

***In vitro* Rescue of Nucellar Embryos and Micropropagation of Citrus Rootstocks**

Arti Yadav, Navneet Kaur, Teg Bahadur Singh, Krishan Kant, Shalu Gupta, Parnika Jindal, Mukta Satsangi, Akbar Ali

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ABSTRACT

Nucellar embryony is a boon for the clonal propagation of *Citrus* rootstocks. Poly-embryonic seeds of *Citrus* rootstocks possess several non-zygotic embryos of nucellar origin that are identical to the maternal genotype and is a heritable trait. Since Citrus seeds are recalcitrant the nucellar embryos need to be rescued immediately after harvest and put for germination. In the present study, rescued nucellar embryos were germinated *in vitro* on Murashige and Skoog medium (MS) supplemented with cytokinins, Benzyl-amino purine (BAP) or Kinetin (Kn) at various concentrations. *In vitro* germinated

nucellar seedlings were micro-propagated further. Best nucellar embryo germination was obtained on MS medium fortified with Kn [Troyer Citrange (1.0 mgL⁻¹), Rough Lemon collection-6 (0.5 mgL⁻¹) and Sour Orange (0.75 mgL⁻¹)] or BAP [Trifoliate Orange (0.75 mgL⁻¹)]. Among the four Citrus rootstocks, germination percentage was maximum in Troyer Citrange and minimum in Rough Lemon Collection-6. Epicotyl segments from two-week-old axenic nucellar seedlings were cultured on MS medium supplemented with different concentrations of BAP alone and in combination with NAA (α -Naphthalene acetic acid) and IAA (Indole-3-acetic acid). In all the Citrus cultivars used, *de novo* shoot regeneration was obtained within two weeks of culture with highest shoot buds formed in Troyer Citrange followed by Trifoliate Orange, Rough Lemon Collection-6 and least in Sour Orange. Shoots of all the rootstocks rooted best on MS + NAA (0.5 mgL⁻¹) and were acclimatized and transferred to pots with almost 90% survival.

Keywords Citrus rootstock, Sour orange, Rough lemon collection-6, BAP, Nucellar embryo.

INTRODUCTION

Citrus (Family, Rutaceae) is an important fruit crop grown in the tropical and subtropical areas of the world with a production of 124 million tons (FAO 2017). In India Citrus ranks third after mango and banana, in area and production, accounting for about 12% and 10.4 %, respectively (Tripathi 2016, Ward-

Arti Yadav¹, Navneet Kaur², Teg Bahadur Singh³, Krishan Kant⁴, Shalu Gupta⁵, Parnika Jindal⁶, Mukta Satsangi⁷, Akbar Ali^{8*}

^{1,8}Assistant Professor, ³Guest Faculty

^{1,2,3,4,5,6,7,8}Plant Physiology and Biochemistry Lab, Department of Botany, Dayalbagh Educational Institute (Deemed to be University), Agra 282005, UP, India

¹RB (PG) College, Kalindi Vihar, Agra 282006, UP, India

³Sw. Smt. Indira Gandhi Government Girls College, Shivpuri 473551, MP, India

Email: akbarali@dei.ac.in

*Corresponding author

han *et al.* 2022). Despite cultivation on large scale, production is not enough due to several factors such as heterozygosity, inbreeding depression, sexual incompatibility, long juvenility, uneven tree size, sensitivity to many biotic and abiotic stresses (Naqvi 2000, Guo and Deng 2001, Ferguson 2002, Grosser and Gmitter 2005, Altaf *et al.* 2008, Sagouti *et al.* 2022). Quality rootstocks are very important for any *Citrus* orchard. Success of any rootstock depends on its tolerance/resistance to prevailing conditions of soil, climate and diseases. The conventional method through seed propagation for *Citrus* cultivars does not work efficiently to develop true to type plants. Plant tissue culture on the other hand when used for mass production is a cost-effective technique to produce true-to-type plants (Bhojwani and Dantu 2013, Iqbal *et al.* 2019). Micropropagation of several *Citrus* species has been obtained using a variety of explants (Jajoo 2010, Kaur 2018). However, very little work has been done on tissue culture of the *Citrus* rootstocks, Troyer Citrange, Trifoliolate Orange, Sour Orange and Rough Lemon Collection-6. The present paper explores the possibility of rescuing nucellar embryos to develop true-to-type healthy plants from these *Citrus* rootstocks and develop a method for micro-propagating the *in vitro* germinated nucellar seedlings.

