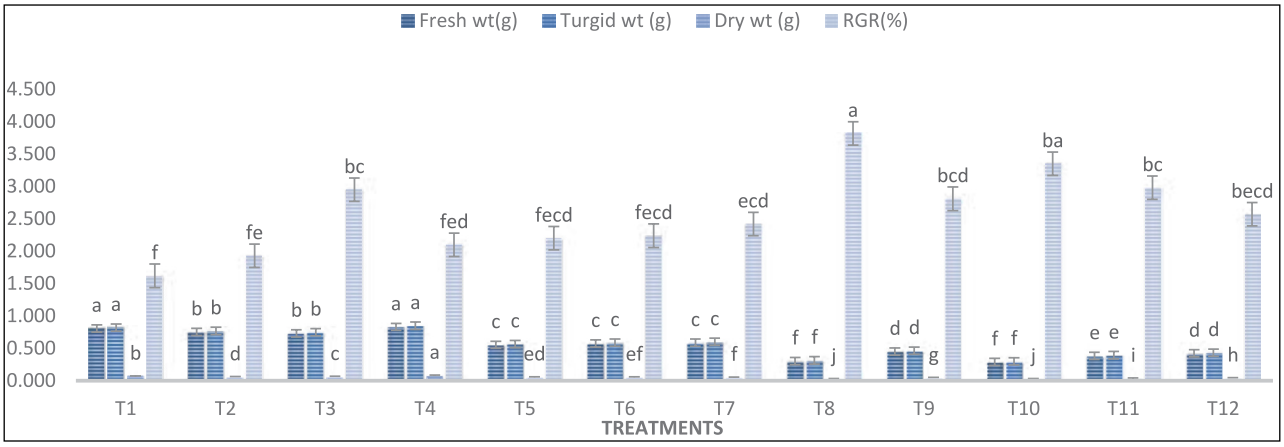

Fig. 1. Effect of different treatments on callus physical parameters (fresh weight, turgid weight, dry weight) and Relative growth rate (RGR).

Table 2. Effect of interactions among PGRs and explant type on *in vitro* callus induction and morphological parameters in sweet orange cv. Mosambi.

