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Impact of Encapsulation on Plantlet Regeneration from *in vitro* Grown Shoot tips of *Citrus aurantifolia* (Lime)

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Abstract

In order to conserve diverse species of citrus, an experiment on *in vitro* micropropagation and production of synthetic seeds from *in vitro* regenerated plant propagules of the species; *Citrus aurantifolia* (Lime) was carried out in which shoot tips were found to be suitable for excapsulation of artificial seeds. Highest rate of germination was obtained from the shoot tips when MS was supplemented with 1 mg/l BAP. Beaded shoot tips produced maximum germination (81.43%). Germinated synthetic seeds with well established roots and shoots were were taken out from the culture bottles and transferred in plastic cups containing a mixture of sterile soil: sand and farmyard manure at a ratio of 1:1:1. Seedlings were further shifted in earthen pots and kept in a partial shed net house for 7 days. Those seedlings were finally transferred under the field conditions for acclimatization.

Introduction

The North Eastern region of India is a part of the centre of origin and is rich in diversity of citrus with wild and endangered species. The present situation of citrus genetic diversity is in an alarming rate due to its enormous destruction in the natural habitat. *C. aurantifolia* (Lime) is one of the commercially important citrus fruits, but they are grown on relatively smaller scales. *Citrus* is being propagated mainly by conventional techniques and is usually dependent on particular season and availability of mother plant, which restricts faster adoption and replacement of new varieties due to unavailability of sufficient quantity of propagules (Rathore et al. 2007, Goswami et al. 2013, Fanta et al. 2016).

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In vitro mass-multiplication using biotechnological tools is a powerful alternative for propagation. There are different biotechnological tools for rapid mass-multiplication of *Citrus* spp. viz., somatic embryogenesis, adventitious shoot bud production, and axillary enhancement which are being routinely used. Amongst these, axillary bud proliferation (micropropagation from nodal sections and shoot tips culture) is commonly practiced for *in vitro* mass-multiplication of *Citrus* because it ensures maximum genetic uniformity of the resulting plants (Rathore et al. 2007, Goswami et al. 2013). In addition, *in vitro* grown plantlets are apparently considered to be diseased free (Grosser and Chandler 2000).

Considering the importance of *Citrus* wealth of East and North-Eastern states of India, major emphasis had been laid on standardization of biotechnological tools for direct mass-multiplication through synthetic seed production using shoot tips of popular species of citrus- *C. aurantifolia* (Lime).

Synthetic seed is produced by enclosing viable plant materials such as somatic embryos, androgenic embryos, pre-embryos, embryos-like-structure (Roy and Mandal 2006, 2008) and protocorms (Bhattacharjee et al. 1998), protocorm-like-bodies (Balilashaki et al. 2015), axillary buds (Ganapathi et al. 1992), meristem (Kamada et al. 1989), shoot segments (Brischia et al. 2002), shoot tips (Gholami and Kavani 2018), etc. in alginate with nutrient sources. For encapsulation, plant propagules were mixed with sterilized sodium alginate (3% w/v) solution, particularly prepared in suitable tissue culture basal medium and supplemented with sucrose. Propagules were then picked up individually and dropped into sterilized aqueous solution of 3% (w/v) calcium salt solution [CaCl2 or (CaNO3)2] with occasional agitation (Roy and Mandal 2008). Calcium alginate beads were formed within 15-30 minutes. Size of the beads depends upon the inner diameter of the pipette nozzle. In the present experiment, shoot tips were considered to be most suitable explants for encapsulation and preparation of synthetic seeds as it exhibited true-to-type planting materials.

Shoot tips which convert into plantlet directly without callus formation would reduce the risk of somaclonal variation (Wang and Den 2001) thereby making the plantlets available round the year (Reed 2002). In this endeavor, an effort was taken to standardize the protocol for preparation of synthetic seeds from shoot tips of *in vitro* grown plantlets of *C. aurantifolia* (Lime).

Materials and Methods

Seeds were collected and extracted manually from matured fruits of *C. aurantifolia* (Lime) and were cut with a sharp knife. Extracted seeds were surface sterilized with 0.1% $HgCl_2$ for 10 minutes followed by 3 - 5 times washing with sterilized distilled water. De-coating of surface-sterilized seeds were done under the laminar airflow cabinet and inoculated on basal MS medium. The culture bottles were incubated in a culture room at 25 ± 2°C with 16/8 hrs light and dark phases for germination, growth, and establishment of

seedlings. After six weeks of inoculation, emergence of *in vitro* grown seedlings were observed and further used as a source of explants for preparation of synthetic seeds.

