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Full Length Research Paper

Effects of adenine sulphate, glutamine and casein hydrolysate on *in vitro* shoot multiplication and rooting of Kinnow mandarin (*Citrus reticulata* Blanco)

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***In vitro* shoot regeneration was carried out for Kinnow mandarin (*Citrus reticulata* Blanco) through shoot tip explants obtained from *in vitro* germinated seedling. The medium supplemented with 2.5 mg/L benzylaminopurine (BAP) supported maximum shoot proliferation (2.45 shoots/explant). This rate was further enhanced (7.23 shoots/explant) by adding 25 mg/L glutamine, 50 mg/L adenine sulphate and 100 mg/L casein hydrolysate. From the *in vitro* proliferated shoots, the shoot apices and nodes were excised and sub-cultured on four different medium namely: SCM-1, SCM-2, SCM-3 and SCM-4 for six consecutive months. Problems like defoliation, chlorosis and loss of vigour in shoots were observed at different stages of subculture. These problems were successfully controlled by SCM-4 having 1.5 mg/L of BAP, 0.5% charcoal, 25 mg/L glutamine, 50 mg/L adenine sulphate and 100 mg/L casein hydrolysate. The SCM-4 also maintained the high proliferation rate throughout the subculturing process. Rooting was best induced on 2 mg/L indole-butyric acid (IBA) in combination with 0.1 mg/L indole-3-acetic acid (IAA). Rooting was significantly enhanced by 100 mg/L casein hydrolysate. The *in vitro* raised plantlets were acclimatized under culture room conditions in different potting mixture, of which the combination of garden soil, sand and vermiculite mixture in 1:1:2 ratio was found most supportive. After 30 days of acclimatization, plantlets were transferred to soil, where established plants showed more than 90% survival.**

Key words: *In vitro* shoot regeneration, micro-shoots, enhanced shoot multiplication, subculture, acclimatization.

INTRODUCTION

Kinnow mandarin (*Citrus reticulata* Blanco), member of family Rutaceae, is a very popular fruit crop among various citrus species. The fruits are known for its good processing quality, excellent source of vitamin C, fresh consumption, aromatic flavor and low content of saturated fat, cholesterol and sodium. Kinnow mandarin is reported to be a hybrid between King tangor and Willow leaf mandarin. Among various citrus species, it has remarkable heat tolerance capability, a character inherited from its parent cultivar King and this helps it to survive in harsh hot summer with maximum temperatures around 48°C (Altaf et al., 2008). It is currently grown commercially, to a major extent in India, West Pakistan and to some extent in California, Arizona and Florida.

Kinnow is an economically important cash crop of India with great deal of production and export. Kinnow mandarins have vigorous growth and heavy yield with favorable growing conditions in the orchards, but only for initial 7 to 8 years. After that, due to various biotic and

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Abbreviations: PGRs, Plant growth regulators; **BAP,** benzylaminopurine; **Kin,** kinetin; **Zea,** zeatin; **SMM,** shoot multiplication medium; **SCM,** sub-culture medium; **IAA,** indole acetic acid; **IBA,** indole butyric acid; **CH,** casein hydrolysate; **Gln,** glutamine; **ADS,** adenine sulphate.

abiotic factors, the decline of the trees start with sparse appearance, yellowing of leaves, sickly look, dried up growth, less number of fruits and smaller size of fruits, resulting in decline in productivity and hence huge financial losses (Naqvi, 2004). Kinnow mandarins are mainly propagated by bud grafting, which allow the transmission of unhealthy planting material to next generation. Further, propagation becomes limited to the season when buds are available.

Micropropagation system with high multiplication rate is not only an important asexual method for the production of healthy clonal plants, but also forms the basis for the introduction of genetic variation by genetic transformation or mutagenesis. A number of *in vitro* studies have been made on various other citrus cultivars (Rathore et al., 2007; Abdulaziz and Al-Bahrany, 2002; Carimi et al., 1999; Savita et al., 2010). Few studies have also reported the *in vitro* shoot regeneration in Kinnow mandarin either through direct shoot organogenesis (Usman et al., 2005; Altaf, 2006; Al-Khayri and Al-Bahrany, 2001; Altaf et al., 2009) or through somatic embryogenesis (Parveen et al., 2005; Gill et al., 1995). The explants, excised from a mature tree, generally suffer from some bacterial, fungal or viral infections, which are very frequent in Kinnow trees making the establishment of aseptic cultures a tedious process. Sometimes additional steps of chemotherapy are also being carried out (Sharma et al., 2007) and the results are also not consistent and reproducible. The initiation of cultures from *in vitro* raised seedlings can be a good alternative to the explants from mature tree. Hassanein and Azooz (2003, 2004) reported various factors for *in vitro* seed germination of *Citrus reticulata* Blanco, but the study lacked the discussion about frequency of shoot regeneration and proliferation rate of shoots under various *in vitro* conditions. Further no discussion has been made regarding shoot multiplication during repeated sub-cultures, which is required for round-the-year supply of healthy planting material. So there is a need to further modify the micropropagation protocol for Kinnow mandarin which can fulfill the commercial demand of healthy planting material of this tree.

Adenine, in various forms enhances the growth of isolated meristem tips, induces the proliferation of axillary shoots in shoot cultures and promotes the adventitious shoot formation indirectly from calli or directly from the explants (Van Stedan et al., 2008). Many reports specify that organic nitrogen source like amino acids instead of inorganic source like nitrates or ammonium sulphates, can improve the cell proliferation as well as regeneration in specific genotypes (Vasudevan et al., 2004; Asad et al., 2009). Some studies report mixture of amino acids (like Casein hydrolysate), rather than a single amino acid, as very supportive for shoot multiplication even in prolonged cultures (Nasir et al., 2011). In the present study we have examined the effects of different concentrations and combinations of adenine sulphate (ADS), glutamine (Gln) and casein hydrolysate (CH) during different stages of micropropagation of Kinnow mandarin; no reports being available for this till date. We report the accomplishment of a highly efficient micropropagation system (with high, sustainable shoot multiplication rate, efficient rooting and acclimatization) for *C. reticulata* Blanco using explants from *in vitro* raised seedling.

