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Preparation of Synthetic Seeds of *Citrus jambhiri* Using *in vitro* Regenerated Multiple Plantlets

Priyanka Sharma[1] and Bidhan Roy[1*]

[1]Department of Seed Science and Technology, Faculty of Agriculture, Uttar Banga Krishi Viswavidyalaya, Pundibari, Cooch Behar, 736165, West Bengal, India.

Authors' contributions

This work was carried out in collaboration between both authors. Author BR designed the experiments, performed the statistical analysis and taken part in writing the manuscript. Author PS conducted the experiments, compiled the data, taken part in statistical analysis and writing the manuscript. Both authors read and approved the final manuscript.

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ABSTRACT

Biotechnological tools are useful for true-to-type propagation. Shoot tips encapsulation is potential for plant development from pre-existing meristematic tissue. MS medium fortified with 1 and 2 mg/L of BAP (6-bezylaminopurine) was found to be suitable for *in vitro* mass-multiplication of plantlets (10.18 and 13.05 plantlets/explant, respectively) of *Citrus jambhiri* from nodal segments. Nodal segments were more appropriate than the shoot tips for *in vitro* multiplication of plantlets. Synthetic seeds were prepared using 2.5% sodium alginate dropping into 3.0% $CaCl_2$ solution. Maximum germination was recorded when beaded shoot tips were cultured on MS medium fortified with 1 and 2 mg/L of BAP (96.67 and 100.00%, respectively). However, the germination of synthetic seeds was found to be comparatively high than the earlier findings. The results support the use of encapsulated unipolar explants for synthetic seed preparation. These type of capsules could be useful in exchange of sterile material between laboratories, germplasm conservation and direct plant propagation.

**Corresponding author: E-mail: bcroy10@yahoo.com;*

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1. INTRODUCTION

Synthetic seed is produced by enclosing viable plant materials such as somatic embryos, androgenic embryos [1,2], pro-embryos [1], embryos-like-structure [1], protocorms [3], protocorm-like-bodies [4], axillary buds [5], meristem [6], shoot segments [7] and shoot tips [8]. in alginate with nutrient sources. For encapsulation, plant propagules are mixed with sterilized sodium alginate (3% w/v), which is prepared in tissue culture basal medium supplemented with sucrose. Propagules are then picked up individually and dropped into sterilized aqueous solution of 3% (w/v) calcium salt solution [$CaCl_2$ or $(CaNO_3)_2$] with occasional agitation [2]. Calcium alginate beads are formed within 15-30 minutes. Ion-exchange reaction occurs and sodium ions are replaced by calcium ions forming calcium alginate beads or capsules surrounding the embryo/bud. The size of the bead depends on the inner diameter of the pipette nozzle.

Shoot tips are most suitable for encapsulation for preparation of synthetic seeds as they produce true-to-type planting materials. Commercial preparation of synthetic seeds using shoot tips helps in minimizing the cost of micro-propagation [9]. Shoot tips converts into plantlet directly without callus formation which reduces the risk of somaclonal variation [10] and making the plantlets available round the year [11].

Citrus is one of the important plant genus worldwide which provides many species suitable for different uses. Citrus as fruit (*Citrus sinensis* (L.) Osbeck, *Citrus limetta* Risso, *Citrus paradisi* Macf. etc.), table purpose (*Citrus limon* (L.) Osbeck, *Citrus assamensis* R. M. Dutta & Bhattacharya etc.), pharmaceutical use (*Citrus medica* L., *Citrus jambhiri* etc.). Citrus bear recalcitrant seeds and usually it is being asexually propagated. Plant tissue culture tools are useful for true-to-type propagation and this technology can be used for mass-multiplication. *C. jambhiri* (Rough lemon) is preferred for rootstock for lemons, oranges, mandarins, grape fruits and kinnows, because of its high vigour and well adaptation ability. In this communication effort was taken to standardize protocol for *in vitro* mass-multiplication of *C. jambhiri* and subsequently preparation of synthetic seeds from shoot tips of *in vitro* grown plantlets.

