Content list available at http://epubs.icar.org.in, www.kiran.nic.in; ISSN: 0970-6429

Indian Journal of Hill Farming

Special Issue 2022,Volume 35, Page 26-32

Micropropagation of *Citrus macroptera Montr.* using explants from in vitro generated seedlings

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ARTICLE INFO

Article history:
Received:31 March, 2022
Revision:17 July, 2022
Accepted:17 August, 2022

Key words: Chambil, endangered citrus, in vitro propagation, Garo Hills, Meghalaya, Nokrek Biosphere Reserve.

DOI: 10.56678/iahf-spl2022.4

ABSTRACT

Citrus macroptera Montr. known as 'Chambil' in Garo language is an endangered species which grows in wild and semi wild conditions in Meghalaya. It has great significance in the life of the Garo tribe of Meghalaya for its culinary and medicinal uses, as well as for its use in traditional rituals. The slow natural regeneration, recalcitrant seeds, lack of commercial cultivation and clearing of forest area for various developmental activities are a threat to this species and need attention for conservation. Considering these facts, i*n vitro* trials were conducted for standardization of protocol for mass multiplication of *Citrus macroptera* with five explants from *in vitro* germinated seedlings. Among the treatments evaluated for nodal segment, MS medium incorporated with 0.5 mg/l BAP showed highest response of 91.49% with 6.04 shoots of 2.05 cm length in 6.16 days. Out of the treatments tried with shoot tip, highest response (66.24%) was observed in MS medium supplemented with BAP 1.0 mg/l producing 2.08 shoots of 1.04 cm length within 5.75 days. Among the treatments assessed for cotyledon explants, MS medium with BAP 1.0 mg/l produced the highest number of 3.88 shoots of 2.10 cm length in 19.77 days showing 73.77 % response. With callus initiation rate of 63.33%, leaf discs cultivated on full strength MS media with 0.5 mg/l of 2, 4-D produced viable callus in 7.33 days. The highest shoot bud proliferation (73.33%) was seen in this callus when grown on full strength MS medium with BAP 1.0 mg/l, which produced 11.13 shoots. Root tips of *in vitro* seedlings did not produce any shoots. The rooting percentage of *in vitro* produced *Citrus macroptera* shoots cultured on full strength MS medium with IBA 1.0 mg/l + NAA 1.0 mg/l was 94.44% with 5.55 roots per shoot of 4.5 cm length in 11.94 days, the highest of the many treatments taken into consideration. Seventy per cent *in vitro* rooted plantlets survived in open conditions. Results indicate that protocol developed for *in vitro* plantlet regeneration from nodal segments of *in vitro* raised seedlings, can be used for mass multiplication of *Citrus macroptera*.

1. Introduction

India's north-eastern area is an essential component of the world's biodiversity hot spots, with its diverse climatic conditions, altitudinal changes, and biological niches that have an impact on its rich biodiversity. Out of the Citrus species found in the northeast, significant numbers thrive in untamed, uncontrolled environments, especially in Meghalaya's Nokrek Biosphere Reserve. Over the past few years, large scale deforestation for various developmental activities, shifting or jhum cultivation and cultivation of commercially important species by farmers has led to loss in *Citrus* genetic diversity from its natural habitat in north-eastern India. *Citrus macroptera Montr.* is one out of the seven Indian *Citrus* species listed as endangered (Singh and Singh, 2003; Malik *et al.*,2006). During their explorations, Malik *et al.* (2006) observed that *Citrus macroptera* was in highly endangered state in Shella valley and Jaintia Hills of Meghalaya bordering Bangladesh. According to the National Research Centre for Citrus (NRCC), Nagpur (2013), *Citrus macroptera* plants were noticed in semi-wild settings in Shella and Dawki close to Cherrapunji, Sasatgre and Sakalgre close to Nokrek, Dalu and Ranggira close to Tura in the West Garo Hills, and Silkigri, Rongsu close to Siju in the South Garo Hills of Meghalaya.

