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Encapsulation of shoot tips and nodal segments of *Cleistanthus collinus* for short term storage and germplasm exchange

Elangomathavan. R*, Nancy Beaulah. S, Hariharan. S & P. Kalaivanan

Department of Biotechnology, PRIST University, Thanjavur, Tamilnadu, India.
*Corresponding author: **Dr. R. Elangomathavan,** Assistant Professor, Department of Biotechnology, PRIST University, Thanjavur, Tamilnadu, INDIA
E-mail: *relangomathavan@gmail.com* Telephone No: +919884289207

Abstract

The encapsulation of nodal segments and shoot tips from *in vitro* raised *Cleistanthus collinus* (Roxb.) Benth and Hook (Euphorbiaceae) was performed using alginate (Gelling matrix) and calcium chloride (Complexing agent) for short term storage and germplasm preservation. The shoot re-growth frequency of encapsulated nodal segments and shoot tips after storage at 4ºC for different time periods (up to 8 weeks) were also assessed. Different combination and concentration of sodium alginate and calcium chloride was tested for the proper bead formation. The perfect synthetic seeds were obtained through a combination of 4% sodium alginate and 100 mM calcium chloride in half strength Murashige and Skoog medium with 3% sucrose. The maximum shoot re-growth response of encapsulated nodes (95%) and shoot tips (90%) were obtained in MS medium supplemented with 1.0 mg/l BAP after 6 weeks of culture. Encapsulated explants stored at 4ºC also produce maximum shoot re-growth frequency of 5.3 shoots / bead for nodal segments and 3.3 shoots / bead for shoot tips in subsequent weeks of transfer. There was higher response in nodal segments compared to shoot tip explants. The re-grown shoots from the encapsulated explants rooted best on half strength MS medium with 0.5 mg/l IAA. The regenerated plants were successfully hardened and acclimatized.

Keywords: Synthetic seeds, *Cleistanthus collinus*, shoot tips, node, encapsulation, Shoot re-growth.

Introduction

Cleistanthus collinus (Roxb.) Benth and Hook (Euphorbiaceae), commonly known as 'oduvanthalai' is a poisonous plant available on the dry hills in various parts of India (Subramanian and Krishnamurthy, 1975; Modi and Subrahmanyan, 1999). Glycosides such as Cleistanthin A and B are the principle toxins isolated from this poisonous plant, as all parts of this plant is reported as highly toxic (Annapoorani et al., 1984). These cleistanthins have been reported to have anticancer property *in vitro* and *in vivo* and may have the probable to become lead compounds to treat cancer (Prabhakaran et al., 1996; Pradheepkumar et al., 2000; Pradheepkumar et al., 1999). The leaves, roots and fruits are severe gastro intestinal irritants. It is also used as cattle and fish poison. The leaves are considered as an abortifacient (Modi and Coius, 1940).

There is a significant amount of arylnaphthalide lignans such as diphyllin, collinusin and many other related compounds present in the plant *C. collinus* (Pinho and Kijjoa, 2007; Satyanarayana et al., 1984). Cleistanthin A and B produce DNA strand breaks in *in vitro* studies by reducing the viability of the cells. These also induce apoptosis in cultured cells. The cells are injured by the induction of oxygen radical and it is

considered as a major factor for the toxicity of *C. collinus*. There is also a reduction in glutathione levels in various tissues of rats and rabbits when dose dependent level of *C. collinus* leaf extract is given (Sarathchandra et al., 1997). Mortality rate is also reduced in rats by the help of cysteine, when administered with *C. collinus* leaf extract (Annapoorani et al., 1986).

C. collinus plant has normal resistance to the predators which are exposed to browsing which leads to the afforestation of hills .The wood is stronger and is not attacked by ants. The tree sprouts from the cut stumps (Quraishi et al., 1996). This plant has insecticidal activity and used as insecticides in rice fields against *Tribolium castaneum*, red flour beetle (Harwansh et al., 2010 and Gupta et al., 2010), and also used as antifeedant against the larvae of *spodoptera litura* (Selvamuthukumaran et al., 2008 (a and b); Selvamuthukumaran and Arivudainambi, 2010). The leaf extracts of *C. collinus* also has larvicidal activity against vector mosquitoes. Hence, it has higher effectiveness in reducing mosquito threat by controlling young stages of vector mosquitoes (Arivoli and Samuel, 2011).

