ISSN 1817-3721, E-ISSN 1818-8745

Plant Tissue Cult. & Biotech. **30**(1): 87-96, 2020 (June)

PTC&B

Evaluation of explants for *in vitro* propagation of *Citrus indica* Tanaka - An Endangered Species

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Key words: *Citrus indica*, Micropropagation, Callus induction, Shoot tip, Nodal segment

Abstract

Of the five explants *viz*., shoot tip, nodal segment, leaf disc, cotyledon and root tip excised from exegenic seedlings of *Citrus indica* Tanaka shoot tip cultured in MS supplemented with 0.5 mg/l of BAP generated shoots within 4.74 days, exhibiting highest percentage of response (85.82%) with highest number of shoots (8.9) and shoot length (3.04 cm). On the other hand nodal segment cultured in MS supplemented with 1.0 mg/l BAP showed 80% response in 5.16 days with a shoot number of 5.41 and shoot length of 2.43 cm. Cotyledon explants inoculated on MS supplemented with1.0 mg/l of TDZ produced shoots in 20 days with the highest response of 69.88%, with 3.77 shoots per cotyledon and shoot length of 2.03cm. Viable callus was obtained from leaf disc cultured on half strength MS medium with less Ca^{++} with 2, 4-D 0.5 mg/l + Kn 0.25 mg/l. This callus when inoculated on half strength MS medium with Kn 1.5 mg/l showed highest shoot bud proliferation of 66.66% with 10.06 shoots per callus. Root tip explant failed to produce any shoots. *In vitro* raised shoots of *Citrus indica* when cultured on half strength MS medium supplemented with NAA (1.0 mg/l) showed 80 % rooting in 5.66 days, with highest number of roots (6.16 per shoot) and longest root (3.78cm). Ninety per cent of *in vitro* rooted plantlets of *Citrus indica* survived in open conditions.

Introduction

Citrus indica commonly known as wild indian orange is the most primitive and perhaps the progenitor of all cultivated *Citrus* (Singh 1981). The presence of this species in the buffer zone of Nokrek Biosphere Reserve spreading in the east, west and south Garo Hills of Meghalaya was reported by Malik et al. (2006) and in the core zone of Nokrek

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DOI: https://doi.org/10.3329/ptcb.v30i1.47794

Biosphere Reserve, foot hills of Nokrek, community forests of south Garo Hills and villages surrounding these forests by Upadhyay et al. (2016). *Citrus indica* is a dwarf shrub bearing small bright orange fruits. The fruit is called 'Memang Narang' in Garo language which means ghost (Memang) orange (Narang). The fruits are used by the Garo tribe during last rites to drive away the ghost of the departed soul, by placing the fruit on the dead body (Malik et al. 2006). Upadhyay et al. (2016) reported that the fruits are used to cure jaundice, small pox, hypertension, stomach diseases of humans and domestic animals, and as an antidote for snake bite and any kind of food poisoning. The juice is used as energy drink for relief from fatigue and dehydration.

The embryos of this *Citrus* species are mostly underdeveloped and show poor natural germination. Moreover, the seeds are recalcitrant and lose viability on drying. *Citrus indica* is one of the seven Indian *Citrus* species listed as endangered which needs to be conserved due to its endemism and high degree of threat perception (Singh and Singh 2003, Malik et al. 2006). During the past few years, slow regeneration of this species in its natural habitat, increasing human intervention around the Biosphere Reserve and practice of shifting cultivation in the region have led to rapid decline in numbers. To protect this endemic and endangered species that has great significance in the socio-economic structure of the tribal population of this region, various *in situ* and *ex situ* methods need to be adopted for effective conservation (Malik et al. 2006)). *In vitro* propagation is one of the *ex situ* methods used for enhancing biomass and conserving germplasm of rare and threatened plants (Kapai et al. 2010). *In vitro* propagation can be successfully used when wild plants are difficult to propagate through other conventional methods, when the population in nature is very less, or when a species has poor reproduction capability (Kapai et al. 2010). *In vitro* culture techniques have been used in many germplasm repositories all over the world to supplement other *ex situ* methods for conservation of plant species which are vegetatively propagated, produce recalcitrant seeds or are rare/endangered (Bapat et al. 2008). Many workers have carried out *in vitro* regeneration of plantlets in various *Citrus* species like *Citrus hystrix* (Eng et al. 2014) and *C. reticulata* (Waghmare and Pandhure 2015) using shoot tip explants; in Kinnow mandarin (Singh et al. 2018) using nodal segments; in *C. grandis* (Ibrahim 2012) and *C. tangerina* (Nwe et al. 2014) using cotyledons; in *C. indica* (Laskar et al. 2009) from leaf induced callus.

