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In vitro direct shoot and root regeneration of Indian Wild Orange (Citrus indica Tanaka) using nodal segments excised from mature tree

Kiran Chhetri • B. Mathew* • Lolly S. Pereira

Department of Rural Development and Agricultural Production,
North-Eastern Hill University, Tura Campus, Chasingre, Tura-794002, Meghalaya, India.

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ABSTRACT

Citrus indica Tanaka, commonly known as the "Indian Wild Orange" and locally called 'Memang Narang' is believed to be the most primitive and perhaps the progenitor of all *Citrus* species. It is a rare, endemic and endangered species grown in the Garo Hills of Meghalaya, India. Its short fruit-bearing period and sparse number of seeds per fruit make its natural regeneration difficult. Due to slow natural regeneration and increasing human intervention, the survival of *C. indica* has become a serious threat and deserves adequate attention for its conservation. Therefore, an experiment was laid down to develop a suitable protocol for *in vitro* direct regeneration and mass multiplication of *C. indica* using nodal segments excised from mature trees, inoculated in Murashige and Skoog (MS) medium fortified with various plant growth hormones in different concentrations and combinations. Treatments showed significant positive effects with respect to its shoots and roots regeneration. MS media fortified with 6-Benzylaminopurine (BAP) 1.5 mg/L and Kinetin (Kn) 0.5 mg/L were found to be the best treatments for shoot initiation, while MS medium supplemented with 1-Naphthalene Acetic Acid (NAA) 0.5 mg/L + Indole-3-Butyric Acid (IBA) 0.5 mg/L and Kn 0.5 mg/L + BAP 1.5 mg/L + NAA 1.5 mg/L were found to be the best treatments for root induction. 100 per cent survivability was achieved when vermicompost was used as the hardening medium.

1. Introduction

Citrus indica Tanaka, locally (Garo) known as *'Memang Narang'* is an endangered species that is believed to be the most primitive *Citrus* species. It is found growing wild in the buffer zone of Nokrek Biosphere Reserve extending up to the South Garo Hills region of Meghalaya, *i.e.*, in Chokpot, Silkigre, Rongsu area near Siju and Baghmara. The fruits are small and deep orange in colour, mostly used for medicinal purposes both for human and animal ailments. It is locally used to cure jaundice, kidney stone, smallpox, stomach diseases and is used in traditional rituals. As it is a non-commercialized fruit, it is of little economic value. Low genetic diversity and destruction of its natural habitat pose a serious threat to *C. indica*. Therefore, conservation of this endemic and endangered species is highly essential to prevent the loss of *Citrus* biodiversity. Due to slow regeneration and. presence of recalcitrant seeds, *in situ* conservation methods alone are inadequate

In the past, regeneration of *in vitro* plants using leaf callus (Laskar *et al.*, 2009) and nodal segments collected from *in vitro* germinated seedlings (Sangma *et al.*, 2020) were reported. However, due to spares availability of seeds per fruit of *Citrus indica*, this method does not serve good for the purpose of mass multiplication. Moreover, callus induces plantlets and seedlings poses concerns of genetic variations. Hence, an alternative approach of micropropagation using nodal segments excised directly from mature trees to produce true-to-type plantlets all year round was attempted on MS medium fortified with various growth hormones *viz.*, 6-Benzylaminopurine (BAP) 1.5 mg/L and Kinetin (Kn), 1-Naphthalene Acetic Acid (NAA), Indole-3-Butyric Acid

*Corresponding author: drbmathew@gmail.com

(IBA) and Indole-3-Acetic Acid (IAA) in different concentrations and combinations. For hardening and acclimatization of rooted plantlets, the locally available media *viz.*, sand, vermicompost and soil were used in different ratio and their effect on survivability of the rooted plantlets was recorded.

2. Materials and methods

Collection of plant material

Actively growing nodal segments from young and healthy plants of *Citrus indica* were collected from the gene bank maintained in the instructional farm of the Department of Rural Development and Agricultural Production, North-Eastern Hill University, Tura Campus, Meghalaya. Nodal segments of about 1.5-2 cm in length were excised from the plant using sterile blades. The collected nodal segments were immersed in distilled water and brought to the tissue culture laboratory. The explants were then washed in running tap water and were again washed thoroughly adding a few drops of Tween-20 to remove the dust particles present on the surface as well as fungal and bacterial spores. This was followed by rinsing with distilled water and moved to the Laminar Air Flow (LAF) cabinet. Under laminar flow, the explants were surface sterilized by immersing in 0.1% (w/v) mercury (II) chloride, ($HgCl_2$) for 2 minutes and rinsing with sterile distilled water three times to remove all remains of sterilant.