MATERIALS AND METHODS

Plant material

Ripened fruits of Troyer Citrange (TC), Trifoliolate Orange (TrO), Sour Orange (SO) and Rough Lemon Collection-6 (RLC-6) rootstocks were collected from the *Citrus* orchard at Indian Agricultural Research Institute, New Delhi in November 2015 and stored at 4°C until further use.

Preparation of explant

Healthy seeds of the four *Citrus* rootstocks were extracted from freshly collected fruits or those stored at 4°C. Seeds were washed thoroughly under tap water to remove mucus present on seed coat surface and soaked in 1% Savlon (Johnson and Johnson, UK) for 5 min. Savlon traces were washed away by washing the seeds under running water and surface sterilized first by rinsing in 70% ethanol for 30 sec followed

with 0.1% mercuric chloride solution for 5 min. Seeds were rinsed five to six times with sterilized double distilled water to remove all traces of mercuric chloride. Further handling of the seeds was done in the laminar air flow chamber. Nucellar embryos were excised from the seeds by removing the outer hard and inner soft seed coats using sterile pointed forceps for inoculation on germination medium.

Epicotyls from two-week-old nucellar seedlings were cut into 20 mm pieces and used for shoot regeneration. The *de novo* obtained shoots were put for rooting.

Preparation of culture medium

Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962) was used for all experiments supplemented with various growth regulators in different combinations. The pH of the medium was set at 5.7 and gelled with 0.8% agar, poured into 25 mm × 150 mm rimless tubes and stoppered with polypropylene caps (Polylab). Media in tubes were sterilized by autoclaving at 1.0546 kg cm⁻² and 121 for 20 minutes and allowed to set as slants at room temperature.

Germination medium: The nucellar embryos were put for germination on MS medium supplemented with kinetin (Kn) or 6-benzylaminopurine (BAP) at different concentrations (0, 0.25, 0.5, 0.75 and 1.0 mgL⁻¹).

Shoot induction medium: To induce shoots on the epicotyls MS basal medium was supplemented with 44 combinations of BAP or Kn (0, 1.0, 2.0, 3.0 and 4.0 mg L⁻¹) without or with Indole-3-acetic acid (IAA) or Naphthalene acetic acid (NAA) (0, 0.25, 0.5, 0.75 and 1.0 mg L⁻¹).

Root induction medium: MS basal medium supplemented with 0.25 and 0.5 mg L⁻¹ NAA or IAA was used for root induction. Media was poured in 25 mm × 150 mm rimless culture tubes (Borosil) and sterilized.

Selection of nucellar embryo

Citrus seeds generally possess 2-5 mm sized embryos.

In the present study 4-5 mm nucellar embryos were used to establish initial cultures. Nucellar embryos of 2-3 mm sizes were also cultured to check their germination response.

Establishment of *in vitro* cultures

All inoculations were carried out in a laminar air flow chamber under aseptic conditions. Nucellar embryos were placed horizontally on the surface of Germination medium and incubated in dark at 24 ± 2 for one week. One-week-old nucellar seedlings were then shifted to 16/8 h photoperiod condition. Epicotyls from two-week-old nucellar seedlings were cut into 0.2 to 0.5 cm segments and placed vertically in the Shoot Induction medium. Cultures were incubated at 24 ± 2 and 16 h cool white fluorescent light ($30 \text{ E/m}^2/\text{s}$) and 8 h darkness.

Rooting and acclimatization

In vitro formed shoots were inoculated in the Rooting medium and incubated for three weeks at 24 ± 2 in 16 h light. Rooted plants were washed to remove agar from roots and acclimatized by transferring to small pots filled with sterilized cocopeat and dry paddy husk (1:1). These plants were initially maintained for 15 days at 100% humidity and subsequently shifted to lower levels of 75% and 50% humidity for two days each before finally transferring to field conditions. Plants were irrigated every 3rd day with $\frac{1}{4}$ MS Major and Minor salt solution mixed with 1% Bavistin to prevent fungal contamination.

Statistical analysis

Germination of nucellar embryos was observed at weekly intervals, their numbers noted and percent germination calculated. Number of branches per nucellar embryo was observed. Observations for *de novo* shoot regeneration from epicotyl segments was also taken at weekly intervals. Number of *de novo* shoot buds formed and their length were recorded. All treatments consisted of 24 replicates and every experiment was replicated thrice. All results were pooled and subjected to one way ANOVA Duncan's multiple range test at 0.05 significance level using SPSS software version 17.

