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Encapsulation of Nodal Segments for Propagation and Short-term Storage of Giloe (*Tinospora cordifolia* Willd.): A Medicinally Important Plant Species

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims and Objectives: Standardization of the concentration of encapsulation matrix and hardening solution owing to the proportion of germination to crop idyllic artificial seeds of *Tinospora cordifolia* followed by plantlet regeneration.
Study Design: Completely Randomized Design (CRD) was adopted to find out significant differences among different treatment combinations.
Place and Duration of the Study: The present study was conducted at Plant Tissue Culture & Genetic Transformation Laboratory, Department of Plant Molecular Biology & Biotechnology, College of Agriculture, Gwalior, Rajmata Vijayaraje Scindia Krishi Vishwa Vidyalaya, Gwalior, M.P., India during 2017 to 2020.
Methodology: A protocol was established for encapsulation of nodal segments of *Tinospora cordifolia* excised from 18-month-old *in vitro* raised plants for short- term conservation and further

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propagation. Diverse concentrations and combinations of gelling matrix *viz*., sodium alginate and complexing agent *i.e*., Calcium chloride were experienced to optimize combination to get uniform beads.

Results: The best gel composition was accomplished employing 2.5% sodium alginate and 100 mM calcium chloride. The maximum conversion response of encapsulated beads was achieved on Murashiage and Skoog's medium supplemented with 2.0 mgl^{-1} BAP in combination with 0.2mgl^{-1} NAA. Well, developed regenerated plantlets were successfully hardened, acclimatized and established first under net house conditions followed in the field.

Conclusions: In this study, synthetic seed production technique in giloe (*Tinospora cordifolia*) has been established. Present method ensures viable strategy for multiplication, conservation and germplasm exchange through synthetic seed development.

Keywords: Giloe; synthetic seed; micropropagation; encapsulation; germplasm conservation; plantlet formation.

ABBREVIATIONS

$CaCl_2.2H_2O$: Calcium chloride, BA: Benzyl adenine, IBA: Indole-3-butyric acid, NAA: α-naphthalene acetic acid, MS: Murashiage and Skoog medium, PGR: Plant growth regulators.

1. INTRODUCTION

Tinospora cordifolia (Willd.) Miers is commonly known as giloe extensively spreading, glabrous, succulent, climbing shrub belonging to the family Menispermaceae [1,2]. It is distributed throughout the tropical region of Nepal, India, Sri Lanka and China ascending to an altitude of 1,200 m asl. It thrives in the tropical region in forests and other habitat. Stems, roots, leaves and starch acquired from the roots and stems are used for medicinal purpose, especially in Ayurveda [2,3]. In recent Covid pandemic, this was the most sought plant species as immune modulator to boost immunity. Due to the presence of immense medicinal properties [2] the plant has been over exploited by pharmaceutical companies and folk people for traditional remedies and lack of organized cultivation led to the acute scarcity of this plant to meet the present-day demand. Moreover, viability of seeds is very less, poor seed set and germination of seeds are the main problems associated with its traditional propagation. Conservation strategies need to be adopted for continuous supply to meet the ever-increasing demands and sustainable utilization of resources. Plant cell, tissue and organ culture techniques is proving very convenient to solve these problems [4,5]. It could be employed for massive *in vitro* propagation [5,6,7,8,9,10,11,12,13], synthetic seed production [14,15], raising embryogenic cell suspension cultures [16,17,18,19,20,21,22], production of useful secondary metabolites [23,24,25] *in vitro* selection in conjunction with somaclonal variation against different biotic [26,27] and abiotic [28] stresses including quality improvement [29].

An amended synthetic seed production procedure is considered a treasured substitute knowledge of propagation in numerous economically imperative crop plants including giloe and a noteworthy method for mass propagation of elite plant genotypes. The production of plant clones multiplied by tissue culture and disseminated as synthetic seeds could be a convenient alternative to the costly crop plants. The conveyance of artificial seeds also enables problems like enterprise numerous traditions for ascending up *in vitro* cultures and acclimatization to *ex vitro* conditions [30,31]. Synthetic seeds/artificial seeds or syn-seeds are artificially have been developed by various researchers in different crops by encapsulating an array of explants such as somatic embryos [32], shoot buds/tips [33,34,35,36,37], nodal segments [38,39,40,41,42,43], cell aggregates [44], auxiliary buds [44,45] and micro shoots/cutting [14,46,47,48] that can be further propagated as a seed and transformed into a plant under *in vitro* or *in vivo* situations.