MATERIALS AND METHODS

Fruit samples were collected during the months of November, 2009 to February, 2010, from a healthy looking young tree growing in the orchard of Department of Horticulture (Government of Haryana State), Sirsa, India. The fruits were peeled off and seeds were collected in a beaker having the fresh juice/pulp of these fruits (to avoid drying of the seed coats during the entire process of fruit peeling and seeds removal). After complete isolation, seeds were thoroughly washed under tap water for 15 min to remove mucus and sugars present on the seed coat. The seeds were dipped in 0.2% Tween-20 solution for 10 min followed by thorough rinsing with double distilled water. Then the seeds were soaked in sterilized double distilled water for 30 min. This was followed by peeling of seeds under aseptic conditions in Laminar Air Flow chamber. Peeled seeds were then surface sterilized with 0.1% $HgCl_2$ for 5 to 7 s and subsequently thorough rinsing of seeds with sterilized double distilled water was carried out for 3 to 4 times to remove the traces of $HgCl_2$.

Medium preparation

The Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) was used throughout the study. The basal media were modified by adding measured quantity of growth regulators (Aldrich Sigma Chemical Company, St. Louis, USA) and medium additives (Aldrich Sigma Chemical Company, St. Louis, USA), as required. The pH of the medium was adjusted to 5.8 using pH meter (pH tester 30), before adding Agar-agar Type-I (0.8%) (Aldrich Sigma Chemical Company, St. Louis, USA). The medium was melted in a microwave till the agar was fully dissolved in the solution. The media was poured equally (75 to 80 ml) in the flasks/magenta boxes (Tarsons products pvt. Limited) with constant shaking to avoid formation of agar clump. Sterilization of medium was carried out in an autoclave at 15 Psi and 121°C, for 15 to 20 min.

In vitro seed germination

The surface sterilized seeds were placed on MS basal medium without any growth regulator and were incubated in culture room at 25 ± 2°C. The photoperiod conditions were varied, some were exposed to a photoperiod of 16 h (60 µmol m^{-2} s^{-1}) while some were incubated in complete dark conditions. Germination of seed in percent, length of the seedling was recorded after four weeks of cultures (Table 1). The average initiation day for germination was also recorded. For both the above treatments, 30 explants (seeds) were used and the experiment was repeated thrice.

Effect of plant growth regulators (PGRs) and different additives on shoot regeneration and multiplication

The shoot tips (ST) were excised from 28 days old *in vitro* germinated seedlings and were inoculated on MS basal medium supplemented with various concentrations (0.5, 1.0, 1.5, 2.0, 2.5

Table 1. Effect of photoperiod on *in vitro* seed germination of Kinnow mandarin.

S/N	Condition	Germination frequency (after 28 days)	Length of seedling (after 28 days) (cm)	Initiation day for germination
1	Complete dark	93.33 ± 04.63	04.31 ± 00.15	10.23 ± 00.20
2	16 h photoperiod	83.33 ± 06.92	03.35 ± 00.15	16.48 ± 00.21

Values represent means ± SE of 30 replicates.

Table 2. Effect of different concentrations of benzylaminopurine (BAP), kinetin (KIN) and zeatin (ZEA), supplemented individually to Murashige and Skoog basal medium, on shoot organogenesis after 4 weeks of culture.

Concentration (mg/L)	Cytokinin	Number of shoots per explant	Length of shoots	Number of nodes per shoot
-	-	1.13 ± 0.23^{b}	0.79 ± 0.10^{c}	1.41 ± 0.16^{bcd}
	BAP	1.63 ± 0.26^{ab}	1.47 ± 0.26^{abc}	1.78 ± 0.22^{b}
0.5	kin	1.38 ± 0.32^{b}	1.69 ± 0.21^{ab}	1.00 ± 0.29^{def}
	Zea	1.21 ± 0.49^{b}	1.09 ± 0.13^{bc}	0.67 ± 0.09^{efg}
	BAP	1.75 ± 0.25^{ab}	1.45 ± 0.25^{abc}	1.26 ± 0.25^{bcd}
1.0	Kin	1.15 ± 0.36^{b}	1.69 ± 0.21^{ab}	0.44 ± 0.09^{g}
	Zea	1.76 ± 0.35^{ab}	1.5 ± 0.27^{ab}	1.07 ± 0.27^{de}
	BAP	1.40 ± 0.19^{b}	1.55 ± 0.31^{ab}	1.64 ± 0.10^{bc}
1.5	Kin	1.39 ± 0.42^{b}	1.49 ± 0.17^{ab}	0.63 ± 0.14^{efg}
	Zea	1.22 ± 0.49^{b}	1.00 ± 0.14^{bc}	0.49 ± 0.08^{fg}
	BAP	2.01 ± 0.23^{ab}	1.62 ± 0.14^{ab}	2.64 ± 0.10^{a}
2.0	Kin	1.38 ± 0.32^{b}	1.01 ± 0.16^{bc}	1.22 ± 0.20^{cd}
	Zea	1.37 ± 0.30^{b}	1.09 ± 0.13^{bc}	0.51 ± 0.04^{fg}
	BAP	2.45 ± 0.14^{a}	2.11 ± 0.29^{a}	2.62 ± 0.12^{a}
2.5	Kin	1.18 ± 0.40^{b}	1.63 ± 0.26^{ab}	0.98 ± 0.16^{defg}
	Zea	1.28 ± 0.11^{b}	1.34 ± 0.19^{bc}	1.04 ± 0.14^{def}