Overexploitation, habitat disturbance from the wild and reproductive failure forced this plant species under threatened and it has been declared as critically vulnerable. Hence there is an urgent need to develop an alternate method to save this plant. The *in vitro* techniques offer an efficient method for multiplication and conservation of threatened plants. Synthetic seed technology helps in carrying the propagules without the loss of viability to distant places for the shoot regeneration in micropropagation techniques (Hung and Trueman, 2012; Lata et al., 2012; Reddy et al., 2012). The encapsulation technique provides the well-organized short and long term storage. There are many studies on encapsulation of somatic embryos. Alternatively, efforts have also been made to encapsulate non- embryogenic *in vitro* derived meristems, nodal segments and axillary buds. The non- embryogenic vegetative propagules of huge valuable crops can also been used for the mass propagation at low rate, when there is lack of somatic embryos for encapsulation. Additionally this could be useful for the conservation of the elite germplasm or plants which are in threat of extinction (Rai et al., 2009).

It is the most important application of micropropagation which helps in the germplasm exchange among laboratories and also provides unproblematic handling (Chand and Singh, 2004). It also helps in easy release of the *in vitro* regenerated plants into green house (Rai et al., 2009). Hence, proper storage conditions and specific storage period is necessary to preserve these synthetic seeds and its viability (Sharma et al., 2013). The different approach to preserve the germplasm is the production of synthetic seeds by sodium alginate encapsulation with *in vitro* derived shoot tips or nodal segments in calcium alginate beads (Rai et al., 2009). This is most simpler and efficient method for direct plantlet development.

There are many varieties of woody plants that has been re-grown from both shoot tips and nodes encapsulated by sodium alginate such as *Hibiscus moscheutos, Olea europaea and Dalbergia sissoo* (West and Preece, 2009; Ikhlaq et al., 2010; Chand and Singh, 2004). The encapsulated explants with alginate coat helps to protect the tissues from physical and environmental harm. During short term storage it also reduces dehydration, and holds the explants by providing mechanical pressure inside the gel matrix. The synthetic seed is able to convert into complete plantlets with shoots and roots (Ara et al., 2000; Ikhlaq et al., 2010). However, there is no protocol so far for the production of synthetic seeds or artificial seeds in *C. collinus* plant. Hence, the present study focus on the proper encapsulation techniques for the short-term storage and distribution of *C. collinus* using shoot tips and nodal segments as explants. The study also determines the appropriate culture medium for the shoot re-growth from short term (4ºC) stored synthetic seeds and *in vitro* conversion under aseptic conditions.

Materials and Methods

Plant material

C. collinus mother plant was collected from Ariyalur (11.1401° N, 79.0786° E), Tamilnadu, India and maintained in the institute herbal garden. Individual nodal segments were excised from the healthy growing mother plant and washed for ten minutes under running tap water followed by teepol solution for about five minutes and afterwards rinsed three or four times with distilled water. The nodal segments were then treated with 70% ethanol for 30 seconds and again rinsed with distilled water followed by surface sterilization in 1% sodium hypochlorite for two minutes and finally rinsed thoroughly with double distilled water. Further sterilization process was done under laminar air flow chamber. The explants were treated with 0.1% mercuric chloride ($HgCl_2$) for 5 min

and rinsed well with sterile distilled water to remove all traces of chemicals. The surface sterilized explants were inoculated in Murashige and Skoog medium supplemented with 0.5 mg/l BAP and 30 g/l sucrose. The media was adjusted to pH 5.8 prior to addition of 0.8%agar and autoclaved at 121°C for 20 min. The cultures were maintained at 25 ± 1°C with a 16 h light / 8h dark photoperiod cycle with 45 $\mu mol\, m^{-2}\, S^{-1}$ irradiation (Philips, India). Shoot tips and nodal segments (4–6 mm in length) were then aseptically excised from the *in vitro* grown healthy plants and used for alginate encapsulation.

Encapsulation matrix and complexing agent

The gelling agent sodium alginate (Qualigens, India) was used to encapsulate the explants (shoot tips and nodal segments) at various concentrations (1 - 5% w/v). Calcium chloride at a range of 25 - 200 mM was used as a complexing agent. Both encapsulation matrix and complexing agent was prepared in half strength liquid MS medium with 3% sucrose.

Encapsulation

Nodal segments and shoot tips were dissected from the healthy growing *in vitro* plant and suspended in low viscosity sodium alginate prepared by half strength MS medium with Sucrose 3% (w/v) and pH was adjusted to 5.8 using 1.0 N NaOH or 1.0 N HCl. Plant growth regulator (0.5 mg/l BAP) was also supplemented along with the medium. Aliquots of the alginate solution, each has one shoot tip or nodal segment was dropped into 100 mM $CaCl_2$ using a sterile Pasteur pipette. All these process were done under aseptic conditions. All media and solutions were autoclaved and cooled before starting the procedure. For proper polymerization, the droplets containing shoot tips or nodal explants were maintained in $CaCl_2$ solution for almost 30 minutes for proper polymerization. Hence, transparent capsules were formed with appropriate bead formation. Then calcium chloride solution was decanted and the capsules were washed thoroughly with sterile double distilled water or half strength MS medium and subsequently treated with pinch amount of streptomycin to avoid bacterial contaminations. These capsules were transferred to petri dish with filter papers to remove excess of water droplets. Then well formed beads were transferred to different strength MS medium (Full, half and quarter strength MS medium) without plant growth regulators to find out the maximum response of re-growth. The optimum strength of MS medium was then supplemented with plant growth regulators (BAP, KIN and TDZ at the range of 0.5 - 5.0 mg/l) to evaluate the optimum hormone concentration for the maximum shoot re-growth and multiple shootlet production of both explants (shoot tips and nodal explants). The percentage with re-growth of shoots, maximum shoot length and number of multiple shoot production from each beads were determined.