Keeping in view the fact that *Citrus indica* Tanaka is the progenitor of all cultivated *Citrus* and considering the endangered status, medicinal value and significance in the socio economic life of the local population, it was felt imperative to develop an *in vitro* protocol for mass multiplication and conservation of this species. Thus, a research programme was framed to standardize protocols for *in vitro* plantlet regeneration using various explants *viz*., shoot tip, nodal segment, leaf disc, cotyledon and root tip from *in vitro* raised seedlings; and to evaluate the ideal explant for *in vitro* multiplication of this endangered plant species of Meghalaya.

Materials and Methods

Five explants *viz*., shoot tip, nodal segment, leaf disc, cotyledon and root tip of *Citrus indica* Tanaka were evaluated for *in vitro* development of plantlets. Shoot tips and nodal segments (1.0 - 1.5 cm long), leaf discs (2 × 4 mm), cotyledons (0.5 - 1cm) cut at both ends and root tips (1 - 2 cm long) were excised from 2 months-old *exegenic* seedlings of *Citrus indica.* MS (Murashige and Skoog 1962) with 3% sucrose, 8% agar-agar and vitamins was used for culturing the explants. The pH of the medium was adjusted to 5.8 and autoclaved at 121ºC at 15 psi pressure for 15 min. Cultures were maintained at 25 ± 2ºC with 10 hrs photoperiod and light intensity of 1000 lux at 80% relative humidity.

Shoot tips and nodal segments were inoculated on MS supplemented with BAP and Kn singly (concentrations ranging from 0.25 - 1.5 mg/l) or in combinations. Cotyledons were cut on both ends and inoculated on MS supplemented with BAP, Kn or TDZ solely at concentrations ranging from 0.25 - 1.5 mg/l. Explants producing shoots were sub-cultured after 4 weeks and observations recorded at 8 weeks. Leaf discs were cultured on full strength and half strength MS medium supplemented with different concentrations (0.25 - 1.5 mg/l) and combinations of BAP, Kn and 2,4-D (2,4-dichlorophenoxy acetic acid) and kept under dark condition for 7 days for callus initiation at temperature of 25 ± 2ºC and 80% relative humidity. Later cultures were maintained at temperature of 25 ± 2ºC with 10 hrs photoperiod, light intensity of 1000 lux and 80% relative humidity for callus proliferation. For shoot bud induction and multiplication, calli were cultured on half strength MS with less Ca^{++} (strength which produced viable callus) supplemented with various concentrations (0.25 - 2 mg/l) of BAP and Kn. Root tips were cultured on MS (full and half strength) supplemented with different concentrations (0.25 - 1.5 mg/l) and combinations of 2,4-D, Kn and BAP maintaining same culture conditions as leaf disc.

For *in vitro* root induction, exegenic shoots (3 - 4 cm height) of *Citrus indica,* were cultured on full strength and half strength MS with 3% sucrose, 8% agar-agar and vitamins incorporated with different concentrations (0.25 - 1.5 mg/l) of auxins *viz*., IBA, IAA and NAA. The pH of the medium was adjusted to 5.8 and was autoclaved at 121ºC at 15 psi for 15 min. Cultures were maintained at 25 ± 2ºC with 10 hrs photoperiod, light intensity of 1000 lux and 80% relative humidity. Observations were recorded after 4 weeks of culture.

