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A Significant Footprint of *In-vitro* Micropropagation on Growing Opportunities of Endangered Citrus Species in India

Ruchi Verma and Susmita Shukla*

Applied Plant Biotechnology Lab, Centre for Plant and Environmental Biotechnology, Amity Institute of Biotechnology, Amity University, Noida, Uttar Pradesh, India-201313
*E-mail: *sshukla3@amity.edu*

Abstract—*Citrus is a very important commercially significant crop where India leads in its production among many countries in the world. However, due to the influence and interference of human activities on natural habitat has become a matter of concern for growing population which has caused depletion of many plant species. Diverse alternatives are accessible to confront this issue among which tissue culture has great potential for germplasm preservation which has attained minor attention till now. Seven Citrus varieties of medicinal and horticultural importance found in the natural habitat of north eastern region of India are categorized as vulnerable and endangered for which efforts are being made widely to develop strategies, methods and technology for their conservation and protection, where in-vitro micro propagation method of plant tissue culture is playing key role in maintaining successful sustainable and commercial production of Citrus. Conventional vegetative methods of citrus plant propagation is time consuming and is dependent on the planting material availability as well as on the season which not only hinders in faster acquisition but also in renewal of new citrus varieties therefore, there is a great need for attention to be laid towards plant tissue culture techniques like micro propagation. Citrus crop isnot only commercially important but also contributes to the environment as it carries the capacity to hold excess storm water, reduces nutrient runoff and its participation in the production of oxygen is major. The basic aim of micro propagation is to acquire genetically identical, ethnically normal, physiologically consistent and virus free seedlings on large scale production in short span of time. Fresh juice and fruits are the most significant products along with some high value products like pectin, essential oils, and dried rinds etc, which are commercially significant. Citrus demand for use as ornamental plants is also considerably increasing worldwide leading to economic development of the nation. Different protocols have been standardized to propagate citrus plantlets on commercial scale through in-vitro propagation methods like micro grafting and micro budding using various parameters to enhance the growth and production of dietary, medicinally and industrially important species which could further improve the economic conditions through establishment of entrepreneurs as well as ensure nutritional food security via diversification from regular agricultural practices. Keeping in view the importance of the endangered species and its conservation the research work is focused on culture initiation ofendangered Citrus species and further on mass multiplication.*

Keywords: *Horticultural; Conservation; Endangered; Micro-propagation; Commercial; Sustainable; Micro-grafting; Medicinal; Nutritional food security.*

1. INTRODUCTION

The conservation of plant biodiversity is an essential issue referring to the human population around the world. The protection of plant species can be done both in-situ as well as ex-situ. In situ approach likein- vitro methods can notably increase collecting efficiency of plantlets raised throughmicropropagation techniques (F. Engelmann, 2011). Through the strategies of tissue culture , collection , multiplication along with the storage of plant germplasm can be achieved as well as it plays key role in the conservation of plant biodiversity including rare and endangered plant species also, genetic resources of recalcitrant seed and vegetatively propagated species as well as elite genotypes (Bunn et al. 2007).In India, there are approximately 30 species of *Citrus* out of which almost nine species are found throughout the India,where as 17 species are only confined to the North-Eastern states of India, which are threatened in their natural habitat according to the International Union for Conservation of Nature and Natural Resources (IUCN). There are Seven Indian Citrus species which has been categorized under endangered zone, including *C. Indica*, *C. macroptera*, *C. latipes*, *C. assamensis*, *C. ichangensis*, *C. megaloxycarpa* and *C. rugulosa*.

The science of plant tissue culture is a method in which aseptic cultivation of plant tissues or whole plantlet using different culture media, plant growth regulators as well as culture conditions like light, temperature and humidity. Various in-vitro protocols for rapid cloning of medicinally and horticulturally important rare, endangered citrus species of north –eastern region of India, employing different explants of field grown plant and also in-vitro grown seedlings through seed culture, were developed for the purpose of their conservation.

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Citrus macroptera (Melanesian papeda) is much significant endangered wild species which is locally popular as Satkara and Hatkara. Commercially it is being cultivated in few areas like Aizawl, Kolasib, Mamitof Mizoram and in the Jumpui hills of Tripurabecause of its raising demand among people of north-east as the fruits of this species are utilized for medicinal, dietary and culinary purposes. Unfortunately, its unsatisfactory production is not meeting the demand of the population hence forth , there is a requirement for the development of rapid multiplication methods like tissue culture techniques which possess a great prospective for excessive mass multiplication as observed while doing the production of multiple shoots from certain explants of few known citrus varities (Baralass and Skene1982)

*In-vitro*micro-propagation of *Citrus macroptera,C. latipes*, *C. assamensis*, *C. ichangensis*and *C. rugulosa*has not yet beenreported in India while few species of citrus have been mass multiplied through various regeneration protocolsand through the findings of the literature an effort has been.With the help of these findings, attempt has been made to initiate and develop a protocol for multiplication and direct regeneration from the explants of*in-vitro* grown seedlings.