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Citrus macroptera is a wild relative of domesticated Citrus species including lime and lemon, as reported by Hazarika (2012). *Citrus macroptera*, also known as "Chambil" in the Garo language, is a tall, evergreen tree with many branches that bears large, spherical fruits that mature to a yellow colour. It has dense foliage and long, sharp thorns. Garo dishes and pickles are made from the fruit's thick rind and juice. The fruit is used by the Garo tribe to treat high blood pressure, cold and cough. For any sort of food poisoning in humans, animals and livestock, juice is utilised as an antidote. The fruit plays a significant part in the most well-known folk dance of the Garo tribe known as the "Wangala dance," which is performed during the harvest festival. In the dance's "Chambil moa" section, the fruit is tied with string to a man's back and rhythmically swung to frighten birds and animals away from crop fields (Upadhyay et al., 2016).

Citrus macroptera seeds are difficult to germinate naturally because the embryos are typically undeveloped and recalcitrant. Many germplasm repositories have adopted *in vitro* culture techniques to supplement other *ex-situ* means of conserving plant species, particularly those that are vegetatively propagated, yield difficult-to-germinate seeds, are rare, and endangered (Bapat *et al.*, 2008). According to Kapai *et al.* (2010), when the population of a plant species is relatively limited in nature, or when a species has poor reproductive capacity or difficult to replicate using conventional methods, *in vitro* propagation can be used. Malik *et al.* (2006) gathered indigenous technical knowledge on the use and socio-economic importance of *Citrus macroptera* which indicated tremendous commercial potential in north east India. Given its medicinal and culinary value, significance to the tribal population of the area in terms of sociocultural life, and endangered status, an effort was made to standardize *in vitro* propagation procedures using five explants from *in vitro* seedlings of *Citrus macroptera* with the aim of determining the best explant for mass multiplication.

2. Materials and Methods:

Five explants excised from two-month-old *Citrus macroptera* in vitro seedlings, including shoot tips (1.0 to 1.5 cm), nodal segments (1.0 to 1.5 cm), leaf discs (2-4 mm), root tips (1-2 cm) and cotyledons cut at both ends (0.5-1 cm) were assessed for in vitro propagation. Explants were cultured on MS (Murashige and Skoog, 1962) medium for shoot and root regeneration under standard culture conditions as maintained by Sangma *et al.* (2020) in *Citrus indica.* Since no rooting was obtained in single auxin concentrations, combinations of IBA and NAA were tried in full and half strength MS medium, and observations were recorded after 4 weeks. *In vitro* rootedplantlets were hardened in partial shade for 4 months and transferred to open conditions and survival rate recorded after one month.

The data were statistically analysed using Fisher's analysis of variance (Panse and Sukhatme, 1989), and significant differences were compared using Least Significant Differences (LSD). The level of significance used in 'F' test was $P \leq 0.01$.

3. Results and Discussion:

Among the different concentrations (0.25- 1.5 mg/l) and combinations of cytokinins (Kn and BAP) used for shoot induction from nodal segments of *in vitro* seedlings of *Citrus macroptera,* the highest response of 91.49% with highest number of shoots/explant (6.04) and longest shoots (2.05 cm) was recorded in 6.16 days from MS medium fortified with BAP 0.5 mg/l (T_3) (Fig.1). Similar concentrations and combinations of cytokinins were used in MS medium for shoot tip explants wherein earliest shoot initiation (5.75 days), highest response (66.24%) and highest number (2.08) of shoots per explant were obtained from MS medium supplemented with BAP 1.0 mg/l (T_4); while maximum shoot length (1.35cm) was obtained from MS medium with BAP 0.25 mg/l + Kn 0.25mg/l (T_{10}) followed by 1.04cm from MS medium with BAP 1.0 mg/l (T_4) which was at par with T_{10}(Fig.2). It was noted that cytokinin BAP showed better shoot induction compared to Kinetin (Kn). *In vitro* trials with nodal segments and shoot tips were conducted by several researchers on various Citrus species showing positive and potent effect of BAP on shoot induction which is in agreement with the observations of the present study. Also, it was observed that combination treatments with cytokinins (Kn and BAP) were less effective in shoot tip and nodal segment explants, indicating that higher amounts of cytokinins were deterrent to shoot induction.