Treatment	Days taken for callusing	Callusing (%)	Fresh wt. (g)	Turgid wt. (g)	Dry wt. (g)	CWC (%)	RGR (%)
T_1×E	14.88^{edfc}	70.28^{mkjiln} (57.00)*	0.80^{cbd}	0.82^{cbd}	0.07^{cbd}	9.25^{ghji}	1.71^{bdc}
T_2×E	13.74^{edf}	71.92^{mkjil} (58.02)	0.70^{fed}	0.71^{fed}	0.05^{ilkmj}	12.67^{ced}	1.89^{bdc}
T_3×E	13.01^{ef}	93.65^{cbd} (75.46)	1.10^{a}	1.12^{a}	0.04^{lkmj}	21.80^{a}	1.42^{d}
T_4×E	10.48^{f}	99.70^{a} (88.19)	1.21^{a}	1.23^{a}	0.11^{a}	9.83^{ghfji}	1.98^{bdc}
T_5×E	15.92^{ebdfc}	77.92^{gjih} (62.02)	0.57^{jhigk}	0.59^{jhigk}	0.05^{ilhkj}	9.74^{ghfji}	2.26^{bdc}
T_6×E	17.96^{ebdac}	80.61^{gfih} (63.88)	0.65^{feg}	0.67^{feg}	0.04^{lnm}	13.24^{cbd}	2.28^{bdc}
T_7×E	16.06^{ebdfc}	86.36^{gfed} (68.52)	0.79^{cbd}	0.80^{cbd}	0.06^{feg}	10.82^{gcfed}	1.67^{bdc}
T_8×E	16.87^{ebdc}	96.57$_{b}$ (79.56)	0.35^{onp}	0.36^{poq}	0.03^{prq}	10.28^{ghfe}	4.25^{bac}
T_9×E	14.69^{edfc}	85.08^{gfeh} (67.40)	0.62^{fhg}	0.63^{fhg}	0.06^{ifheg}	9.34^{ghji}	2.23^{bdc}
T_{10}×E	13.67^{edf}	94.16^{cb} (76.07)	0.47^{mk}	0.48^{lnomk}	0.04^{lnom}	9.86^{ghfji}	2.31^{bdc}
T_{11}×E	14.00^{edfc}	76.07^{kjih} (60.78)	0.38^{onm}	0.39^{pno}	0.04^{lnom}	7.87^{hkji}	3.16^{bdac}
T_{12}×E	18.82^{ebac}	72.38^{kjih} (58.31)	0.39^{nm}	0.40^{no}	0.03^{proq}	10.80^{gfed}	2.56^{bdac}
T_1×C	17.91^{ebdac}	49.56^{qr} (44.75)	0.86^{cb}	0.87^{cb}	0.08^{cb}	9.69^{ghji}	1.65^{bdc}
T_2×C	19.22^{bdac}	67.49^{mkjoln} (55.27)	0.65^{fheg}	0.66^{fheg}	0.06^{ifhegj}	9.96^{ghfi}	1.67^{bdc}
T_3×C	19.06^{bdac}	71.48^{mkjil} (57.73)	0.48^{jlmk}	0.50^{jlnimk}	0.05^{ifhgj}	7.27^{kj}	5.15^{a}
T_4×C	15.99^{ebdfc}	59.33^{qpon} (50.39)	0.75^{ced}	0.77^{ced}	0.08^{b}	7.99^{hkji}	2.39^{bdac}
T_5×C	19.52^{bac}	65.52^{mkpoln} (54.05)	0.64^{fheg}	0.66^{fheg}	0.70^{ced}	8.25^{ghkji}	2.12^{bdc}
T_6×C	19.35^{bdac}	60.81^{mqpon} (51.25)	0.66^{feg}	0.67^{feg}	0.06^{fed}	8.57^{ghkji}	2.16^{bdc}
T_7×C	16.64^{ebdc}	60.63^{mqpon} (51.14)	0.47^{lmk}	0.48^{jlnomk}	0.04^{pnom}	10.45^{ghfe}	2.93^{bdac}
T_8×C	21.28^{ba}	57.80^{qpo} (49.49)	0.41^{nm}	0.42^{nom}	0.03^{pnoq}	10.03^{ghfi}	3.31^{bdac}
T_9×C	18.15^{ebdac}	66.51^{mkpoln} (54.66)	0.59^{jfhigk}	0.60^{jfhigk}	0.06^{ifheg}	8.90^{ghkji}	2.65^{bdac}
T_{10}×C	16.69^{ebdc}	18.91^{uvw} (25.64)	0.25^{rqp}	0.26^{srq}	0.02^{rs}	9.10^{ghji}	3.48^{bdac}
T_{11}×C	16.45^{ebdc}	62.12^{mpoln} (52.02)	0.55^{jlhigk}	0.56^{jlhigk}	0.04^{pnom}	12.34^{cfed}	2.20^{bdc}
T_{12}×C	16.50^{ebdc}	55.56^{qp} (48.20)	0.60^{jfhig}	0.61^{jfhig}	0.05^{ihkgj}	9.37^{ghji}	1.67^{bdc}
T_1×R	16.90^{ebdc}	75.17^{kji} (60.19)	0.75^{ced}	0.76^{ced}	0.04^{lnm}	15.60^{b}	1.49^{dc}
T_2×R	23.08^{a}	40.45^{sr} (39.49)	0.90^{b}	0.92^{b}	0.06^{fheg}	13.41^{cb}	2.23^{bdc}
T_3×R	18.79^{bdac}	92.90^{cbd} (74.61)	0.60^{fhig}	0.62^{fhig}	0.08^{b}	6.46^{k}	2.27^{bdc}
T_4×R	14.59e^{dfc}	96.09^{b} (78.69)	0.53^{jlhik}	0.54^{jlhimk}	0.05^{ilhkj}	9.06^{ghkji}	1.92^{bdc}
T_5×R	19.54^{bac}	87.78^{cfed} (69.75)	0.43^{lnm}	0.44l^{nom}	0.04^{lnm}	8.47^{ghkji}	2.22^{bdc}
T_6×R	20.71^{ba}	58.39^{qpo} (49.84)	0.40^{nm}	0.41^{no}	0.04^{lnkm}	7.58^{kji}	2.28^{bdc}
T_7×R	18.69^{ebdac}	89.45^{ced} (71.09)	0.49^{jlimk}	0.50^{jlnimk}	0.04^{lnom}	10.13^{ghfei}	2.65^{bdac}
T_8×R	17.44^{ebdac}	10.74^{w} (19.13)	0.14^{r}	0.15^{s}	0.011^{tu}	9.97^{ghfi}	3.90^{bdac}
T_9×R	18.91^{bdac}	23.47^{ut} (28.92)	0.14^{r}	0.14^{s}	0.01^{tsu}	8.41^{ghkji}	3.54^{bdac}
T_{10}×R	18.92^{bdac}	20.58^{uv} (26.95)	0.13^{r}	0.14^{s}	0.01^{u}	10.07^{ghfi}	4.28^{ba}
T_{11}×R	16.88^{ebdc}	12.46^{vw} (20.59)	0.21^{rq}	0.22^{sr}	0.02^{trs}	8.01^{hkji}	3.59^{bdac}
T_{12}×R	17.56^{ebdac}	31.17st (33.94)	0.27^{oqp}	0.28^{prq}	0.02^{rq}	8.22^{ghkji}	3.48^{bdac}
Mean	17.19	64.97 (55.08)	0.55	0.57	0.05	10.06	2.58
LSD (P≤0.05)	5.74	6.95	0.11	0.12	0.01	2.61	2.77