RESULTS

Nucellar embryo germination

Isolation of nucellar embryos

Initial culture establishment using nucellar embryos was very critical, since these had to be carefully separated from the zygotic embryo. Nucellar embryos in most of the Citrus seeds are present at micropylar end surrounding the single creamy white zygotic embryo. The nucellar embryos are greenish white and are distinguishable from zygotic embryo. The number and size of nucellar embryos vary among Citrus seeds and are smaller in size as compared to zygotic embryo (unpublished data).

Impact of nucellar embryo size on germination

Embryo size influenced its germination. Healthy seeds of poly-embryonic Citrus rootstocks usually contain 2-5 mm sized nucellar embryos (Fig. 1 A). In the present study approximately 4-5 mm sized nucellar embryos were used for germination. Nucellar embryos smaller than 4 mm showed delayed (after three weeks) or very poor germination. One-week-old seedlings showed a significant increment in height upon shifting to light.

Germination of nucellar embryos

Excised nucellar embryos were cultured on either MS basal medium or supplemented with four different concentrations of Kinetin or BAP to check effect of cytokinin type and concentration on germination. MS basal medium supported good nucellar embryo germination but presence of Kn or BAP promoted early and better germination in all rootstocks. Kn was better than BAP in all cultivars except Trifoliolate Orange. Optimal nucellar embryo germination was obtained on MS medium fortified with varying concentrations of Kn that varied with cultivars, 1.0 mgL^{-1} in TC, 0.5 mgL^{-1} in RLC-6, 0.75 mgL^{-1} in SO and 0.5 mgL^{-1} in TrO. When BAP was used in place of Kn, optimal nucellar embryo germination was observed at 0.75 mgL^{-1} in TC, TrO and RLC-6 while 0.5 mgL^{-1} BAP was optimal for SO. Although all four cultivars responded to both types of cytotoki-

Table 1. Germination percentage in nucellar embryos of four *Citrus* rootstock cultivars on MS medium size of nucellar embryo at the time of inoculation was 4-5 mm, data obtained after one weeks of inoculation, values are the means of nucellar embryo germination percentage. Means followed by different letters are significantly different at $p=0.05$.

Medium used		RLC-6	SO	TC	TrO
Control (MS basal)		66.00 ± .57	88.66 ± .88	92.66 ± .88	82.00 ± 1.73
BAP (mg L ⁻¹)	0.25	77.66 ± 1.45	99.00 ± .57	98.33 ± .33	97.00 ± 1.52
	0.5	83.00 ± .57	99.66 ± .33	100.00 ± .00	100.00 ± .00
	0.75	82.66 ± 1.45	100.00 ± .00	100.00 ± .00	100.00 ± .00
	1.0	86.66 ± 1.85	100.00 ± .00	100.00 ± .00	95.66 ± .88
Kn	0.25	80.0 ± 0.57	100.00 ± .00	100.00 ± .00	80.66 ± 2.18
(mg L ⁻¹)	0.5	100.0 ± 0.00	99.66 ± .33	100.00 ± .00	83.66 ± 1.76
	0.75	90.66 ± .88	100.00 ± .00	100.00 ± .00	82.33 ± 2.84
	1.0	96.00 ± 2.30	99.00 ± .57	100.00 ± .00	83.33 ± 1.45

nins, TrO showed better germination with multiple branches at 0.75 mgL⁻¹ BAP while the remaining three cultivars responded best in various concentrations of Kn (Table 1). Among the four *Citrus* rootstocks germination percentage was maximum in Troyer Citrange followed by Trifoliate Orange, Sour Orange and minimum in Rough Lemon Collection-6. Besides the effect on nucellar embryo germination the cytokinins also influenced number of branches per seedling when one-week-old seedlings were shifted from dark to light for two weeks. Highest numbers of branches were recorded in Troyer Citrange (6.47) and minimum in Rough Lemon Collection-6 (3.53) (Table 1, Fig. 1 (B-E) and Fig. 2.

