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Micropropagation, *in-vitro* conservation and genetic stability studies in pummelo (*Citrus maxima*)

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ABSTRACT

Pummelo (*Citrus maxima* L.) is a natural, mono-embryonic and a true fruit species of genus Citrus cultivated widely in India. The study aimed at developing *in vitro* technique for conservation of *Citrus maxima* germplasm using shoot tip and nodal section as explants. Various concentrations and combination of hormones were used for successful *in-vitro* conservation. The shoots sprouted within 9-20 days when inoculated on MS medium supplemented with 0.5 mg/l BAP + 0.1 mg/l NAA growth hormones and above 86% germination was obtained with maximum numbers of shoots (8) and nodes (8.67). Highest shoot elongation (82%) was observed in MS medium supplemented with concentration of 0.6 mg/l GA_3 in combination with 0.2 mg/l BAP, shoot length ranged between 1.17 to 6.90 cm and the number of nodes was 2.33-8.33 per plantlet. 1/2 MS medium supplemented with 0.5 mg/l BAP +1.0 mg/l NAA along with 2.5 g phytagel showed best result for rooting (78.33%). The regenerated plantlets did not show any detectable morphological variation at genetic level as evidenced by SSR profiles of plantlets. The developed protocol can be successfully employed for large-scale multiplication and conservation of Pummelo germplasm.

Key words: Pummelo, Root induction, Shoot tips, Sub-culture, Tissue culture.

In-vitro multiplication and conservation has been a successful technique for obtaining virus free woody plants and has been widely practiced for short- to medium-term germplsm conservation of woody species (Engelmann 1997). This method has come out to be complementary conservation strategy to the field gene banks useful for exchange of germplasm and also a source of explants for cryopreservation. In the previous studies, citrus plant recovery and genetic stability have been taken into the consideration (Chetty and Rao 1990). Prime focus of *in vitro* establishment and multiplication is to protect the genetic integrity of the genotype and protection from various diseases, abiotic and biotic stresses (Taskin *et al*. 2013). The complete information of re-growth pattern of sub-cultures, sub-culture period, rooting of the cultures and hardening of plantlet in the field is required for achieving successful *in vitro* conservation. In the previous studies it was stated that the specific ratio of auxin-cytokinin controls the root and shoot formation during *in vitro* culture (Engelke *et al*. 1973, Can *et al.* 1992). Mass multiplication of shoots appeared to be affected by the concentration of cytokinin in *C. megaloxycarpa* and maximum number of shoots were induced using MS medium supplemented with 0.25 mg/l BA + 0.50 mg/l NAA or 1.0 mg/l BA with 0.50 mg/l kinetin (Haripyaree *et al.* 2011).

In *Citrus* species several explants like shoot tips, stem sections, root sections, leaf sections, stem internodes, epicotyl segments and transverse thin cell layer have been used for micropropagation followed by *in vitro* conservation and cryopreservation using various techniques in several laboratories of the world (Rohini *et al*. 2016, Liu and Deng 2007, De Carlo and Lambardi 2005).

These collections may be vulnerable to numerous stresses or diseases which may be fatal for important genotypes (Damania 1996). On the other hand, seeds of *Citrus* species are non-orthodox and lose viability within a short time depending on the species and storage condition (Malik *et al.* 2012). Therefore, it is very important to develop long term cryopreservation protocols for seeds/ embryos or to develop *in-vitro* multiplication, maintenance and conservation protocols for specific cultivars. The conserved indigenous and exotic germplasm have great utility in *Citrus* industry and crop improvement programmes.

The genetic conservation of various varieties is essential for the future prospect (Marin and Duran-vila 1991, Withers 1980) as deforestation, destruction to the natural habitat, over utilization of the species and climate change is leading to loss of diversity. The genetically uniform varieties are also being replaced with highly diverse local cultivars and landraces of traditional agro-ecosystems.

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Therefore, there is an urgent need to collect, characterize and conserve the existing vast genetic resources of Citrus for safe conservation and utilization in crop improvement programmes of existing genotypes and rootstocks based on both conventional and biotechnological methods. The present study on pummelo (*Citrus maxima* L.) on *in vitro* multiplication and conservation have been carried out to achieve short to medium term conservation and also to develop ready explants for longterm conservation using cryopreservation.

MATERIALS AND METHODS

Diverse accessions of *C. maxima* were collected from various parts of India and fruits brought to the laboratory at NBPGR in New Delhi. Seeds collected from the fruits, were sterilized with 1% sodium hypochlorite (Qualigens) for surface sterilization after which they were washed thrice with doubled distilled water for 15 min each in the laminar air flow. These seeds were then dried on blotted filter paper for removal of extra water content. Embryonic axes were excised from the sterilized seeds and inoculated on basal MS medium (Murashige and Skoog 1962) with 3% sucrose and 0.8% agar for 5-10 days. Then these axes were transferred to two different mediums, *viz*. MS+ 1.0 mg/l 6- benzylaminopurine (BAP) +1.0 mg/l α-Naphthalene acetic acid (NAA) + 0.8% agar + 2.5% activated charcoal and same medium composition without activated charcoal for the further growth of the plantlets.