Citrus macroptera nodal segments and shoot tips were employed by Miah *et al.* (2008) for direct multiple shoot regeneration. The nodal segment produced the most shoots (4.88) in MS medium containing 1.0 mg/l BAP compared to 2.84 shoots from shoot tip explants. Results of the present experiment revealed that nodal segments produced more shoots (6.04) per explant with lesser concentration of BAP (0.5 mg/l) compared to *Miah et al. (2008);*and it was observed that increasing concentrations of BAP led to significant decrease in shoot regeneration. The highest percentage of shoot induction was 91.49% in MS medium with BAP 0.5 mg/l (T_3) while it was drastically reduced to 68.91% when the concentration of BAP was increased to 1.0 mg/l (T_4) which further decreased to 60.58%. when the concentration of BAP was further increased to 1.5 mg/l (T_5). Similarly, Komal *et al.* (2013) in their *in vitro* trials with *Citrus limon* L. cv. Kaghzi Kalan, reported a decrease in shoot proliferation with increase in concentration of

cytokinin BAP. Similar *in vitro* trials were also conducted by Kim *et al.* (2001) using nodal explants of *Citrus junos,* by Adhikarimayum *et al.* (2011) on *Citrus megaloxycarpa,* using shoot tips; by Eed*et al.* (2011) on *Citrus limonia*using nodal segments and shoot tip explants; by Waghmare and Pandhure (2015) from shoot tip explants of *Citrus reticulata,* by Singh *et al.* (2018) from nodal segments of Kinnow mandarin and by Sangma*et al.* (2020) using nodal segments and shoot tips of *Citrus indica.*

Cotyledons of *Citrus macroptera* were inoculated on MS medium fortified with various concentrations (0.25 mg/l to 1.5 mg/l) of cytokinins (TDZ, BAP and Kn). It was noted that MS medium with BAP 1.0mg/l (T_{12}) generated the highest response of 73.77% with highest number of shoots per explant (3.88) and longest shoots (2.10cm) in 19.77 days. Out of the three cytokinins assessed, BAP showed better results (Fig.3). Similarly, cotyledons were used as explants by Sharma *et al.* (2011) in *Citrus reticulata,* Ibrahim (2012) in *Citrus grandis,* Nwe *et al.* (2014) in *Citrus tangerina* and Sangma *et al.* (2020) in *Citrus indica.*

Citrus macroptera leaf discs were cultured in full strength and half strength MS media that contained different concentrations (0.25-1.5 mg/l) and combinations of BAP, Kn, and 2,4-D. Full strength MS medium with 2,4-D 0.5 mg/l (T_3) showed highest callus initiation of 63.33% in 7.33 days which was creamy white, friable and healthy. No callus was produced by leaf discs cultured on MS media at half strength. Viable calli from full strength MS medium were transferred to fresh MS media with different concentrations (0.25–2.0 mg/l) of BAP and Kn for the regeneration of shoots. In full strength MS media with BAP 1.0 mg/l (T_4), the maximum shoot bud proliferation (73.33%) and the highest number of shoots (11.13) per explant were observed. It was noticed that Kn showed poor responses compared to BAP (Fig. 4). Similar experiments with leaf discs were also conducted by Kamruzzaman *et al.* (2015) and Khan *et al.* (2019) in *Citrus reticulata,* Kasprzyk-Pawelec*et al.* (2015) in *Citrus limon,* Mumtaz*et al.* (2015) in Troyer citrange (*Poncirustrifoliata*× *Citrus sinensis*), Laskar*et al.* (2009) and Sangma*et al.* (2020) in *Citrus indica.*

Even after a month of culture, root tip explants from *Citrus macroptera in vitro* seedlings, cultured on full and half strength MS medium with auxins (IBA, NAA, and 2,4-D) and cytokinins (Kn and BAP) in various concentrations (0.25-1.5 mg/l) and combinations did not develop shoots.