This prompted to develop synseed method for propagation, short term conservation as well germplasm exchange using encapsulation [14,35,39,41]. Considerable efforts have been made for *in vitro* plantlet regeneration exploiting different strategies of micropropagation in giloe [5,6,7,12] however to our knowledge till date no, protocol for artificial-syn seeds has been reported in giloe. During present investigation, an

effort has been made to compute the optimum quantity of encapsulation matrix and hardening solution for encapsulation of nodal segments and appropriate concentrations and combinations of plant growth regulators to be added in culture medium for germination of encapsulated nodal segments.

2. MATERIALS AND METHODS

2.1 Explant Source

In vitro cultures of the *Tinospora cordifolia* were initiated from plant material grown at net house at Department of Plant Molecular Biology & Biotechnology, College of Agriculture, RVSKVV, Gwalior ($26^0$13'5.8332" N $78^0$10'58.1916" E) in cultivated soil beds.

2.2 Culture Media

MS [49] basal medium formulated by Murashige & Skoog (1962) was employed as basal medium in present experimentation. Besides MS basal macro and micro salts, vitamins and 7.5 g l^{-1}agar, cytokinin *viz*., BAP in varying concentrations (as sole) as well as in combination with an auxin *i.e*., NAA were added to amend MS media for culture establishment. Readymade basal medium and all other ingredients were procured from Hi-media Laboratories, Mumbai, India.

2.3 Preparation of Culture Media

All initial culture media were made using readymade basal MS medium (HiMidia™) and supplemented with different types of plant growth regulators in various concentrations and combinations, 30.0 gl^{-1} sucrose and the final volume was made to 1000 ml and pH was adjusted to 5.8±0.1 with 1N KOH solution. After adjusting the pH, agar @ 7.5 gl^{-1} was added to the media as a semi-solidifying agent. Warm culture media, still in liquid state was poured into baby food bottles (50-60 ml / bottle) and culture tubes (15-20 ml / tube) tracked by autoclaving at 121 °C under 15 psi pressure for 25 minutes. However, in case of pouring in petridishes, autoclaved warm culture media was dispensed into pre-sterilized 100x17 mm glass petridishes (25-30 ml/dish) under aseptic conditions of Laminar Flow Clean Air Cabinet.

2.4 Culture Establishment

Shoot tip explants collected from net house planted plants were washed systematically under running tap water tracked by adding a few drops of detergent (Tween-20) chased by surface sterilization with 0.1% (w/v) mercuric chloride for 5 min pursued by rinsing 3 to 4 times with sterile double distilled water and inoculated on MS medium supplemented with plant growth regulators. Periodic subculturing at an interval of 28 days was carried out to maintain the proliferative shoot cultures. These cultures were further utilized for experimentation of development of synthetic seeds.

2.5 Preparation of Gel Matrix

The encapsulation was attempted by suspending the nodal segments on modified MS medium (devoid of calcium chloride) supplemented with different concentrations (1, 2.0, 2.5 and 3% m/v) of sodium alginate and 0.5 M sucrose. This mixture was dispensed with a micropipette into different concentrations of calcium chloride solution (50, 75 and 100 mM) and left for 30 min for complex formation and encapsulation. The sodium alginate and calcium chloride solutions were prepared and sterilized by autoclaving at 121 °C and 1.05 kg/cm^2 pressure for 25 min.

2.6 Encapsulation of Nodal Explants

Nodal explants (0.3-0.5 cm) were excised from *in vitro* proliferated multiple shoots aseptically, dried using sterile filter papers and encapsulated by mixing the freshly excised individual node micro cuttings in various concentrations of sodium alginate solution (1-3%w/v). The encapsulated explants were then thrown down in diverse concentrations of complexing solution *namely* calcium chloride in range of 50-100 mM using cut micropipette tips of 0.5 cm diameter. Each beaded drop containing single nodes were left in calcium chloride solution for 30 min for polymerization. The calcium chloride solution was carefully decanted off and the encapsulated beads retrieved were washed 3-4 times with sterile double distilled water, blotted dry on sterilized filter paper and inoculated on MS nutrient medium fortified with different concentrations of BA or BA in combinations with NAA to facilitate shoot organogenesis.