De novo plant regeneration

Green epicotyls of young *in vitro* germinated seedlings were used for establishing shoot cultures on

Shoot Induction medium. Presence of growth regulators was necessary for *de novo* shoot buds formation from the epicotyl segments in all the *Citrus* cultivars. Within two weeks of culture light green shoot buds developed on upper surface of epicotyl segments that grew into individual shoots. Shoot regeneration was better in MS supplemented with BAP and NAA in three cultivars while BAP with IAA was better for the fourth cultivar (Fig. 3 A-D). Among the four *Citrus* rootstocks, highest shoot buds were formed in Troyer Citrange (10.86) followed by Trifoliate Orange (8.7) and Rough Lemon Collection-6 (6.32) while, least shoot buds were formed in Sour Orange (5.37) (Fig. 3 (A-D) and Fig. 4 A-E).

Rooting of shoots and field establishment

Nucellar embryo derived shoots of all the *Citrus* rootstocks varieties were transferred to Rooting medium.

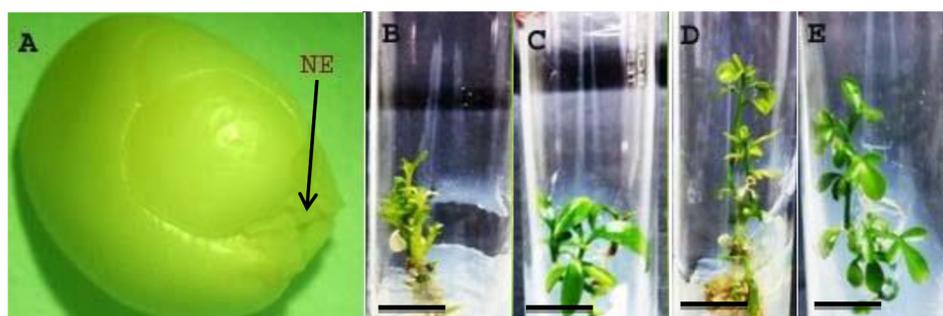


Fig. 1. Nucellar seedlings of *Citrus* rootstocks showing multiple branches: (A) Uncoated *Citrus* Seed showing many nucellar embryos (NEs) at micropylar end (B) Multi branched nucellar seedling of RLC-6 in MS + 0.5 Kn, (C) Multi branched nucellar seedling of sour orange (SO) in MS + 0.75 Kn, (D) Multi branched nucellar seedling of TC in MS + 1 Kn and (E) Multi branched nucellar seedling of trifoliate orange (TrO) in MS + 0.75 BAP (at 28 days). Performed DMRT at Significant level $p \leq 0.05$. Bar a= 0.2 cm, b=0.63 cm, c= 0.68 cm, d= 1.18 cm, e= 1.15 cm.

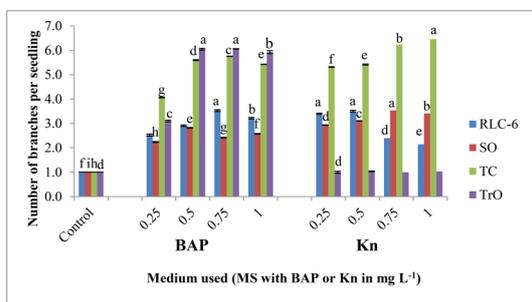


Fig. 2. Effect of various concentrations of BAP and Kn on multiple branch emergence from nucellar embryo derived *in vitro* seedling. One week old single branched seedlings were transfer to light condition. Performed DMRT at significant level $p \leq 0.05$.

Nearly 2-4 roots per shoot were formed within two weeks in MS medium supplemented with 0.5 mg L⁻¹ NAA (Fig. 4F). Rooted plants were transferred to hardening media for acclimatization. Plants were successfully acclimatized under high humidity condition and transferred to soil pots with 90% survival (Fig. 4G).

DISCUSSION

Nucellar embryo development in *Citrus* is a unique feature as several adventitious embryos arise from nucellus tissue (Xu *et al.* 2021). For nursery people

