Original Research Article **Biotechnology**

International Journal of Pharma and Bio Sciences

ISSN 0975-6299

PROPAGATION OF *RHINACANTHUS NASUTUS* (L.) KURZ., THROUGH ENCAPSULATED SHOOT TIPS AND NODAL SEGMENTS FOR GERMPLASM EXCHANGE AND DISTRIBUTION

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ABSTRACT

An encapsulation protocol to obtain synthetic seed was standardized in *Rhinacanthus nasutus*. *In vitro* grown shoot tips and nodal segments were encapsulated at different concentration of sodium alginate (2-5 % w/v) and calcium chloride (25 to 200 mM) was used as a gelling agent. Synthetic seeds were stored at 4 °C for a period of 6 months and re-growth frequency were assessed at different strength of MS medium (Full strength, half strength and quarter strength).The maximum response of *in vitro* grown encapsulated shoot tips was found to be the highest percentage of multiple shoot re-growth, when compared to nodal segments in the case of production of multiple shoot re-growth. Maximum shooting re-growth response (95%) of encapsulated shoot tips produced multiple shoots (6.0 ± 1.8) re-growth was observed on Murashige and Skoog (MS) medium supplemented with 6- Benzylaminopurine BAP (2.0 mg/l) and nodal segments re-growth response (85%) was observed with of multiple shoots (3.5 ± 0.5) cultured on MS medium with BAP (2.0 mg/l). The healthy shoots retrieved from the encapsulated shoot tips and nodal segments were rooted on half-strength MS medium augmented with Indole-3-butyric acid IBA (0.5 mg/l).

KEYWORDS: **Synthetic seeds, Shoot tips, Nodal segments, Cold storage and Shoot re-growth.**

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Received on : 27-08-2016
Revised and Accepted on : 07-11-2016
DOI: http://dx.doi.org/10.22376/ijpbs.2017.8.1.b98-105

INTRODUCTION

Rhinacanthus nasutus (L.) Kurz is belonging to the family Acanthaceae distributed in India, China and Thailand[1]. The potential task of *R. nasutus* in remedial diseases like peptic ulcer, liver disorder and obesity had been well known [2]. In addition, plant leaves and stems are used to cure hypertension [3], antidiabetic effects in rats[4]. Whole *R.nasutus* plant parts have been used to treat various types of diseases. It contains rich therapeutics of diabetes, hepatitis, pulmonary tuberculosis, eczema and skin diseases[5,6]. The major bio-active compounds of rhinacathin (C, D and N) was found to have more effectual antiviral [7], anticancer [8], antifungal [9], antibacterial [10], antiallergic and antimutagenic [11], antioxidant and anti-inflammatory [12] properties. In north India rural peoples commonly used this plant for hair dyes and coloring mats. Large scale cultivation of vegetative propagation of this plant uses for health drink industry in Thailand. For this reason, this plant had been destructed from its wild population. Natural propagation of seeds became a bottleneck due to low viability and poor germination, Significant efforts have been made for direct and indirect regeneration of root, Somatic embryogenesis and synthetic seed germination[13], leaf [14] and nodal [15] segments. However, there is no investigation has so far been performed on encapsulated shoot tips and nodal segments storage at low temperature 4°C about 6 months in this plant. The synthetic seed production technology could be useful for the germplasm exchange for the conservation of elite plant genotypes and axenic plant materials axenic plant materials between laboratories and pharmaceutical industries[16,17]. Recently synthetic seed technology was also recommended in high frequency for large scale propagation from encapsulated tissue explants and protection of plant species at low temperature of economically valuable plant genotypes throughout the year, A synthetic seed is a bipolar embryogenic (i.e., somatic embryo or microbulblet) or several unipolar non-embryogenic vegetative propagule (i.e., shoot tips, nodal segments or some other meristematic tissue) encapsulated in an alginate gelling matrix coating that could be used for easy handled and real seed transport, storage and sowing, for that reason, would ultimate develop either *in vitro* or *ex vitro* into an established plantlet[18,19,20]. The use of unipolar propagules for synthetic seed production has been recorded in several plant species including [21,22,23]. Non-embryogenic propagule is more valuable in such plant genotypes where somatic embryogenesis is not well predictable. In the current study, we report the optimized protocol for synthetic seed propagation using shoot tips and nodal segments of *R. nasutus*, which can be used for the germplasm preservation and conservation.