Values represent means ± SE of 8 replicates, those representing similar letter in the appropriate column are not significantly different (ANOVA, $P < 0.05$), as analyzed by DMRT.

mg/L) of three different cytokinins (benzylaminopurine (BAP), kinetin (Kin) and zeatin (Zea)), individually (Table 2). The cultures were kept in culture room at a photoperiod of 16 h. The percent response, mean number of shoots per explant, average length of shoot and mean number of nodes per shoot was observed after four weeks of culture, to select the most suitable PGR regime. To this, three additives glutamine (Gln), adenine sulphate (ADS) and casein hydrolysate (CH) were added in different concentrations and combinations to define the optimal shoot multiplication medium (SMM) (Table 3). Observations were recorded as reported in Table 3. For each of the treatment, 8 explants were used and the experiment was repeated thrice.

Effect of medium components on shoot multiplication during repeated sub-culturing

The optimal shoot multiplication medium (SMM), from the study, was further modified to four different sub-culture medium (SCM) namely: SCM-1, SCM-2, SCM-3 and SCM-4. These four media were having the same concentrations of glutamine (that is, 25 mg/L), ADS (50 mg/L) and charcoal (0.5%) but differed in concentrations of BAP (2.5 mg/L for SCM-1and 1.5 mg/L for each SCM-2, SCM-3, SCM-4) and CH (100 mg/L for SCM-1 and 2, while 150 mg/L for SCM-3 and 200 mg/L for SCM-4).

The shoots regenerated on the SMM were taken for first sub-culturing experiment. Nodes and shoot tips were excised from the shoots and were inoculated on the mentioned four media. Observations were recorded after four weeks of culture. After that, the shoots regenerated on each of the four medium were again separated into nodal segments and shoot apices, which were then cultured on respective medium, making it second sub-culture event. This process was repeatedly carried out consecutively six times, after a regular interval of 28 days. The effect of media components and sub-culturing event on shoot proliferation was recorded, after 28 days of each sub-culture (Table 4). Culture conditions and observations recorded were the same as reported above. For each of the above treatment 20 explants (nodes and shoot apices

Table 3. Effect of glutamine (Gln), adenine sulphate (ADS) and casein hydrolysate (CH), supplemented in different concentrations and combinations to MS medium having 2.5 mg/L BAP, on shoot multiplication after 4 weeks of culture.

Gln (mgL^{-1})	ADS (mgL^{-1})	CH (mgL^{-1})	Number of shoots	Length of shoots (cm)	Number of nodes
Control	-	-	2.45 ± 0.14^{i}	2.11 ± 0.29^{de}	2.62 ± 0.12^{bc}
25	-	-	4.03 ± 0.26^{fg}	2.34 ± 0.30^{cd}	3.25 ± 0.39^{ab}
50	-	-	3.38 ± 0.12^{h}	2.09 ± 0.14^{de}	3.38 ± 0.35^{ab}
100	-	-	1.20 ± 0.07^{j}	1.58 ± 0.22^{e}	3.25 ± 0.31^{ab}
-	50	-	5.97 ± 0.13^{c}	2.38 ± 0.31^{cd}	2.63 ± 0.25^{bc}
-	100	-	3.94 ± 0.19^{g}	1.99 ± 0.33^{de}	3.26 ± 0.27^{ab}
-	150	-	4.88 ± 0.07^{de}	2.87 ± 0.17^{bc}	2.38 ± 0.11^{c}
-	-	50	4.50 ± 0.13^{ef}	2.97 ± 0.19^{bc}	2.65 ± 0.08^{bc}
-	-	100	5.18 ± 0.05^{d}	3.14 ± 0.19^{b}	3.12 ± 0.16^{abc}
25	50	100	7.24 ± 0.19^{a}	4.13 ± 0.27^{a}	3.88 ± 0.17^{a}
25	100	100	6.59 ± 0.30^{b}	2.88 ± 0.17^{bc}	3.38 ± 0.23^{ab}
25	150	100	3.75 ± 0.29^{gh}	3.02 ± 0.18^{bc}	3.37 ± 0.38^{ab}

Values represent means ± SE of 8 replicates, those representing similar letter in the appropriate column are not significantly different (ANOVA, $P < 0.05$), as analyzed by DMRT.

Table 4. Effect of various combinations of benzylaminopurine (BAP) and casein hydrolysate (CH) supplemented to MS medium having 25 mg/L glutamine (Gln), 50 mg/L adenine sulphate (ADS) and 0.5% charcoal, on shoot proliferation of *Citrus reticulata*, during repeated six subculture (SC).