Citrus macroptera in vitro shoots were cultured on full strength and half strength MS media with varied concentrations of auxins, such as IBA, NAA, or IAA (0.25–1.5 mg/l) (T_1–T_{13}), but no root induction was seen. Consequently, combinations of IBA and NAA (T_{14}–T_{21}) were tried on full and half strength MS medium. In both the strengths of MS media, the same treatment T_{15} (IBA 1.0 mg/l + NAA 1.0 mg/l) had the best rooting performance. Full strength, however, produced the best results. IBA 1.0 mg/l + NAA 1.0 mg/l in full strength MS media (T_{15}) exhibited 94.44% rooting in just 11.94 days with 5.55 roots and 4.5 cm in length, but the same treatment at half strength showed only 66.66% rooting with 2.05 roots per shoot of 2.05 cm in length in 15.66 days (Fig.5). Similarly, Saini *et al.* (2010) and Kaur (2018) observed highest rooting in *Citrus jambhiri* with IBA 1.0 mg/l + NAA1.0 mg/l. Similar works on *in vitro* root induction were also reported in C*itrus indica* Tanaka by Laskar *et al.* (2009) and Sangma *et al.* (2020), in *Citrus megaloxycarpa* by Adhikarimayum *et al.* (2011), in *Citrus jambhiri* by Savita *et al.* (2011) and Kour and Singh (2012), and in *Citrus reticulate* by Sarma *et al. (*2011).*Citrus macroptera* plantlets that had been *in vitro* rooted were put in small pots or polybags with sterilised mixtures of soil, manure, sand, and coco peat in the proportion of 3:2:1:1 and kept for four months in partial shade before being moved to open condition. Seventy per cent plantlets survived after one month under open conditions.

Researchers have carried out a number of experiments on the *in vitro* plantlet regeneration of several citrus species, but very little study has been done on the *in vitro* propagation of the endangered *Citrus macroptera*. For large-scale multiplication and conservation of rare or endangered plants that are challenging to propagate using other conventional methods or have recalcitrant seeds, in vitro propagation can be an effective tool.In the present investigation nodal segment explants exhibited 91.49% shoot regeneration while cotyledon and leaf disc explants showed 73.77% and 73.33% shoot regeneration respectively. Shoot tip explants showed only 66.24% shoot regeneration while root tip explants did not produce any shoots. Highest number of 11.13 shoots was generated from leaf induced callus but the response was low (73.33%) and also the intervening stage of callus formation was time taking. Nodal segments produced 6.04 shoots per explant while cotyledons produced 3.88 shoots and shoot tips produced only 2.08 shoots per explant. Hence, it can be inferred that protocol developed for *in vitro* plantlet regeneration from nodal segments of *in vitro* seedlings of *Citrus macroptera Montr.* can be used for mass multiplication of this endangered species.

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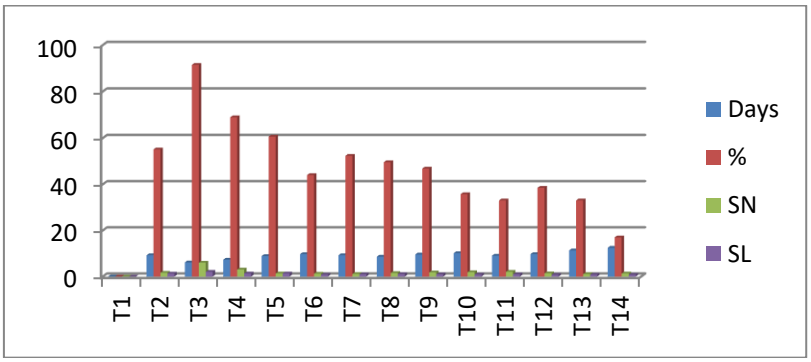

Fig. 1. Effect of different concentrations of cytokinins (BAP and Kn) on shoot induction from nodal segment of *Citrus macroptera*.