Data presented in parenthesis are Arc Sin √% transformed values; Mean values in a column are separated by different alphabetical letter(s) in superscript are significant as per Tukey's test

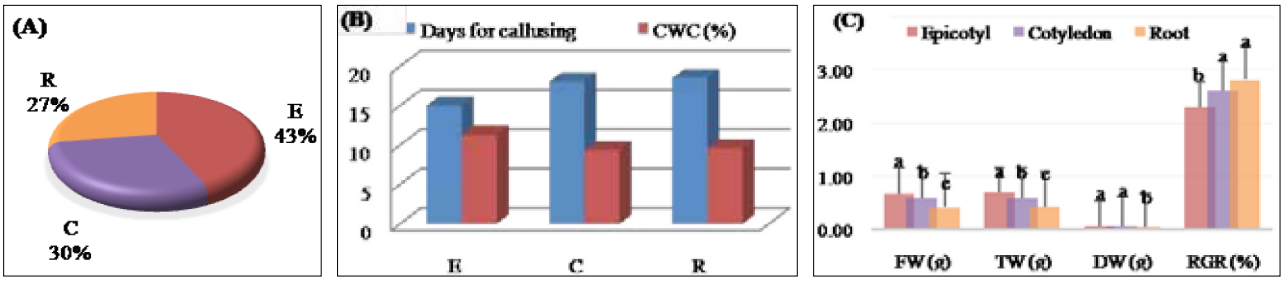

Fig. 2. (A) Effect of callus induction among different explants. (B) Comparison among different explant types on days taken for callus induction and CWC (callus water content) (C) Physical callus data among different explants. E- Epicotyl; C-Cotyledon; R-Root.

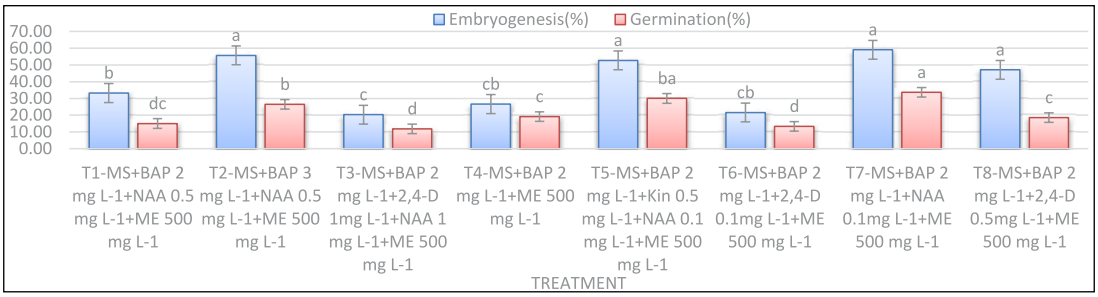

Fig. 3. Effect of different treatments on embryogenesis and germination on the epicotyl derived callus cultures