Subculture event	Medium name	BAP (mg/L)	CH (mg/L)	Number of shoots per node	Length of shoots (cm)	Number of nodes	Comment
First SC	SCM-1	2.5	100	8.80 ± 0.34^{c}	3.37 ± 0.11^{b}	3.25 ± 0.23^{b}	+
	SCM-2	1.5	100	9.50 ± 0.57^{b}	3.65 ± 0.11^{b}	3.10 ± 0.23^{b}	+
	SCM-3	1.5	150	11.65 ± 0.49^{b}	3.59 ± 0.11^{b}	3.30 ± 0.30^{b}	++
	SCM-4	1.5	200	13.30 ± 0.30^{a}	4.20 ± 0.10^{a}	4.25 ± 0.27^{a}	+++
Second SC	SCM-1	2.5	100	9.30 ± 0.33^{d}	3.65 ± 0.10^{b}	3.15 ± 0.24^{b}	-
	SCM-2	1.5	100	10.20 ± 0.58^{c}	3.13 ± 0.12^{c}	4.15 ± 0.29^{a}	-
	SCM-3	1.5	150	12.45 ± 0.34^{b}	4.08 ± 0.16^{a}	4.05 ± 0.23^{a}	++
	SCM-4	1.5	200	13.80 ± 0.43^{a}	4.34 ± 0.17^{a}	4.20 ± 0.30^{a}	+++
Third SC	SCM-1	2.5	100	11.40 ± 0.49^{b}	4.01 ± 0.15^{a}	4.65 ± 0.40^{a}	-
	SCM-2	1.5	100	12.05 ± 0.49^{ab}	3.35 ± 0.14^{b}	3.15 ± 0.28^{b}	-
	SCM-3	1.5	150	13.10 ± 0.44^{a}	3.55 ± 0.12^{b}	4.30 ± 0.19^{a}	++
	SCM-4	1.5	200	13.10 ± 0.57^{a}	4.21 ± 0.18^{a}	4.30 ± 0.33^{a}	+++
Fourth SC	SCM-1	2.5	100	6.85 ± 0.42^{d}	2.55 ± 0.12^{b}	3.40 ± 0.34^{b}	-
	SCM-2	1.5	100	7.75 ± 0.49^{c}	3.36 ± 0.16^{ab}	3.30 ± 0.23^{b}	-
	SCM-3	1.5	150	13.55 ± 0.33^{b}	3.72 ± 0.14^{ab}	3.25 ± 0.27^{b}	++
	SCM-4	1.5	200	15.00 ± 0.46^{a}	3.92 ± 0.10^{a}	4.40 ± 0.38^{a}	+++
Fifth SC	SCM-1	2.5	100	5.70 ± 0.40^{d}	3.40 ± 0.13^{b}	3.25 ± 0.26^{b}	-
	SCM-2	1.5	100	7.75 ± 0.44^{c}	3.48 ± 0.10^{b}	3.20 ± 0.25^{b}	-
	SCM-3	1.5	150	12.65 ± 0.59^{b}	4.01 ± 0.17^{a}	4.10 ± 0.31^{ab}	+
	SCM-4	1.5	200	14.30 ± 0.39^{a}	3.66 ± 0.15^{ab}	4.70 ± 0.39^{a}	+++
Sixth SC	SCM-1	2.5	100	5.99 ± 0.66^{c}	3.01 ± 0.11^{b}	3.10 ± 0.28^{b}	-
	SCM-2	1.5	100	5.15 ± 0.43^{c}	3.11 ± 0.15^{b}	4.15 ± 0.23^{a}	-
	SCM-3	1.5	150	11.70 ± 0.47^{b}	3.61 ± 0.11^{a}	4.00 ± 0.21^{a}	+
	SCM-4	1.5	200	14.10 ± 0.45^{a}	3.80 ± 0.05^{a}	4.55 ± 0.37^{a}	+++

Values represent means ± SE of 20 replicates, those representing similar letter in the appropriate column are not significantly different (ANOVA, $P < 0.05$), as analyzed by DMRT. - Early leaf fall and pale green leaves, weak stem, sickly appearance, + No chlorosis, Leaf fall at low frequency, sickly appearance, ++ No leaf fall, No chlorosis, healthy shoots, +++Healthy thick shoots with enlarged leaves.

Table 5. Effect of different concentrations and combinations of naphthalene acetic acid (NAA), indole butyric acid (IBA), indole acetic acid (IAA) and casein hydrolysate (CH) on rooting frequency, length of the longest root and root number, after four weeks of culture.

NAA (mgl^{-1})	IBA (mgl^{-1})	IAA (mgl^{-1})	CH (mgl^{-1})	Rooting (%)	Longest root length (cm)	Number of roots
Control	-	-	-	00.00 ± 00.00^{f}	00.00 ± 00.00^{e}	00.00 ± 00.00^{d}
0.1	1.0	-	-	55.00 ± 11.41^{de}	1.51 ± 0.12^{d}	00.45 ± 00.11^{cd}
0.5	1.0	-	-	65.00 ± 10.94^{bcde}	3.01 ± 0.31^{bc}	00.55 ± 00.15^{cd}
-	1.0	0.1	-	65.00 ± 10.94^{bcde}	3.19 ± 0.16^{b}	00.65 ± 00.15^{c}
-	1.0	0.5	-	60.00 ± 11.23^{cde}	2.90 ± 0.14^{bc}	01.05 ± 00.19^{bc}
0.1	2.0	-	-	70.00 ± 10.51^{abcd}	2.52 ± 0.12^{c}	00.95 ± 00.21^{bc}
0.5	2.0	-	-	40.00 ± 11.23^{e}	2.86 ± 0.11^{bc}	00.65 ± 00.23^{c}
-	2.0	0.1	-	90.00 ± 06.88^{ab}	3.22 ± 0.23^{b}	01.45 ± 00.26^{b}
-	2.0	0.5	-	85.00 ± 8.19^{abc}	2.84 ± 0.16^{bc}	01.35 ± 00.23^{b}
-	2.0	0.1	50	90.00 ± 06.88^{ab}	04.88 ± 00.12^{a}	02.80 ± 00.26^{a}
-	2.0	0.1	100	95.00 ± 05.00^{a}	03.78 ± 00.18^{b}	02.30 ± 00.16^{a}

Values represent means ± SE of 20 replicates, those representing similar letter in the appropriate column are not significantly different (ANOVA, $P < 0.05$), as analyzed by DMRT.