In vitro rooted plantlets of *Citrus indica* were taken out from the flasks and washed thoroughly brushing off the medium adhered to the roots and then transferred to small pots or polybags containing sterilized mixture of soil + manure + sand + coco peat in the ratio of 3 : 2 : 1 : 1. These potted plants were kept under partial shade for 4 months and later transferred to open conditions. The survival rate was recorded after one month of transferring to open condition.

The data collected during the investigation was statistically analysed by Fisher's ANOVA (Panse and Sukhatme 1989) using Ag. Res. Statistical Software, (c) 1994 Pascal Intl Software Solutions, Version 3.01 and significant differences were compared by LSD.

The level of significance used in 'F' test was $p \leq 0.01$. Critical difference was calculated for comparison wherever the 'F' test was found significant.

Results and Discussion

Among the various concentrations (0.25 - 1.5 mg/l) and combinations of BAP and Kn incorporated in MS for shoot induction from shoot tip explants of *Citrus* indica, earliest shoot initiation (4.74 days), highest percentage of response (85.82), highest number of shoots per explant (8.9) and highest length of shoots (3.04cm) was recorded from MS supplemented with BAP 0.5 mg/l (T_3, Fig. 1) which was also the best performing treatment among the explants tried in this experiment. Significant difference was observed among the treatments evaluated for shoot initiation from shoot tip explant. It was also noted that combinations of BAP and Kn were less effective than the single concentrations. Similar *in vitro* trials were carried out on other *Citrus* species where shoot tip explant was noted to give optimum response of shoot regeneration. Eng et al. (2014) reported that shoot tip of *Citrus hystrix* cultured on MS incorporated with 2.22 µM BAP produced highest number of 3.42 shoots/explant. Waghmare and Pandhure (2015) developed highest number of *in vitro* multiple shoots from shoot tip explants of *Citrus reticulata* on MS supplemented with BAP (2.6 mg/l) + IAA (0.4 mg/l).

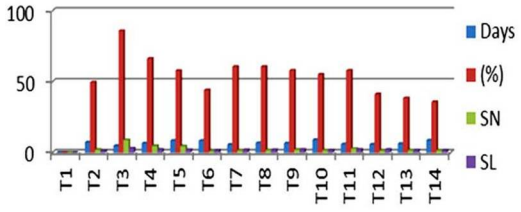

Fig. 1. Effect of different concentrations and combinations of BAP and Kn on shoot induction from shoot tip explant of *Citrus indica.*

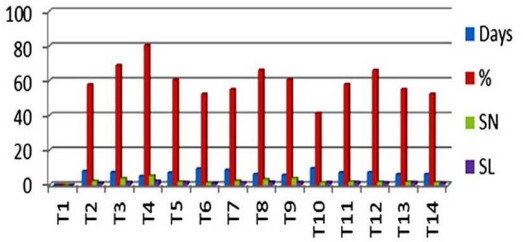

Fig. 2. Effect of different concentrations and combinations of BAP and Kn on shoot induction from nodal segment of *Citrus indica.*

Treatments: T_1 = Blank, T_2 = (BAP 0.25 mg/l), T_3 = (BAP 0.5 mg/l), T_4 = (BAP 1.0 mg/l), T_5 = (BAP 1.5 mg/l), T_6 = (Kn 0.25 mg/l), T_7 = (Kn 0.5 mg/l), T_8 = (Kn 1.0 mg/l), T_9 = (Kn 1.5 mg/l), T_{10} = (BAP 0.25 mg/l + Kn 0.25 mg/l), T_{11} = (BAP 0.5 mg/l + Kn 0.25 mg/l), T_{12} = (BAP 1.0 mg/l + Kn 0.25 mg/l), T_{13} = (BAP 0.25 mg/l + Kn 0.5 mg/l), T_{14} = (BAP 0.25 mg/l + Kn 1.0 mg/l). Days = Days taken for shoot initiation, % = Percentage of response, SN = Shoot number, SL = Shoot length (cm).