Low temperature storage

After encapsulation, beads were transferred to Petri plates moistened with sterile double distilled water and tightly covered with parafilm to avoid dehydration and microbial contamination. The encapsulated beads and non encapsulated beads were then stored in a refrigerator at 4°C and seven different low temperature exposure period (up to 8 weeks) were tested to determine the percentage of shoot re-growth after short term storage. After every storage time, each beads (encapsulated and non encapsulated) were taken from the petri plates and cultured on agar solidified Full strength MS medium (Murashige and Skoog, 1962) containing optimal concentration of plant growth regulators and kept under light in culture room for 4 weeks and the percentage of shoot re-growth for both encapsulated and non encapsulated beads were determined after 6 weeks of culture. The recovered shoots were transferred to root induction medium with IAA, IBA and NAA (0.5 – 3.0 mg/l).

Hardening and Acclimatization

The healthy shoots with well developed roots were removed from the rooting medium and washed with sterile water to remove the adhering agar derbies from the root and transferred into poly cups containing garden soil mixed with sand and vermi-compost (1:1 ratio). The cups were covered with polythene cover and watered regularly to sustain humidity and maintained at 25 ± 1°C with 16-h photoperiod for one month in the culture room. The established plantlets were transferred to earthen pots containing garden soil mixed with sand (2:1 ratio). The well grown plants were transferred to field.

Statistical analysis

The multiple shoot formation from alginate encapsulated shoot tips and nodal segments were evaluated. All the treatments were repeated three times with ten replicates for each experiment. Statistical analysis was performed utilizing SPSS 13 (SPSS Inc., Chicago, IL, USA) and Excel 2007 (Microsoft, Redmond, WA, USA) software. Data were subjected

to ANOVA analysis to consider for significant and Least Significant Differences test was utilized to compare means at $p<0.05$ level.

Results and Discussion

In the present study, Shoot tips and nodal segments excised from the *in vitro* grown shoots were used as explants for producing synthetic seeds in *C. collinus.*

Sodium Alginate encapsulation

The nodal segments and shoot tips of healthy growing *in vitro* plants of *C. collinus* were used for the production of synthetic seeds and different concentration of cytokinin was used to investigate the shoot re-growth, multiple shootlet formation. Encapsulated shoot tips were compared with nodal segments encapsulated for their shoot re-growth and conversion. There is highest percentage of response in nodal segments compared to shoot tips. Multiple shootlet production was also higher in nodal segments. Of the various concentrations, 4% sodium alginate and 100 mM calcium chloride was found to be optimum for the proper bead formation after submerging aliquots of sodium alginate with shoot tips and nodal segments in calcium chloride solution for 30 minutes. These produces uniform hardening of the beads and the nodal segments and shoot tips were covered with a coat that was sufficient for handling and also it allows maximum re-growth of the plantlets. Similar results have been produced during encapsulation of mulberry and tea, where 4% of sodium alginate was found optimum to form clear beads with soft texture for germination (Pattnaik and Chand, 2000; Mondal et al., 2002).

The most advantage of synthetic seed technology is their ability to maintain its viability for long periods. The shoot re-growth and conversion ability is also retained after encapsulation. In the present study the best combination for hydrogel formation is 4% sodium alginate and 100 mM calcium chloride, which produce hard and clear beads. But the beads formed from lower concentrations of sodium alginate (1, 2 and 3 %) and Calcium chloride (25, 50 and 75 mM) were breakable, prolonged the polymerization and not easy to handle during transfer to re-growth media. The decline in gelling capacity due to lower concentration of sodium alginate has previously reported by Larkin et al., (1998). On the other hand, higher concentrations resulted in too hard and compact which leads to delay in germination or sprouting. This result is similar with the findings of Sundararaj et al., (2010) where 4% sodium alginate is the critical concentration for the appropriate bead formation in *Zingiber officinale*. In most studies 3% sodium alginate is also suitable for proper capsule formation (Ozudogru et al., 2011; Mishra et al., 2011).