The seedlings were taken out from the culture tubes and 1.5-2 cm of nodal section and shoot tips excised from the seedlings were used as an explants for this present study. These explants were inoculated on basal MS medium for 5-10 days and then transferred to shooting, multiplication, elongation and rooting medium. The surface sterilized explants were inoculated on to semi-solid Murashige and Skoog medium (MS) (1962) supplemented with varying concentrations and combinations of PGRs-including 0.1-1.0 mg/l benzylaminopurine (BAP), 0.2-1.0 mg/l Gibberellic acid (GA_3) and 0.1-1.0 mg/l α-Naphthalene acetic acid (NAA). The pH of each medium was adjusted to 5.8 prior to autoclaving at 121ºC for 20 min. All cultures were maintained at 25º ± 2ºC under a 16 h photoperiod at a light intensity of 35 µmol photons/m^2/s.

For shoot induction and multiplication, Murashige and Skoog medium (1962) without growth regulators was used as a basal medium. The explants were placed vertically in the culture tubes (25×150 mm) containing sterilized MS medium supplemented with different combination of BAP (0.1, 0.5 and 1.0) and NAA (0.1 and 1.0) @ mg/l to optimize growth, differentiation and multiplication. DKW and WPM (Woody plant medium) medium also used with these PGRs for shoot induction and multiplication. MS basal medium without any PGRs was used as a control. Shoot induction was recorded fortnightly while percentage shoot induction and number of shoots per explants was recorded after 30 days of culturing.

The plantlets were then aseptically cut into 1.5 cm segments with nodes in middle and the shoot tips and these explants were inoculated in shooting and multiplication medium. The shoot initiation and multiplication varied with different concentration and combination of BAP and NAA. A total of seven different medium supplemented with different combination and concentration of PGRs were used in the present experiment viz, MS+ 3% sucrose + 0.8% Agar/Phytagel + 2.5% charcoal (0.1 mg/l BAP+0.1 mg/l NAA, 0.1 mg/l BAP+1 mg/l NAA, 0.5 mg/l BAP+0.1 mg/l NAA, 0.5 mg/l BAP+1 mg/l NAA, 1 mg/l BAP+0.1 mg/l NAA), DKW and WPM medium.

For shoot elongation, sub-cultures were developed using nodal stem as an explant after 30 days of inoculation to the MS medium supplemented with different combinations of BAP (0.2, 0.6 and 1.0) and GA_3 (0.2, 0.4, 0.6, 0.8, and 1.0) @ mg l^{-1}. The shoot length was recorded after 40 days of sub-culturing.

The cultures were transferred to half MS medium supplemented with various concentrations of auxins like IBA (0.1, 0.5 and 1.0) and NAA (0.1, 0.5 and 1.0) @ mg/l. The medium was maintained at 5.7 pH and was solidified using 0.8% agar and 2.5% phytagel, autoclaving for 15 min at 121ºC. The rooting parameters viz., number of roots per explants and length of roots were also recorded after 20 days of initiation of roots.

Well-developed, rooted plantlets were washed under the running tap water and transplanted to pots containing a 1:1:1 (v/v/v) admixture of farm manure : vermiculite : soilrite and covered with air-tight clear plastic sheet to maintain a high humidity, initially. After 1 day, three to four little pores (1 – 2 cm in diameter) were made in these covered ploythene sheets to remove excess condensed water drops. These covered pots were transferred to a shaded greenhouse at the NBPGR, New Delhi, India. The polythene sheets were finally removed 10 – 15 days after transfer in the green house.

In each experiment, 25 explants were used per treatment and each experiment was repeated twice. Standard errors (SE) of the arithmetic means were calculated for each treatment. The data were analysed by analysis of variance (ANOVA) in SPSS software for Windows (Release 15.0; SPSS Inc., Chicago, IL, USA) to measure the effect of basal MS medium and the various concentrations and combinations of PGRs on *C. maxima* shoot proliferation, elongation and root induction. Significant differences between means were assessed using Duncan's multiple range test (DMRT) at $P \leq 0.05$ (Gomez and Gomez 1976).

Total genomic DNA was isolated from 100 mg leaves collected from fresh *in vitro* sub-cultures using cetyl trimethyl ammonium bromide (C-TAB) protocol (Choudhary et al. 2013). DNA quality and quantity were determined with NanoDrop 2000 Spectrophotometer and quality was assessed on 0.8% (w/v) agarose gel. Simple sequence repeat (SSR) analysis was carried out by SSR primers summarized in Supplementary Table 1. Twelve SSR primer pairs were used in the analysis of the DNA sample of mother plant and *in vitro* cloned (7) plants. SSR amplification was