In the present investigation, similar treatments as in shoot tip explant were also used for shoot induction from nodal segments of *Citrus indica.* Fig. 2 clearly indicates that MS supplemented with BAP 1.0 mg/l (T_4) showed the best response among the various treatments evaluated, with earliest shoot initiation (5.16 days), highest percentage of response (80.33), highest number of shoots/explant (5.41) and longest shoots (2.43 cm). The treatments showed significant variation among themselves and the single

concentrations were more effective than combinations of BAP and Kn. Han and Han (1994) studied the effect of cytokinins on multiple shoot production of *Citrus* species 'Sambokam' and 'Byungkyool' from nodal explants of exegenic seedlings and observed that MS supplemented with BAP (1.0 mg/l) was the most effective for multiple shoot production with highest number of leaves in 'Sambokam' than 'Byungkyool'. Kim et al. (2001) reported that nodal explants of *Citrus junos* cultured on MS medium with BAP (1.0 mg/l) showed efficient multiple shoot induction. *In vitro* shoot regeneration of Kinnow mandarin was carried out by Singh et al. (2018) from nodal segments on MS with BAP (2.9 mg/l) + NAA (0.06 mg/l) exhibiting shoot number of 3.25 with 2.65 cm shoot length.

Cotyledon explants of *Citrus indica* were inoculated on MS supplemented with BAP, Kn and TDZ in concentrations varying from 0.25 mg/l - 1.5 mg/l wherein significant differences were noted in responses. The treatment TDZ at 1.0 mg/l (T_{12}) produced shoots in 20 days with the highest response of 69.88%, highest number of 3.77 shoots per cotyledon and the longest shoots of 2.03 cm. Among the three cytokinins evaluated for shoot induction from cotyledon, BAP and Kn were less effective (Fig. 3). Similar trials with cotyledon explants were also carried out by Sharma et al. *(*2011) in *Citrus reticulta,* Ibrahim (2012) in *Citrus grandis* and Nwe et al. *(*2014) in *Citrus tangerina*.

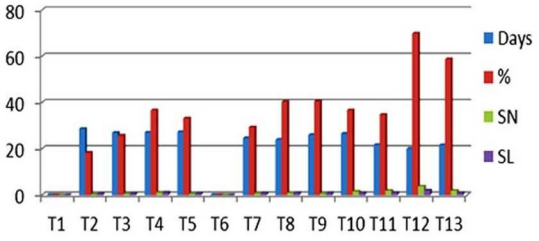

Fig. 3. Effect of different concentrations of BAP and Kn on shoot induction from cotyledon explants of *Citrus indica*.

Treatments: T1 = Blank, T2 = (BAP 0.25 mg/l), T3 = (BAP 0.5 mg/l), T4= (BAP 1.0 mg/l), T5= (BAP 1.5 mg/l), T6 = (Kn 0.25 mg/l), T7 = (Kn 0.5 mg/l), T8 = (Kn 1.0 mg/l), T9 = (Kn 1.5 mg/l), T10 = (TDZ 0.25 mg/l), T11 = (TDZ 0.5 mg/l), T12 = (TDZ 1.0 mg/l), T13 = (TDZ 1.5 mg/l). Days = Days for shoot initiation, % = Percentage of response; SN = Shoot number; SL = Shoot length (cm).

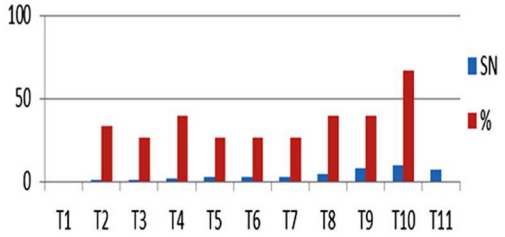

Fig. 4. Effect of various concentrations of BAP and Kn on morphogenetic responses from leaf induced callus of *Citrus indica.*

Treatments: T1 = Blank, T2 = (BAP 0.25 mg/l), T3 = (BAP 0.5 mg/l), T4 = (BAP 1.0 mg/l), T5 = (BAP 1.5 mg/l), T6 = (BAP 2.0 mg/l), T7 = (Kn 0.25 mg/l), T8 = (Kn 0.5 mg/l), T9 = (Kn 1.0 mg/l), T10 = (Kn 1.5 mg/l), T11 = (Kn 2.0 mg/l), % = Percentage of response, SN = Shoot number.