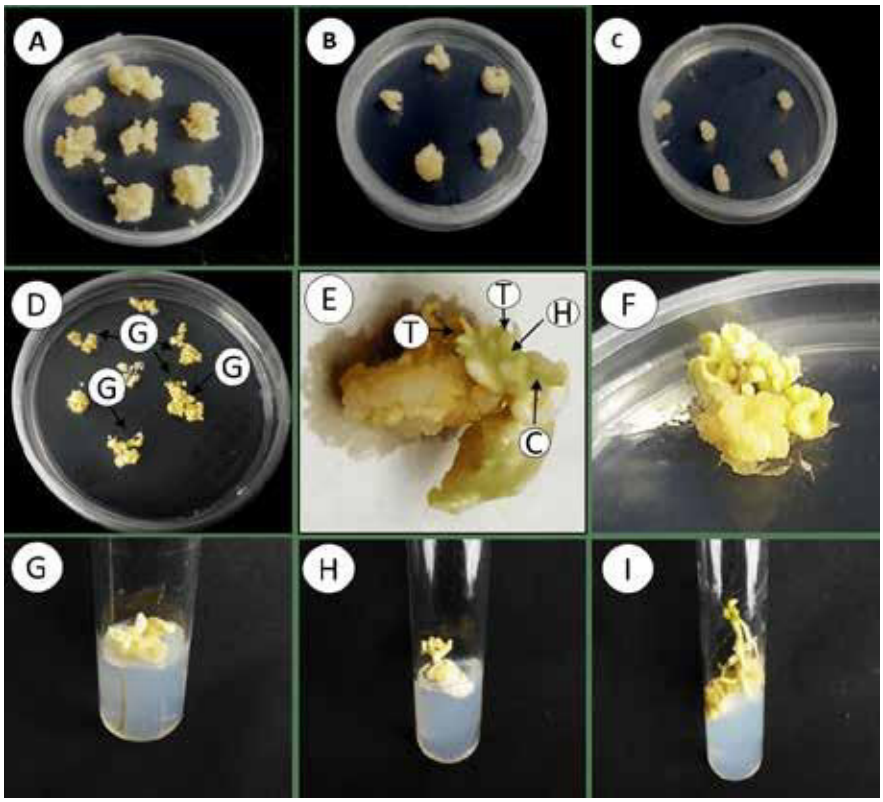

Fig. 4. Callus induction among different explants (A) Epicotyl (B) Cotyledon (C) Root segments (D) Initiation of embryogenesis with formation of globular embryos (E) Different stages of embryo development (F) No. of embryos/ callus mass(G) Germination of embryos initiated (H) Germination of embryo to plantlet (I) Plantlet development. G-Globular; H-Heart; T-Torpedo; C-Cotyledon shaped embryos.

lacking malt extract. The present report on regeneration is in line with the findings of Gill *et al*. (6) and Carimi (2).

The effects of different carbon sources (sucrose, lactose, maltose, galactose and glycerol) supplied at varying concentrations imparted significant influence on embryo regeneration (Table 3).The lowest period for somatic embryogenesis (125.30 days) was recorded in lactose 5% which showed statistical parity with lactose 6% (124.77 days), while it was minimum (83.09 days) in maltose 5%. The highest number of somatic embryo/ callus mass (73.26), embryogenesis (65.27%) and plantlet formation (68.77%) was recorded in glycerol 5%. Considering the concentrations, glycerol, galactose and sucrose at 5% each, and maltose and lactose each at 6% showed the best effect on embryogenesis. The process of embryogenesis takes place as a result of the stress factor, and carbon sources act as a precursor for the induction of embryogenic calli, embryo formation and germination. In the present study, sucrose had a significant effect on production of advanced stages of embryos (heart, torpedo and cotyledons). This was due to the increased sucrose concentration, which might have changed the osmotic potential of the medium leading to osmotic stress and acting as an aid for embryogenesis. The positive effect of glycerol as the carbon source for embryogenesis has also been reported in different citrus genotypes as compared to sucrose (Feher, 4). The increased regeneration of embryo in glycerol is reported due to its role during the induction phase of embryogenesis, which altered its glucose metabolism and the tissues reported to have more sucrose and hexose as compared to others (Bellettre *et al*.,1), as also observed during the course of present study. The effect of maltose on embryogenesis was found better than lactose and sucrose, and lesser than glycerol, which might be due to the source of two glucose molecules, while lactose, a disaccharide with glucose and galactose as the end product might have developed toxicity on embryogenesis. The lowest somatic embryogenesis percentage or transformation of callus to somatic embryo (27.14%) was reported in galactose 4%, as galactose exhibited toxic effect on the process of somatic embryogenesis due to accumulation of galactose-1-phosphate, inhibiting conversion of UDP-glucose to glucose1-phosphate. It is also reported to reduce auxin transport and synthesis of cell-wall components (Rosellini, 14).

