In vitro propagation of *Citrus megaloxycarpa*

Adhikarimayum Haripyaree[1]*, Kshetrimayum Guneshwor[1], Huidrom Sunitibala[2], Maibam Damayanti[1]

[1]Department of Life Sciences, Manipur University, Imphal, 795003, India
[2]Institute of Bioresources and Sustainable Development, Takyelpat, Manipur, India

*Corresponding author, E-mail: adhikarimayum_devi@yahoo.in

Abstract

An efficient micropropagation protocol was established for *Citrus megaloxycarpa* Lush., a highly acidic citrus cultivar of Manipur, India, through shoot tip explant culture. The shoot tip explants produced multiple shoot buds when cultured on agarized Murashige and Skoog's medium containing 0.25 to 2 mg L^{-1} N6 benzyl adenine (BA) alone, and in combination with 0.50 mg L^{-1} naphthalene acetic acid (NAA) or with 0.50 mg L^{-1} kinetin. The maximum number of shoots was induced on medium containing 0.25 mg L^{-1} BA together with 0.50 mg L^{-1} NAA or 1 mg L^{-1} BA with 0.50 mg L^{-1} kinetin. The separated shoots were rooted and elongated on medium containing 1 to 2 mg L^{-1} indole acetic acid, indole butyric acid and NAA. NAA was superior to other growth regulators for *in vitro* root induction. Axillary shoots were induced from *in vitro* raised plantlets by decapitating them. Axillary shoot tip explants were used for further multiple shoot bud induction. A maximum of about 120 plantlets were obtained from a single seedling. Rooted plantlets were successfully established in the soil. Efficient mass multiplication of this important fruit was achieved.

Key words: axillary shoot induction, *Citrus megaloxycarpa*, MS medium, shoot tip, multiple shoot induction.
Abbreviations: BA, N6 benzyl adenine; IAA, indole acetic acid; IBA, indole butyric acid; KN, kinetin; MS, Murashige and Skoog; NAA, α-napthalene acetic acid.

Introduction

Citrus megaloxycarpa Lush., commonly known as sour pummelo, is an indigenous citrus cultivar with a potential for commercialization in warm humid climates. Presently, it is mainly cultivated in homestead gardens of Manipur, a north-east state of India (23°47'- 25°41' NL; 93°61' – 94°47' EL; 300-3600 m above mean sea level; 1600-3430 mm annual rainfall), for its flavour and sour fruit juices. The juicy pulp of sour pummelo is either eaten raw or used for juice. It is indigenous to India as similar fruits do not seem to have been reported to exist elsewhere (Woodford 2005). The fruits sour pummelo are highly acidic and are esteemed for medicinal properties.

Although tissue culture of citrus species is well studied (Paudyal, Haq 2000; Alkhayri, Aziz 2001; Altaf et al. 2008), several publications report a strong genotype dependence (Paudyal, Haq 2000). Moreover, citrus tissue culture is mostly confined to more common species like *C. reticulata, C. aurantifolia, C. jambhiri, C. aurantium* etc. There have been few reports on micropropagation of *C. grandis* (Paudyal, Haq 2000) and no information for *C. megaloxycarpa*. In pummelo (*C. grandis*), morphogenesis and shoot regeneration are obtained through secondary organogenesis of callus derived from immature ovules (Song et al. 1991) and shoots (Zhong et al. 1991), but rooting in the greenhouse is limited by poor shoot elongation. Moreover, plants obtained from secondary organogenesis from disorganized tissues such as callus may not be true to type due to somaclonal variation. Rapid cloning of elite genotype through *in vitro* adventitious shoot propagation is extensively employed for many fruit species (Zimmerman 1986). Attempts to propagate pummelo from shoot tip explants of *in vitro* grown seedling have been made (Baruha et al. 1995).

Most of the existing citrus collections are conserved in field gene banks in different citrus growing countries (Khawle, Singh 2005). Such collections are vulnerable to biotic and abiotic hazards (Damania 1996). Ageing seeds of *Citrus* species are recalcitrant and lose viability within a short time. Therefore, bearing in mind the problems associated with conventional propagation and the need to develop *in vitro* generation protocols for specific cultivars, the aim of the present study was to develop an efficient protocol for *in vitro* clonal mass multiplication and conservation of germplasm of this elite citrus cultivar by inducing multiple shoots on shoot tip explants. Further multiplication was achieved using axillary shoots induced with regenerated plantlets by decapitating them.