performed as described by Barkley et al. (2006) with minor modifications. The PCR-amplification was carried out in 25 ml reaction volume containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.0–2.5 mM $MgCl_2$, 0.2 mM dNTP each, 1.0 U Taq DNA polymerase (G-Biosciences, India), 0.2 mM primer and 20–25 ng genomic DNA. The DNA amplification was carried out in a BioeR Xp thermocycler with reaction conditions programmed as initial pre-denaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at optimized temperature for 1 min, and extension at 72°C for 1 min. A final 7 min extension at 72°C followed the completion of 35 cycles. For SSR analysis, the amplification products were separated by electrophoresis on 3% agarose gels stained with Gel view stain at 120 V for 3 h and bands were visualized and documented under UV gel doc system Amplified bands were scored as present (1) or absent (0) homologous bands across all the accessions studied. Molecular weight of the amplified bands was estimated using 1 kb DNA ladder as a standard. A pair wise similarity matrix of all the accessions was estimated based on Jaccard's coefficient (Jaccard 1908) and a dendrogram was generated based on the un-weighted pair-group method for arithmetic mean (UPGMA) using the software NTSYS version 2.10e (Rohlf 2000).

Fig 1 *In-vitro* micropropagation of *Citrus maxima*. [A] Shoot induction, [B] Shoot apices used as an explants, [C] Shoot induction, [D] Multiplication, [E] Elongation, [F] Rooting, [G] Hardening and [H] Plantlets transfer to field.

RESULTS AND DISCUSSION

In-vitro recovery and sub-culture

In the present study, the aim was to develop a successful protocol for *in vitro* multiplication using shoot tips and nodal sections as explants for regeneration of healthy and normal plantlets. The study revealed that the ratio of cytokinin and auxins have substantial effect on the regeneration of plantlets.

Within 7-10 days, the embryonic axes regenerated and 100% shoot and root formation was observed with the average shoot length and root length of 5.83 cm and 7.53 cm, respectively, along with maximum nodes (8.67) without callus formation using MS medium supplemented with 1 mg/l BAP and 1 mg/l NAA with activated charcoal (Table 1 and Fig 1A).

Shoot formation and multiplication

The plantlets were then aseptically cut into the 1.5 cm segments with nodes in middle and the shoot tips which were taken as explants (Fig 1B). The highest shoot induction (86.63%) was recorded in the MS Medium supplemented with 0.5 mg/l BAP + 0.1 mg/l NAA (Fig 1C). In all the combination of hormones, the shoot induction was observed from 9-20 days and the maximum number of nodes (8.67) were found in the MS medium supplemented 0.5 mg/l BAP +0.1 mg/l NAA and least number of nodes recorded in DKW and WPM medium with no intervening callus except in DKW media. The maximum numbers of shoots (8) were achieved in the MS medium supplemented with 0.5 mg/l BAP+0.1 mg/l NAA (Table 2 and Fig 1D).

Similarly, Paudyal (2000) also reported thatMS medium supplemented 0.5 mg/l BAP +0.1 mg/l NAA was most effective to give 86%% shoot formation with maximum 8 adventitious shoots and 8.33 number of nodal segments without intervening callus after 8 weeks of culture. The

Table 1 *In vitro* culture of embryonic axes to establish mother cultures

Medium	Time taken for germination	Shoot formation (%)	Shoot length (cm)	Root formation (%)	Root length (cm)	No. of nodes	Callus
Basal medium The embryonic axes were inoculated for 5 days							
MS+1 mg l^{-1} BAP+ mg l^{-1} NAA + 0.8% Agar + 2.5% charcoal	7- 10 (days)	100 (±0.00)[a]	5.83 (±0.15) [a]	100 (±0.00) [a]	7.53 (±0.18) [a]	8.67 (±0.33) [a]	-
MS+1 mg l^{-1} BAP+ 1 mg l^{-1} NAA + 0.8% Agar	5-10 days	99.33(±0.67) [a]	5.03 (±0.09)[b]	98.67 (±1.33) [a]	5.83 (±0.12)[b]	7.33 (±0.33)[b]	-

All values are means ± SD (n = 3). Mean values in each column followed by the same lower-case letters are not significantly different at $P \leq 0.05$ by Duncan's multiple range test. BAP, benzylaminopurine; NAA, α-Naphthalene acetic acid

Table 2 Effect of growth regulators on multiple shoot induction in shoot tip explants of *C. maxima*

BAP (mg l^{-1})	NAA (mg l^{-1})	Time for initiation	Shoot formation (%)	No of shoots	Shoot length (cm)	Root formation (%)*	Root length (cm)	Nodes	Callus
MS+ 0.1	0.1	9-13	76.78 (±0.7)^{c}	03	3.63 (±0.12)^{d}	0.00 (±0.00)	0.00 (±0.00)	5.33 (±0.33)^{cd}	-
MS + 0.1	1.0	11-13	78 (±0.01)^{b}	05	4.80 (±0.63)^{c}	66 (±0.24)^{a}	5.80 (±0.15)^{a}	5.7 (±0.12)^{c}	-
MS + 0.5	0.1	13-20	86.63 (±1.63)^{a}	08	6.20 (±0.17)^{a}	0.00 (±0.00)	0.00 (±0.00)	8.67 (±0.33)^{a}	-
MS + 0.5	1.0	13-20	75. 5(±0.58)^{cd}	05	5.63 (±0.03)^{b}	0.00 (±0.00)	0.00 (±0.00)	7.3 (±0.33)^{b}	-
MS + 1.0	0.1	10-15	65 (±1.53.)^{d}	03	3.03 (±0.19)^{e}	0.00 (±0.00)	0.00 (±0.00)	4.80 (±0.58)^{d}	-
WPM alone	0.0	9-13	76.67 (±1.86)^{c}	01	3.17 (±0.23)^{e}	0.00 (±0.00)	0.00 (±0.00)	4.67 (±0.33)^{de}	-
DKW alone	0.0	9-13	77.33 (±1.20) ^{bc}	01	2.33 (±0.18)^{f}	0.00 (±0.00)	0.00 (±0.00)	4.33 (±0.33)^{e}	+