2.7 Culture Conditions

The cultures were maintained in culture tubes and kept in the culture room at a temperature regime of 25±2°C, 65±5% Relative Humidity and at a photoperiodic regime of 16/8 hr light/dark periods provided by cool white fluorescent tubes of intensity 1600-2000 lux. Regenerated multiple

shoots having well developed roots were harvested from the culture tubes, washed under running tap water to remove adhering agar and transferred to plastic cups containing autoclaved sand. Initially, the plastic cups were covered with thin polythene bags with minor holes to maintain humidity and incubated at 25±2°C. After two weeks, plantlets were transferred under net house conditions and maintained for further growth under 30±2°C and 65±5% RH for 15-30 days for adaptation. Acclimatized plants were then transferred to Net House for 30 days for hardening before shifting them to the field.

2.8 Experimental Design and Data Analysis

Experiments were conducted in CRD (completely randomized design) and the data were analyzed as suggested by Snedecor and Cochran [50]. The significant difference between different treatments was experimented by Duncan's multiple range test (DMRT) at $p < 0.05$. The same letters in one treatment represent non-significant differences at $p < 0.05$.

3. RESULTS AND DISCUSSION

Artificial seeds are encapsulated tissue culture resultant micropropagules that hold plant conversion potential and is employed for conservation, germplasm exchange and distribution. The use of nodal segments as explant for syn seed production abolishes the fortuitous of somaclonal variation [51]. Artificial seed production using nodal segments in *Tinospora cordifolia*, a medicinal plant becoming extinct owing to high commercial exploitation is not yet reported.

3.1 Synthetic Seed Formation

The artificial seeds are bounded by a defensive coating, which delivers essential protection throughout storage, handling and transportation. The coating could integrate plant nutrients or plant growth regulators that aid its conversion into plantlet [51]. In the investigation, nodal segments from the *in vitro* cultures were encapsulated using sodium alginate (1, 2 2.5 and 3 5% m/v) in modified MS medium and calcium chloride (50, 75 and 100 200 mM). The beads were formed by exchange of ions, Na^{+} ions of sodium alginate being exchanged with Ca^{++} ions forming calcium alginate. Encapsulation hardiness is strongminded by the optimal ion exchange of Na^{+} and Ca^{++} ions, which may vary with propagules as well as with plant species [51,52,53].