Shoot re-growth

The shoot tips and nodal segments were encapsulated using 4% sodium alginate and 100 mM calcium chloride. The beads with shoot tips and nodal segments were cultured on MS medium and their re-growth efficiency was evaluated. The percentage of shoot re-growth was higher in encapsulated nodal segments compared to shoot tips. The beads with shoot tips and nodal segments were cultured on different strength MS medium before storage (half, quarter and Full strength MS medium) and their re-growth efficiency was evaluated. Of the three strength MS medium, maximum response was noticed in Full strength medium with 1.0 mg/l BAP for encapsulated explants (Table 1). The regeneration frequency was increased when BAP is added in the regeneration medium and shoots emerged out within 2 weeks of culture (Fig. 1 & 2D). The maximum number of shoots (5.3 shoots / bead) was obtained in encapsulated nodal explants followed by shoot tips (3.3 shoots / bead) before low temperature storage, Table 2).

The encapsulated explants (i.e. synthetic seeds) were formed without delaying the shoot re-growth by 4% sodium alginate and 100 mM $CaCl_2$, that produce consistent capsules which was very clear and transparent (Rai et al., 2008; Singh et al., 2010). These beads can be stored for 8 weeks at 4ºC and there is high chance of shoot re-growth of these stored beads when cultured on full strength MS medium. The preservation of the encapsulated shoot tips and nodal segment was ineffective in storage medium without nutrients and sucrose. The gel matrix and impact of capsule formation is one of the most important properties for efficient plant propagation of many plants. This was influenced by sodium alginate and calcium chloride which forms ion exchange between Na^+ and Ca^{2+} which helps in the hardness of the capsule. This was different with various propagules and reported in many plant species (Singh et al., 2009; Rai et al., 2009).

Table 1: Response of different medium strength on plant re-growth from encapsulated nodal segments and shoot tips of *C. collinus*.

Medium strength without Plant growth regulators	Frequency of plantlet conversion (%)	
	Encapsulated Nodal segments	Encapsulated Shoot tips
Full strength MS medium	75.0	70.0
½ MS medium	65.0	60.0
¼ MS medium	40.0	30.0

Table 2: Response of different plant growth regulators on plant re-growth from encapsulated nodal segments and shoot tips before low temperature storage at 4ºC.

Concentration of Plant Growth Regulators (mg/l)		Encapsulated Nodal segments			Encapsulated Shoot tips		
		Shooting response (%)	Mean No. of Shootlets / explant ± S.D*	Mean height of Shootlet ± S.D	Shooting response (%)	Mean No. of Shootlets / explant ± S.D*	Mean height of Shootlet ± SD
Control	0.0	75	1.2±0.6^{bc}	1.1±0.4	70	1.2±0.4^{bc}	1.1±0.5
BAP	0.5	80	1.7±0.6^{b}	1.4±0.5	75	1.6±0.7^{b}	1.4±0.8
	1.0	95	5.3±0.6^{a}	3.5±0.8	90	3.3±0.6^{a}	3.2±0.7
	2.0	75	2.0±0.7^{b}	1.5±0.4	75	1.8±0.7^{b}	1.2±0.8
	3.0	70	1.6±0.7^{b}	1.3±0.3	65	1.4±0.5^{b}	1.0±0.5
	4.0	65	1.0±0.0^{c}	1.0±0.0	60	1.0±0.0^{c}	1.0±0.0
Kinetin	0.5	70	1.4±0.5^{b}	1.4±0.5	60	1.4±0.5^{b}	1.2±0.6
	1.0	75	1.9±0.8^{b}	1.9±0.8	70	1.9±0.7^{b}	1.4±0.8
	2.0	65	1.5±0.5^{b}	1.4±0.5	60	1.5±0.5^{b}	1.2±0.5
	3.0	55	1.4±0.4^{b}	1.3±0.3	50	1.4±0.4^{b}	1.1±0.5
	4.0	50	1.0±0.0^{c}	1.0±0.0	45	1.0±0.0^{c}	1.0±0.0
TDZ	0.5	45	1.4±0.6^{b}	1.1±0.4	40	1.5±0.5^{b}	1.2±0.6
	1.0	60	1.3±0.6^{b}	1.2±0.4	55	1.4±0.5^{b}	1.2±0.6
	2.0	55	1.2±0.6^{bc}	1.1±0.6	45	1.3±0.6^{b}	1.2±0.7
	3.0	45	1.0±0.5^{c}	1.0±0.5	40	1.2±0.6^{bc}	0.9±0.4
	4.0	30	1.0±0.0^{c}	1.0±0.0	25	1.0±0.0^{c}	1.0±0.0

*Values are mean ± SD (n=10) dissimilar letters indicated significant difference between means within the treatment at $p<0.05$ level based on LSD mean separation