Table 6. Effect of different potting mixture viz. P_1 (sand:soil in 1:1), P_2 (sand:soil:vermiculite mixture in 1:1:1 ratio) and P_3 (sand:soil:vermiculite mixture in 1:1:2 ratio) on plant survival and plant growth, after one month of acclimatization of *in vitro* regenerated plantlets of Kinnow mandarin.

S/N	Potting mixture	Survival percentage	Number of leaves after one month	Height after one month (cm)
1	P_1	45.00 ± 11.41^{b}	02.65 ± 00.29^{b}	02.80 ± 00.14^{b}
2	P_2	65.00 ± 10.94^{ab}	03.15 ± 00.26^{b}	03.24 ± 00.19^{b}
3	P_3	80.00 ± 09.18^{a}	05.30 ± 00.41^{a}	04.50 ± 00.13^{a}

Values represent means ± SE of 20 replicates, those representing similar letter in the appropriate column are not significantly different (ANOVA, $P<0.05$), as analyzed by DMRT.

excised from the *in vitro* regenerated shoots) were used and the experiment was repeated thrice.

Rooting, acclimatization and transplantation

The full strength MS medium having 0.5% charcoal along with different concentrations and combinations of indole acetic acid (IAA), indole butyric acid (IBA) was used for *in vitro* rooting (Table 5). To the most suitable PGR regime, selected from this study, casein hydrolysate (CH) was added in two concentrations namely: 50 and 100 mg/L for exploring the effects on rooting (Table 5). Percent root induction, number of roots and the length of longest root per shoot were recorded after four weeks of culture. For each of the above treatment, 20 shoots were used and the experiment was repeated thrice.

Acclimatization and transplantation

The plantlets with well developed roots were removed from culture, washed thoroughly with sterile water and transferred to three different potting mixture- P1 (garden soil and sand in 1:1 ratio), P2 (garden soil, sand and vermiculite mix in 1:1:1 ratio) and P3 (garden soil, sand and vermiculite mix in 1:1:2 ratio) (Table 6). For each treatment, 20 replicates were used and the experiment was repeated thrice. The plantlets were covered with transparent plastic to maintain the high humidity and were watered once a day and kept in culture room. The plastic sheets were removed periodically and after 25 to 30 days the plantlets were shifted to the soil.

Statistical analysis

The experiments were set up in completely randomized design (CRD) and repeated thrice. The data were analyzed by Analysis of Variance (ANOVA) followed by Duncan multiple range test. Data analysis was carried out by using SPSS version 18.

RESULTS

In vitro germination of seeds

Seeds cultured on MS basal medium, germinated successfully after a mean of 10.23 days, when incubated in complete dark conditions while incubation in a photoperiod of 16 h, took an average of 16.48 days for initiation of germination (Table 1). Frequency of seed germination was higher (93.33%) under dark conditions as compared to under 16 h photoperiodic incubation (83.33%), with seedlings reaching to a height of 4.31 and 3.35 cm respectively after 28 days (Table 1).

Figure 1. Shoots proliferating from shoot tip explant on MS medium supplemented with 2.5 mg/L BAP.

Effect of cytokinins and additives on shoot proliferation

The shoot tip (ST) explant responded with hundred per cent frequencies on all PGR regimes used in the present study. Amongst various concentration of the three cytokinins, BAP at the concentration of 2.5 mg/L induced maximum number of shoots (2.45) per explant (Table 2) (Figure 1). None of the concentrations of kin and Zea were found suitable for shoot multiplication. The proliferated shoots reached to a maximum average height of 2.11 cm with significantly higher nodes (mean number of 2.62) per shoot (Figure 1), on medium having 2.5 mg/L BAP. The shoots, on kinetin supplemented medium were having less number of nodes but longer inter-nodal space as compared to shoots obtained on BAP/Zea fortified medium.

The inclusion of Gln, ADS and CH, individually as well as in combination, significantly affected the shoot multiplication rate, when added to the MS medium having 2.5 mg/L BAP (the most suitable cytokinin level for shoot proliferation as reported above, taken as control for this study) (Table 3). Gln and ADS, when supplied individually, favored the shoot multiplication at low level namely: 25 mg/L of Gln and 50 mg/L of ADS each, while CH was found favoring the shoot proliferation at higher level (100 mg/L), amongst the various concentrations of each used in the study (Table 3). The higher concentration of Gln (100 mg/L) was found inhibitory for shoot proliferation as well as elongation. The combination of 25 mg/L Gln, 50 mg/L ADS and 100 mg/L CH exhibited a synergistic positive impact on shoot multiplication, more than their individual's affect; the shoot multiplication rate was increased by three fold (7.23 shoots/explant) (Figure 2) from that on control (2.45 shoots/explant) (Table 3). The same combination also resulted in elongated shoots (4.13 cm, on an average) with enlarged leaves and more number of nodes per shoot (3.88) (Table 3) (Figure 2). This medium having 2.5 mg/L BAP, 25 mg/L Gln, 50 mg/L ADS and 100 mg/L CH was considered as optimal SMM.

Shoot multiplication during repeated sub-culture

The individual shoots were isolated from multiple shoot clumps, obtained in the above study. Nodal segments and shoot tips were excised from separated shoots and cultured on each of the four sub-culture medium viz. SCM-1, SCM-2, SCM-3 and SCM-4, repeatedly for the six consecutive months. No significant difference in the response of nodal segments and shoot apices was observed and so response of each was combined together for calculating the average value recorded in Table 4.

For each sub-culture, the combination of higher BAP (2.5 mg/L) and lower CH (100 mg/L) level namely: SCM-1 was found to be least supportive for shoot proliferation and the shoots proliferated on this medium suffered with problems like leaf fall, chlorosis, less vigour etc. (Table 4). Rather, the medium having lower BAP (1.5 mg/L) and highest level of CH (200 mg/L) was found most suitable for shoot multiplication as well as for healthy growth of the shoots, for each sub-culture.