2. HISTORICAL BACKGROUND

Plant tissue culture was first proposed by the German Botanist Gottlieb Haberlandt in 1902 .He is regarded as the father of plant tissues culture. He principally worked on palisade tissue and developed them on Knob's salt arrangement with sucrose and monitored the development of cells. Hanning (1904) extracted developed embryos of the crucifers and effectively developed them on the mineral salt and sugar solution. The incipient organism culture was additionally created by over back (1941). This ended up being a defining moment in plant tissue culture. In 1972, Carlson and other created the first somatic hybrid with physical half and half between Nicotianagluca and N.langschorffii by intertwining their protoplast. Tissue culture first utilized on extensive scale by the orchid business in 1950s.

Types of Plant Tissue Culture includes Apical meristem culture, Axillary bud culture, Callus culture, Cell culture, Suspension culture, Protoplast culture, Embryo culture. By exploiting any of these technique one can regenerate the whole plant from a mass of totipotent cells.

The attention of tissue culture in citrus has paid long back and amply emphasized by Bitter and Murashige (1967) and Kochba and Spiegel-Roy (1976). The wide significance of tissue culture in citrus breeding for improvement and augmenting production was discussed by Kochba and Spiegel-Roy (1977) and various other aspects of citrus tissue culture by Button and Kochba (1977) and Spiegel-Roy and Kochba (1980).

In the field of micropropagation in citrus, Murashige (1972) developed shoot tip culture in citrus. They mainly developed the technique for virus free propagative bud wood to reduce the losses occurring by citrus virus and virus like diseases. The spectacular success achieved till date in ornamental and fruit crops which have revolutionized the tissue culture. (Debergh P *et al.* 1990;Hussey., 1997; Jona R. and Menini U, 1987)

In recent years micropropagation is increasingly used as clonal propagation, restoration of endangered species andgermplasm conservation.

2.1: National and International Scenario

Citrus rank among top three fruits of the world with respect to area and production. It is grown commercially in more than 140 countries in all over the world, world production of citrus fruit has experienced continuous growth in the last decades of the twentieth century with total annual citrus production over 105 million tons between 2000 and 2004. These fruits are commercially important contributing $6-8 billion (US) annually to the world economy and providing jobs to millions of people around the world in harvesting, handling, transportation, storage and marketing.

Post harvest biology and technology of citrus fruits is gaining importance as the therapeutic value of citrus fruits is realized and supported by the increase in health awareness among the general public. Global production of citrus counts for an important market for grapefruit, orange and lemon juicewhich is contributing to enhance world economy.

Table 1: *In vitro* regeneration protocol of some Endangered Citrus species.

Species	Explant	Medium	Plant Growth Regulator for Shoot formation	Plant Growth Regulator for Root formation	References	Country
C. indicaTanaka	Leaves	MS and WPM	0.5mg/L BAP, 0.25 mg/L TDZ, &0.25 mg/L NAA	1.0 mg/L NAA	Rao C S et al., 2009	India
C. megaloxycarpa	Shoot Tips	MS	0.25 mg/L BAP & 0.50 mg/L NAA	1 to 2 mg/L IAA, IBA & NAA	DamyantiMaibamet al., 2011	India
C. halimmi	Shoot tips, Nodes, Hypocotyls	MS	0.14 to 0.72 mg/L BAP	0.17 mg/L NAA	Normah M.N. et al., 1997	Malaysia

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C. chrysocarpa L.	Mature seed embryos	MS	0.84 mg/L BAP	0.65 mg/L IBA	Prodhan H. Shamshulet al., 2016	Bangladesh

2.3: Micropropagation an important tool for conservation of threatened and endangered Citrus species worldwide