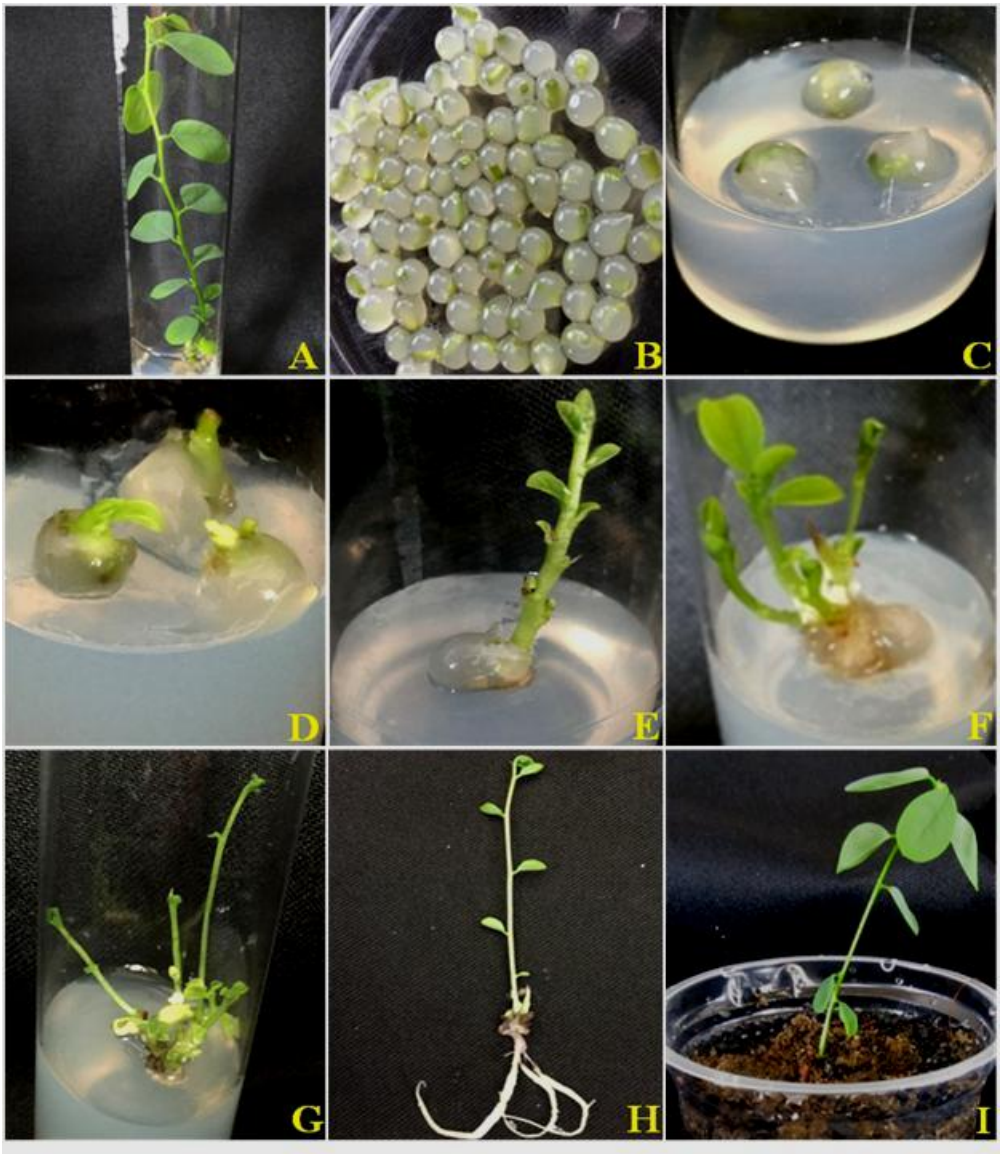

Fig. 1: Plantlet regeneration from encapsulated nodal segments of *C. collinus*. (A) *In vitro* raised plant used for encapsulation; (B) Encapsulated nodal segments in calcium alginate beads; (C) Culture of stored beads (4^0C) in MS medium supplemented with 1.0 mg/l BAP; (D&E) Emergence of shoot from encapsulated nodal segments; (F&G) Multiple shoot re-growth from encapsulated beads; (H) Plantlet with well-developed roots in 0.5 mg/l IAA; (I) Acclimatized plantlets from encapsulated nodal segments.