Preparation of culture medium

A ready-made powdered form of Murashige and Skoog (Murashige and Skoog, 1962) medium, was used for inoculating the explants on different concentrations of growth hormones. The medium was already fortified with 3% sucrose and essential vitamins. The pH of the medium is adjusted to 5.8 with 1.0 N HCl and 1.0 N NaOH and the final volume of the medium was adjusted to 1 L using double distilled water. MS basal medium was solidified using 1% agar-agar supplemented with various combinations and concentrations of growth hormones like 6-benzylamino purine (BAP), Kinetin (Kn), 2,4-Dichlorophenoxyacetic acid (2,4-D), 1-Naphthalene Acetic Acid (NAA), Indole-3-Acetic Acid (IAA) and Indole-3-Butyric Acid (IBA). The medium was then dispensed into sterilized culture vessels and the mouth of the vessels were plugged with non-absorbent cotton plugs. The aseptic manipulations were carried out in the Laminar Air Flow (LAF) chamber fitted with ultraviolet light and HEPA (High-Efficiency Particulate Air) filter.

All the culture vessels were kept in the culture room at a temperature of 25±2 ºC, with 60±5 per cent relative humidity under 35µmol/ms photosynthetic photon flux white fluorescent light emitted by 40W tubes, programmed for 16-hour photoperiod and 8-hour dark condition.

Shoot and Root multiplication

Nodal segments of *Citrus indica* were inoculated and multiplied on MS medium (1% agar-agar) supplemented with varying concentrations (0.5, 1 and 1.5 mg/l) and combinations (BAP and Kinetin) of growth hormones. The sub-culturing of multiplied shoots was done at an interval of four weeks. Micro-shoots of about 2-3 cm in length were excised and were further inoculated on MS medium fortified with either cytokinins only or along with auxins (2,4-D and NAA) of various concentrations and combinations for further multiplication.

Micro-shoots of 1.5 to 2 cm in length were excised at different stages of sub-culturing and then transferred to MS medium supplemented with different concentrations (0.5-1 mg/l) of auxins (NAA, IBA and IAA) singly as well as in combination with cytokinins (BAP and Kinetin) for root induction. The cultures were incubated at 25±2ºC under 16-hour photoperiod. After four weeks of incubation different observations were recorded.

Hardening and acclimatization

Well-developed plantlets were removed from the MS medium and the roots were gently washed in water to remove the sticky agar medium. The plantlets were then transferred to small plastic cups containing different hardening mixtures *viz.*, sand, vermicompost and soil and their combinations. The suitability of the hardening mixtures and their combinations on the survivability of the rooted plantlets was assessed. Acclimatization was achieved by keeping the plantlets under controlled light and temperature for 30 days following which they were exposed to the natural environment. Subsequently, the plantlets were kept in shade conditions for eight weeks to avoid direct sunlight after which they were transferred to larger pots.

Experimental details

The experiment was laid out in the Completely Randomized Design with six explants per treatment repeated thrice. The parameters *viz.* number of days required for shooting, the average number of shoots developed per nodal segments, mean length of shoot, number of days required for rooting, average number of roots developed per shoot and the mean length of root were recorded. Data were examined by Analysis of Variance (ANOVA) among means for all treatments were tested at $P<0.01$% followed by Least

Significant Difference (LSD) using SPSS (Statistical Package for Social Sciences, SPSS Inc., Chicago, IL, USA) program for statistical analysis.

3. Results and Discussion

Shoot induction in cytokinins: Data presented in Table 1 revealed that the shortest time required for initiation of shoots (15 days) was observed in MS medium fortified with BAP 1 mg/L + Kn 1.5 mg/L (T_{13}). The result of the Least Significant Difference analysis revealed that they were statistically at par with each other. The number of shoots and the length of shoots were recorded after 30 days. The highest number of shoots (4.33) were recorded in T_{16} (BAP 1.5 mg/L + Kn 1.5 mg/L) and T_6 (Kn 1.0 mg/L) followed by T_{13} (BAP 1.0 mg/L + Kn 1.5 mg/L) and T_{14} (BAP 1.5 mg/L + Kn 0.5 mg/L). Micro-shoot having the highest length (4.23 cm) was recorded when explants were inoculated in MS medium fortified with a combination of BAP 1.0 mg/L + Kn 1.0 mg/L (T_{12}) followed by T_5 (Kn 0.5 mg/L) and T_{13} (BAP 1.0 mg/L + Kn 1.5 mg/L). The highest weight of shoots was recorded in T_5 (Kn 0.5 mg/L) followed by T_{16} (BAP 1.5 mg/L + Kn 1.5 mg/L) and T_{12} (BAP 1.0 mg/L + Kn 1.0 mg/L). All the leaves were dark green in colour. While considering all the parameters under the study, it was observed that T_4 (BAP 1.5 mg/L), T_5 (Kn 0.5 mg/L), T_{12} (BAP 1.0 mg/L + Kn 1.0 mg/L) and T_{13} (BAP 1.0 mg/L + Kn 1.5 mg/L) were at par and the best treatments for shoot initiation of *Citrus indica* from nodal segment.