MATERIALS AND METHODS

Mother plants of *R. nasutus* were collected from a wild population from Ooty, India. Individual nodal segments were excised and washed under running tap water, followed by 1.0 % (v/v) deetol (India) for 7 min and washed in distilled water, consequently rinsed with 70 % ethanol for 30 s followed by surface sterilization in 1 % sodium hypochlorite (NaOCl) for 2 min and finally washed well with sterile distilled water. Explants were surface sterilized with 0.1% (w/v) mercuric chloride ($HgCl_2$) for 5 min inside laminar airflow chamber and rinsed three times with sterile distilled water. The surface sterilized explants were cut into small pieces (1.0 - 2.0 cm) in height prior to inoculation on MS[24]. medium supplemented with 30 g/l sucrose and BAP (0.5 mg/l) for the culture initiation after 25 days. The medium wash adjusted to pH 5.8 and gelled with 0.8% agar before autoclaving at 121^{0}C for 20 min. The cultures were maintained at 25 ± 1^{0}C with a 16 h light /8 h dark photoperiod cycle with 45 µmol m^{-2} S^{-1} irradiation (Philips, India). Shoot tips and nodal segments (3–4 mm in size) were excised from *in vitro* derived shootlets (Fig.1&2a) and used for synthetic seed production.

Encapsulation

Shoot tips and nodal explants were collect and suspended in a matrix of MS medium with different concentrations of sodium alginate (2-5 % w/v) as a gelling agent. For, complexation various concentrations of (25, 50, 75, 100 and 200 mM) calcium chloride solutions were prepared in liquid MS medium. Both, sodium alginate and calcium chloride complex agents were sterilized separately autoclaving at 1.06 kg cm^{-2}(121 °C) for 15 min after adjusting the pH to 5.8, whereas sodium alginate containing each shoot tips and nodal segments were gently dropped into sterile calcium chloride solutions, so as to encapsulate the whole shoot tips and nodal segments. The droplet beads containing shoot tips and nodal segments were gentle shaken by using a shaker at 25 minutes for proper polymerization and alginate beads formation. The alginate beads were washed twice in sterile double distilled water. Consequently treated with a diluted streptomycin solution to avoid bacterial contamination and placed beads on sterile petri-plates containing filter paper to remove excess water droplets. All these experiments were done in a laminar airflow chamber with the sterile condition in order to avoid contamination. For, re-growth of synthetic seeds under *in vitro* cultures, encapsulated shoot tips and nodal segments were cultured on the different strength of MS medium (Full strength, half strength and quarter strength MS medium) containing 0.8 % agar supplemented with the different concentrations of BAP, Kn and TDZ (0.5 – 5.0 mg/l).

Low temperature storage

This experiment compared the multiple shoot re-growth ability of encapsulated shoot tips and nodal segments were stored at 4°C cultured on different strength of MS medium (Full strength, Half strength and Quarter strength MS medium) supplemented with BAP, Kn and TDZ (0.5 – 5.0 mg/l) after short-term storage period of one month. Twenty shoot tips and nodal beads were used for each set and the experiment was repeated thrice. After finding out the suitable storage temperature at 4°C and full strength of MS medium for highest frequency of multiple shoot re-growth efficiency, Synthetic seeds were transferred into sterile petri-plates and tightly covered with Para film to prevent microbial contamination and stored at 4 °C for 0, 1, 2, 3, 4, 5 and 6 months. After each storage period, encapsulated shoot tips and nodal segments were cultured on MS medium supplemented with BAP (2.0 mg/l). Percentage

of multiple shoot re-growth was assessed after 35 days of inoculation. Thereafter, re-growth healthy shoots were transferred to half strength MS medium supplemented with various concentrations of IBA, IAA and NAA (0.5-3.0 mg/l) for rooting.

Regeneration of shoots and In vitro culture establishment

The well developed rooted shootlets were taken out of the culture bottles and washed thoroughly with running tap water to remove the excess of agar medium and then transferred into small plastic cups containing garden soil mixed with sand (1:1) ratio. The cups were covered with transparent polythene bags with perforation to maintain higher humidity for initial two weeks in the culture room. After four weeks established plantlets were then transferred to pots containing garden soil mixed with sand (2:1) ratio. The well developed plantlets were transferred to the field.