For the prolonged maintenance of proliferating cultures, the potential of the four sub-culture medium for shoot proliferation during repeated sub-culturing was compared. The shoots proliferated with different rate and

Figure 2. Enhanced shoot multiplication from shoot tip explant on optimal shoot multiplication medium (SMM) having 2.5 mg/L BAP along with 25 mg/L Gln, 50 mg/L ADS and 100 mg/L CH.

different quality after each sub-culture, on the respective medium. The medium SCM-1, having the same composition as the optimal shoot proliferation medium in the primary cultures that is, SMM (2.5 mg/L BAP + 25 mg/L Gln + 50 mg/L ADS + 100 mg/L CH) but with additional 0.5% charcoal, resulted in higher shoot multiplication in the initial sub-cultures event (8.80 shoots/explant in first sub-culture, 9.30 shoots/explant in second sub-culture and 11.30 shoots/explant in third sub-culture, Table 4), compared to that on SMM (7.23 shoots/explant, Table 3). But after third sub-culture, shoot proliferation rate decreased drastically on SCM-1 and the shoots obtained were unhealthy also. Lowering of BAP concentration to 1.5 mg/L, keeping all other components same, to formulate the SCM-2 medium did not serve any good. Like the SCM-1, it also performed poorly, both in terms of multiplication rate as well as in terms of quality (shoots appeared sick exhibiting chlorosis, early leaf fall and less vigor) and this started after the second sub-culture and continued the same way till the end Keeping the BAP same as in SCM-2 (1.5 mg/L), when CH concentration was increased to 150 and 200 mg/L in SCM-3 and SCM-4, respectively, the shoot proliferation rate increased and quality of shoots improved after every sub-culture. The medium SCM-4, having the highest CH concentration (200 mg/L) was found to support the highest shoot multiplication rate (ranging from 13.10 to 15.00 shoots per explant, on an average), consistently for the six consecutive sub-cultures (Table 4). Shoots proliferated on the same medium (that is, SCM-4) were very healthy, having thick, elongated stem with enlarged leaves, after every sub-culture (Figure 3a and b). Another medium SCM-3 (150 mg/L CH and 1.5 mg/L BAP) also supported good proliferation rate (ranging from 11.65 to 13.55 shoots per explant, on an average) during the consecutive six sub-cultures but shoots obtained on this exhibited the healthy signs only till the fourth sub-culture, after that the shoots gave a sickly look and exhibited leaf fall at low frequency (Table 4).

Rooting, acclimatization and transplantation

Rooting of the *in vitro* regenerated shoots was not observed on PGRs free medium. Roots were induced in the shoots with different frequencies on different concentrations and combinations of naphthaleneacetic acid (NAA), IBA and IAA (Table 5). Significantly higher frequencies of root induction were observed on medium having 2.0 mg/L IBA in combination with 0.1 mg/L IAA (90 %) and on medium having 2.0 mg/L IBA along with 0.5 mg/L IAA (85%) (Table 5). The average length of the longest root was maximum (3.22 cm) on the combination of 2.0 mg/L IBA with 0.1 mg/L IAA and the same medium supported maximum number of roots per shoot (1.45, on an average) (Figure 4a). Addition of CH to this medium (having 2.0 mg/L IBA and 0.1 mg/L IAA), significantly enhanced the *in vitro* rooting. Amongst the two concentrations of CH used, 100 mg/L concentration was found most suitable; it resulted in enhanced rooting frequency (95%), more number of roots per shoot (2.30) and increased length of the longest root (3.78) (Figure 4b, Table 5).