Effect of different combinations of cytokinins and along with auxins on shoot induction:

Different concentrations and combinations of cytokinins and auxins did not exhibit significant difference in the number of days required for root induction (Table 2). When 2,4-D was used as a combination, highest shoot induction (4.33) was observed in T_4 (Kn 0.5 mg/L + BAP 1.0 mg/L + 2,4-D 0.5 mg/L) followed by T_2 (Kn 0.5 mg/L + BAP 0.5 mg/L + 2,4-D 1.0 mg/L). When NAA was used as a combination, highest shoot induction (3.67) was observed in Kn 0.5 + BAP 1.0 mg/L + NAA 1.5 mg/L (T_{15}) followed by T_{13} (Kn 0.5 mg/L + BAP 1.0 mg/L + NAA 0.5 mg/L). When 2,4-D was used as a combination, micro-shoots having the highest length (3.37 cm) were recorded in MS medium fortified with Kn 0.5 mg/L + BAP 1.0 mg/L + 2,4-D 0.5 mg/L (T_4). Similar results were observed in T_2 (Kn 0.5 mg/L+ BAP 0.5 mg/L + 2,4-D 1.0 mg/L). When NAA was used as a combination, the longest micro-shoots (3.77 cm) were recorded in Kn 0.5 mg/L + BAP 0.5 mg/L + NAA 1.5 mg/L (T_{12}) followed by T_{18} (Kn 0.5 mg/L + BAP 1.5 mg/L + NAA 1.5 mg/L) which were statistically at par with each other. The highest weight of shoots was recorded when 2,4-D was used as a combination, was recorded in T_4 (Kn 0.5 mg/L + BAP 1.0 mg/L + 2,4-D 0.5 mg/L). Similar response was recorded when NAA was used as a combination (0.5 Kn + 1 BAP + 0.5 NAA). The leaves obtained were all dark green in colour.

In the present study, it was observed that MS medium fortified with cytokinins (BAP 1.0 mg/L + Kn 1.5 mg/L) exhibited earliest shoot initiation (15 days) while the combination of auxins (NAA and 2,4-D) and cytokinins did not improve the days of shoot initiation. MS medium fortified with Kn 1.0 mg/L was found to be the best treatment for multiple shoot induction. Similar effects of kinetin were also recorded in *in vitro* regeneration and multiple shoot induction from nodal segments of *Citrus reticulata* Blanco (Mukhtar *et al.*, 2005). When only cytokinins were used, a higher number of shoots were recorded when the combination of BAP 1.0 mg/L along with Kn 1.5 mg/L and when BAP 1.5 mg/L along with Kn 0.5 mg/L were used. These results were in conformity with Al-Khayri and Al-Bahrany (2005) who reported similar response work in *Citrus aurantifolia*. BAP has been reported to stimulate shoot induction from nodal segments in different Citrus species. Sangma *et al.* (2020) reported the highest shoot induction from nodal segments of *in vitro* seedlings of *Citrus indica* when MS media was supplemented with BAP 1.0 mg/L. The presence of BAP alone was found to be essential for the development of adventitious buds in *Citrus macrophylla* and *Citrus aurantium* (Tallon *et al.*, 2012). Tzatzani *et al.* (2018) reported the highest number of shoots in commercial Citrus rootstocks *viz.* *Poncirus trifoliata* and *Citrus aurantifolia* (Christmas) Swing using BAP 0.5 mg/L and 2.0 mg/L.

When the cytokinins were used along with auxins *viz.* 2,4-D and NAA, the results varied significantly ($P<0.01$). The highest number of micro-shoots were observed when MS medium was fortified with 0.5 mg/L+ BAP 1.0 mg/L + 2,4-D 0.5 mg/L and Kn 0.5 mg/L + BAP 1.0 mg/L + NAA 0.5 mg/L. Similar results were observed when the same concentration of cytokinins was maintained and NAA was increased to 1.5 mg/l. This hormonal combination has been seen to be effective to promote shoot multiplication from nodal explants in *Citrus aurantifolia*, *Citrus reticulata* and *Citrus limon* (Singh *et al.*, 1994) where the combination of Kn 0.5 mg/L+ BAP 1.0 mg/L + NAA 0.5 mg/L caused the highest shoot proliferation. Chamandoosti (2020) reported BAP 1.0 mg/L and NAA 0.01 mg/L to be the best phytohormonal combination for multiple shoot induction on *Citrus latifolia* nodal explants. Thus, we can infer that in addition to the cytokinins, auxins also play a major role in the shoot initiation process of Citrus species.