Materials and methods

In micropropagation of *C. megaloxycarpa*, the culture medium consisted of Murashige and Skoog (MS; 1962)

basal medium, B_5 vitamins, 2% (w/v) sucrose, 0.8% (w/v) agar and various concentrations of N6 benzyl adenine (BA), α-napthalene acetic acid (NAA), kinetin (KN), IAA indole acetic acid (IAA), andindole butyric acid (IBA). The pH of the medium was adjusted to 5.7 with 1N NaOH or HCl and autoclaved (121 °C, 105 kg cm^{-2}) for 20 min. The shoots tips taken as explants were first washed with labolene and then treated with 0.1% Dhanustin 50 (fungicide) for 10 to 15 min. This was followed by surface sterilization with 70% ethanol for 4 min, then 0.2% $HgCl_2$ for 7 min and rinsed five times with sterile distilled water. The shoot tips were trimmed by removing the expanded leaves and cultured in culture tubes (35 mm × 200 mm) containing 20 mL of bud induction medium consisting of MS basal medium supplemented with 0.25 to 2 mg L^{-1} BA alone, and in combination with 0.50 mg L^{-1} KN or with 0.50 mg L^{-1} NAA. The number of multiple shoot buds and percentage of explants forming multiple shoot buds were estimated after four weeks. The shoot buds were then separated and cultured on rooting medium consisting of MS basal medium supplemented with different concentration of auxins (1 and 2 mg L^{-1} IAA, 1 and 2 mg lL^{-1} IBA, 1 and 2 mg L^{-1} NAA). After four weeks of culture, data on number of roots per microshoot, shoot length and root length were measured.

Four-week old rooted plantlets were decapitated to induce axillary shoot development by cutting the tips with a sterile blade. Axillary shoots developing in the axils of leaves of the decapitated plantlets were used for further multiple shoot bud induction by culturing them on medium containing 0.25 to 0.50 mg L^{-1} BA alone, and in combination with 0.50 mg L^{-1} NAA or 0.50 to 1 mg L^{-1} BA with 0.50 mg L^{-1} KN. After four weeks, the numbers of shoot buds were counted. The shoot buds formed were cut and rooted in MS basal medium with 2 mg L^{-1} NAA. The cultures were incubated at 25 ± 2 °C under 28 μmol m^{-2} s^{-1} illumination from cool white fluorescent tubes (Philips, India) at a 16/8 h light/dark photoperiod.

Four-week old rooted plantlets were transferred into small plastic pots (7.5 × 8.0 cm) filled with peat, perlite and sand (1:1:1, v/v) and were kept inside the seed propagator of a culture room at 25 to 30 °C, 12 h photoperiod. Each plantlet was fed twice a week with 10 ml of 1 mg L^{-1} NAA. After four weeks the plantlets were transferred to a greenhouse at 30 to 40 °C, relative humidity 70%, 12 h photoperiod.

All the experiments were set up in a completely randomized design. Six cultures were raised for micro shoot induction and ten for root induction and axillary shoot multiplication experiments. The data were subjected to one way analysis of variance (ANOVA) and means were evaluated at p = 0.01 level of significance using the Duncan's multiple range test. All the experiments were repeated three times.

Results and discussion

Studies on the regeneration systems of citrus species reported so far had shown the critical effect of cytokinin concentration and type or cytokinin-auxin ratios in regeneration from various types of explants (Duran-Villa 1989; Paudyal, Haq 2000; Silva et al. 2006). Use of shoot tips as the explants was found to be an effective method for *in vitro* clonal multiplication of *Citrus aurantifolia* (Al-Khayri, Aziz 2001). We studied the morphogenetic response of shoot tip explants of *Citrus megaloxycarpa* cultured on MS medium supplemented with different concentrations of BA alone, and in combination with NAA or KN. After four weeks, shoot buds developed from shoot tip explants (Fig. 1A). The maximum number of buds were produced in medium supplemented with 0.25 mg L^{-1} BA, 0.25 mg L^{-1} BA with 0.5 mg L^{-1} NAA, and 1mg L^{-1} BA with 0.5 mg L^{-1} KN (Table 1). Among these 0.25 mg L^{-1} BA in combination with 0.50 mg L^{-1} NAA proved to be the most effective, followed by 1 mg L^{-1} BA with 0.50 mg L^{-1} KN and 0.25 mg L^{-1} BA. However, Altaf et al. (2008) showed that 2 mg L^{-1} BA with 0.50 mg L^{-1} NAA induced multiple buds from *Citrus jambhiri*. Addition of 0.50 mg L^{-1} NAA to the medium containing relatively high concentration of BA