Citrus which is woody perennial plant has been one of the most widely studied plants for in-vitro culture (Gmitter et al.,1992)where in India, few studies on the efficient regeneration protocols of endangered citrus species have been reported so for where different explants and the expository effects of the various concentrations of the cytokinins on them has been shown (Silva *et al*., 2006) used like for C. *indica*, the work was started with only 20 seeds and the leaf explants were taken as the starting material for setting up the experiments (Laskar M.A. *et al*., 2009) while in C. *megaloxycarpa*, shoot tips were taken as explants which were inoculated in the MS basal medium with B_5 vitamins for its propagation (HaripyareeAdhikarimayum*et al*., 2011). Some reports on micro-propagation of endangered citrus species of South- East Asia has been reported where successful protocol for direct organogenesis of C. *halimmi* has been established with the help of shoot tips, young leaves, nodes, internodes, hypocotyls and root tips explants (Normah M. N. *et al*.,1997) while in Bangladesh, an efficient in-vitro propagation of wild type Indian orange *C. chrysocarpa*L. has been developed using seed embryos as explants (ProdhanShamsul H. *et al*., 2006) while there has been a single report on micropropagation of one of the Australian species, C. australasica (Ling and Iwamasa 1997) where shoot tips were used as expalnts.

3. ESTABLISHMENT OF INITIATION STAGE OF ENDANGERED SPECIES AT APPLIED PLANT BIOTECHNOLOGY LAB, AIB, AUUP

The present study is mainly dealing with the *in vitro* studies of endangered Citrus species. The various factors affecting the culture initiation such as explants selection, surface sterilization, germination rate etc which has been studied resulted in standardization of culture initiation and establishment

3.1: Explant Selection

In the present study, authenticated seeds collected from the North eastern region and nodal segments excised into 0.5 cm from the in-vitro grown seedlings of the Citrus *macroptera*were taken and cultured onto different media compositions (WPM, MS, Gamborg's B5 medium and MT) alone and supplemented with different concentrations of BAP (0.5,1.0,1.5,2.0mg/L) for direct multiple shoot regeneration. The propagated shoots were further on the same media for further multiplication and the observation was made every five week interval for data collection.

3.2: Culture Conditions

After inoculation, seed cultures were incubated in the dark room of plant growth chamber maintained at 25±2 ^{0}c for 2 weeks and after germination transferred to culture racks with 16/8 h (light/dark) photoperiod while the media inoculated with nodes were incubated directly in the growth room with 16/8 h (light/dark) photoperiod at 25±2 ^{0}c and the cultures were sub cultured at an interval of 15 days.

3.3: Surface Sterilization of Seeds

Seeds were soaked for 2 hours in the distilled water and 2-4 drops of 1% Tween 20 is added into it with continuous stirring until it becomes foamy for 10 minutes and then washed 3-4 times to remove the foam, after that under sterilized conditions, seeds were dipped in 5% NaOCl (Sodium hypochlorite) and stirred for 5 minutes followed by three times rinsing with autoclaved distilled water and then using forceps and knife with gentle care the seed coats were removed. 0.1% $HgCl_2$ is then added and stirred gently for 3 minutes followed by three times rinsing with autoclaved distilled water. Finally, the seeds were treated with 70% ethanol for 1 minute and rinsed three times with autoclaved distilled water and the sterilized seeds were then inoculated into the different media compositions (WPM, MS, Gamborg's B5 medium and MT).

3.4: Mediastanderdization

To initiate seed germination and multiple shoot proliferation various media compositions were experimented namely, Woody plant medium(WPM), Gamborg's B5 medium, Murashige and Skoog medium (MS) and Murashige and Tucker medium(MT) were used alone for seed germination and supplemented with BAP (0.5,1.0,1.5,2.0mg/L) for initiation of multiplication protocol. The pH of medium was adjusted to 5.7-5.8 before autoclaving the media.

Nodal explants from grown seedlings of 4-5 cm long were obtained after 15 days of seed germination and cultured onto different freshly prepared medium (WPM, MS, Gamborg's B5 medium and MT) containing BAP at 0.5,1.0,1.5 and 2.0mg/L concentrations for initiation of direct multiple shoot proliferation.

4. RESULT AND DISCUSSION

Previous studies on seed germination of *C. indica*showed 60% germination in MS medium (Laskar M A *et al*., 2009) while in the present study, seed germination in different media composition suggested thatthe seed germination is slow and non-uniform and all the media compositions experimented were capable of producing seedlings, though the seed germination percentage was higher in Gamborg's B5 medium (66.6%) but the seedling produced werecomparatively shorter in height with fewer number of nodes, leaves and more number of stronger roots while in the MS and WPM medium the seedlings obtained were of 5.5 and 5 cm height

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respectively with more number of nodes and leaves and the MT medium showed moderate growth of seedlings compared to above media compositions, so it was conclude that the best media respective to seed germination rate was Gamborg's B5 medium while the seeds germinated in MS and WPM were capable of producing more planting material to further set up of the experiments.