Effect of different concentrations of auxins on root initiation:

Data presented in Table 3 revealed that different concentrations and combinations of auxins did not exhibit significant differences in the number of days required for root initiation which ranged between 16.67 to 20.33 days on an average. Highest root induction (6 nos.) was observed in T_8 (NAA 0.5 mg/L + IAA 0.5 mg/L), T_9 (NAA 0.5 mg/L+ IAA 1.0 mg/L), T_{12} (NAA 1.0 mg/L+ IAA 0.5 mg/L) and T_{15} (NAA 1.0 mg/L + IBA 1.0 mg/L). Similar results were also seen when NAA was kept constant but the concentration of IAA was increased to 1.0 mg/l. The number of roots induced was seen to be statistically at par when the concentration of NAA was kept constant at 1.0 mg/L and IAA was used at 0.5 mg/L, respectively. The longest roots (4.13 cm) were observed in MS media supplemented with NAA 0.5 mg/L + IBA 0.5 mg/L (T_{10}). The number of roots and length of roots were highest when the MS medium was fortified with NAA 0.5 mg/L + IBA 0.5 mg/L (T_{10}).

Effect of different combinations of cytokinins and auxins on root induction:

In Table 4, when NAA was used in combination with cytokinins, the earliest root induction (17.33 days) was observed when Kn 0.5 mg/L+ BAP 1.0 mg/L + NAA 1.5 mg/L (T_6) were used followed by T_7 (Kn 0.5 mg/L+ BAP 1.5 mg/L + NAA 0.5 mg/L). The highest number of roots (5.33) were observed in Kn 0.5 mg/L + BAP 1.5 mg/L + NAA 1.5 mg/L (T_9). When IAA was used in combination with the cytokinins, highest number of roots (5.33) was obtained in MS media fortified with Kn 0.5 mg/L + BAP 0.5 mg/L + IAA 0.5 mg/L (T_{10}). This combination also gave early induction of roots (18 days). Similar results were obtained when the concentration of IAA was increased to 1.0 mg/l and the cytokinins were kept constant. The longest roots (6.8 cm) were attained when Kn 0.5 mg/L + BAP 1.5 mg/L + IAA 1 mg/L (T_{17}) were used. Of all the combinations, T_{17} (Kn 0.5 mg/L + BAP 1.5 mg/L + IAA 1mg/L) was found to be most suitable as it gave early root induction and higher number of long roots

Highest root induction in plantlets was observed in NAA 0.5 mg/L + IAA 0.5 mg/L, NAA 0.5 mg/L + IAA 1.0 mg/L, NAA 1.0 mg/L + IAA 0.5 mg/L and NAA 1.0 mg/L + IBA 1.0 mg/L. The combination of cytokinins did not show any significant improvement in root initiation of *Citrus indica*. Sangma *et al.* (2021) and Laskar *et al.* (2009) reported highest root induction in explants derived from *in vitro* seedlings of *Citrus indica* when MS medium was supplemented with 1.0 NAA mg/L. The positive effects of NAA either with combinations or alone, have been reported in other *Citrus* species like *Citrus limon* (Kasprzyk-Pawelec *et al.*, 2015), *Citrus reticulata* (Blanco) (Mukhtar *et al.*, 2005) and *Citrus jambhiri* Lush (Saini *et al.*, 2010). Longest roots were observed in MS media supplemented with NAA 0.5 mg/L+ IBA 0.5 mg/L when auxins were used alone. Similar reports were recorded when NAA and IBA were used as a combination for *Citrus aurantifolia* (Al-Bahrany, 2002). From the observations made in the present study and perusal of published research work on various Citrus species, the efficiency of NAA as a competent rooting hormone probability for Citrus species can be deduced.

Hardening and acclimatization of regenerated plantlets:

Data in Table 5 shows that out of the four different treatments used for hardening *viz.*, sand (T_1), vermicompost (T_2), sand + vermicompost (1:1) (T_3) and sand + vermicompost + soil (1:1:1) (T_4). In addition to soil and sand, vermicompost was chosen as a hardening medium since it is locally available and a proven source of non-toxic nutrients. 100 % survivability was recorded among plantlets hardened in vermicompost (T_2) alone after eight weeks (Table 5). The plantlets were then transferred to larger pots and acclimatization was achieved gradually in outdoor conditions where the rate of survival of the plantlets was 100%. Vermicompost was found to be the most suitable hardening mixture for the acclimatization of the *in vitro* raised plantlets of *C. indica*. This could be due to the presence of a high level of humic substance present in the vermicompost that possesses growth hormone-like property thereby enhancing the growth and development of plants (Atiyeh *et al.* 2002 and Musculo *et al.* 1996).