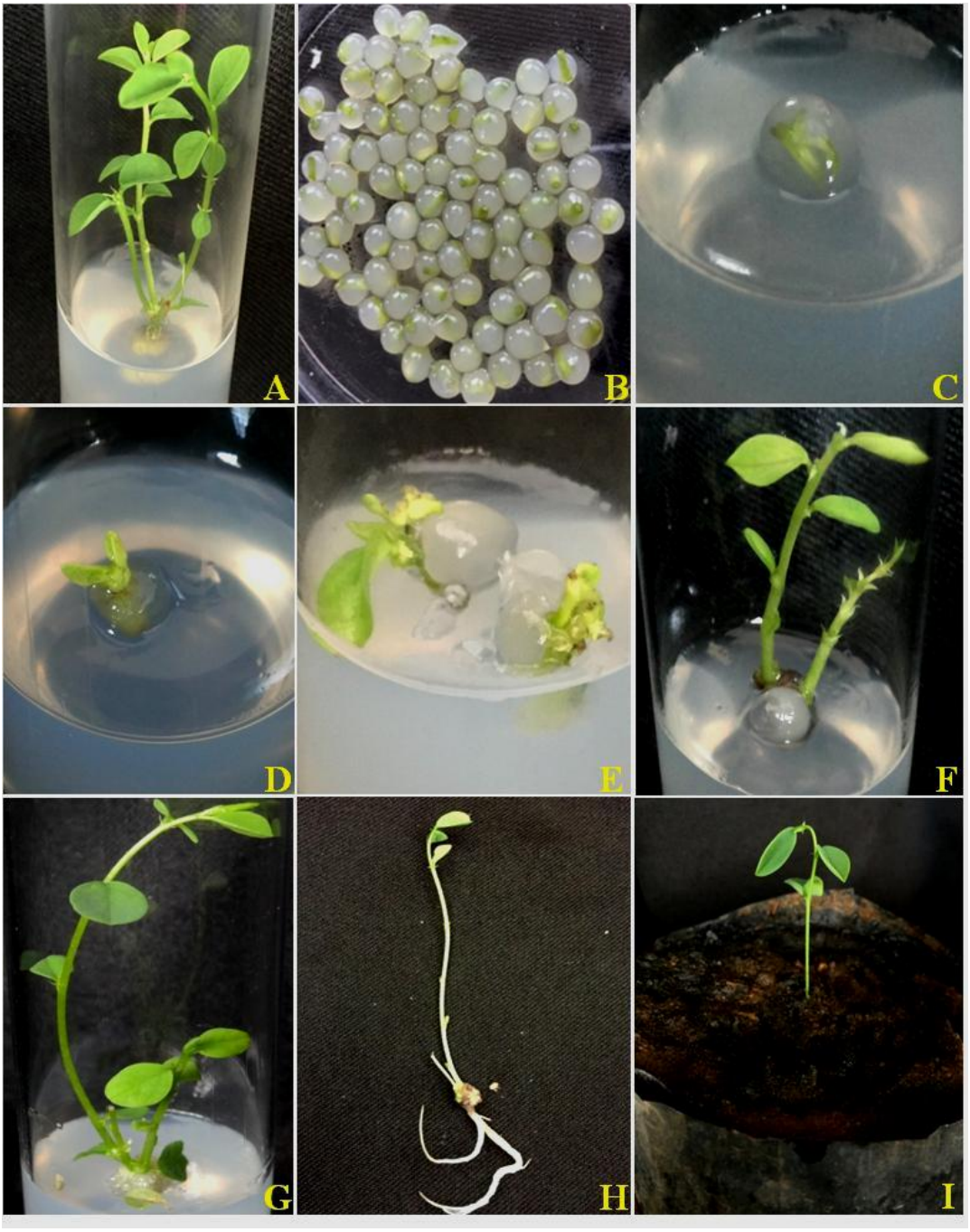

Fig. 2: Plantlet regeneration from encapsulated Shoot tips of *C. collinus*. (A) *In vitro* raised plant used for encapsulation; (B) Encapsulated shoot tips in calcium alginate beads; (C) Culture of stored beads (4^0C) in MS medium supplemented with 1.0 mg/l BAP; (D&E) Emergence of shoot from encapsulated shoot tips; (F&G) Multiple shoot re-growth from encapsulated beads; (H) Plantlet with well-developed roots in 0.5 mg/l IAA; (I) Acclimatized plantlets from encapsulated shoot tips.

Low temperature storage and shoot re-growth

The encapsulated shoot tips and nodal segments were stored at low temperature 4ºC for about 8 weeks to evaluate the re-growth frequency at every week interval. Non encapsulated explants were also stored at the same time period at same temperature.. After each storage periods, the beads were cultured on full strength MS medium which was optimized already. The maximum re-growth frequency 95% for nodal segments and 90% for shoot tips were obtained on MS medium supplemented with BAP 1.0 mg/l. The encapsulated and non encapsulated explants without cold storage (control) produced maximum regeneration frequency. After 6 weeks of culture, encapsulated beads produced 5.3 shoots as average with 95% regeneration response for nodal segments and 3.3 mean shoots with 90% response for shoot tip explants in optimum regeneration medium (1.0 mg/l BAP). The re-growth percentage was decreased with increased storage period. After 6 weeks of storage there was a gradual decrease with 2.4 and 1.6 mean number of shoots per bead from nodal and shoot tip explants respectively. On the other hand, non encapsulated explants also showed a sharp decline in the regeneration frequency (10%) for both encapsulated explants, produced 0.5 and 0.3 number of shoots / bead from both type explants.