Leaf discs were cultured on full strength MS in varying concentrations (0.25 - 1.5 mg/l) and combinations of 2, 4-D (auxin), BAP and Kn and highest percentage of callus initiation (53.33 %) was observed in 7.66 days from MS supplemented with 2, 4-D (0.5 mg/l) + Kn (0.25 mg/l) but the nature of callus was white, hard and non-friable with medium intensity which failed to regenerate plantlets. Leaf discs were further cultured in half strength MS with less Ca^{++} with same treatments as in full strength MS. Half strength

MS containing the same combination of 2, 4-D (0.5 mg/l) + Kn (0.25 mg/l) showed callus initiation on the 7th day of culture when kept in dark condition initially. 73.33% of creamy white, friable callus was observed after 4 weeks of culture. It was observed here that combination treatments were more effective than single concentrations of 2, 4-D, Kn and BAP. In a similar experiment conducted by Laskar et al. (2009), regenerative calli were developed on full strength MS supplemented with TDZ (0.01 mg/l) and NAA (0.1 mg/l) from leaf discs of *Citrus indica*. Kamruzzaman et al. (2015) observed 90% callus induction from leaf explants of *C. reticulata* on MS medium + 2, 4-D (1.0 mg/l). Khan et al. (2019) observed highest calli initiation (86.7%) from leaf disc of *Citrus reticulata* on MS medium supplemented with 2, 4-D (3.0 mg/l).

Viable callus obtained from half strength MS supplemented with 2, 4-D (0.5 mg/l) + Kn (0.25 mg/l) was transferred to half strength MS supplemented with BAP and Kn at concentrations varying from 0.25 - 2.0 mg/l which exhibited significant differences in shoot initiation responses. Among the various treatments tested, half strength MS with Kn 1.5 mg/l (T_{10}) showed highest shoot bud proliferation of 66.66% with maximum shoot number of 10.06 per callus (Fig. 4). It was observed that BAP showed poor morphogenetic responses from leaf discs compared to Kn. Laskar *et al.* (2009) reported 4.42 shoots per callus on WPM medium supplemented with BAP (0.5 mg/l) + TDZ (0.25 mg/l) + NAA (0.25 mg/l) in *Citrus indica* which was quite less than the present observation.

Root tips from *in vitro* raised seedlings of *Citrus indica* cultured on full strength and half strength MS supplemented with 2,4-D, IBA, NAA, Kn and BAP in varying concentrations (0.25 - 1.5 mg/l) and combinations did not show shoot initiation even after one month of observation. Similarly, Mishra et al. (2018) tested various explants *viz.,* leaf, internode, hypocotyls and root of seedlings of *Tectona grandis* for shoot organogenesis and observed that the root explants were least responsive.

Although several *in vitro* trials have been conducted in many *Citrus* species, very negligible research has been carried out on *in vitro* propagation of *Citrus indica. In vitro* propagation is an effective method that can be used for mass multiplication and conservation of rare or endangered plant species which are difficult to propagate through other conventional methods or have very low population in nature or have recalcitrant seeds. Development of *in vitro* protocol can be of great help in producing virus free and uniform planting material, which can be introduced into its natural habitat for *in situ* conservation. In the present investigation the highest percentage of shoot regeneration response (86.82) was observed from shoot tip explant closely followed by 80.33 from nodal segment but the number of shoots per nodal segment was only 5.41 compared to 8.9 from shoot tip. However, the highest number of shoots (10.06) was recorded from leaf induced callus but the percentage of response was the lowest (66.66) and also the intervening callus formation stage was time consuming. Hence, it can be inferred from the observations that shoot tip is the ideal explant for *in vitro* shoot regeneration of *Citrus indica*.