The findings of the present study suggest a well-developed protocol for indirect somatic embryogenesis from the epicotyl segment of *C. sinensis* cv. Mosambi. Among the formulated PGRs treatment and explants were undertaken, treatment MS + 2,4-D (1.5 mg L^{-1}) + BAP (1 mg L^{-1}) + ME (500 mg L^{-1}) and epicotyl proved their superiority in the induction of embryogenic callus

Table 3. Effect of carbon sources on somatic embryogenesis on epicotyl callus in sweet orange cv. Mosambi.

Treatment (%, w/v)	Days taken for embryogenesis	Embryogenesis (%)	No. of somatic embryo / callus mass	Plantlet formation (%)
Sucrose 4%	106.84^{dc}	56.79^{dc} (48.91)*	26.39^{gf}	44.87^{e} (42.06)*
Sucrose 5%	108.80^{c}	57.91^{bc} (49.56)	20.19^{h}	57.26^{c} (49.17)
Sucrose 6%	108.33^{c}	53.64^{dce} (47.09)	15.05^{j}	52.59^{d} (46.49)
Lactose 4%	118.64^{b}	42.97^{f} (40.96)	30.04^{f}	56.24^{dc} (48.59)
Lactose 5%	125.30^{a}	44.28^{f} (41.71)	35.50^{e}	47.18^{e} (43.38)
Lactose 6%	124.77^{a}	52.09^{e} (46.19)	36.00^{e}	47.06^{e} (43.31)
Maltose 4%	88.19^{hg}	62.17^{ba} (52.06)	41.41^{d}	65.73^{a} (54.18)
Maltose 5%	83.09^{h}	63.93^{a} (53.10)	42.95^{d}	61.84^{b} (51.85)
Maltose 6%	86.14^{h}	51.38^{e} (45.79)	56.60^{c}	61.30^{b} (51.53)
Galactose 4%	94.14^{fg}	27.14^{h} (31.39)	18.78^{i}	57.21^{c} (49.15)
Galactose 5%	95.11^{fe}	31.79^{g} (34.32)	22.74^{h}	68.00^{a} (55.56)
Galactose 6%	100.92^{de}	27.80^{hg} (31.78)	26.30^{gf}	66.77^{a} (54.80)
Glycerol 4%	84.46^{h}	55.14^{dce} (47.95)	61.76^{b}	65.53^{a} (54.07)
Glycerol 5%	94.80^{f}	65.27^{a} (53.90)	73.26^{a}	68.77^{a} (56.03)
Glycerol 6%	94.03^{fg}	52.58^{de} (46.48)	54.96^{c}	55.78^{dc} (48.33)
Mean	100.90	49.66 (44.75)	37.46	58.41 (49.90)
LSD (P≤0.05)	5.96	2.53	5.06	2.21

Data presented in parenthesis are Arc Sin √%transformed values; Mean values in a column are separated by different alphabetical letter(s) in superscript are significant as per Tukey's test

and correlated well with morpho-physical parameters. Calli derived from the epicotyl segment when subjected to embryogenesis and maturation, the best regeneration was reported in the treatment MS+BAP (2 mg L^{-1}) + NAA (0.1 mg L^{-1}) + ME (500 mg L^{-1}). Supplementation of different carbon sources to the standardized regeneration media at different concentrations showed its effectiveness for glycerol at 5%, thus proving its potential for enhancing regeneration of calli derived epicotyl segments. The protocol can further be used in citrus improvement such as *in vitro* conservation, transformation and *in vitro* mutagenesis study.

AUTHORS' CONTRIBUTION

Conceptualization of research (OPA); Designing of the experiments (OPA& SK); Contribution of experimental materials (OPA); Execution of field/lab experiments and data collection (RP); Analysis of data and interpretation (RP, OPA, SK & CB); Preparation of the manuscript (RP, OPA & SK).

DECLARATION

The author declares no conflict of interest.

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