All values are means ± SD (n = 3). Mean values in each column followed by the same lower-case letters are not significantly different at $P \leq 0.05$ by Duncan's multiple range test.

addition of 0.10 mg/l NAA to medium with 2.5 mg/l BAP did not improve the rate of shoot proliferation in pummelo. Begum (2003) reported that half strength of MS medium supplemented with only cytokinin 1 mg/l BAP obtained maximum percentage with 5.3 shoots per explants and the maximum length of shoots was 3.2 cm which is quite lower than the results obtained in the present study. Ibrahim *et al*. (2012) also showed that the nucellar embryo cultured on MS medium supplemented with 2 or 4 mg/l BA with 0.1 mg/l NAA gave adventitious 88% shoots directly with average length of 3 cm with 9.33 shoots per explant and for cotyledonary segments cultured on MS medium supplemented with 4 mg l^{-1} BA with 0.1 mg/l NAA gave white callus after eight weeks. With the length of 3cm with 6.3 shoots per explant as well as 80% of that formation callus sub-cultured on MS medium supplemented with 1 or 2 mg/l BA with 0.1 mg/l NAA gave adventitious shoots indirectly after six weeks of culture. Baruha *et al*. (1995) obtained most 3.2 shoots per explant when pomelo shoot tips excised from *in-vitro* grown seedlings were cultured on MS medium supplemented with 0.37 mg/l BA. However, in the study of other citrus species reported by Altaf *et al.* (2008) showed that 2 mg/l BA with 0.50 mg/l NAA induced multiple buds from *Citrus jambhiri.* Haripyaree, (2011) showed similar results when *Citrus megaloxycarpa* shoot tip explant culture produced 100% shoot formation When cultured on MS medium supplemented with 0.25 mg/l BAP and 1 mg/l BAP with 0.50 mg/l NAA and the maximum number of shoots 4.7 was induced on medium containing 0.25 mg/l BA together with 0.50 mg/l NAA or 1 mg/l BA with 0.50 mg/l kinetin. Kumar *et al*. (2014) reported the multiplication in *C. resini*. The maximum survival of explants (90%) on BAP 1.0 mg/l with kinetin 0.5 mg/l minimum time required to bud breaking (19.50 days) on BAP 0.5 mg/l with kinetin 0.5 mg/l maximum number of shoot (7.30) on BAP 2.0 with mg/l kinetin 1.0 mg/l maximum length shoot (2.40 cm) on BAP 0.5 mg/l with kinetin 2.0 mg/l.

Elongation

The effect of GA_3 alone and in combination with BAP was analysed on elongation of plantlets using shoot tips and nodal sections as an explant. The use of GA_3 alone in the medium have resulted low percentage elongation of plantlets in comparison to when used in the combination of BAP, where it has been quite effective. The approximately 80% elongation has occurred in 3 different concentrations of GA_3 (0.6 mg/l, 0.8 mg/l and 1.0 mg/l in combination with 0.2 mg/l BAP). The length of shoot formation ranged 6-7 cm with 7-8 nodes in the plantlet and no intervening callus was recorded. Interestingly, there was a reduction in elongation of the plantlets when the GA_3 was used in combination with 0.6 mg/l BAP instead this led to the formation of callus and pale yellow plantlets (Table 3 and Fig 1E). Only GA_3 in the medium have low percentage in elongating the plantlet but with the combination of BAP it has been effective. The 80% elongation has occurred in 3 different concentration of GA_3 (0.6 mg/l , 0.8 mg/l and1 mg/l in combination with 0.2 mg/l BAP). The length of shoot formation ranged 6-7 cm with 7-8 nodes in the plantlet and no intervening callus was recorded. There was reduction in elongation of the plantlet when the GA_3 in combination with 0.6 mg/l BAP was used, which lead to the formation of callus and pale yellow plantlet. Paudyal (2000) reported that addition of 5.8 mM gibberellic acid in shoot-proliferation medium during the second subculture improved shoot elongation significantly. Shoot multiplication increased 3.5 fold in each successive subculture which is quite related to the present study.