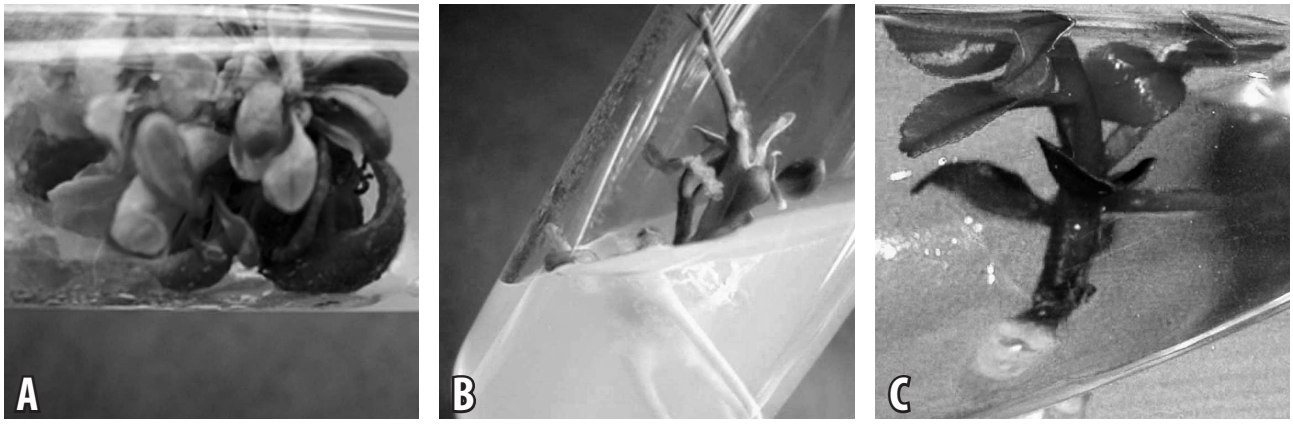

Fig. 1. *In vitro* propagation of *C. megaloxycarpa*. (A) multiple shoot induction from shoot tip explants; (B) rooted plantlets; (C) induction of axillary shoot by decapitation.

Table 1. Effect of growth regulators on multiple shoot induction on shoot tip explants of *C. megaloxycarpa*. Means ± SE, n = 6. Means followed by the same letter are not significantly different at p = 0.01 according to the Duncan's multiple range test. *Maximum number obtained was 6

Growth regulators (mg L^{-1})			Explants forming shoots (%)*	Shoot number
BA	NAA	KN		
0.25	–	–	100	3.6 ± 0.5 cd
0.25	0.50	–	100	4.7 ± 0.5 d
0.25	–	0.50	33.3	1.6 ± 0.2 ab
0.50	–	–	83.3	3.0 ± 0.3 c
0.50	0.50	–	100	2.6 ± 0.2 bc
0.50	–	0.50	33.3	1.6 ± 0.4 ab
1.00	–	–	83.3	2.6 ± 0.4 bc
1.00	0.50	–	100	2.8 ± 0.3 bc
1.00	–	0.50	83.3	4.4 ± 0.4 d
2.00	–	–	66.6	1.5 ± 0.4 de
2.00	0.50	–	66.6	1.2 ± 0.2 a
2.00	–	0.50	83.3	1.6 ± 0.2 ab

(2 mg L^{-1}) did not improve the rate of shoot proliferation in sour pummelo. This result is in agreement with that of Omura and Hidaka (1992), who found that the addition of 0.50 mg L^{-1} NAA is not as critical as that of different concentration of BA for shoot tip culture of satsuma mandarin. In addition, Paudyal and Haq (2000) found that addition of low concentration (0.10 mg L^{-1}) NAA to medium containing relatively high concentration of BA (2.5 mg L^{-1}) did not improve the rate of shoot proliferation in pummelo. One reason for shoot proliferation without addition of any auxin to the medium may be due to the ability of tissues to synthesize the required amount of auxin endogenously (Smith, Murashige 1970).