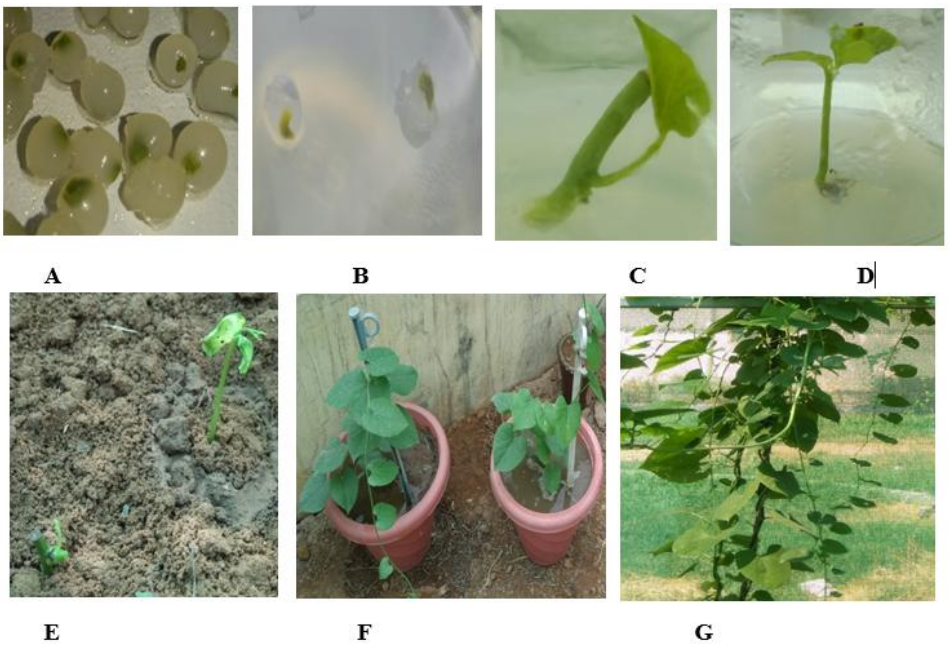

Fig. 1. Encapsulation of *in vitro* raised nodal segments of *Tinospora cordifolia* and germination A. Encapsulated nodal explants in 2.5% (w/v) sodium alginate solution; B-C. Emergence of shooting buds from encapsulated beads on MS2B.2N medium; D. *In vitro* rooting in MSIB medium; E. Acclimatize and hardening of plants in autoclaved sand; F. Plantlet in sterile soil after 4 weeks; and G. Plantlets transferred in net house conditions

Sodium alginate beads with encapsulated nodal segments varied morphologically with reverence to texture, shape and transparency with diverse concentrations of sodium alginate and calcium chloride (Table 1). Amongst the diverse concentrations of sodium alginate (1 - 3.0%) and calcium chloride (50-100 mM) employed in the investigation, a combination of 2.5 % sodium alginate and 100 mM calcium chloride followed by 2.5% sodium alginate in combination with 75mM calcium chloride were evidenced to be the best gel complexation medium and was appropriate for the foundation of secure quality beads which could be effortlessly handled as well (Fig.1A). Alteration of encapsulated nodal segments into plantlets was efficaciously accomplished (Fig.1 B-C). Higher magnitude of sodium alginate (3%) subdued the alteration of encapsulated owing to toughness of beads while lower concentrations occasioned in the foundation of fragile encapsulated beads that were tough to grip. The beads, which were shaped employing 2.5% (w/v) sodium alginate solution was of uniform in size, clear, firm and round shaped and were proved perfect for further regeneration. Similar findings have also been addresses by Sharma *et al*. [14] in *Bacopa monnieri,* by Castillo *et al*. [54] in *Carica papaya* and Prakash et al. [51] in *Plumbago rosea* whereas, successful germination in *Rhodiola kirilowii* at 4% and 5% of sodium alginate has also been possible [44].

3.2 Synthetic Seed Germination/ Regeneration

Higher germination/regeneration frequency has been documented from synthetic seeds on MS medium supplemented with 2.0 mgl^{-1} BA in combination with 0.2 mgl^{-1} NAA intimately followed by culture medium amended with 2.0 mgl^{-1} BA in combination with 0.4 mgl^{-1} NAA and/or medium fortified with 1.0 mgl^{-1} BA in conjunction with 0.2 mgl^{-1} NAA. Thus, it may be concluded that for germination of synthetic seed nutrient medium fortified with cytokinin solely supplemented in the MS medium (BA alone), did not give as much proliferation of shoots as with BA-NAA combination. This indicates the synergistic effect of BA and NAA on shoot proliferation. Parallel reports have also been documented by various other scientists in an array of crop plants [5,8,9,10,13,51,55,56].

Table 1. Effect of different concentrations of sodium alginate and calcium chloride on beads formation and germination from encapsulated explants

S. No.	$CaCl_2.H_2O$ concentration (m/M)	Sodium alginate concentration (%)	*Explants Responded (%)	Texture
1.	50	1	0.00^{g}	Failed to coat nodal explants
2.	75	1	4.80^{f}	Very fragile and soft to handle
3.	100	1	7.20^{e}	Fragile and soft
4.	50	2	8.66^{e}	Too soft and very fragile
5.	75	2	9.34^{e}	Soft and very fragile
6.	100	2	13.38^{d}	Poor bead formation
7.	50	2.5	24.28^{b}	Solid texture
8.	75	2.5	26.14^{b}	Uniform in size & solid
9.	100	2.5	34.58^{a}	Firm quality beads, clear, round and uniform in size
10.	50	3	14.28^{d}	Rigid & solid texture
11.	75	3	19.78^{c}	Solid
12.	100	3	17.27^{c}	Uniform in size, solid and short tail at the surface

a, b values within the column followed by different letters are significantly different, and the same letters are not different at a 5% probability level by Duncan's multiple range test