Rooting

Root formation initiated within 13-17 days after the regenerated plantlets transferred to the rooting medium. Various combinations of hormones using auxins (NAA) were utilized for rooting. The plantlets with 2.5 cm length were inoculated on MS + 0.8% Agar/Phytagel with 1.5% sucrose. The best result of root formation with 81% rooting with no intervening callus was observed in response to 1/2MS+ 0.1 mg/l BAP+1 mg/l NAA+ 2.5 gm phytagel medium (Fig 1F). The plantlets were acclimatized and survived after the transfer to the fields (Figs 1G-H). The root formation regenerated from the shoots was best achieved in response against half strength of MS+ 0.1 mg/l BAP+1 mg/l NAA+ 2.5 gm phytagel. The rooting percentage was higher on half strength of MS medium than on full strength

Table 3 Effect of growth regulators on multiple shoot elongation of *C. maxima*

Elongation medium MS+0.3% Sucrose+2.5% Phytagel						
GA_3 (mg l^{-1})	BAP (mg l^{-1})	Time for initiation	Shoot elongation (%)	Shoot length (cm)	Nodes	Callus
0.0	0.0	25	0.00 (±0.00)	0 (±0.00)	0.00 (±0.00)	-
0.2	0.0	21	20.67 (±0.88)^{f}	3.37 (±0.09)^{fg}	3.67 (±0.33)^{e}	-
0.4	0.0	21	34.00 (±1.16)^{e}	3.43 (±0.32)^{f}	4.67 (±0.33)^{d}	-
0.6	0.0	21	43.00 (±0.58)^{de}	3.63 (±0.22)^{ef}	4.67 (±0.33)^{d}	-
1.0	0.0	21	46.00 (±1.16)^{d}	3.70 (±0.31)^{e}	5.67 (±0.33)^{cd}	-
0.2	0.2	25	67.00 (±1.42)^{c}	4.57 (0.19)^{d}	6.40 (±0.33)^{c}	-
0.4	0.2	25	79.33 (±2.41)^{b}	5.63 (0.15) ^{c}	7.10 (±0.33) ^{c}	-
0.6	0.2	15-17	82.00 (±0.63)^{a}	6.97 (0.12)^{a}	7.33 (±0.33)^{bc}	-
0.8	0.2	15-17	81.20 (±0.13)^{ab}	6.70 (0.12)^{ab}	8.33 (±0.33)^{a}	-
1.0	0.2	15-17	81.09 (±0.45)^{ab}	5.93 (0.09)^{b}	7.67 (±0.33)^{b}	-
0.2	0.6	20-25	77.67 (±1.86)^{b}	4.60 (0.15)^{d}	7.33 (±0.33)^{bc}	-
0.4	0.6	25-30	41.00 (±1.53)^{de}	3.73 (0.12)^{e}	4.67 (±0.33)^{d}	+
0.6	0.6	25-30	23.67 (±2.19)^{ef}	3.40 (0.26)^{f}	4.33 (±0.33)^{de}	++
0.8	0.6	25-30	05.67 (±0.88)^{h}	2.13 (±0.38)^{g}	3.67 (±0.33)^{e}	+
1.0	0.6	25-30	0.00 (±0.00)	0 (±0.00)	0 (±0.00)	++
0.2	1.0	25-30	0.00 (±0.00)	0 (±0.00)	0 (±0.00)	+++
0.4	1.0	25-30	13.00 (±1.16)^{f}	1.17 (±0.20)^{i}	2.33(±0.33)^{g}	++
0.6	1.0	25-30	09.67 (±0.33)^{g}	1.67 (±0.12)^{h}	2.33 (±0.33)^{g}	++
0.8	1.0	25-30	08.67 (±1.20)^{gh}	2.10 (±0.17)^{g}	2.33 (±0.33)^{g}	+++
1.0	1.0	25-30	0.00 (±0.00)	0 (±0.00)	2.67 (±0.33)^{f}	+++

Where, (-)= No callus formation, (+)= partial callus formation, (++)= 50% callus formation, and (+++) = 80% callus formation. All values are means +/– SD (n = 3). Mean values in each column followed by the same lower-case letters are not significantly different at $P \leq 0.05$ by Duncan's multiple range test.

of MS medium (Choudhary *et al*. 2015). The root formation initiated within 13-17 days, with 81% root formation and no intervening callus was observed. In the previous studies of *C. maxima* (Paudyal 2000) stated that NAA was superior to indolebutyric acid (IBA) for *in vitro* root induction which is an agreement with the present study. 75% of the shoots developed roots when transferred to half-strength MS medium with 1.3, 2.7, or 5.4 mM NAA. The results with maximal rooting at 2.0 mg/l NAA, and a decrease in the frequency of rooting below of NAA concentration 2.0 mg/l. Begum (2003) reported for rooting half strength of MS medium with 0.1 mg/l NAA with 100% rooting within 20-25 days after culture with average length of 3.1 cm and established 95 plantlets. The rooting percentage was similar to the present study but varied on the basis of the length of the root which was quite low. Ibrahim *et al*. (2012) cultured shoots on half strength of MS medium supplemented with 0.2 mg/l NAA with 0.1 mg/l BA gave roots after six weeks. Haripyaree (2011) also studied rooting in *C. megaloxycarpa* and achieved in MS medium containing 1.0 mg/l or 2.0 mg/l of IBA, IAA and NAA. The highest number of roots was produced with 2.0 mg/l NAA with the maximum of 4.4 roots. The maximum (90%) survival of micro-shoot for rooting and length of root (6.28 cm) were recorded on MS medium modified with NAA 0.5 mg l^{-1} with IBA 0.5 mg l^{-1}. The minimum time taken to root induction (22.00 days) on NAA 0.5 mg l^{-1} with IBA 0.1 mg l^{-1} and maximum number of root (5.60) on NAA 0.5 mg l^{-1} with IBA 1.0 mg l^{-1} were observed (Table 4).