it is quite difficult to screen and separate nucellar plants from zygotic one (Das *et al.* 2000). On the other hand plant tissue culture is an efficient technique to produce a large number of healthy nucellar plants in less time and space. To obtain contamination-free embryos, seeds are a good source as it is difficult for the pathogens to reach inside of the seed even when the plant is highly infected (Singh *et al.* 2011, Zhang *et al.* 2024). Nucellar embryos were used as explant in the present study as in the earlier studies of Das *et al.* (2000) Jajoo (2010) and Zhang *et al.* (2024). *In vitro* propagation of *Citrus* plants using nucellar embryo as a starting material is recommended to achieve true to type plants. The effect of plant growth regulators on nucellar embryo germination and *de novo* shoot regeneration was studied. Nucellar embryos germinated within one week of culture on MS medium fortified with Kn or BAP. Nucellar embryo germination was early (within 3-5 days of culture) and 100% on MS medium supplemented with Kn in all rootstocks except TrO. However, the concentration of Kn varied among rootstocks: TC (1.0 mgL⁻¹), RLC-6 (0.5 mgL⁻¹) and SO (0.75 mgL⁻¹). On the other hand, BAP (0.75 mgL⁻¹) was optimum for fast embryo germination (100%) in TrO. The present study was comparable to the previous findings on *Citrus* by Das *et al.* (2000), Costa *et al.* (2002), Ali and Mirza (2006), and Gill *et al.* (2012), who

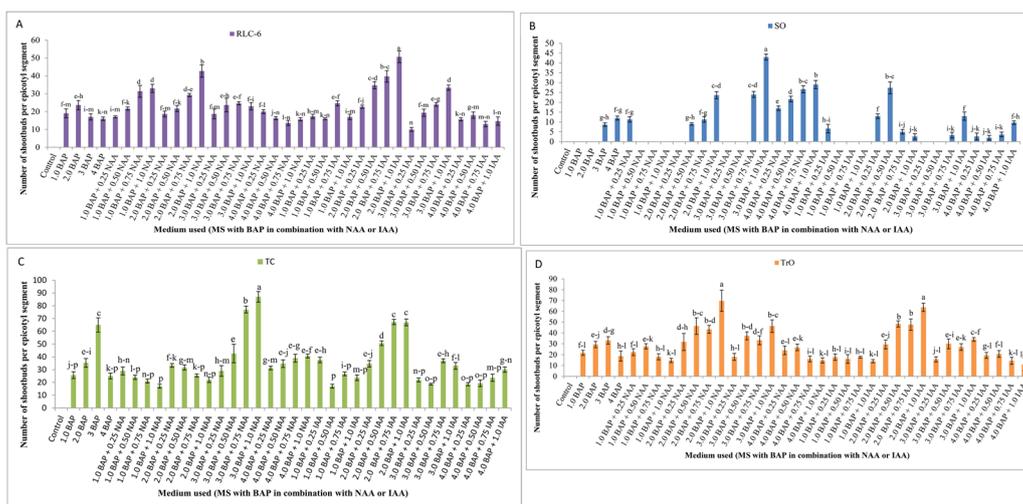


Fig. 3. Effect of plant growth regulators on multiple shoot bud induction from single epicotyl A-D (Data obtained after 14th day of inoculation). Performed DMRT at Significant level $p \leq 0.05$.

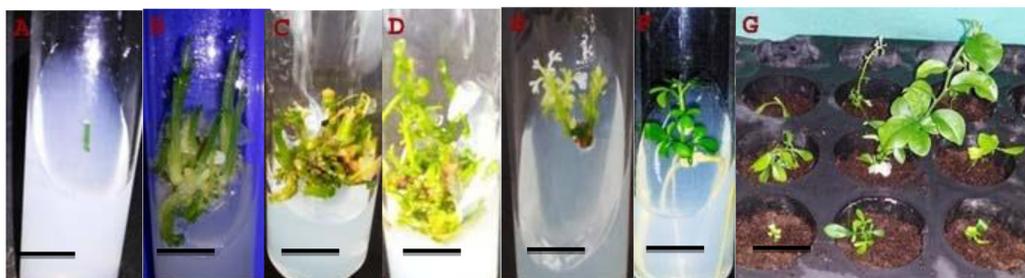


Fig. 4. *In vitro* shoots from epicotyl segment of four *Citrus* rootstock cultivars. (A) Epicotyl segment at the time of inoculation, (B) Multiple shoots in rough lemon collection-6 (RLC-6), (C) Multiple shoots in sour orange (SO), (D) Multiple shoots in troyer citrange (TC) (E) Multiple shoots in trifoliolate orange (TrO), (F) Root formation in 1-2 cm long shoots, (G) *In vitro* rooted plants after 10 weeks of hardening and acclimatization. Performed DMRT at Significant level $p \leq 0.05$. Bar a=1.06 cm, b=1.12 cm, c=1.09 cm, d=1.14 cm, e=0.96 cm, f=1.24 cm, g=0.48 cm.