Statistical analysis

The high frequency of multiple shoot re-growth from encapsulated shoot tips and nodal segments response was evaluated. For, each experiment 20 cultures were used and repeated three times. The response of multiple shoot numbers produced per culture of shoot tips and nodal segments from synthetic seeds was recorded after 35 days culture. The data was presented as mean and its standard deviation (Mean ±SD).

RESULTS AND DISCUSSION

Encapsulation

In the present investigation, shoot tips and nodal explants were excised from *in vitro* grown shootlets of *R. nasutus* (Fig.1a and 2a) and used as non-embryogenic propagules for the production of synthetic seeds. The potential of *R. nasutus* explants type used for synthetic seed and their multiple shoot re-growth was evaluated. The response to encapsulated multiple shoot re-growth development of shoot tips was much higher than the encapsulated nodal segments (Table 2). Recently, *in vitro* propagation through encapsulated shoot tips and nodal segments has also been reported in several medicinal plants[25,26,27,28]. Critical estimation of various concentrations of sodium alginate and calcium chloride, which influences the gel matrix and capsule impact, is one of the major characteristics for efficient plant propagation through synthetic seed encapsulation methods. Synthetic seed hardness depends upon the standardized ion exchange between Na^{+} and Ca^{2+} and it can diverse with various propagules as well as reported with various plant species[29]. Lower concentrations of sodium alginate (1, 2 and 3%) and Calcium chloride (25, 50 and 75 mM) were not suitable for good quality bead production and synthesis. On the diverse, higher concentrations of sodium alginate (4–5 %) produced with hard in nature this may affects the budding shoot re-growth. This confirms with the results of [30,31b] who recorded 4 % sodium alginate as a critical concentration for gel matrix bead formation in *Pogostemon cablin* and *Zingiber officinale* correspondingly.In addition to this, 3 % sodium alginate with 100 mM calcium chloride produced the most uniform beads for easy handling (Fig.1e). It was reported by several workers that 3 % sodium alginate with 100 mM calcium chloride was the most suitable combination for synthetic seed production[32,33,34,35,36]. This variation in sodium alginate concentrations for proper bead formation in various plant species might be due to the variation in commercial source from which the chemicals were purchased as reported earlier *Ocimum* species[37].

Low temperature storage and shoot re-growth

Encapsulated shoot tips and nodal segments were stored at 4°C for about one month. The response of re-growth frequency of plant conversion among the suitable storage temperature was noticed. Meanwhile, response of non stored encapsulated shoot tips and nodal segments controls showed 100 % re-growth compared to encapsulated shoot tips and nodal segments beads which were stored at 4 °C was observed (Fig 3). Earlier, low temperature storage 4 °C was reported to be a potential re-growth of plant conversion response in various plant species[38,39,40]. After a storage period of 6 months synthetic shoot tips and nodal segments beads were cultured on three different strength MS medium (Full strength, half strength and Quarter strength MS medium) with cytokinins BAP, Kn and TDZ (0.5 – 5.0 mg/l). Various kinds of media composition, encapsulated shoot tips and nodal explants developed multiple shoot re-growth after 2-3 weeks.The re-growth of encapsulated shoot tips and nodal segments into plantlets was achieved on full, half and quarter strength MS medium however, conversion percent varied with various media composition. Maximum percentage response (95% shoot tips and 80%nodal segments) for re-growth of encapsulated shoot tips and nodal segments into plantlets was achieved on full strength MS medium. It was observed that encapsulated shoot tips showed higher conversion percentage response than nodal segments under the same culture conditions. The use of half and quarter strength of MS medium reduced the re-growth percentage response as compared to full strength MS medium (Table 1). The reason for these differences may be due to the higher requisite of MS salts and vitamins. Similar observations were also reported in banana[41,42b]. The maximum mean number of re-growth multiple shootlets (6.0 ± 1.8) and (3.5 ± 0.5) was obtained from synthetic shoot tips and nodal beads cultured on MS medium with BAP (2.0 mg/l) with a 95 % and 85 % shooting response (Table 2; Fig. 1d and 2d) followed by TDZ (1.0 mg/l) supplemented medium enhanced the formation of (3.3 ± 0.3) and (2.3 ± 0.2) shootlets re-growth per synthetic bead culture and kinetin (2.0 mg/l) response of (2.8 ± 0.2) and (1.9 ± 0.3) shootlets re-growth per synthetic bead culture. Average length of shoots was observed after 35 days of shoot culture. For, encapsulated shoot tips average maximum shoot length was observed 3.3cm in BAP 2.0 mg/l followed by nodal average length 2.5 cm was recorded BAP 2.0 mg/l. The caulogenic response and number of shoots re-growth declined with an enhancement in cytokinins concentration beyond the optimum. The maximum response of multiple shoot re-growth of about 80% was achieved from synthetic shoot tips, beads and response 65 % of multiple shoot re-growth was observed from synthetic nodal beads after 6 months of storage. These results revealed that, better