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 initiation; % = percentage of response; SN = shoot number; SL = shoot length.

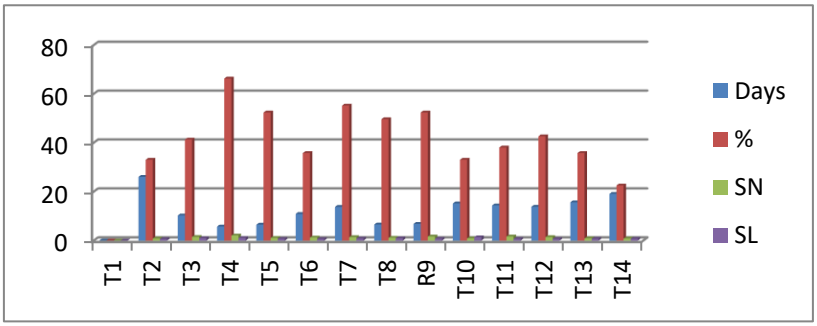

Fig. 2. Effect of different concentrations of cytokinins (BAP and Kn) on shoot induction from shoot tip of *Citrus macroptera*.

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 initiation; % = percentage of response; SN = shoot number; SL = shoot length

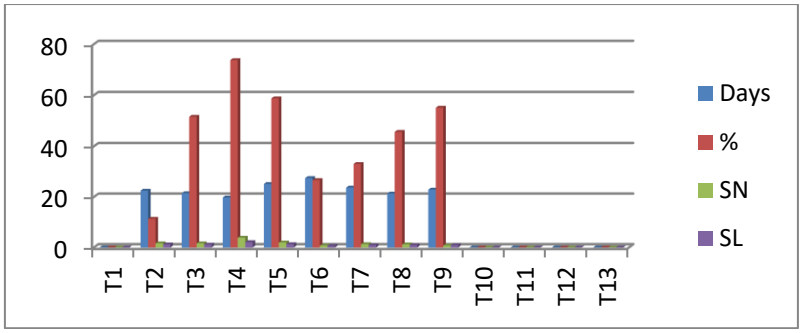

Fig. 3. Effect of different concentrations of cytokinins (BAP, Kn and TDZ) on shoot induction from cotyledons of *Citrus macroptera*.

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}=** TDZ(0.25 mg/l); **T_{11}=** TDZ (0.5 mg/l)**; T_{12}=** TDZ (1.0mg/l); **T_{13}=** TDZ (1.5 mg/l);

Days= days taken for initiation; % = percentage of response; SN = shoot number; SL = shoot length.

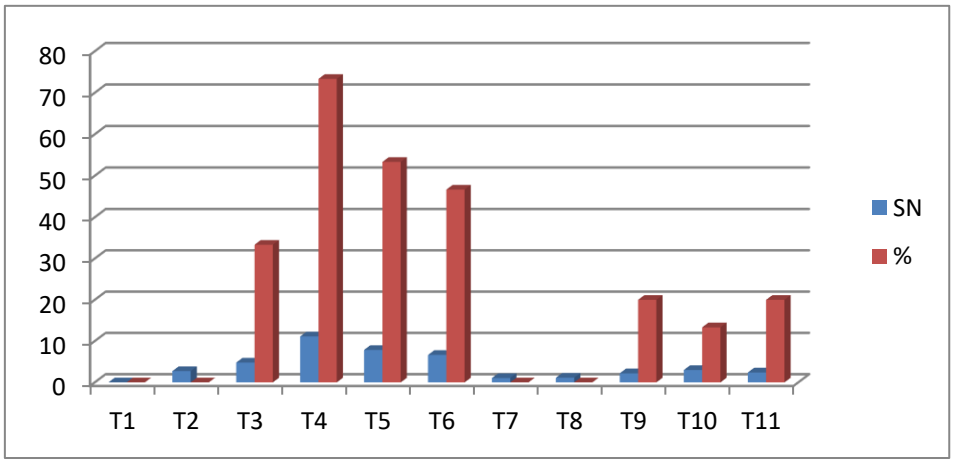

Fig. 4. Effect of various concentrations of BAP and Kn on shoot induction from leaf induced callus of *Citrus macroptera.*