4. Conclusion

From the investigations made in the study, it was observed that for shoot initiation use of cytokinins singly was more effective than the combined effect of cytokinins and auxins. The most suitable media for mass-multiplication from nodal segments excised from mature trees were MS media fortified with Kinetin 0.5 mg/L and BAP 1.5 mg/L for shoot initiation, while MS media fortified with NAA 0.5 mg/L + IBA 0.5 mg/L and Kn 0.5 mg/L + BAP 1.5 mg/L + NAA 1.5 mg/L were the best media for root initiation from the *in vitro* developed shoots of *C. indica*.

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TABLE 1. Effect of different cytokinins on shoot initiation of nodal segments of *Citrus indica* cultured in MS medium

TREATMENTS	No. of days for shoot initiation	No. of shoots	Length of shoots (in cm)	Weight of shoots (in gm)	Colour
T_1 (Control)	20.67 ± 0.33^{gh}	1.33 ± 0.33^{d}	1.33 ± 0.33^{h}	0.77 ± 0.01^{ab}	Dark Green
T_2 (0.5 BAP)	20 ± 0.57^{gh}	2.67 ± 0.33^{bcd}	2.63 ± 0.11^{efg}	0.83 ± 0.03^{ab}	Dark Green
T_3 (1.0 BAP)	18 ± 1.15^{cde}	3.67 ± 0.33^{abc}	2.47 ± 0.33^{fg}	0.83 ± 0.06^{ab}	Dark Green
T_4 (1.5 BAP)	17 ± 0.57^{abcde}	3.67 ± 0.33^{abc}	3.67 ± 0.07^{abc}	0.83 ± 0.03^{ab}	Dark Green
T_5 (0.5 Kn)	16 ± 0.33^{a}	3.33 ± 0.66^{abc}	3.73 ± 0.12^{a}	1.10 ± 0.10^{a}	Dark Green
T_6 (1.0 Kn)	18.67 ± 0.33^{efgh}	4.33 ± 0.33^{a}	2.97 ± 0.11^{bcdefg}	0.93 ± 0.06^{ab}	Dark Green
T_7 (1.5 Kn)	16.67 ± 0.66^{abcde}	2.33 ± 0.33^{cd}	3.57 ± 0.05^{abc}	0.7 ± 0.02^{b}	Dark Green
T_8 (T_2+T_5)	18.33 ± 0.33^{defg}	3.33 ± 0.33^{abc}	3.37 ± 0.12^{bcde}	0.80 ± 0.05^{ab}	Dark Green
T_9 (T_2+T_6)	21.67 ± 0.33^{h}	2.67 ± 0.33^{bcd}	2.83 ± 0.17^{cdefg}	0.7 ± 0.02^{b}	Dark Green
T_{10} (T_2+T_7)	17.67 ± 0.66^{cdef}	2.33 ± 0.33^{d}	3.43 ± 0.11^{bcde}	0.7 ± 0.02^{b}	Dark Green
T_{11} (T_3+T_5)	15.33 ± 0.33^{a}	1.67 ± 0.66^{d}	2.73 ± 0.11^{def}	0.9 ± 0.05^{ab}	Dark Green
T_{12} (T_3+T_6)	15.67 ± 0.66^{abc}	3.33 ± 0.33^{abc}	4.23 ± 0.12^{a}	1.0 ± 0.05^{a}	Dark Green
T_{13} (T_3+T_7)	15 ± 0.05^{a}	4^{ab}	3.7 ± 0.12^{ab}	0.90 ± 0.05^{ab}	Dark Green
T_{14} (T_4+T_5)	16.33 ± 0.33^{a}	4 ± 0.57^{ab}	2.8 ± 0.11^{defg}	0.86 ± 0.03^{ab}	Dark Green
T_{15} (T_4+T_6)	16.33 ± 0.33^{a}	2.67 ± 0.33^{bcd}	3.47 ± 0.09^{ab}	0.76 ± 0.03^{ab}	Dark Green
T_{16} (T_4+T_7)	16.33 ± 0.33^{a}	4.33 ± 0.33^{a}	3.2 ± 0.06^{bcdefg}	1.04 ± 0.07^{a}	Dark Green

Columns with similar letters are not significantly different according to Least Significant Difference

TABLE 2. Effect of different cytokinins and auxins on shoot initiation of nodal segments of *Citrus indica* cultured in MS medium