MS was prepared specifically with 30g of sucrose and 8 g of plant tissue culture-grade agar powder. The pH of the medium was adjusted to 5.8. Sterilization of the prepared media was obtained by autoclaving at 121 degrees Celsius under 104 kPa for 15 minutes. This sterilized nutrient MS medium was used for further regeneration of *in vitro* multiple shoots from shoot tips encapsulated synthetic seeds.

Shoot portion of *in-vitro* grown seedlings were cut into small pieces keeping a few nodes. Shoot tips of the seedlings were also used for regeneration of multiple plantlets. An individual culture bottle was inoculated and comprised of two explants with a total of 30 explants for each treatment. The experiment was repeated two times. Inoculated culture bottles were kept in culture room at 25 ± 2°C with 16/8 h light and dark phases at optimum photosynthetic flux provided by cool fluorescent lamps for sprouting and regeneration of multiple plantlets.

Preliminary step for synthetic seed production is the preparation of Sodium alginate solution (2.5% w/v) of Sigma Aldrich Chemical by mixing 200 ml of liquid MS fortified with 30 g of sucrose. Standard protocols developed by Roy and Mandal (2008) and Roy (2020) were used for preparation of a 3.0% aqueous solution of calcium chloride (Sigma Aldrich Chemical) in a 500 ml conical flask. Both the solutions were autoclaved at 121 degree Celcius under 104 kPa for 15 minutes for sterilization. Shoot tips of well-grown plantlets were cut under the laminar airflow cabinet and used as propagules for preparation of synthetic seeds by mixing with sodium alginate solution. Individual propagule was picked up with a graduated dropper and dropped in a sterile aqueous solution of calcium chloride with occasional agitation. Calcium alginate beads were formed within 15-20 minutes. Beads were taken out by decanting off $CaCl_2$ solution, washed with sterile double distilled water, surface dried with sterilized blotting paper and temporarily placed in clean sterilized Petri plates. Freshly prepared synthetic seeds were directly transferred and cultured on MS medium fortified with different concentrations and combinations of treatments which are mentioned below. Seedling emergence and regeneration of plantlets were recorded after two weeks of inoculation. 40 beads were employed in three replications for each treatment. The experiment was conducted entirely under a controlled environment using a laminar airflow cabinet.

Treatments used were full strength MS, ½ MS, ¼ MS, 1.0 mg/l of BAP, 2 mg/l of BAP, 4.0 mg/l of BAP, 1.0 mg/l of IBA+1.0 mg/l of IAA and 1.0 mg/l of IBA+1.0 mg/l of IAA+ 1.0 mg/l of NAA.

For interpretation of results, a completely randomized design (CRD) experiment was laid out for the laboratory experiment. Data were subjected to standard statistical methods of analysis of variance (ANOVA) using Ag Res Statistical Software, (c) 1994 Pascal Intl Software Solutions, Version 3.01, and significant differences were compared

by LSD at p = 0.05. Analysis of data was used to interpret the results and draw conclusions.

Results and Discussion

Data on germination of synthetic seeds were taken after six weeks of inoculation (Table 1). Germination of synthetic seeds of *C. aurantifolia* (Lime)derived from shoot tips varied from 64.62% to 81.43% with a grand mean of 71.36%. All the treatments showed high percentage of germination. However, maximum germination was obtained on MS medium supplemented with 1 mg/l of BAP (81.43%) which is insignificant with 2 mg/l of BAP (79.67%). The treatments 1 mg/l of IBA + 1 mg/l of IAA (74.50%), 4 mg/l of BAP (71.33%) and 1 mg/l of IBA + 1 mg/l of IAA + 1 mg/l of NAA (70.33%) were also found to be insignificant among themselves.

Table 1. Germination of synthetic seeds of *C. aurantifolia* (Lime) on different plant growth regulators under *in vitro* conditions.

Treatment	Germination (%)
MS	64.62 de
½ MS	66.75 cd
¼ MS	62.26 e
MS + 1 mg/l of BAP	81.43 a
MS + 2 mg/l of BAP	79.67 a
MS + 4 mg/l of BAP	71.33 b
MS + 1 mg/l of IBA + 1 mg /l of IAA	74.50 b
MS + 1 mg/l of IBA + 1 mg/l of IAA + 1 mg/l of NAA	70.33 bc
Range	64.62-81.43
Mean	71.36

Values bearing same letter in the column are not significantly different at p = 0.05 of LSD.