2. MATERIALS AND METHODS

2.1 Plant Materials

Mature fruits of *C. jambhiri* were cut with sharp knife and seeds were extracted manually. Seeds were surface sterilized with 0.1% $HgCl_2$ for 10 minutes followed by 3-5 times washing with sterilized distilled water. Surface sterilized seeds were de-coated under laminar air flow cabinet and inoculated on basal Murashige and Skoog (MS) [12]. For germination and establishment of seedlings, the culture bottles were incubated in culture room at 25±2°C with 16/8 h light and dark phases. After six weeks of inoculation, the *in vitro* grown seedlings were used as explants.

2.2 Medium Preparation

Medium was prepared using the individual chemical compounds listed by Murashige and Skoog [12]. Individual stock solutions were prepared and stored in separate bottles for ready-to-use during the preparation of culture media. MS medium was prepared with 3% sucrose and 0.8% agar. The pH of the medium was adjusted to 5.8. The media were sterilized by autoclaving at 121°C under 104 kPa for 15 minutes. The sterilized nutrient MS medium was used for *in vitro* multiple shoots regeneration from shoot tips and nodal segments.

2.3 Shoots Regeneration

About 30 ml medium composing MS fortified with different combinations and concentrations of plant growth regulators (Table 1) was dispensed into culture bottles for multiple shoots regeneration and plantlet growth. The media were autoclaved at 121°C under 104 kPa for 15 minutes for sterilization. Explants were prepared from *in vitro* grown seedling (Fig. 2A). Shoot portion of the seedlings were cut into small pieces keeping 1 or 2 nodes (Fig. 1A, B & C). Shoot tips of the seedlings were also used for regeneration of multiple plantlets (Fig. 1D, E & F). Two explants were inoculated per culture bottle and a total of 30 explants were inoculated for each treatment. The experiment was repeated two times. The inoculated culture bottles were kept in culture room at 25±2ºC with 16/8 h light and dark phases at optimum photosynthetic flux provided by cool fluorescent lamps for multiple plantlet regeneration.

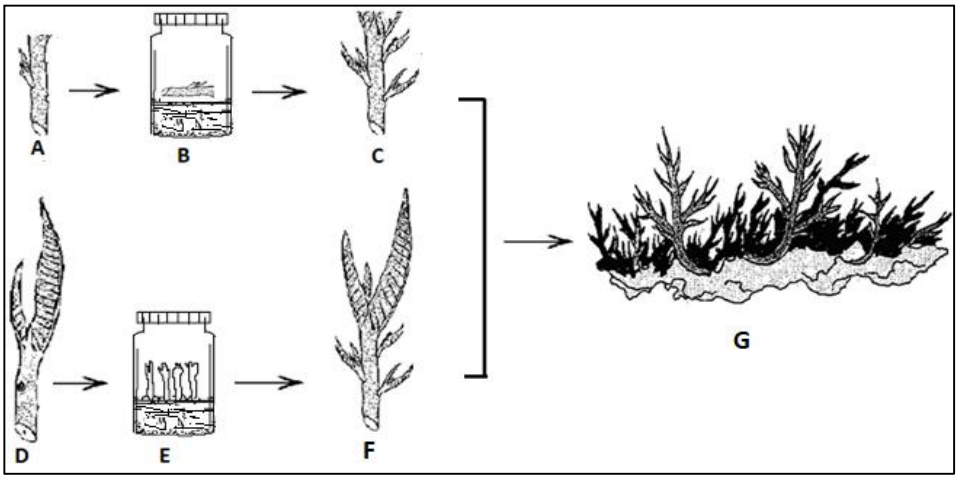

Fig. 1. Multiple shoots regeneration from nodal sections and shoot tips (modified from Roy [13]. A) Nodal section; B) Nodal sections inoculated on MS medium supplemented with different concentrations and combination of plant growth regulators; C) Sprouting of buds on inoculated nodal section; D) Shoot tip; E) Shoot tips inoculated on MS medium supplemented with different concentrations and combination of plant growth regulators; F) Sprouting of buds on inoculated shoot tips; G) Multiple shoots regeneration