In vitro generated shoots of *Citrus indica* (3 - 4 cm height) were cultured on full strength and half strength MS incorporated with IBA, NAA or IAA in concentrations varying from 0.25 - 1.5 mg/l for *in vitro* root induction. MS incorporated with NAA 1.0 mg/l exhibited the best rooting response with earliest root initiation (7.16 days), highest percentage of rooting (66.08), highest number of roots (4.24) and longest roots (2.34 cm). On half strength MS also, the same treatment of NAA 1.0 mg/l (T_8) showed the best response for rooting with earliest (5.66 days) root initiation, highest rooting percentage (80.16), highest number of roots (6.16) and longest root (3.78 cm, Fig. 5). Comparing the rooting results of full and half strength MS, it can be concluded that half strength MS supplemented with NAA 1.0 mg/l is the best treatment for *in vitro* root induction on *in vitro* generated shoots of *Citrus indica.* Similar results were also reported by Laskar et al. (2009) where regenerated microshoots of *Citrus indica* were rooted on MS supplemented with 1.0 mg/l NAA giving 2.5 roots per shoot which was quite less than the values observed in the present experiment. Adhikarimayum et al. (2011) recorded best rooting response in microshoots of *Citrus megaloxycarpa* on MS containing 2 mg/l NAA. Savita et al. (2011) reported the best rooting response of 91.67% in regenerated shoots of *Citrus jambhiri* on half strength MS supplemented with NAA (0.5 mg/l). Kour and Singh (2012) observed that in *vitro* multiplied shoots of *Citrus jambhiri* could be best rooted in half strength MS supplemented with IBA (1.0 mg/l) and NAA (1.0 mg/l).

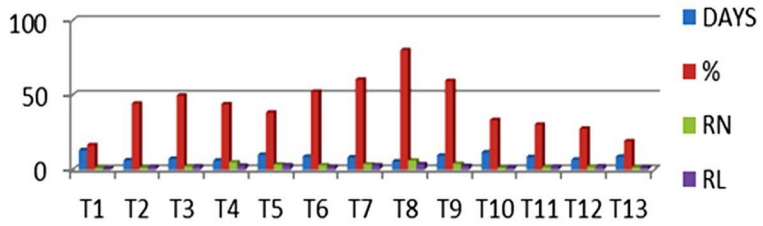

Fig. 5. Effect of different concentrations of IBA, NAA or IAA on root induction from *in vitro* exegenic shoots of *Citrus indica* on half strength MS.

Treatments: T1 = Blank; T2 = (IBA 0.25 mg/l), T3 = (IBA 0.5 mg/l), T4 = (IBA 1.0 mg/l), T5 = (IBA 1.5 mg/l), T6 = (NAA 0.25 mg/l), T7= (NAA 0.5 mg/l), T8 = (NAA1.0 mg/l), T9 = (NAA 1.5 mg/l), T10 = (IAA 0.25 mg/l), T11 = (IAA 0.5 mg/l), T12 = (IAA 1.0 mg/l), T13 = (1.5 mg/l). Days = Days for root initiation; % = Percentage of response, RN = Root number; RL = Root length (cm).

In vitro rooted plantlets of *Citrus indica* were transferred to small pots or polybags containing a sterilized mixture of soil + manure + sand + coco peat in the ratio of 3 : 2 : 1 : 1 and kept under partial shade for four months and then transferred to open conditions. After one month of transferring to open conditions, 90% plantlets of *Citrus indica* survived.

Out of the five explants evaluated for *in vitro* propagation of *Citrus indica*, shoot tip explant showed the best shoot regeneration response followed by nodal segment, cotyledon and leaf explants while root tip explant failed to produce any shoots. Shoot tip explants from *in vitro* raised seedlings of *Citrus indica* inoculated on MS incorporated