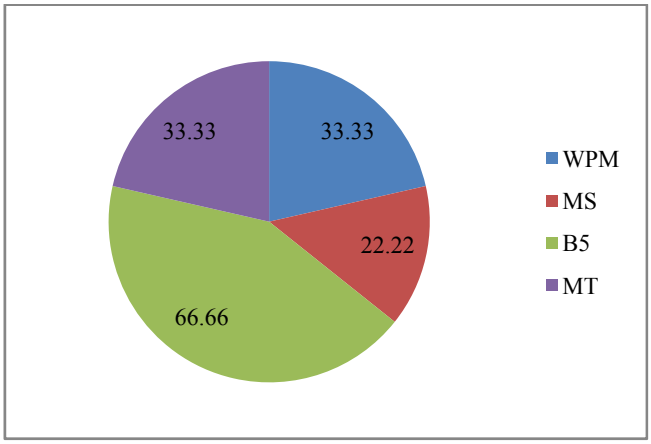

Fig1: Effect of WPM, MS, B5 & MT medium on percentage seed germination.

4.1: Effect of BAP on multiple shoot initiation and regeneration

The results shown in the (Table1) suggested thatthenodal explants were capable of producing multiple shoots (Islam et al. 2008) and highest bud initiation percentage (93.33%) was found in MS+BAP(1mg/L) also, maximum no. of shoots per explants (4.33) were noted in the same set of experiment. The attempts for establishment of initiation protocol for the multiple shoot regeneration were based on the successful regeneration protocols of various endangered citrus species like C. *indica*(C.S. Rao et al. 2009) and C. *megaloxycarpa* (MaibamDamyanti et al. 2011).

MS+BAP Conc.(mg/L)	% of Explants Responded	No. of Shoots obtained per explant
MS(0.0)	33.33	0.93±0.37
MS(0.5)	91.33	3.26±0.64
MS(1.0)	93.33	4.33±0.70
MS(1.5)	53.33	1.73±0.21
MS(2.0)	41.66	1.13±0.36

Table2: Effect of different concentrations of MS+BAP (0.5, 1.0, 1.5, 2.0 mg/L) on initiation of multiple shoot proliferation from nodal explants of in-vitro grown seedlings of C.*macroptera.* Data as (Mean ± S.E.) were recorded after five weeks of culture.

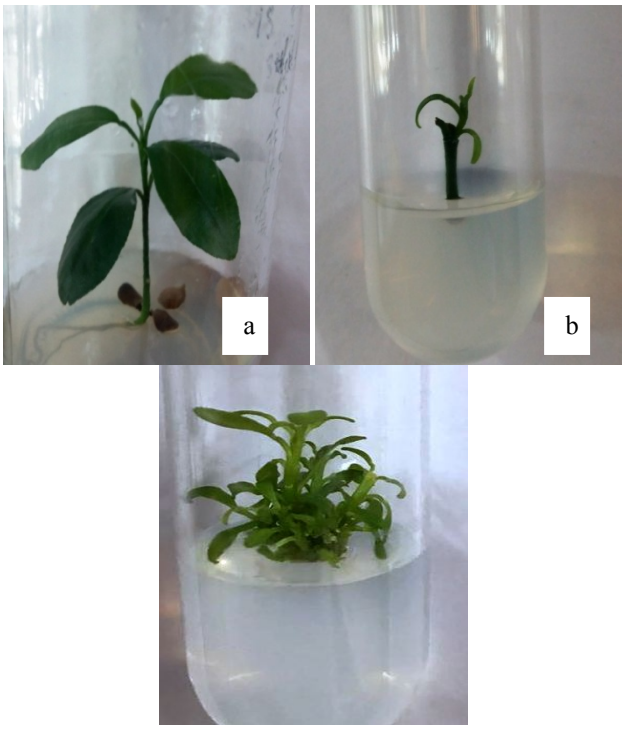

Fig 2(a-c): a. *Invitro* grown seedling of *C. macroptera*from seeds; b. Bud initiation; c. Initiation of multiple shoots

5. CONCLUSION

In vitro plant propagation for the conservation of plant species is a biotechnological approach through which economically important crops of recalcitrant seeds could be preserved along with the production of virus free plants. Introduction of regenerated plantlets directly into its natural habitat could be done using in situ conservation**.** One of the major issues with the tissue culture of endangered and threatened plant species is the limited quantity of planting material for setting up the experiments in replications for standardization of protocols (Campos and Pais, 1996, McComb, 1985). Along with it collection of plant material is quite troublesome because the palnts may be situated in distant areas from where collection may be difficult and costly. Inspite of all the above hindrances, there has been a lot of work done on micropropagation of endangered species which is worthy to mention for preservation of germplasm and to maintain genetic diversity (Pence,1999).

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