Genetic stability

Maintenance of true-to-type or clonal fidelity is one of the important aspects to be looked into while attempting *in vitro* conservation of plant species. SSR markers were successfully applied for the detection of genetic similarities or dissimilarities in between *C. maxima* mother plant and sub-cultured clonal plantlets. Out of 20 SSR primers used in the initial screening, only 12 primers produced clear and reproducible loci. The 12 SSR primers produced 57 distinct and scorable loci in the size range of (UCM20) 75 – 550bp. A maximum number of 8 loci were confined within the ladder size of 90 to 210 bp. Furthermore, no difference was observed in banding patterns of any of the sample population for a particular primer with their respective mother plants, indicating absence of any genetic variation among the *in vitro* raised plantlets (Fig 2). The overall banding profile obtained from the total of 57 bands (number of plants analyzed × total number of scorable loci from SSR primers) was generated from the mother plant and 7 *in vitro* raised clones. All banding profiles from micropropagated plants

Table 4 Effect of growth regulators on root induction of *C. maxima.*

Rooting medium 1/2MS+1.5% sucrose+2.5% Phytagel				
BAP (mg l^{-1})	NAA (mg l^{-1})	Root formation (%)	Root length	Callus
0.0	0.0	0.00(±0.00)	0(±0.00)	-
0.1	1.0	37.67(±0.88)^{e}	2.80(±0.21)^{c}	+
0.1	0.1	32.67(±0.87)^{e}	1.50(±0.29)^{d}	-
0.1	0.5	65.67(±0.88)^{b}	3.37(±0.09)^{b}	-
0.1	1.0	76.00(±1.53)^{ab}	3.73(±0.37)^{a}	-
0.5	0.1	49.67(±0.88)^{d}	2.57(±0.12)^{c}	-
0.5	0.5	60.67(±0.78)^{c}	2.60(±0.31)^{c}	-
0.5	1.0	78.33(±1.20)^{a}	3.73(±0.38)^{a}	-
1.0	0.1	44.33(±0.77)^{d}	3.67(±0.33)^{ab}	++
1.0	0.5	56.00(±1.16)^{cd}	3.20(±0.09)^{bc}	+
1.0	1.0	64.00(±0.58)^{bc}	3.40(±0.15)^{b}	+

Where, (-)=No callus formation, (+)= partial callus formation, (++)= 60% callus formation during rooting. All values are means +/– SD (n = 3). Mean values in each column followed by the same lower-case letters are not significantly different at $P \leq 0.05$ by Duncan's multiple range test.

were monomorphic and similar to those of the mother plants (Fig 2). The possible reason may be multiple shoot bud differentiation without intervening callus phase the condition which is least vulnerable to genetic changes. A similarity matrix based on Jaccard's coefficient revealed that the pair-wise value between the mother plant and the plantlets derived from different explants was 1, indicating 100% similarity. Genetic variation studies among the *in vitro* regenerated plantlets have been undertaken using cyto-morphological and molecular marker analysis, in spite of morphological similarities in several species (Seth and Panigrahi 2018). DNA based markers are relatively proficient and trustworthy for genetic fidelity appraisal. Thus, different types of DNA markers including ISSR were employed to assess the genetic stability of *in vitro* derived plantlets in several plants, such as, *Dioscorea* (Mandal *et al*. 2008), *Abutilon indicum* (Seth and Panigrahi, 2018) *Rhinacanthus nasutus* (Cheruvathur et al., 2012); *Ceropegia evansii* (Chavan *et al*. 2012) and *Hylocereus undatus* (Fan et al. 2013). Hence, it becomes imperative to regularly check the genetic purity of the micropropagated plants in order to produce clonally uniform progeny while using different techniques of micropropagation. Our results further suggest that the molecular marker approach is a useful tool in the evaluation of the genetic stability of *in vitro* propagated plants. Similar results have been reported by Choudhary *et al.* (2013) in *Morus* spp after cryopreservation. Using organized and well-differentiated explants like apical shoots and avoiding a callus phase minimizes the genetic variations in regenerating plants (D'Amato 1985). But a detailed study of the other markers and relative suitability of each one of them is warranted. Owing to the importance of genetic stability, similar protocol appears to be highly stable and