Table 3: Effect of storage at 4ºC for different time periods on re-growth of encapsulated and non-encapsulated nodal segments and Shoot tips of *C. collinus* on MS medium containing BAP (1.0 mg/l).

Type of explant	Storage period (weeks)	Nodal segments			Shoot tips		
		Shooting response (%)	Mean No. of Shootlets/ explant ± S.D*	Mean height of Shootlet ± S.D	Shooting response (%)	Mean No. of Shootlets/ explant ± S.D*	Mean height of Shootlet ± S.D
Encapsulated	0.0	95	5.3 ± 0.6^{a}	3.5 ± 0.8	90	3.3 ± 0.6^{b}	3.2 ± 0.7
	1.0	90	5.2 ± 0.7^{a}	3.2 ± 0.7	85	3.2 ± 0.7^{b}	3.0 ± 0.8
	2.0	85	4.4 ± 0.7^{b}	2.4 ± 0.7	80	2.2 ± 0.7^{c}	2.1 ± 0.7
	4.0	80	3.3 ± 0.6^{c}	2.2 ± 0.7	75	1.9 ± 0.7^{c}	1.6 ± 0.5
	6.0	70	2.4 ± 0.5^{d}	1.7 ± 0.8	70	1.6 ± 0.7^{c}	1.4 ± 0.6
	8.0	65	1.0 ± 0.0^{e}	1.0 ± 0.0	55	1.0 ± 0.0^{d}	1.0 ± 0.0
Non-encapsulated	0.0	98	6.2 ± 0.6^{a}	3.5 ± 0.8	95	4.4 ± 0.7^{a}	4.3 ± 0.6
	1.0	50	3.2 ± 0.7^{c}	2.2 ± 0.7	55	3.4 ± 0.5^{b}	3.0 ± 0.8
	2.0	40	1.4 ± 0.8^{d}	1.2 ± 0.7	45	1.4 ± 0.8^{c}	1.4 ± 0.8
	4.0	25	1.0 ± 0.0^{e}	1.0 ± 0.0	30	1.0 ± 0.0^{d}	1.0 ± 0.0
	6.0	15	0.8 ± 0.5^{e}	0.7 ± 0.3	25	0.4 ± 0.2^{d}	0.3 ± 0.2
	8.0	10	0.5 ± 0.2^{e}	0.6 ± 0.3	10	0.3 ± 0.2^{d}	0.2 ± 0.1

*Values are mean ± SD (n=10) dissimilar letters indicated significant difference between means within the treatment at $p<0.05$ level based on LSD mean separation

After one week of storage most encapsulated nodal segments and shoot tips of *C. collinus* produced emergent shoots. The percentage response, mean number of shoots per explants and shootlet length was higher in nodal segments compared to shoot tips. Similar results were also obtained in *Ficus carica*, where the higher response was noticed in nodal segments than shoot tips. This is due to the difference between the physiological stages of the buds along the stem and also there is strong apical dominance in the shoot tips which stopped the bud sprouting and multiplication of shoots (Shiwali Sharma et al., 2014; Lakshmanan et al., 1997).

In fact shoot re-growth frequency was reduced after 8 weeks of storage as most of the beads become brownish and failed to germinate. There is also drastic loss of sprouting. The reason behind this is the inhibition of the tissue respiration by the alginate medium or the loss of moisture content in the beads (Danso and Ford-Llyod, 2003; Faisal and Anis, 2007). Low temperature storage at 4ºC was optimum for shoot re-growth for *C. collinus*. This was also reported in various plants *Sterculia urens* Roxb and *Eclipta alba* L. (Subhashini Devi et al., 2014; Ray et al., 2010), where 4ºC is suitable for plant re-growth. Our results are in confirmation with the previous results of Ahmed et al. (2015), the conversion of nodal

encapsulated explants were efficient in *Vitex trifolia* at storage temperature of 4ºC. Similar results of storage were also experienced by Mangal et al., (2015) in *Withania coagulans* and Kavyashree et al. (2006), in mulberry plants. Short term storage is also reported in *Decalepis hamiltonii* by Sharma and Shahzad, (2012).

The storage of encapsulated beads in cold temperature (4ºC) is most effective than room temperature in *C. collinus* plant. This was also reported in few plants such as *Coelogyne breviscapa* and *Olea europaea* (Mohanraj et al., 2009; Micheli et al., 2007). The re-growth efficiency was higher in encapsulated explants than non-encapsulated explants due to the addition of MS salts in the gelling matrix, which serves as an artificial nutrient to the explants. Hence the storage of encapsulated explants for a limitable period of time helps in the conservation of germplasm and used for the regeneration of complete plantlets.