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= (BAP 2.0 mg/l); T_7= (Kn 0.25 mg/l); T_8= (Kn 0.5 mg/l); T_9= (Kn 1.0 mg/l); T_{10}= (Kn 1.5 mg/l); T_{11}= (Kn 2.0 mg/l).

SN= shoot number; %= percentage of response

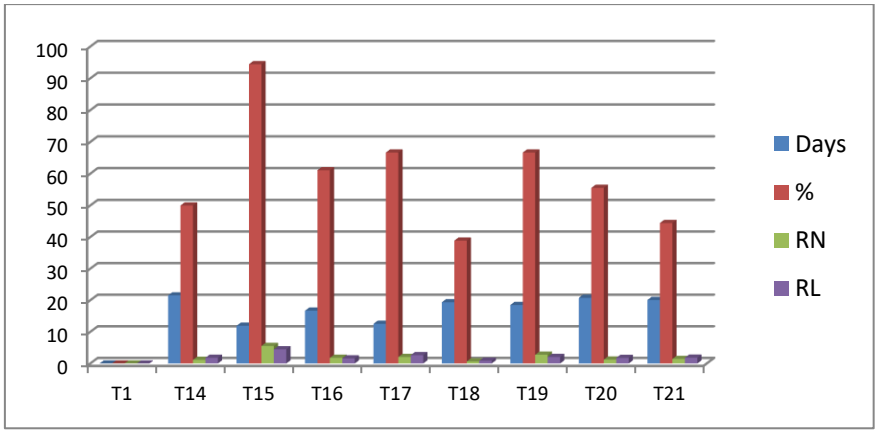

Fig. 5. Effect of different combinations of auxins (IBA and NAA) on root induction from *in vitro* raised shoots of *Citrus macroptera.*

Treatments: T_1 = Blank; T_{14}= (IBA 1.0 mg/l + NAA 0.5 mg/l); T_{15}= (IBA 1.0 mg/l + NAA 1.0 mg/l); T_{16}= (IBA 1.0 mg/l + NAA 1.5 mg/l); T_{17}= (IBA 1.0 mg/l + NAA 2.0 mg/l); T_{18}= (IBA 1.5 mg/l + NAA 0.5 mg/l); T_{19}= (IBA 1.5 mg/l + NAA 1.0 mg/l); T_{20}= (IBA 1.5 mg/l + NAA 1.5 mg/l); T_{21}= (IBA 1.5 mg/l + NAA 2.0 mg/l).

Days= days taken for root initiation; % = percentage of response; RN = root number; RL = root length