reported that percent embryo germination was less and delayed using MS basal medium. Jajoo (2010) and Oh *et al.* (2024) reported delayed nucellar embryo germination (after two weeks of inoculation) on different medium. Among the four *Citrus* rootstocks germination percentage was maximum in Troyer Citrange and minimum in Rough Lemon Collection-6. The effect of cytokinin was favorable in early nucellar embryo germination and multiple branch formation after transfer of cultures to light as was observed by Jajoo (2010) and Singh *et al.* (2020) in different *Citrus* species. Embryo size was found to influence germination (Gill *et al.* 2012, Boccaccini *et al.* 2024). In the present study, approximately 4-5 mm sized nucellar embryos developed into seedlings while, nucellar embryos less than 4 mm size were very poor in germination. In all four *Citrus* cultivars epicotyl segments were used for shoot bud regeneration as in earlier studies (Costa *et al.* 2002, Devi and Rattanpal 2018). *In vitro* formed shoot buds were reported to be *de novo* in the earlier studies (Peña and Navarro 1999, Cervera *et al.* 2000, Dominguez *et al.* 2000) and in the present study as well. *De novo* shoot bud regeneration was directly influenced by concentration of plant growth regulators (Rani and Dantu 2012, 2016, Bhojwani and Dantu 2013, Papry *et al.* 2021). In all *Citrus* rootstocks used in the study, BAP at 2.0 mg L⁻¹ and 3.0 mg L⁻¹ was favorable for multiple shoot bud induction. Effect of BAP in combination with NAA and IAA on multiple shoot bud induction in the present study supported by earlier studies (Das *et al.* 2000, Chikhale *et al.* 2002, Costa *et al.* 2002, Gill and Gosal 2002, Devi and Rattan pal 2018, Haradzi

et al. 2021). However, Yadav *et al.* (2017) used Kn (1.0 mg L⁻¹) for *in vitro* shoot regeneration in rice but in recent study of Setiadi *et al.* (2025) used kinetin (1.0 mg L⁻¹) in combination with NAA (0.5 mg L⁻¹) for shoot induction in vanilla. A good rooting percentage (87%) with maximum roots per plant (2-4 roots/plant) was obtained on MS medium fortified with NAA (0.5 mg L⁻¹) which was comparable with control and other treatments (Unpublished data). Only one auxin (NAA, 0.5 mg L⁻¹) was sufficient for the formation of roots in the present study. The results of this study are in agreement with Kaneyoshi *et al.* (1994) Ali and Mirza (2006) and Abdi *et al.* (2023), however, earlier studies by Germanà *et al.* (2008), Jajoo (2010) Devi and Rattan pal (2018) and Silvina *et al.* (2025) have used combination of a cytokinin (BAP) and auxin (IAA, NAA) for root induction in other *Citrus* cultivars. Other previous studies reported poor rooting efficiency in several tree species of *Citrus* (Duran-Vila *et al.* 1989), Sweet orange (Pena *et al.* 1995). All the rooted plants were transferred onto hardening media for acclimatization. Plants were acclimatized well under high humidity and transferred to clay pots with 90% survival.

CONCLUSION

In the present study a simple and efficient *de novo* shoot regeneration protocol was established from nucellar embryo of four most promising *Citrus* rootstocks. The study found notable effects of Kn on nucellar embryo germination in TC, RLC-6, SO and BAP on germination of TrO. Kn promoted

germination in all rootstocks except TrO which may be a genotypic response. However, TrO showed second highest germination percentage when cultured on MS medium supplemented with BAP (0.75 mg L⁻¹). Among the four *Citrus* rootstocks TC showed maximum nucellar embryo germination while least germination was observed in RLC-6. Epicotyls from all rootstocks successfully developed numerous *de novo* shoots within two weeks of culture. Maximum percentage of shoot buds were obtained in TC (10.86) while SO was least responsive (5.37). Present study showed a simple and cost-effective regeneration protocol in which 100% nucellar embryo germination was achieved in salt tolerant *Citrus* rootstocks. Similarly excellent *de novo* shoot regeneration was observed, where 97% epicotyls regenerated. Rooting (87%) was induced in all rootstock cultivars using 0.5 mg L⁻¹ NAA. The *in vitro* plants obtained in this study could be used in micropropagation, micrografting, protoplasts fusion and culture and genetic transformation studies.

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