TREATMENTS	No. of days for shoot initiation	No. of shoots	Length of shoots (in cm)	Weight of shoots (in gm)	Colour
T_1 (0.5 Kn + 0.5 BAP +0.5 2,4-D)	17.67±0.66	2.33±0.33^{cd}	1.930.28^{cde}	0.83±0.03^{b}	Dark Green
T_2 (0.5 Kn + 0.5 BAP + 1.0 2,4-D)	17.33±0.33	3.67±0.33^{abc}	3.27±0.71^{ab}	0.86±0.03^{b}	Dark Green
T_3 (0.5 Kn + 0.5 BAP + 1.5 2,4-D)	17.33±0.33	2.67±0.33^{bcd}	2.57^{abcd}	0.86±0.08^{b}	Dark Green
T_4 (0.5 Kn + 1.0 BAP + 0.5 2,4-D)	16.33±0.33	4.33±0.33^{a}	3.37±0.12^{a}	1.26±0.06^{a}	Dark Green
T_5 (0.5 Kn + 1.0 BAP + 1.0 2,4-D)	17.33±0.33	2.67±0.33^{bcd}	2.43±0.01^{bcd}	1.06±0.06ab	Dark Green
T_6 (0.5 Kn + 1.0 BAP + 1.0 2,4-D)	17.67±0.66	3^{bcd}	1.83±0.09^{de}	0.96±0.031^{ab}	Dark Green
T_7 (0.5 Kn + 1.5 BAP + 0.5 2,4-D)	17	3.33±0.33^{bcd}	2.77±0.09^{abcd}	0.93±0.06^{ab}	Dark Green
T_8 (0.5 Kn + 1.5 BAP + 1.0 2,4-D)	15.33±0.33	3.33±0.33^{bcd}	3.17^{ab}	0.93±0.13^{ab}	Dark Green
T_9 (0.5 Kn +1.5 BAP + 1.5 2,4-D)	14.67±0.66	2.33±0.33^{cd}	3.03±0.08^{ab}	0.90±0.10^{ab}	Dark Green
T_{10} (0.5 Kn+ 0.5 BAP + 0.5 NAA)	16±0.52	1.67^{bcd}	3.3±0.13^{abc}	0.96±0.06^{ab}	Dark Green
T_{11} (0.5 Kn + 0.5 BAP + 1.0 NAA)	16±0.52	2.67±0.33^{bcd}	2.27±0.11^{e}	0.93±0.08^{ab}	Dark Green
T_{12} (0.5 Kn + 0.5 BAP + 1.5 NAA)	17±0.57	3±0.33^{bcd}	3.77±0.11^{a}	1±0.08^{ab}	Dark Green
T_{13} (0.5 Kn + 1 BAP + 0.5 NAA)	15±0.57	3.33±0.33^{bcd}	2.6^{cde}	1.26±0.03^{a}	Dark Green
T_{14} (0.5 Kn + 1 BAP + 1.0 NAA)	17.33±0.33	2±0.33^{bcd}	3.1±0.11^{abcd}	1.03±0.08^{ab}	Dark Green
T_{15} (0.5 Kn + 1 BAP + 1.5 NAA)	16.67±0.66	3.67±0.577^{a}	2.73±0.05^{bcde}	1.13±0.06^{ab}	Dark Green
T_{16} (0.5 Kn + 1.5 BAP + 0.5 NAA)	16.33±0.33	2.67±0.33^{b}	2.3±0.11^{de}	1.10±0.011^{ab}	Dark Green
T_{17} (0.5 Kn + 1.5 BAP + 1.0 NAA)	13.67±0.66	2±0.33^{bcd}	2.4±0.01^{de}	1.16±0.14^{ab}	Dark Green
T_{18} (0.5 Kn + 1.5 BAP + 1.5 NAA)	15.67±0.52	2.67^{bc}	3.43^{ab}	1.13±0.06^{ab}	Dark Green

Columns with similar letters are not significantly different according to Least Significant Difference

Table 3. Effect of different auxins on root initiation of *Citrus indica* cultured in MS medium