There is a sudden fall in the regeneration frequency after 8 weeks of cold storage. Only 65% and 55% beads of both explants showed regeneration with single shoot per bead after 6 weeks of culture (Table 3). On the other hand, non-encapsulated shoot tips and nodal segments showed a sudden reduction in the regeneration frequency.

Rooting and Acclimatization

The multiple shoots re-grown from the encapsulated shoot tips and nodal segments were excised and inoculated in rooting medium (1/2 strength MS medium with IAA, IBA and NAA at the range of 0.5-3.0 mg/l). Among the different concentration of plant growth regulators IAA at 0.5 mg/l was found to be the best hormone concentration for rooting which produces thick white root after 2 weeks of culture. This result was in confirmation with the findings of Vijayalakshmi and Giri, (2003); Cheepala et al. (2004), where IAA is found to be the best hormone for the root induction. There is no root induction in basal medium without growth hormones. The optimum concentration for root development was 0.5 mg/l IAA where, the average number of rootlets (3.3) was produced for nodal segments followed by 2.1 for shoot tip explants (Table 4 and Fig. 1 & 2H). Basal callus was developed from the cut ends when higher concentration of auxin is used. The re-grown plant with well developed root was removed from the culture tubes and washed under running tap water to clean all the agar derbies and transferred to poly cups containing garden soil mixed with sand and vermin-compost (1:1 ratio). The cups were kept in same culture conditions and covered with polythene cover. Humidity was maintained by watering regularly and maintained at 25 ± 2°C with 16-h photoperiod for one month in the culture room. The established plantlets were transferred to earthen pots containing garden soil mixed with sand (2:1 ratio).

Table 4: Response of different plant growth regulators on rooting from encapsulated nodal segments and shoot tips of *C. collinus.*

Storage period (weeks)		Encapsulated Nodal segments			Encapsulated shoot tips		
		Rooting response (%)	Number of Rootlets/ explants ± S.D*	Root length/ explant ± S.D	Rooting response (%)	Number of Rootlets/ explants ± S.D*	Root length/ explant ± S.D
control	0.0	0.0	0.0	0.0	0.0	0.0	0.0
IAA	0.5	75	3.3 ± 0.6[a]	1.5 ± 0.2	65	2.1 ± 0.8[a]	1.4 ± 0.2
	1.0	70	1.3 ± 0.6[b]	1.3 ± 0.2	60	1.0 ± 0.4[b]	1.2 ± 0.1
	2.0	55	1.0 ± 0.0[b]	1.0 ± 0.0	50	1.0 ± 0.0[b]	1.0 ± 0.0
	3.0	35	1.0 ± 0.0[b]	1.0 ± 0.0	30	1.0 ± 0.0[b]	1.0 ± 0.0
IBA	0.5	50	1.2 ± 0.5[b]	1.4 ± 0.2	45	1.1 ± 0.5[b]	1.3 ± 0.2
	1.0	40	1.3 ± 0.4[b]	1.3 ± 0.2	35	1.0 ± 0.4[b]	1.2 ± 0.1
	2.0	35	1.0 ± 0.0[b]	1.0 ± 0.0	30	1.0 ± 0.0[b]	1.0 ± 0.0
	3.0	30	1.0 ± 0.0[b]	1.0 ± 0.0	25	1.0 ± 0.0[b]	1.0 ± 0.0
NAA	0.5	40	1.1 ± 0.6[b]	1.3 ± 0.2	35	1.1 ± 0.5[b]	1.3 ± 0.2
	1.0	30	1.0 ± 0.6[b]	1.2 ± 0.1	25	1.1 ± 0.4[b]	1.0 ± 0.0
	2.0	25	1.0 ± 0.0[b]	1.0 ± 0.0	20	1.0 ± 0.0[b]	1.0 ± 0.0
	3.0	20	1.0 ± 0.0[b]	1.0 ± 0.0	10	1.0 ± 0.0[b]	1.0 ± 0.0

*Values are mean ± SD (n=10) dissimilar letters indicated significant difference between means within the treatment at $p<0.05$ level based on LSD mean separation

Conclusion

The present study was successful in producing an efficient method in optimising the parameters to produce synthetic seeds by sodium alginate encapsulation of shoot tips and nodal segments in *C. collinus*. The successful plant re-growth from the encapsulated explants following low temperature storage mostly depends on the plant species, gel matrix, calcium chloride composition and the storage period. The low temperature storage at 4ºC was successful in plant retrieval of *C. collinus.* This protocol could be useful for the *in vitro* preservation and conservation of the germplasm of *C. collinus*.

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