In vitro formed plantlets with well developed root

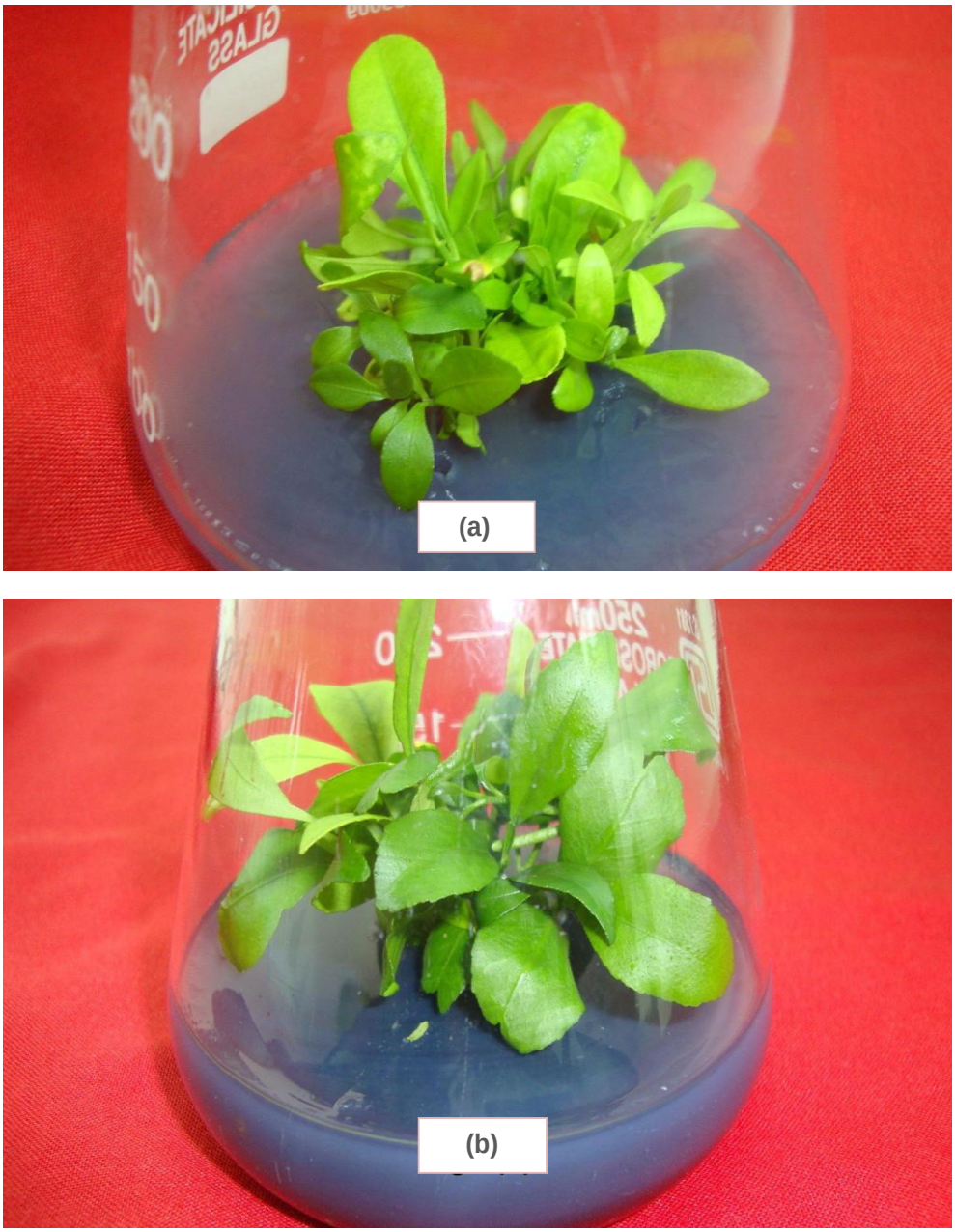

Figure 3. Shoot multiplication on SCM-4 medium (MS medium having 1.5 mg/L BAP, 25 mg/L Gln, 50 mg/l ADS and 100 mg/L CH) after (a) third sub-culture, (b) after sixth sub-culture.

system acclimatized differently in different potting mixture. Plantlets survived poorly on P1 potting mixture(having only garden soil and sand -1:1) (Table 6) (Figure 5). Addition of vermiculite mixture significantly improved the acclimatization process (Table 6). The maximum percent survival (80%), was observed on P3 potting mixture and the plantlets reached to a maximum height of 4.50 cm, on an average, with a mean of 5.30 number of leaves, after one month of acclimatization on the same potting mixture that is, P3 (Table 6 and Figure 5).

DISCUSSION

A highly efficient micropropagation system, capable of sustainable multiplication of shoots, has been developed for *C. reticulata* Blanco. using shoot tip explants obtained from *in vitro* germinated seedling. In this study, the *in vitro* seed germination was favored by incubation of cultures in complete dark conditions, in accordance to the earlier observations recorded by Hassanein and Azooz (2003, 2004). Amongst the three cytokinins used, BAP was found as more suitable cytokinin for shoot

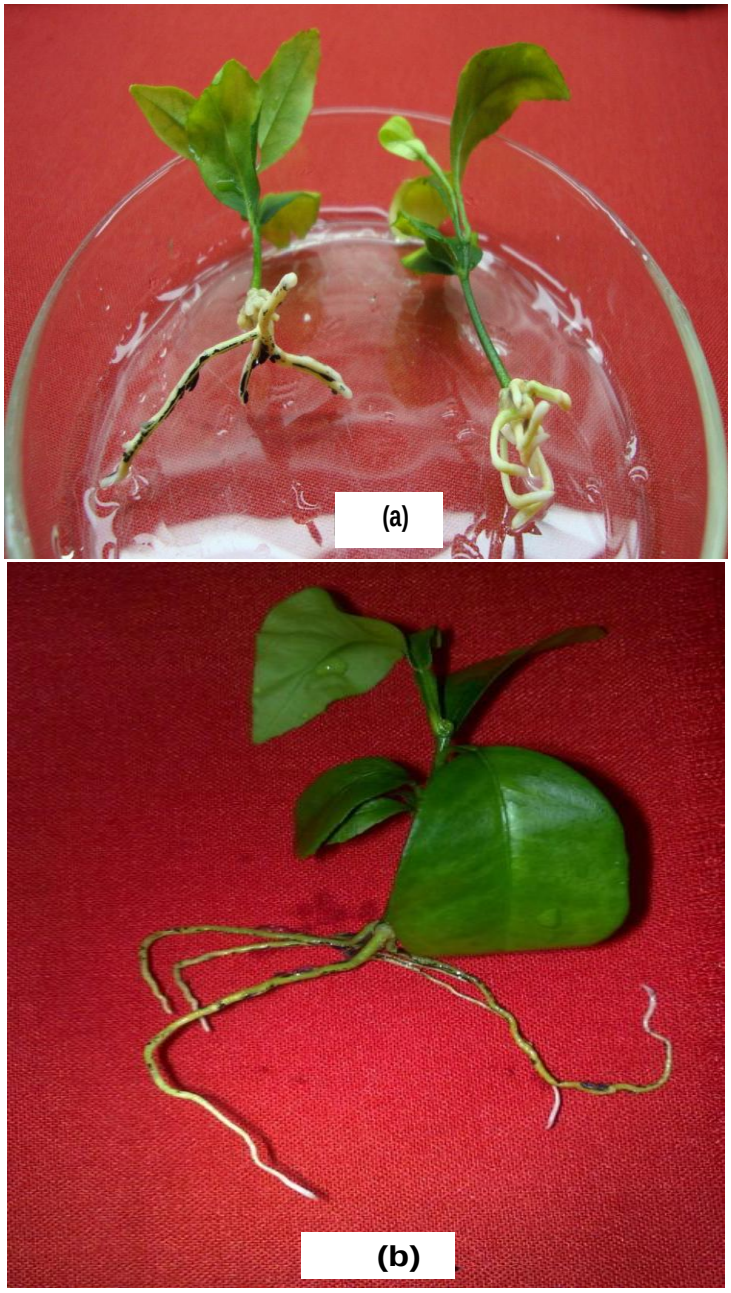

Figure 4. *In vitro* rooting of micro-shoots (**a**) on MS medium having 2.0 mg/L IBA and 0.1 mg/L IAA and (**b**) on MS medium having 2.0 mg/L IBA, 0.1 mg/L IAA and 100 mg/L CH.