TREATMENTS	No of days	No of roots	Length of roots
T_1 (Control)	20.33±0.33	$2±0.25^{g}$	$2.4±0.11^{g}$
T_2 (0.5 NAA)	19.66±0.66	$2.67±0.33^{fg}$	$2.8±0.11^{fg}$
T_3 (1.0 NAA)	17±0.57	$4±0.19^{def}$	$3.53±0.17^{bcd}$
T_4 (0.5 IAA)	18±0.60	$3.67±0.33^{def}$	$3.63±0.17^{abcd}$
T_5 (1.0 IAA)	17.33±0.33	$4.67±0.33^{bcde}$	$2.93±0.21^{fg}$
T_6 (0.5 IBA)	17.67±0.66	$4.67±0.33^{bcde}$	$3.2±0.15^{def}$
T_7 (1.0 IBA)	17.33±0.42	$5.67±0.33^{abc}$	$3.3±0.17^{cdef}$
T_8 (T_2+T_4)	18.33	$6±0.34^{a}$	$3.27±0.22^{cdef}$
T_9 (T_2+T_5)	18±0.33	$6±0.34^{a}$	$3.77±0.13^{c}$
T_{10} (T_2+T_6)	17.33±0.66	$5.67±0.45^{abc}$	$4.13±0.07^{a}$
T_{11} (T_2+T_7)	17.67±0.66	5^{a}	$2.97±0.25^{ef}$
T_{12} (T_3+T_4)	17.67±0.57	$6±0.32^{a}$	$2.83±0.31^{fg}$
T_{13} (T_3+T_5)	18±0.57	$5±0.01^{abc}$	$3.57±0.11^{cd}$
T_{14} (T_3+T_6)	17.33±0.33	$5.67±0.77^{ab}$	$2.93±0.21^{fg}$
T_{15} (T_3+T_7)	17.67±0.66	6^{a}	$2.83±0.12^{fg}$
T_{16} (T_4+T_6)	16.67±0.33	$4.33±0.33^{cde}$	$3.6±0.34^{abcd}$
T_{17} (T_5+T_7)	17.67±0.66	$3.33±0.33^{efg}$	$2.93±0.22^{fg}$
T_{18} (T_5+ T_6)	17.67±0.66	$3.33±0.33^{efg}$	$3.5±0.15^{bcde}$
T_{19} (T_5 + T_7)	17.67±0.66	$4±0.18^{f}$	$3.93±0.27^{a}$

Columns with similar letters are not significantly different according to Least Significant Difference

Table 4. Effect of different cytokinins and NAA and IAA on root initiation of nodal segments of *Citrus indica* cultured in MS medium

TREATMENTS	No. of days for root initiation	Number of roots	Length of roots (in cm)
T_1 (0.5 Kn + 0.5 BAP + 0.5 NAA)	$21±0.57^{cd}$	2.67^{c}	$3.1±0.05^{c}$
T_2 (0.5 Kn + 0.5 BAP + 1.0 NAA)	$19±0.55^{abc}$	$4±0.31^{ab}$	$3.77±0.11^{c}$
T_3 (0.5 Kn + 0.5 BAP + 1.5 NAA)	$18.33±0.33^{ab}$	$3.67±0.31^{c}$	$3.17±0.09^{c}$
T_4 (0.5 Kn + 1 BAP + 0.5 NAA)	$19.33±0.33^{abc}$	$3.33±0.45^{cd}$	$3.5±0.06^{c}$
T_5 (0.5 Kn + 1 BAP + 1.0 NAA)	$19.67±0.66^{bc}$	$4.33±0.33^{ab}$	$4.4±0.12^{b}$
T_6 (0.5 Kn + 1 BAP + 1.5 NAA)	$17.33±0.33^{a}$	$4.67±0.33^{ab}$	$4.57±0.32^{b}$

T_7 (0.5 Kn + 1.5 BAP + 0.5 NAA)	18.33±0.33^{ab}	3.67±0.31^{bc}	4.73±12^{b}
T_8 (0.5 Kn + 1.5 BAP + 1.0 NAA)	20.33±0.33^{bcd}	4.33±0.27^{ab}	4.77±0.15^{b}
T_9 (0.5 Kn + 1.5 BAP + 1.5 NAA)	18.67±1.1^{ab}	5.33±0.32^{a}	5.47±0.15^{bc}
T_{10} (0.5 Kn + 0.5 BAP + 0.5 IAA)	18±1.15^{a}	5.33±0.32^{a}	4.9±0.23^{cd}
T_{11} (0.5 Kn + 0.5 BAP + 1.0 IAA)	18.33±0.33^{a}	4.33±0.31^{ab}	4.73±0.12^{d}
T_{12} (0.5 Kn + 0.5 BAP + 1.5 IAA)	18±0.57^{a}	3.33±0.33^{cd}	5.93±0.31^{b}
T_{13} (0.5 Kn + 1 BAP + 0.5 IAA)	20.33±0.88^{c}	3.33±0.33^{cd}	5.6±0.25^{bc}
T_{14} (0.5 Kn + 1 BAP + 1.0 IAA)	18.33±0.33^{a}	3.0±0.25^{e}	3.43±23^{e}
T_{15} (0.5 Kn + 1 BAP + 1.5 IAA)	18±0.57^{a}	4.0^{ab}	3.8±0.15^{e}
T_{16} (0.5 Kn + 1.5BAP + 0.5 IAA)	19±1.1^{ab}	4.33±0.28^{ab}	3.90±23^{e}
T_{17} (0.5 Kn + 1.5 BAP + 1.0 IAA)	18.67±0.66^{ab}	4.33±0.33^{ab}	6.8±0.26^{a}
T_{18} (0.5 Kn + 1.5 BAP + 1.5 IAA)	17.33±0.66^{a}	4.67±0.57^{ab}	5.17±0.07^{cd}