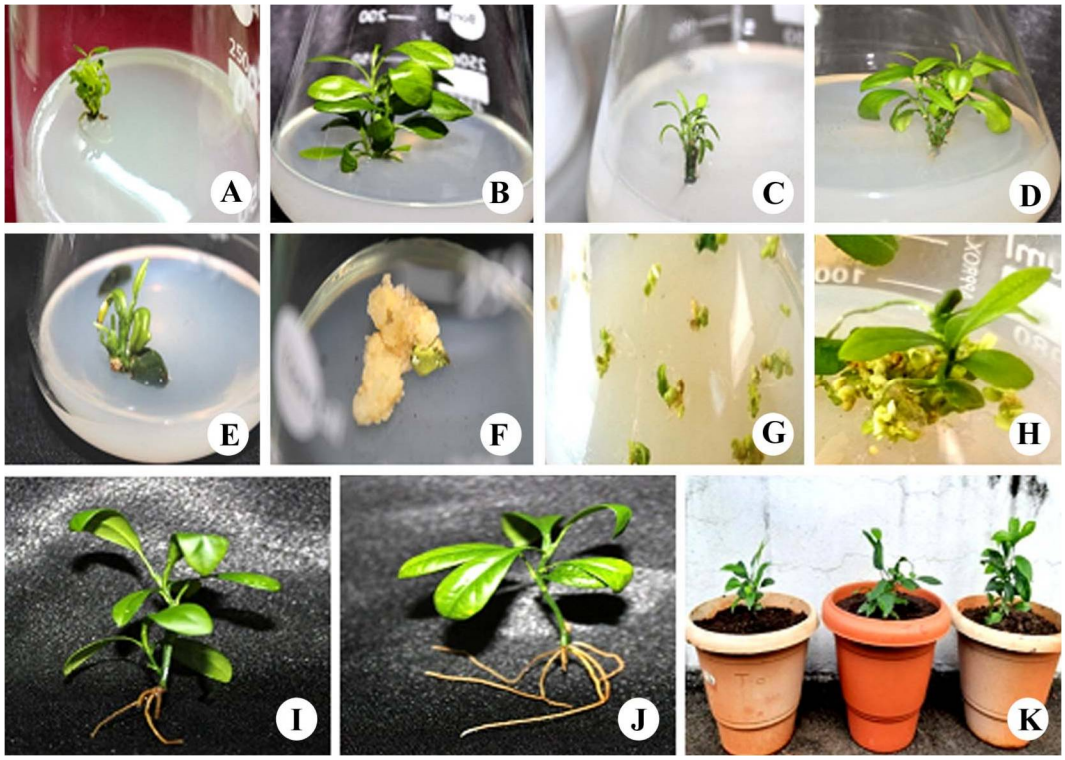

Fig. 6A-K: *In vitro* propagation of *C. indica* using different explants. A. *In vitro* multiple shoot induction from shoot tip on MS + BAP (0.5 mg/l). B. Elongated healthy i*n vitro* shoots from shoot tip. C. *In vitro* multiple shoot induction from nodal segment on MS + BAP (1.0 mg/l). D. Elongated healthy i*n vitro* shoots from nodal segment. E. *In vitro* multiple shoot induction from cotyledon on MS + TDZ (1.0 mg/l). F. Callus initiation from leaf disc on half strength MS (with less Ca^{++}) + 2,4-D (0.5 mg/l) + Kn (0.25 mg/l). G. Shoot regeneration from leaf induced callus on half strength MS (with less Ca^{++}) + Kn (1.5 mg/l). H. Shoot proliferation from leaf induced callus. I. *In vitro* root induction from *in vitro* shoots on full strength MS + NAA 1.0 mg/l; J: *In vitro* root induction from *in vitro* shoots on half strength MS + NAA 1.0 mg/l. K. Hardened i*n vitro* generated plantlets of *Citrus indica*.

with 0.5 mg/l BAP, exhibited earliest shoot initiation, highest percentage of shoot regeneration, highest number of shoots with highest shoot length. *In vitro* raised shoots of *Citrus indica* when cultured on half strength MS supplemented with NAA (1.0 mg/l) produced earliest root initiation, highest rooting percentage, with maximum number of roots and highest root length. Ninety per cent of *in vitro* rooted plantlets of *Citrus indica* survived after one month of transferring to open conditions.

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(Manuscript received on 1 May, 2020; revised on 26 May, 2020)