2.4 Synthetic Seed Production

Sodium alginate (Sigma Chemical) solution (2.5% w/v) was prepared by mixing with liquid MS fortified with 3% sucrose. In a separate conical flask, 3.0% aqueous solution of calcium chloride (Sigma Chemical) was prepared [2,13]. Both the solutions were autoclaved at 121°C under 104 kPa pressure for 15 minutes for sterilization. Individual nodal segment of shoot tip were isolated from the clusters (Fig. 2B & C) *in vitro* regenerated multiple plantlets.

Grown up plantlets were selected for preparation of propagules. Shoot tips of the plantlets were cut in laminar air flow cabinet. Those shoot tips were used as propagules for preparation of synthetic seeds. The shoot tips were mixed with sodium alginate solution. Individual propagule was picked up with suitable 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 the $CaCl_2$ solution, washed with sterile double distilled water, and surface dried with sterilized blotting paper. Freshly prepared beads were directly cultured on MS medium fortified with different levels of synthetic hormones (Table 2). Seedling emergence and plantlet regeneration were recorded after three weeks. For each treatment 40 beads were employed in two replications. The experiment was conducted entirely under control environment using laminar air flow cabinet.

2.5 Statistical Analysis

Completely randomized design (CRD) experiment was laid out for the laboratory experiment. The data were subjected to standard statistical methods of analysis of variance (ANOVA) using AgRes Statistical Software, (c) 1994 Pascal Intl Software Solutions, Version 3.01 and significant differences were compared by LSD at p=0.05. The analysis of data was used to interpret the results and draw conclusions.

3. RESULTS AND DISCUSSION

Effect of different growth regulators in different concentrations and combinations were used to establish the proficiency multiple shoots regeneration from shoot tips and nodal sections of *C. jambhiri* which provided the propagules for preparation of synthetic seeds.

Analysis of variance exhibited high significant variations among the treatments with different growth regulators in different combinations and concentrations for responsiveness of explants, number of plantlets per explant and shoot length for multiple shooting of *C. jambhiri* (Table 1). These result corroborated with the findings of Sarker et al. [14]; Fanta et al. [15]; Kaur [16].

3.1 Regeneration of Multiple Shoots

Responsiveness of the explants varied from 30.00 to 86.67% with a mean of 52.67% (Table 1). Highest response was obtained when nodal sections were inoculated on MS medium fortified with 2 mg/L of BAP (86.67%) followed by 1 mg/L of BAP (73.33%). Highest response for shoot tips was obtained on MS medium added with 1 and 2 mg/L of BAP (60.00%). Lowest response was recorded when nodal segments were cultured on MS medium fortified with 1 mg/L of IBA + 1 mg/L of IAA + 1 mg/L of NAA (30.00%). The response was higher from the nodal sections as compared to shoot tips. Al-Bahrany [17], Usman et al. [18] and Goswami et al. [19] also reported higher number of shoots from nodal section as compared to shoot tips. Taye et al. [20] reported higher shoots induction (98.44%) from nodal sections than shoot tips cultures (96.9%).