response of encapsulated shoot tips BAP (2.0 mg/l) was founded as most effective supplemented full strength MS medium for multiple shoot re-growth higher than nodal segments beads. A high percentage response of conversion of about 80 % was obtained from encapsulated shoot tips and 65 % of retrieved from encapsulated nodal segments after six months of storage period. High efficiency of synthetic seed shoot re-growth were obtained in *Cineraria maritime* L. (82%) [43], *Glycyrrhiza glabra* L[44]. (98%) ,*Khaya senegalensis* [45] 71%–98% and also reported shoot reg-rowth in *Bacopa monnieri* [46] (86.6 %) following six months of storage. In addition, encapsulated shoot tip beads started budding emergence (Fig. 1c) within 10 days after cultured on re-growth MS medium. This confirms the beneficial of using shoot tips for encapsulation, like increased the plant conversion survival percentage of shoot re-growth[47]. Encapsulated derived shoot tips have been found to acquire consistent retrieved ability in various medicinal plants like *Camellia sinensis* L.[48], *Rouvolfia serpentine*[49], *E. alba* [50] and *Ocimum kilimandscharicum* Guerke[51].

Root induction and hardening

The multiple shoots re-growth from encapsulated beads were individually excised measuring a length of about 2.0 - 3.0 cm was a sub-culture on half strength MS medium used for rooting. Rootlets were initiated from the basal cut end of the shootlets placed on IBA supplemented medium after 2 weeks of the time period without any basal callusing. The maximum number of rootlets (9.0) was obtained from encapsulated shoot tips derived shoots while culturing the shoots on half strength MS with 0.5 mg/l IBA followed by half strength MS with 0.5 mg/l IBA induced (6.0) numbers of rootlets from synthetic nodal derived shoots (Table 3; Fig 1&2h). It was clear that higher the concentration of auxin the lesser the number of rootlets formation along with slight basal callus. Consequently, the plantlets growth was also stunted. There was no rooting from the shootlets planted on basal medium. Effectiveness of half strength MS medium for root induction has been well documented in species like *Tylophora indica*[52]. The promotive role of IBA in root induction has been well documented in *R. nasutus*[53]. Well developed healthy rooted plantlets were transferred into the greenhouse for three weeks after hardening and acclimatization in the field.

Table 1

Effect of plant growth regulators on shoot re-growth response of R. nasutus from encapsulated shoot tips and nodal segments cultured on different strength medium after 35 days culture.

Medium strength with Plant growth regulators	Frequency of plantlet conversion (%)	
	Encapsulated Shoot tips	Encapsulated Nodal segments
Control (non-stored encapsulated seeds)	100.00	100.00
Full strength MS medium	95.0	80.0
½ MS medium	75.0	60.0
¼ MS medium	55.0	45.0

Data represents to means ± SD (standard deviation) of 20 replicates per culture and all the experiment was repeated thrice.

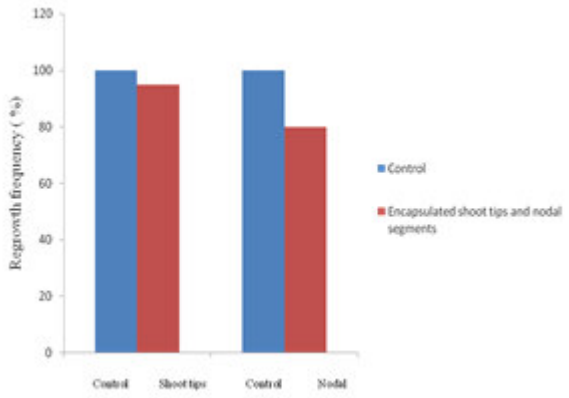

Figure 3

Effect of Storage temperature 4 ºC on frequency of re-growth of encapsulated in vitro propagules of R. nasutus following one month of storage

Table 2

Effect of different Plant growth regulators on shoot re-growth from encapsulated shoot tips and nodal segments of R. nasutus after 35 days of culture.