Table 2. MS media supplemented with different concentrations and combinations of plant growth regulators for germination of synthetic seeds

S. No.	Culture media combination	Plant Growth regulators mgl^{-1}		Days taken to germinate	*Regeneration (%)	*Numbers of sheetlets
		BAP	NAA			
1.	MS	-	-	21 days	$57.56^{h} \pm 1.38$	$1.24^{i} \pm 0.18$
2.	MS0.5B	0.5	-	28 days	$72.26^{f} \pm 1.46$	$1.43^{h} \pm 0.32$
3.	MSB	1.0	-	35 days	$74.04^{ef} \pm 1.56$	$1.63^{h} \pm 0.24$
4.	MS1.5B	1.5	-	35 days	$77.60^{d} \pm 1.58$	$1.93^{g} \pm 0.34$
5.	MS2B	2.0	-	40-45 days	$82.63^{c} \pm 1.62$	$2.16^{g} \pm 0.40$
6.	MS3B	3.0	-	45 days	$80.32^{c} \pm 1.60$	$2.10^{g} \pm 0.38$
7.	MSB0.2N	1.0	0.2	48-50 days	$86.50^{b} \pm 1.88$	$2.96^{cd} \pm 0.44$
8.	MSB0.4N	1.0	0.4	48-50 days	$88.50^{b} \pm 1.92$	$3.12^{c} \pm 0.49$
9.	MS2B0.2N	2.0	0.2	50 days	$94.06^{a} \pm 1.96$	$5.06^{a} \pm 0.52$
10.	MS2B0.4N	2.0	0.4	50 days	$87.90^{b} \pm 1.86$	$4.50^{b} \pm 0.50$
11.	MS3B0.2N	3.0	0.2	50 days	$68.78^{g} \pm 1.44$	$2.32^{f} \pm 0.28$
12.	MS3B0.4N	3.0	0.4	50 days	$56.42^{h} \pm 1.34$	$2.64^{e} \pm 0.22$

** Mean ± standard deviation. of three repeated experiments with ten replicates each. a, b Values within the column followed by different letters are significantly different, and the same letters are not different at a 5% probability level by Duncan's multiple range test*

Variable percentage of germination shown after 21 days of inoculation (Table 2). In all cases synthetic seed germination occurred within 10-15 days of incubation primarily by bursting open sodium alginate matrix resulting in the formation of shootlets after 6-8 weeks of culture (Table 2). This was pursued by the steady appearance of multiple shoots. These shoots were subsequently rooted (Fig. 1D) after transferring in to MS rooting medium supplemented with 1.0 mgl^{-1}IBA. Some of the artificial seeds failed to germinate and encapsulated nodes turned brown within the matrix. Similar reports have also been addressed by Sharma *et al*. [14].

Higher germination proportion in instance of synthetic seeds (deprived of storage) could be owing to the matrix, that not only enabled steady nutrient supply but also dwindling subtle tissue from any mechanical damage while handling and from desiccation. Plantlets regenerated from the encapsulated nodal segments were effectively transferred to pot (Fig. 1E) and hardened off in polyhouse (Fig. 1F) followed by net house (Fig. 1G) before transplanting in the field. The accustomed plants displayed usual growth and development.

The beads can potentially serve as a reservoir of nutrients that may aid survival and speed up growth as earlier reported [14, 57, 58]. The alginate matrix containing nutrients reduced the viscosity and ability of the gel to form solid beads. Propagation through encapsulation of vegetative propagules have been reported in number of medicinally important plant species like *Bacopa monnieri* [14], *Withania somnifera* [34]. *Plumbago rosea* [51] and *Tylophora indica* [59].

4. CONCLUSIONS

This is first report of synthetic seed production in this important medicinal plant of high value. In this study synthetic seed production technique in giloe (*Tinospora cordifolia*) has been established. The developed method provides good procedure to produce plantlets of 'elite' clone and conservation for short-term storage in this important medicinal plant in demand. Hence, this technology can be considered as a promising strategy for the exchange and distribution of *giloe* between laboratories.

NOTE

The study highlights the efficacy of "Ayurveda" which is an ancient tradition, used in some parts of India. This ancient concept should be carefully evaluated in the light of modern medical science and can be utilized partially if found suitable.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and

producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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