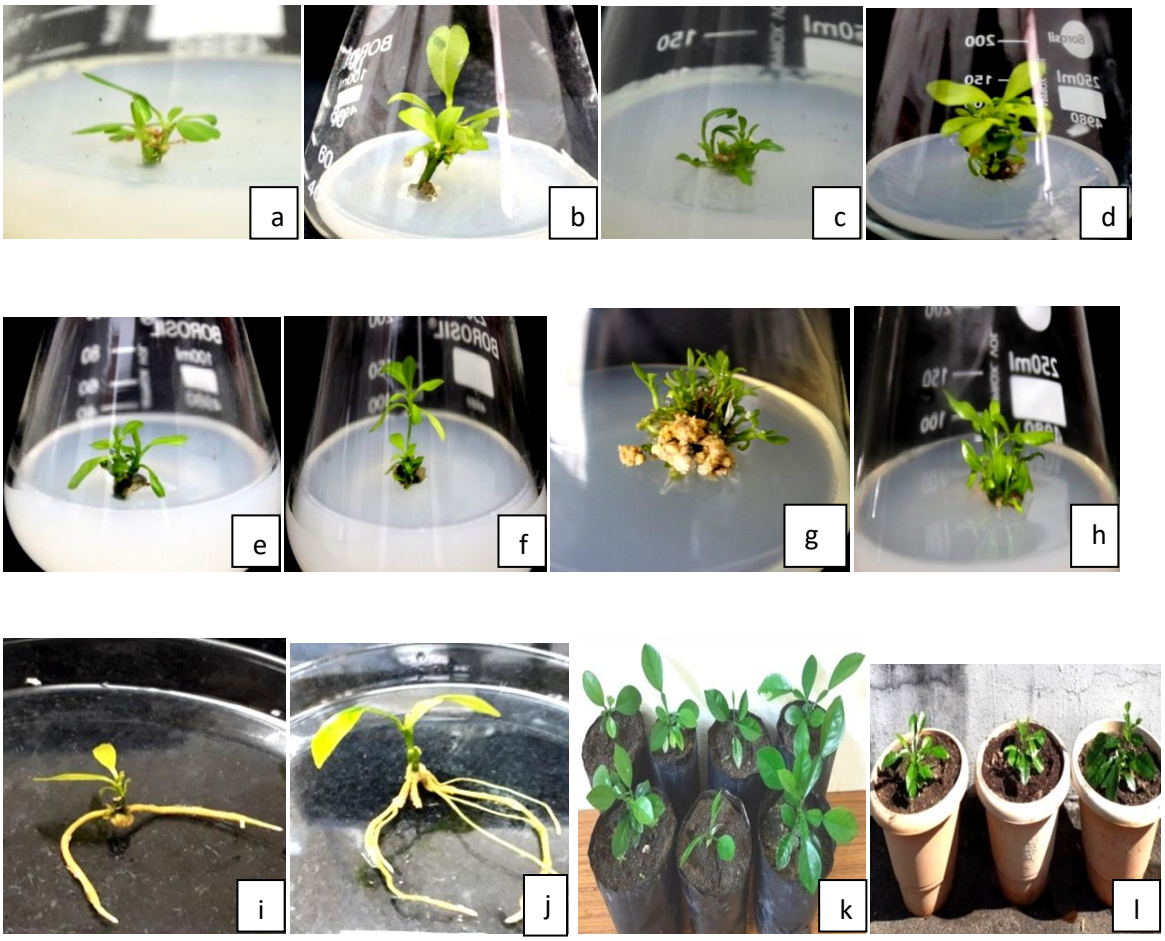

Fig.6.(a-l): *In vitro* propagation of *Citrus macroptera* using different explants. **a:** *In vitro* multiple shoot induction from shoot tip on MS + BAP (1.0 mg/l); **b:** Elongated healthy *in vitro* shoots from shoot tip; **c:** *In vitro* multiple shoot induction from nodal segment on MS + BAP (0.5mg/l); **d:** Elongated healthy *in vitro* shoots from nodal segment; **e:** *In vitro* multiple shoot induction from cotyledon on MS + BAP (1.0 mg/l); **f:** Elongated healthy *in vitro* shoots from cotyledon; **g:** Shoot regeneration from leaf induced callus on MS + BAP (1 mg/l); **h:** Elongated healthy *in vitro* shoots from leaf induced callus; **i:** *In vitro* root induction on full strength MS + IBA (1.0mg/l) + NAA (1.0mg/l); **j:** *In vitro* root induction on half strength MS + IBA (1.0mg/l) + NAA (1.0mg/l); **k:** *In vitro* regenerated plantlets under partial shade; **l:** I*n vitro* regenerated plantlets in open condition.