Concentration of Plant Growth Regulators (mg/l)		Encapsulated Shoot tips			Encapsulated Nodal segments		
		Shooting response (%)	Mean No. of Shootlets/explant ± SD	Mean height of Shootlet ± SD	Shooting response (%)	Mean No. of Shootlets/explants ± SD	Mean height of Shootlet ± SD
Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0
BAP	0.5	65	1.3 ± 0.2	2.1 ± 0.3	60	1.2 ± 0.2	1.9 ± 0.3
	1.0	80	2.2 ± 0.2	2.6 ± 0.3	75	1.8 ± 0.3	2.1 ± 0.3
	2.0	95	6.0 ± 1.8	3.3 ± 0.3	85	3.5 ± 0.5	2.5 ± 0.3
	3.0	75	4.2 ± 0.6	1.7 ± 0.2	70	2.2 ± 0.2	1.4 ± 0.3
	4.0	60	2.3 ± 0.1	1.4 ± 0.3	60	1.6 ± 0.3	1.2 ± 0.2
	5.0	55	1.2 ± 0.2	1.1 ± 0.1	55	1.0 ± 0.0	1.2 ± 0.2
Kinetin	0.5	55	1.2 ± 0.2	1.4 ± 0.3	55	1.0 ± 0.0	1.3 ± 0.2
	1.0	60	1.4 ± 0.1	1.8 ± 0.3	60	1.3 ± 0.3	1.6 ± 0.3
	2.0	75	2.8 ± 0.2	2.2 ± 0.2	75	1.9 ± 0.3	2.1 ± 0.3
	3.0	58	1.5 ± 0.2	1.6 ± 0.3	60	1.4 ± 0.3	1.4 ± 0.3
	4.0	55	1.3 ± 0.2	1.2 ± 0.1	55	1.2 ± 0.2	1.3 ± 0.2
	5.0	40	1.0 ± 0.0	1.3 ± 0.2	40	1.0 ± 0.0	1.2 ± 0.2
TDZ	0.5	60	1.4 ± 0.1	1.3 ± 0.1	70	1.2 ± 0.2	1.2 ± 0.2
	1.0	80	3.3 ± 0.3	1.8 ± 0.3	85	2.3 ± 0.2	1.5 ± 0.2
	2.0	70	2.3 ± 0.2	1.5 ± 0.2	65	1.8 ± 0.3	1.3 ± 0.2
	3.0	55	1.2 ± 0.2	1.4 ± 0.3	55	1.6 ± 0.3	1.2 ± 0.2
	4.0	45	1.0 ± 0.0	1.3 ± 0.1	45	1.3 ± 0.1	1.0 ± 0.0
	5.0	40	1.0 ± 0.0	1.2 ± 0.2	40	1.0 ± 0.0	1.0 ± 0.0

Data represents to means ± SD (standard deviation) of 20 replicates per culture and all the experiment was repeated thrice

Table 3

Rooting of recovered encapsulated shoot tips and nodal segments R. nasutus shootlets cultured on half -strength MS medium containing auxins

Concentration of Plant Growth Regulators (mg/l)		Encapsulated Shoot tips			Encapsulated Nodal segments		
		Rooting response (%)	Number of Rootlets/explants ± SD	Root length/explant ± SD	Shooting response (%)	Number of Rootlets/explants ± SD	Root length/explants ± SD
Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0
IBA	0.5	90	9.0 ± 1.6	4.2 ± 0.7	80	6.0 ± 1.9	2.8 ± 0.4
	1.0	80	6.8 ± 1.4	2.2 ± 0.2	75	4.0 ± 1.2	1.8 ± 0.2
	2.0	65	4.7 ± 1.1	1.5 ± 0.2	60	3.0 ± 1.0	1.3 ± 0.1
	3.0	55	3.0 ± 1.0	1.4 ± 0.3	50	1.2 ± 0.3	1.2 ± 0.1
IAA	0.5	75	5.0 ± 0.8	3.5 ± 0.2	65	4.0 ± 1.2	1.5 ± 0.2
	1.0	70	4.0 ± 1.2	3.0 ± 1.0	60	2.4 ± 0.7	2.2 ± 0.8
	2.0	65	2.4 ± 0.7	2.3 ± 0.2	55	1.8 ± 0.2	1.5 ± 0.2
	3.0	50	1.5 ± 0.2	2.4 ± 0.7	45	1.2 ± 0.1	1.4 ± 0.5
NAA	0.5	65	3.0 ± 1.0	1.3 ± 0.1	50	2.0 ± 0.7	1.4 ± 0.5
	1.0	60	2.2 ± 0.7	1.7 ± 0.2	60	1.4 ± 0.1	1.3 ± 0.1
	2.0	55	1.4 ± 0.3	1.5 ± 0.2	50	1.3 ± 0.1	1.2 ± 0.1
	3.0	45	1.3 ± 0.1	1.4 ± 0.3	40	1.0 ± 0.0	1.0 ± 0.0