Number of shoots regeneration per explant across the explants differed from 1.57 to 13.05 cm with a mean of 6.35 cm (Table 1). Maximum number of plantlets per explant was found when nodal sections were inoculated on MS medium treated with 2 mg/L of BAP (13.05 shoots/explant) followed by nodal sections on 1 mg/L of BAP and shoot tips on 1 mg/L of BAP (Table 1). Some of the nodal sections cultured on BAP supplemented medium produced 50-60 shoots/explant. Lowest number of shoots /explant was recorded when shoot tips were cultured on MS medium fortified with 1 mg/L of IBA + 1 mg/L of IAA + 1 mg/L of NAA (1.57 shoots/explant). Goswami et al. [19] also used BAP for shoot multiplication of *C. limon* L. cv. KaghziKalan. Fanta et al. [15] recorded higher shoot regeneration efficiency on BAP supplemented MS medium. As per the findings of Upadhyay et al. [21], MS invigorated with 2.0 mg/L BAP + 1.0 mg/L Kinetin + 0.1 mg/L NAA was appropriate for mass-multiplication of shoots and elongation of shoots. In contradictory, Sarker et al. [14] observed highest number of shoots per explant when MS medium was supplemented with 1.5 mg/L of BAP and however, at higher concentration (2 mg/L) reduced the plantlets number per explant. Fanta et al. [15] also reported that increased level of BAP and sucrose above 2.5 mg/L and 30 g/L respectively, the shoot number and shoot length decreased considerably. It was observed that large number of shoots regenerated on BAP added medium. Findings of Roy and Mandal [22] correspondingly confirmed that BAP has enhancing effect on shoot regeneration.

Shoot length across the explants varied from 0.71 to 5.21 cm with a mean of 2.46 cm (Table 1). Maximum length was observed when nodal sections were cultured on MS medium fortified with 4 mg/L of BAP (5.21 cm) followed by nodal sections were cultured on 1 mg/L of IBA + 1 mg /L of IAA (3.56 cm) which is at par with the shoot tips cultured on 1 mg/L of IBA + 1 mg /L of IAA (3.51 cm). Conversely, Goswami et al. [19]

Table 1. Mean of regeneration parameters of *Citrus jambhiri* on different concentrations and combinations of plant growth regulators

Explants	Concentrations and combinations of growth regulators	Shoot regeneration responsiveness	No. of shoots/ explant	Shoot length
Shoot tips	1 mg/L of BAP	60.00 c	10.03c	0.71a
	2 mg/L of BAP	60.00 c	12.50ab	1.33b
	4 mg/L of BAP	46.67de	2.65e	1.61bc
	1 mg/L of IBA + 1 mg /L of IAA	43.33e	2.90de	3.51c
	1 mg/L of IBA + 1 mg/L of IAA + 1 mg/L of NAA	36.67f	1.57e	2.38d
Nodal segments	1 mg/L of BAP	73.33 b	10.18bc	1.90de
	2 mg/L of BAP	86.67a	13.05a	1.41ef
	4 mg/L of BAP	43.33e	4.90d	5.21efg
	1 mg/L of IBA + 1 mg /L of IAA	46.67de	3.58de	3.56efg
	1 mg/L of IBA + 1 mg/L of IAA + 1 mg/L of NAA	30.00g	2.18e	2.95g
	Range	30.00-86.67	1.57-13.05	0.71-5.21
	Mean	52.67	6.35	2.46

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

reported contradictory result that the addition of IBA appeared to have negative effect on shoot elongation. Smallest shoots were obtained when shoot tips were cultured on MS supplemented with 1 mg/L of BAP (0.71 cm). Reduced shoot lengths were also observed when the number of shoots per explant was high. This may be due to the competition among the higher number of shoots for space and nutrition.

3.2 Preparation of Synthetic Seeds

In general germination of synthetic seeds was high and ranged from 76.67 to 100.00% with a mean of 87.50% (Table 2). Highest germination was recorded when the MS medium was fortified with 2 mg/L of BAP (100.00%) followed by 1 mg/L of BAP (96.67%). High germination was also observed on MS medium without any plant growth regulators (93.33%). Roy and Mandal [2] also found the enhancing effect of BAP on germination of synthetic seeds. Some researchers obtained maximum viability when synthetic seeds were cultured on MS medium supplemented with 10 mg/L of BAP and 1 mg/L of NAA [8,23].

Synthetic seed technology presents an innovative and potential tool in plant biotechnology for an efficient and cost-effective large scale propagation *in vitro* conservation, germplasm exchange and distribution. Superiority of encapsulated embryos was reported by Shigeta and Sato [24] in horseradish, Datta and Potrykus [25] in microspore-derived embryos of barley, Suprasanna et al. [26] and Roy and Mandal [2] in rice.