Rooting of the regenerated shoot buds was achieved in MS medium containing 1 mg L^{-1} or 2 mg L^{-1} of IBA, IAA and NAA. The highest number of roots was produced with 2 mg L^{-1} NAA (Table 2). A maximum of 4.4 roots was obtained for explants cultured on this medium (Fig. 1B). A study with *Citrus grandis* showed similar results with maximal rooting at 2 mg L^{-1} NAA, and a decrease in the frequency of rooting below of NAA concentration 2 mg L^{-1} (Paudyal, Haq 2000). However, in the study by Parthasarathy and Nagarju (1996), MS medium supplemented with 0.05 mg L^{-1} NAA was found to be the best for rooting in many *Citrus* species, excepting Musambi for which the best concentration was 0.2 mg L^{-1} NAA.

Among the auxins, IBA and IAA were less effective for rooting of *Citrus megaloxycarpa* than NAA. Root length decreased with higher concentration of NAA and the longest root (22 ± 0.22 mm) was found in the 1 mg L^{-1} NAA treatment (Table 2). The length of the roots induced in IBA-supplemented media did not differ significantly between the different concentrations used. The shoots cultured with 1 to 2 mg L^{-1} IAA and IBA produced only one or two roots per shoot at all concentrations. Similarly, various rooting responses of mandarin varieties to different types of auxin was reported by Omura and Hidaka (1992).

The rooted plantlets elongated in the rooting media and maximum elongation of 20 to 40 mm of the regenerated shoots was achieved in medium containing 1 or 2 mg L^{-1} NAA or 2 mg L^{-1} IBA after four weeks of the culture. The length of the shoots was found to increase with increasing concentration of IBA.

After four weeks of culture in rooting medium, the elongated plantlets were decapitated and the effect on the growth of axillary shoots was studied. The decapitated plantlets showed the development of two to five axillary shoots within two weeks of culture (Fig. 1C). Induction of axillary shoots by decapitation of *in vitro* generated plantlets has been reported in many plant species (Hyde, Phillips 1996; Sanatombi, Sharma 2008). However, so far there have been no reports of micropropagation of *Citrus* species from axillary shoot tip induced by decapitation. On culturing the axillary shoot tips on bud-induction medium, they proliferated to produce multiple shoot buds (three to six) on the medium supplemented with 0.25 mg L^{-1} BA, 0.25 mg L^{-1} BA, with 0.50 mg L^{-1} NAA or 1 mg l-1 BA with 0.50 mg L^{-1} KN (Table 3). These shoot buds showed rooting and elongation in medium containing 2 mg L^{-1} NAA. The rooted plantlets were decapitated again and used for further induction of axillary shoot or they were transplanted after

Table 2. Effect of auxin on elongation and rooting of shoot buds of *C. megaloxycarpa*. Means ± SE, n = 10. Means followed by the same letter are not significantly different at p = 0.01 according to the Duncan's multiple range test

Growth regulators (mg L^{-1})			Shoot length (mm)	Number of roots per microshoot	Shoot number
BA	NAA	KN			
1.0	–	–	10.9 ± 0.2 c	1.2 ± 0.2 a	12.5 ± 0.1 b
2.0	–	–	10.6 ± 0.2 c	1.4 ± 0.2 a	15.1 ± 0.2 a
–	1.0	–	18.2 ± 0.3 a	1.2 ± 0.2 a	16.2 ± 0.3 bc
–	2.0	–	22.2 ± 0.3 c	1. 0 ±0.3 a	15.2 ± 0.1 c
–	–	1.0	34.3 ± 0.1 ab	4.0 ± 0.3 b	22.0 ± 0.2 a
–	–	2.0	22.2 ± 0.2 ab	4.4 ± 0.2 b	18.1 ± 0.3 b

Table 3. Effect of growth regulators on multiple shoot bud induction from axillary shoot explants of *C. megaloxycarpa*. Means ± SE, n = 10. Means followed by the same letter are not significantly different at p = 0.01 according to the Duncan's multiple range test

Growth regulators (mg L^{-1})			Explants forming	Shoot number
BA	NAA	KN	shoots (%)	
0.25	–	–	100	3.2 ± 0.2 b
0.25	0.50	–	100	4.6 ± 0.4 a
0.50	–	–	100	2.5 ± 0.4 a
0.50	0.50	–	90	2.2 ± 0.4 b
0.50	–	0.50	80	2.0 ± 0.3 b
1.00	–	0.50	100	4.2 ± 0.3 a

hardening and acclimatization. The regenerated plantlets showed 70% survival when transplanted. The success rate was recorded by emergence of two or three new leaves. Morphologically, these plants were slightly yellowish when compared to the parent plant.