Data represents to means ± SD (standard deviation) of 20 replicates per culture and all the experiment was repeated thrice

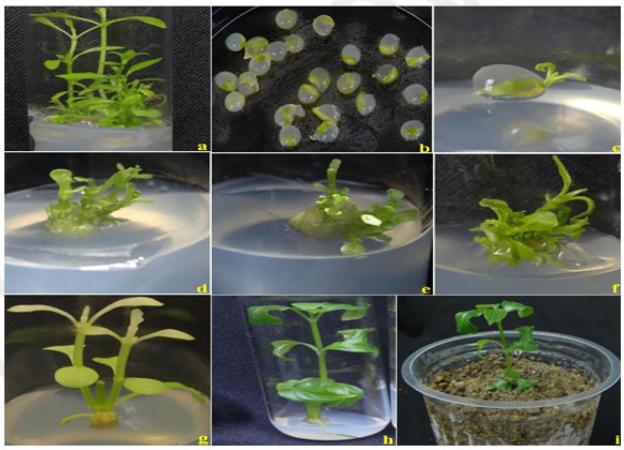

Figure 1

Propagation of plantlets from encapsulated shoot tips storage of R. nasutus: (a) In vitro grown multiple shoots (b) Encapsulated shoot tips (c) Emergence of shoots while storing the encapsulated shoot tips storage at 4ºC (d,e&f) Multiple shoot re-growth from synthetic shoot tips (g) Elongation of shoots (h) Root induction of shootlets (i) Hardening and established plantlets.

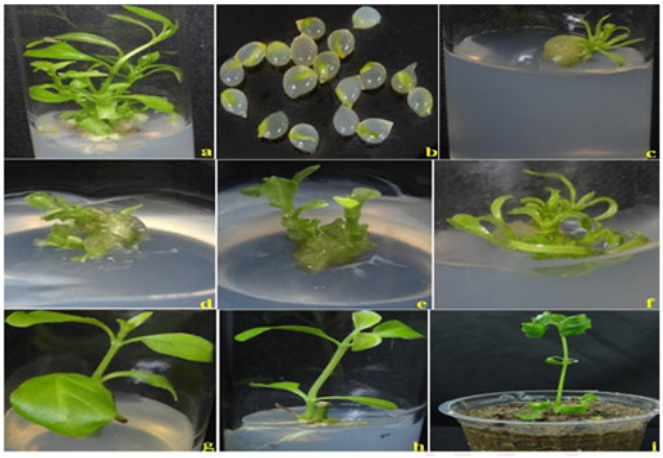

Figure 2

Propagation of plantlets from encapsulated nodal segments storage of R. nasutus: (a) In vitro grown multiple shoots (b) Encapsulated nodal segments (c) Emergence (b) of shoots while storing the encapsulated nodal segments storage at 4ºC (d,e&f) (c) Multiple shoot re-growth from synthetic nodal segments (g) Elongation of shoots (d) (h) Root induction of shootlets (i) Hardening and established plantlets.

CONCLUSION

This is the detailed study on synthetic seed production in *R. nasutus* using shoot tips and nodal segments. The re-growth shoots from encapsulated shoot tips and nodal segments were acclimatized in the natural environment conditions. Hence, synthetic beads could be handled as wild plant seeds or seedling transport as the natural true seeds. This protocol developed could be consistently utilized for mass conservation of this potent multipurpose medicinal plant under low requirements without sub-culturing for the required period of six months and could also be applied as an alternative method of plant propagation.

ACKNOWLEDGEMENTS

Authors are thankful to the Management authority of PRIST University, Thanjavur, Tamilnadu, INDIA for having given permission to perform the research work.

CONFLICT OF INTEREST

Conflict of interest declared none.

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