proliferation; zeatin exhibited very low shoot multiplication rate while kinetin supported poor differentiation of nodes in the stems. The edge of BAP over other cytokinins has also been reported for some other citrus cultivars (Rathore et al., 2007). The shoot proliferation rate was significantly affected by addition of Gln, ADS and CH. The simulative role of ADS in shoot multiplication has been emphasized from time to time in various plants (Husain and Anis, 2004; Dhar and Upreti, 1999; Husain et al., 2008). In our study, we found 50 mg/L of ADS as highly effective for enhancing the shoot multiplication rate (4.88 shoots per explant). The effectiveness of organic nitrogen source like glutamine for enhanced multiplication has been reported for some other plant species also (Sanjaya et al., 2005; Vasudevan et al., 2004). It has been suggested that positive effect of organic nitrogen, in comparison to that of inorganic sources is associated to enhanced mobility of the former at a lower energy cost than the later (Kim and Moon, 2007). No report of use of amino acids in the tissue culture study of Kinnow, in

Figure 5. Plantlets, on different potting mixture namely: P1 (sand and soil in 1:1 ratio), P2 (sand, soil vermiculite mixture in 1:1:1 ratio) and P3 (sand: soil: vermiculite mixture in 1:1:2) after one month of acclimatization.

particular, and citrus species in general, is available, to the best of our survey. In the present study, the low level (25 mg/L) of glutamine in themedium increased the shoot multiplication (4.03 shoots per explant) while higher level (100 mg/L) lowered it (1.25 shoots per explant), indicating the requirement of an optimal amount of the supplied amino acid. In fact, instead of a single amino acid, mixture of amino acids like casein hydrolysate was found more supportive in shoot proliferation in the present study; shoot multiplication increased with increase in concentration of CH when supplied individually. Further the defined combination of ADS, Gln and CH (25 mg/L of Gln, 50 mg/L of ADS and 100 mg/L CH) was more suitable (greatly increased the shoot proliferation rate to 7.23 shoots per explant) than their individual's effect; indicating the enhanced availability of some cytokinin like activity as well as better nourishment, to the *in vitro* cultures at this particular combination.

For the prolonged maintenance of proliferating cultures, high cytokinin concentration (2.5 mg/L BAP), as in SCM-1, performed well for first three cultures, afterwards, it was found unsuitable. In fact high proliferation rate on SCM-1 for the first three sub-cultures (8.8, 9.3, 11.4 shoots/explant after first, second and third sub-culture, respectively) as compared to that on SMM (7.24) can be attributed to the presence of charcoal in SCM-1, as rest of the components were same in these two medium. Growth promoting effects of activated charcoal (AC) have been attributed to its capacity to adsorb unwanted phenolic oxidation exudates accumulated in culture media (Fridborg et al., 1978). Proliferation rate decreased drastically after third sub-culture on the SCM-1 medium. Keeping the concentration of Gln, ADS, charcoal and CH same as in SCM-1, when BAP concentration was lowered to 1.5 mg/L to form the SCM-2, the shoot proliferation rate on it, though better than that on SCM-1, also decreased after fourth sub-cultures. The shoots obtained on SCM-1 and SCM-2 was unhealthy since the second sub-culture event. Occurrence of defoliation of shoots during sub-culturing has also been reported during micropropagation studies on *Citrus limon* (Rathore et al., 2007). The problems of chlorosis and leaf fall can be due to extraordinary requirement of *in vitro* cells for some essential nutrients. This could further be correlated

to the response observed on medium having different CH level namely SCM-2 (100 mg/L CH), SCM-3 (150 mg/L CH) and SCM-4 (200 mg/L CH), all other components being same for these three medium. Quality of shoots improved and the proliferation rate increased, after every sub-culture, with increase in concentration of CH (Table 4). A consistent high shoot multiplication rate and very healthy shoots were obtained on highest CH used in the present study. A similar role of CH in prolonged maintenance of proliferative cultures has been reported for other plants species like rose (Roy et al., 2004), sugarcane (Nasir et al., 2011), *Psoralea corylofolia* L. (Baskaran and Jayabalan, 2010) etc., though no report is present on its effect on *in vitro* culture of Kinnow, to the best of our survey.

The combination of 2.0 mg/L IBA and 0.1 mg/L IAA induced rooting at high frequency (90%) and supported the maximum root elongation (3.22 cm) during the present study. However, Hassanein and Azooz (2003/2004) documented a combination of BAP, NAA and IBA as the most suitable for *in vitro* root induction. The casein hydrolysate was also found promotive for *in vitro* rooting. Acclimatization was successfully carried out in culture room. Vermiculite compost was found to strongly affect the process of acclimatization of the *in vitro* raised plantlets of Kinnow.

Conclusion

To our knowledge there are no reports about the optimal medium requirements during continuous sub-culturing of shoots of Kinnow mandarin. The present protocol is highly efficient in terms of repeated shoot multiplication, rooting and acclimatization. This protocol can be utilized for large scale clonal propagation of this important fruit crop.

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