Thus, the induction of multiple shoot buds from shoot tip explants provided a novel protocol for propagation of *Citrus megaloxycarpa* in tissue culture. It resulted in the regeneration of a large number of plantlets from single explants. This technique, therefore, is an efficient system for germplasm conservation and mass multiplication of this important fruit plant, as compared to propagation by seed.

Acknowledgements

A.H. acknowledges DST, New Delhi for financial assistance provided as Women Scientist WOS-A wide grant no. SR/WOS-A/LS-58/2008 and authors are grateful to the Director IBSD, Takyelpat for providing laboratory facilities.

References

Al-khayri J.M., Aziz A. 2001. *In vitro* micropropagation of *Citrus aurantifolia* (Lime). *Curr. Sci.* 81: 438–445.

Altaf N., Khan A.R., Ali L., Bhatti I.A. 2008. Propagation of rough lemon (*Citrus jambhiri* Lush.) through *in vitro* culture and adventitious rooting in cutting. *EJEAF Chem.* 7: 3326–3333.

Baruha B. 1999. Prospect of citriculture in South East Asia by the year 2000. *FAO Plant Prot. Bull.* 38: 151–173.

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

Duran-Villa N., Ortega V., Navarro L. 1989. Morphogenesis and tissue culture of three *Citrus* species. *Plant Cell Tissue Organ Cult.* 16: 123–133.

Khawle R.N., Singh S.K. 2005. *In vitro* adventitive embryony in citrus: A technique for citrus germplam exchange. *Curr. Sci.* 88: 1309–1311.

Murashige T., Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15: 473–479.

Nafees A., Rehman A., Bhath I.A., Ali L. 2009. Tissue culture of *Citrus* cultivars. *EJEAF Chem.* 8: 43–51.

Omura M., Hidaka T. 1992. Shoot tip culture of citrus. 1. Culture condition. *Bull. Fruit Tree Res. Stat.* 22: 23–26.

Parthasarathy V.A., Nagaraju V. 1996. Rooting of micro-cutting of certain *Citrus* species. *Indian J. Hortic.* 53: 255-258.

Paudyal K.P., Haq N. 2000. *In vitro* propagatipon of Pummelo (*Citrus grandis* L. Qsbeck). *In Vitro Cell. Dev. Biol. Plant* 36: 511–516.

Poutney C., Swietlik D. 1988. The effect of Dropp (TDZ), a urea derivative with cytokinin activity, on sour orange seedling and field grown grape fruit trees. *J. Rio. Granade Valley Hortic. Soc.* 41: 33–39.

Sanatombi K., Sharma, G.J. 2008. *In vitro* propagation of *Capsicun chinense* Jacq. *Biol. Plant.* 52: 517–520.

Silva R.P., Almeida W.A.B., Souza E.S., Filho F.A.A.M. 2006. In vitro organogenesis from adult tissue of 'Bahia' sweet orange (*Citrus sinensis*). *Fruits* 61: 367–371.

Smith R.H., Murashige T. 1970. *In vitro* development of isolated shoot apical meristems of angiosperms. *Amer. J. Bot.* 57: 562–568.

Song W.S., Oh S.D., Cho H.M., Park J.H. 1991. Plant regeneration in *Citrus grandis* Qsbeck through somatic embryogenesis. 1. Effect of plant growth regulators on embryogenetic callus induction and somatic embryogenesis. *Res. Rep. Rural Devel. Admin. Biotech.* 33: 14–21.

Woodford R.C. 2005. *Citrus Classification*. Biotech Books Publisher. India, pp.129.

Zhong Q.S., Shu G.P., Song S.H. 1991. The modification of *Citrus* shoot tip grafting technique. *China Citrus* 20: l1–13.

Zimmerman R.H. 1986. Propagation of fruits, nuts and vegetable-overview. In: Zimmerman R.H., Griesbach R.J., Hammerschlag F.A., Lawson R.H. (eds) *Tissue Culture as Plant Production System for Horticultural Crops*. Martines Nijhoff, Dordrecht. pp.183–200.

Received 4 July 2011; received in revised form 12 August 2011; accepted 17 September 2011