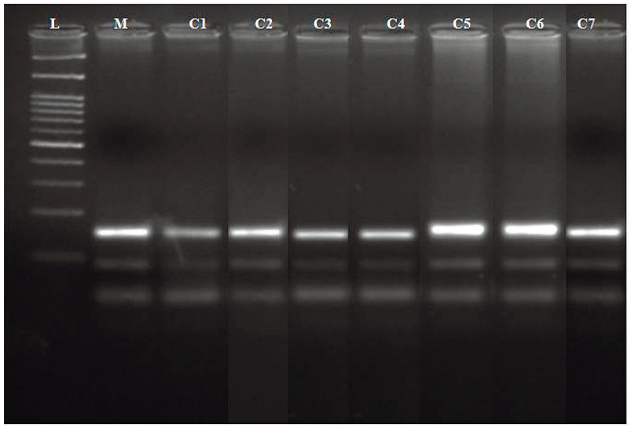

Fig 2 Genetic stability showing photographs using SSR marker (SSR 01) with mother plant (M) and *in vitro* cloned plants (C1 to C7).

supports our claim that micropropagated plantlets are true to type to the donor plants in mulberry (Saha *et al*. 2015). Successful protocol developed for in vitro multiplication, conservation and regeneration for *C. maxima* with no morphological and genetic fidelity would be highly useful for commercialization and promotion of specific genotypes cultivars. Besides, successful *in vitro* multiplication would provide the explants for attempting cryopreservation studies to achieve long term true to type conservation of specific cultivars.

Conclusion

The successful protocol for micropropagation and subculture at certain intervals has lead conservation of various plant species through vegetative propagation. The protocol developed by proliferating the axillary shoots and the nodal section as explants for maintaining the cultures for medium term conservation and successful regeneration/ transfer of plantlets in field is the first attempt in this important Citrus species. The study revealed the best combination of medium used for *in-vitro* conservation of *C. maxima* was 0.5 mg/l BAP+0.1 mg/l NAA in which the cultures can be maintained for about 7 months. *In vitro* conserved germplasm can be considered for backup collections, the most conventional method for regeneration of virus free plantlet through the mother plant and may ensure the availability of healthy germplasm for the storage and exchange purposes.

REFERENCES

Altaf N, Khan A/R, Ali L and Bhatti I/A. 2008. Propagation of rough lemon (*Citrus jambhiri* Lush.) through *in vitro* culture and adventitious rooting in cutting. *EJEAF Chemistry* **7**: 3326–33.

Barkley N A, Roose M L, Krueger R R and Federici C. 2006. Assessing genetic diversity and population structure in a citrus germplasm collection utilizing simple sequence repeat markers (SSRs). *Theoretical Applied Genetics*, **112**: 1519–31.

Baruah A, Nagaraju V and Parthasarathy VA. 1995. Cytokinin mediated response in shoots of *Citrus grandis sb*. *Annals of Plant Physiology*, **9**: 13–6.

Begum F, Amin M N, Islam S, Azad M A K and Rehman M M. 2003. *In vitro* plant regeneration from cotyledon-derived callus of three varieties pummelo (*Citrus grandis* (L.) Osbeck.). *Onine Journal of Biological Sciences* **3 (8)**: 751–759.

Chavan J J, Gaikwad N B, Kshirsagar P R, Umdale S D, Bhat K V, Dixit G B and Yadav S R. 2015. Highly efficient in vitro proliferation and genetic stability analysis of micropropagated Ceropegia evansii by RAPD and ISSR markers: A critically endangered plant of Western Ghats. *Plant Biosystems,* **149**: 442–50.

Cheruvathur M K, Sivu A R, Pradeep N S and Thomas T D. 2012. Shoot organogenesis from leaf callus and ISSR assessment for their identification of clonal fidelity in *Rhinacanthus nasutus* (L.) Kurz., a potent anticancerous ethno medicinal plant. *Industrial Crops and Products* **40**: 122–8.

Choudhary R, Chaudhury R, Malik S K, Kumar S and Pal D. 2013. Genetic stability of mulberry germplasm after cryopreservation by two-step freezing technique. *African Journal of Biotechnology* **12 (41**): 5983–93.

Choudhary R, Chaudhury R and Malik S K. 2015. Development of an efficient regeneration and rapid clonal multiplication protocol for three different *Morus* species using dormant buds as explants. *Journal of Horticulture Sciences and Biotechnology (UK)*, **90 (3):** 245–53.

Damania A B. 1996. Biodiversity conservation: A review of options complementary to standard *ex-situ* methods. *Plant Genetic Resources Newsletters* **107**: 1–18.

D'amato F. 1985. Cytogenetics of plant cell and tissue cultures and their regenerates. *CRC Critical Reviews for Plant Sciences* **3**: 73–112.

De Carlo A and Lambardi M. 2005. Cryopreservation of citrus germplasm. (*In*): *The role of biotechnology*, Villa Gualino, 5-7 March. 2005, Turin, Italy, pp 169–70.

Engelke A I, Hamzi H Q and Skoog F. 1973. Cytokinin-gibberellin regulation of shoot development and leaf form in tobacco plantlets. *American Journal of Botany* **60**: 491.

Engelmann F. 1997. Importance of desiccation for cryopreservation of recalcitrant seed and vegetatively propagated species. *Plant Genetic Resources Newsletter* **112**, 9–18.