Columns with similar letters are not significantly different according to Least Significant Difference

Table 5. Effect of different potting media on the survival of the hardened plants

Media used	Survivability percentage
Sand	13.33
Vermicompost	100
Sand + Vermicompost	94.40
Sand + Vermicompost + Soil	73.30

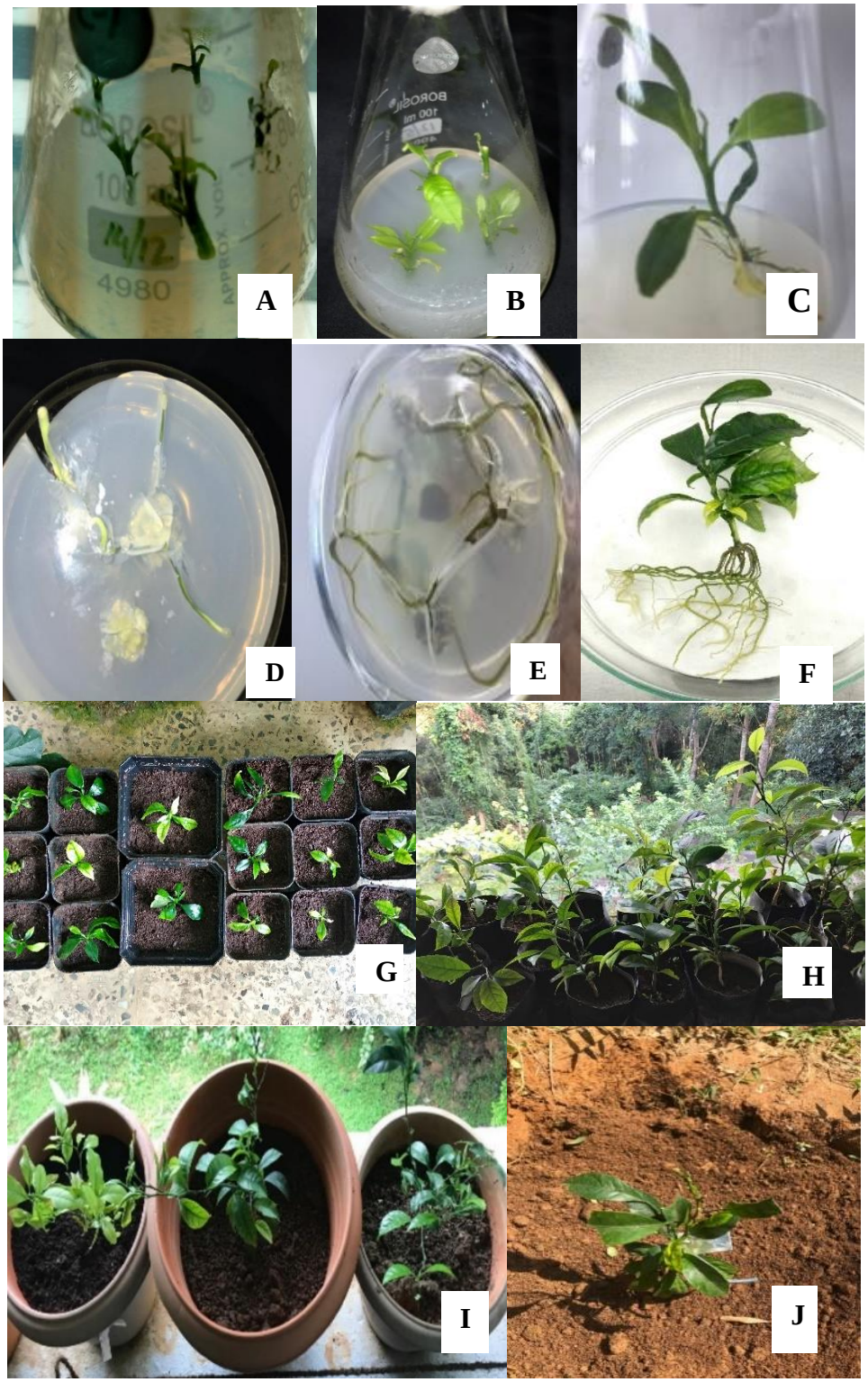

Figure: (A) Initial shoot development from nodal segment in *Citrus indica;* (B) Development of micro-shoots; (C) Regenerated micro-shoots; (D) Initiation of root induction from nodal segment; (E) Root development in nodal segment; (F) Fully developed rooted plantlet; (G-J) Different stages of hardening.