Germinated synthetic seeds were allowed to grow until the roots were well developed. Well grown rooted plantlets from synthetic seeds were taken out from the culture bottles. Medium that adhered with the regenerated plantlets was removed by washing in running water. The plants were transferred to the plastic cups containing a mixture of sterile sand : soil : farmyard manure at a ratio of 1 : 1 : 1 and kept in the plant tissue culture chamber for two weeks. After two weeks, the cups were kept in a room condition at ambient environment for another one week (Fig. 1). Then it was shifted under partial shed-net for two weeks in earthen-pots. Acclimatized seedlings obtained from synthetic seeds were finally planted in the field condition. Based on the results of

germination and emergence of shoots, it clearly signifies that the synthetic seed technology could be used as a true-to-type plant propagation method in mass-multiplication of *Citrus* spp. Such kind of sodium alginate encapsulated plant propagules could be useful in exchange of sterile materials between laboratories due to its small size and relative ease in handling these structures, or in germplasm conservation with proper preservation techniques, conservation through cryo-preservation as per the justifications and observations of the following researchers (Roy and Mandal 2008, Gholami and Alavi 2016, Gholami and Kavani 2018, Amin and Mujeeb 2019) or even in plant propagation and nurseries. Similar kind of results are also in agreement by the work of several researchers such as (Piccioni and Standardi (1995). MS medium fortified with 1.0 mg/l BAP was found to be suitable for *in vitro* mass-multiplication of plantlets of *C. aurantifolia* (Lime) from shoot tips segments of *in vitro* germinated seedlings with a high germination percentage of 81.43%.

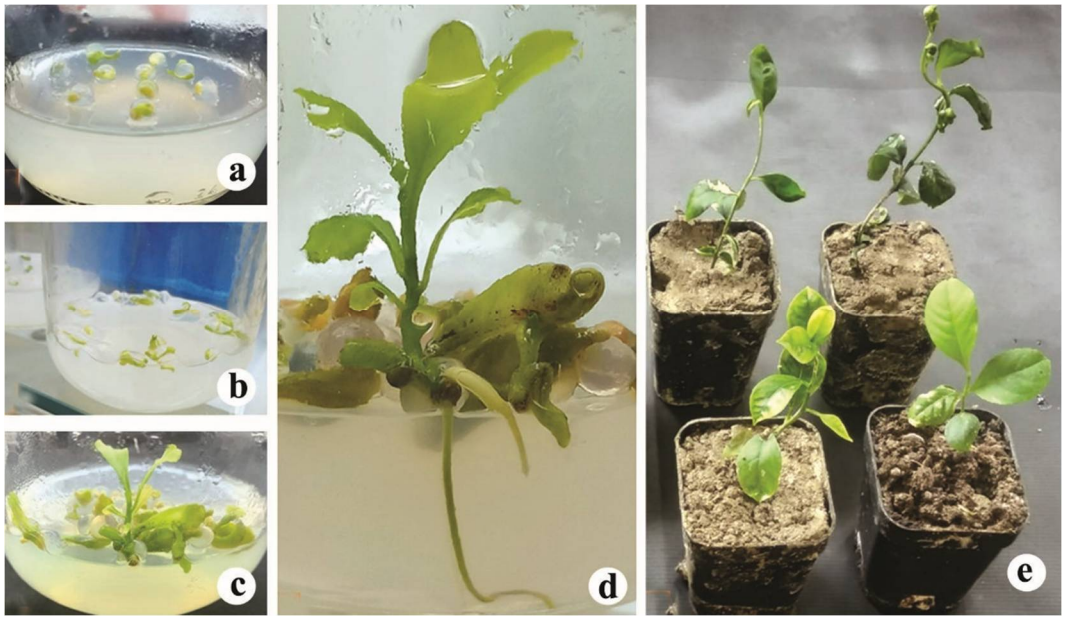

Fig. 1 (a-e). Depiction of Synthetic seeds of *C. aurantifolia* (Lime). (a) Inoculated synthetic seeds on MS supplemented with different combinations and concentrations of plant growth regulators (b) Germinating synthetic seeds (c) Well germinated synthetic seeds (d) Well grown plantlets obtained from synthetic seeds with roots(e) Hardening of plantlets derived from synthetic seeds.

Based on the results of the present study, it could be clearly confirmed that MS medium fortified with 1.0 mg/l BAP was found to be suitable for *in vitro* mass-multiplication of plantlets of *C. aurantifolia* (Lime) from shoot tips segments of *in vitro*

germinated seedlings. Highest germination percentage of synthetic seeds was recorded when beaded shoot tips were cultured on MS medium fortified with 1.0 mg/l BAP (81. 43%). In addition, rate of germination of synthetic seeds was found to be comparatively high on all the treatments consisted of plant growth regulators and at the same time, highest amongst them was also found to be in ½ MS supplemented media. In nutshell, synthetic seed technology could be used as a true-to-type plant propagation method in mass-multiplication of *Citrus* spp.

Shoot tips are suitable for encapsulation studies of artificial seeds as they possess great potential for plant development from pre-existing meristematic tissues. The results encourage the use of encapsulated unipolar explants for synthetic seed technology.

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