Fan Q, Zheng S, Yan F, Zhang B, Qiao G and Wen X. 2013. Efficient regeneration of dragon fruit (*Hylocereus undatus*) and an assessment of the genetic fidelity of in vitro-derived plants using ISSR markers. *Journal of Horticultural Science and Biotechnology* **88**: 631–7.

Gomez K A and Gomez A A. 1976. *Statistical procedures for agricultural research with emphasis on rice.* International Rice Research Institute, Los Banos, the Philippines.

Haripyaree A, Guneshwor K, Sunitibala H and Damayanti M. 2011. *In vitro* propagation of *Citrus megaloxycarpa. Environmental and Experimental Biology* **9**: 129–32.

Ibrahim M A. 2012. *In vitro* plant regeneration of local pummelo (*Citrus grandis* (L.) Osbeck.) via direct and indirect organogenesis. *Genetics and Plant Physiology* **2(3–4):** 187–91.

Jaccard P. 1908. Nouvelles recherches sur la distribution florale. Bulletin Societe, Vaudoise des Sciences Naturelles, **44**, 223–270.

Kumar R, Kaul M K, Saxena S N, Singh A K, Singh J and Lohora S K. 2014. *In vitro* propagation studies of virus tolerant citrus rootstock Cleopatra mandarin (*Citrus reshnii* Tanaka). *Progressive Horticulture* **46**: 202–8.

Liu Y Z and Deng X X. 2007. Citrus breeding and genetics in China. *Asian and Australasian Journal of Plant Science and Biotechnology* **1(1):** 23–8.

Madhava Chetty and Rao K N. 1990. Endemic plants to tirumala hills chittor district of Andhra Pradesh. *Vegetos* **3**: 12–5.

Malik S K, Chaudhury R and Pritchard H W. 2012. Long-term, large scale banking of citrus species embryos: comparisons between cryopreservation and other seed banking temperatures. *CryoLetters* **33 (6):** 453–64.

Mandal B B, Ahuja-Ghosh S and Srivastava P S. 2008. Cryopreservation of *Discorea rotundata* Poir. A comparative study with two cryogenic procedures and assessment of true-to-type of regenerants by RAPD analysis. *CryoLetters* **29**: 399–408.

Marin M L and Duran-Vila N. 1988. Survival of somatic embryos and recovery of plants of sweet orange (*C. sinensis* (L) osb.) after immersion in liquid nitrogen. *Plant Cell Tissue Organ Culture* **14**: 51–7.

Morton J. 1987. Pummelo. (In): *Fruits of Warm Climates*. Julia F. Morton, Miami, FL, pp 147–51.

Murashige T and Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**: 473–97.

NHB Database. 2012. National Horticulture Board (NHB) © Government of India PDES – 256 (E) Controller of Publication 500 – (DSK-III)(www.agricoop.nic.in).

Paudyal K P and Haq N. 2000. *In vitro* propagatipon of pummelo (*Citrus grandis* L. Qsbeck). *In Vitro Cellular Developmental Biology- Plant* **36**: 511–6.

Rohini M R, Malik S K, Choudhary R, Kaur S, Uchoi A and Chaudhury R. 2016. Storage behaviour and Cryopreservation of embryonic axes of rough lemon (*Citrus jambhiri*): a promising rootstock for long-term conservation. *Turkish Journal of Agriculture and Forestry* **40**: 865–73.

Rohlf F J. 2000. NTSYS-pc: numerical taxonomy and multivariate analysis, system, ver. 2.10e, Exeter Ltd., Setauket, NY, USA.

Saha S, Adhikari S, Dey T and Ghosh P. 2016. RAPD and ISSR based evaluation of genetic stability of micropropagated plantlets of *Morus alba* L. variety S-1. *Meta Gene* **7**: 7–15.

Seth S and Panigrahi J. 2018. *In vitro* organogenesis of *Abutilon indicum* (L.) Sweet from leaf derived callus and assessment of genetic fidelity using ISSR markers, *Journal of Horticultural Science and Biotechnology,* DOI: 10.1080/14620316.2018.1447314.

Singh H P and Chadda K L. 1993. Genetic resources of citrus.. (*In*) *Advances in Horticulture-Fruit crops* Vol 2, pp:95–122 (Chadda K L and Pareek O P eds) Malhotra Publishing House, New Delhi.

Styer D J and Chin C K. 1983. Meristem and shoot-tip culture for propagation, pathogen elimination and germplasm preservation. *Horticulture Reviews* **5**: 221–77.

Taskin H, Baktemur G, Kurul M and Buyukalaca S. 2013. Use of tissue culture techniques for producing virus-free plant in garlic and their identification through real-time PCR. The Scientific World Journal, http://dx.doi.org/10.1155/2013/781282, 1–5.

WCSP. 2012. http://ipni.org/urn:lsid:ipni.org:names:30075266-2.

Withers L A and King P J. 1980. A simple freezing unit and cryopreservation method for plant cell suspensions. *Cryoletters* **1**: 213–20.