Sprouting of non-beaded shoot tips was comparatively very low than the beaded shoot tips. It varied from 30.00 to 60.00% with a mean of 45.20% (Table 2). Non-beaded shoot tips also showed enhancing effect of BAP on sprouting. Highest sprouting was recorded when the MS medium was added with 2 mg/L of BAP and 1 mg/L of BAP (60.00%).

3.3 Hardening of Rooted Plantlets

Rooted plantlets from synthetic seeds were removed from the culture bottles. The agar along with the medium which adhered onto the plantlets was removed and transferred to plastic cups containing a mixture of sterile sand, soil and 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 at ambient temperature for another week (Fig. 2F) where after it was placed under partial shed net for two weeks in earthen-pots. Acclimatized seedlings obtained from synthetic seeds were planted under realistic condition established.

Table 2. Germination of synthetic seeds on different concentrations and combinations

Explant	Medium and treatment	Sprouting (%)
Synthetic seeds	MS	93.33b
	½ MS	86.67c
	¼ MS	83.33cd
	1 mg/L of BAP	96.67ab
	2 mg/L of BAP	100.00a
	4 mg/L of BAP	83.33cd
	1 mg/L of IBA + 1 mg /L of IAA	80.00de
	1 mg/L of IBA + 1 mg/L of IAA + 1 mg/L of NAA	76.67e
	Range	76.67-100.00
	Mean	87.50
Non-beaded shoot tips	½ MS	30.00j
	MS	40.00hi
	1 mg/L of BAP	60.00f
	2 mg/L of BAP	60.00f
	4 mg/L of BAP	46.67g
	1 mg/L of IBA + 1 mg /L of IAA	43.33gh
	1 mg/L of IBA + 1 mg/L of IAA + 1 mg/L of NAA	36.67j
	Range	30.00-60.00
	Mean	45.24

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

Fig. 2. *In vitro* shoot multiplication and synthetic seed preparation. A)*In vitro* grown seedlings; B) Multiple shoots regeneration from the nodal segments on MS medium supplemented with 2 mg/L BAP; C) Multiple plantlets regeneration from the shoot tips on MS medium supplemented with 2 mg/L BAP; D) Inoculation of synthetic seeds on MS basal medium for germination; E) Geminated synthetic seeds on MS basal medium; F) Acclimatized plantlet from synthetic seeds

To summarise, shoot tips are suitable for encapsulation studies of artificial seeds as they have potential for plant development from pre-existing meristematic tissue. The results encourage the use of encapsulated unipolar explants for synthetic seed technology. This kind of capsules could be useful in exchange of sterile material between laboratories due to small size and relative ease in handling these structures, or in germplasm conservation with preservation techniques [27], conservation through cryopreservation [28] or even in plant propagation and nurseries.

4. CONCLUSION

Shoot tips encapsulation possess great potential for plant biotechnology in mass-multiplication through synthetic seed preparation. MS medium fortified with BAP (6-bezylaminopurine) @ 1 and 2 mg/L was found to be suitable for *in vitro* mass-multiplication of plantlets (10.18 and 13.05 plantlets/explant, respectively) of *Citrus jambhiri* from nodal segments of *in vitro* germinated seedling. Nodal segments were most appropriate as compared to shoot tips for *in vitro* replication of plantlets. Synthetic seeds were prepared using 2.5% sodium alginate (Sigma chemical) dropping into 3.0% $CaCl_2$ solution. Highest germination of synthetic seeds was recorded when beaded shoot tips were cultured on MS medium fortified with BAP @ 1 and 2 mg/L (96.67 and 100.00%). However, the germination of synthetic seeds was found to be comparatively high on all the treatment with plant growth regulators or on MS basal media. This kind of capsules could be useful in exchange of sterile material between laboratories, germplasm conservation and direct plant propagation.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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