



Calcium alginate encapsulated synthetic seed production in *Plumbago rosea* L. for germplasm exchange and distribution

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Abstract *Plumbago rosea* L. (Plumbaginaceae), is a medicinal shrub commercially exploited for its naphthoquinone principle, plumbagin, extracted from the roots especially for treating skin disorders. As the plant is exploited from the wild without being replenished, conservation of the species becomes inevitable. Synthetic seeds would provide for effective conservation, germplasm exchange and distribution of this species. A reliable protocol for synthetic seed production in *Plumbago rosea* has been developed encapsulating the axillary buds. The axillary buds from *P. rosea* cultures established and multiplied using the nodal explants in Murashige and Skoog (MS) medium supplemented with Benzyl Adenine (BA) 1.5 mg/L and Indole 3-Acetic acid 1.0 mg/L, were used for synseed production. The plantlet conversion efficiency was the highest in synthetic seeds developed with sodium alginate 2.5% in modified MS with 0.4 M sucrose and CaCl₂ 100 mM. This combination gave the earliest bud initiation (9.19 ± 0.39 days) and maximum number of shoots per explant (2.31 ± 0.16 shoots). Microshoots from the culture, when inoculated on to MS medium supplemented with Naphthalene Acetic Acid 1.0 mg/L gave the best rooting response with 10.67 ± 0.94 roots per plant and 5.42 ± 0.29 cm root length. This is the first report of synthetic seed production in *P. rosea* using axillary buds as explant.

Keywords *Plumbago rosea* · Synthetic seed · Germplasm exchange · Conservation

Abbreviations

BA	N6-Benzylaminopurine
Kn	Kinetin (6-furfurylaminopurine)
IBA	Indole 3-butyric acid
IAA	Indole 3-acetic acid
NAA	α-Naphthaleneacetic acid
MS	Murashige and Skoog
SA	Sodium alginate

Introduction

Plumbago rosea L. (Syn. *Plumbago indica*) of the family Plumbaginaceae, is a commercially exploited medicinal plant in the Indian system of medicine, Ayurveda. It is a bushy perennial shrub originated from India and South-East Asia. It is distributed widely in tropical and subtropical regions of the world especially in tropical Africa, tropical Asia and the Pacific region (Chuakul et al. 1999). In India, it is seen in Eastern Himalayas, South India, North East India and Madhya Pradesh (Sharma 2017). Its tuberous roots contains a quinoid constituent, plumbagin. Plumbagin is reported to have antibacterial, antifungal, anti-inflammatory and antiproliferative activities (Anuf et al. 2014; Kaewbumrung and Panichayupakaranant 2014; Xue et al. 2016). The plant is utilized in various Ayurvedic pharmaceutical formulations for treating cough, bronchitis, gastrointestinal disorders, skin disorders etc. Oh et al. (2017) reported the inhibitory effect of plumbagin on pigmentation and its efficacy to be used as a safe component in skin-care cosmetic formulations. In spite of its huge demand in traditional pharmaceutical and cosmetic

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industry, organized cultivation of this plant is very sparse and hence, the demand is being met from the wild. This has led to the decline in its population in the wild and necessitates the need for conservation of this species.

Synthetic seed technology facilitates the development of a clonal propagation system by enabling the vegetative propagules to be stored for long periods of time, and further, regeneration into plant. It is developed by encapsulation of micropropagules viz, somatic embryos, pollen, axillary bud/nodal segments, shoot tip or meristem, in an alginate hydrogel coating. Small size of the synthetic seeds and its relative ease in handling facilitates easy germplasm exchange and distribution. It also enables exchange of pathogen free propagules across international borders avoiding bulk transportation of plants, quarantine and spread of diseases (Bhanuprakash 2015). As the plant conversion ability is retained even after aseptic storage under culture room conditions (25 ± 2 °C) or in liquid nitrogen (-196 °C) it is being used for conservation of many endangered medicinal plant species (Nair and Reghunath 2009; Maqsood et al. 2012, Gantait et al. 2015).

An efficient in vitro regeneration system is indispensable for synthetic seed technology. In vitro propagation via enhanced release of axillary buds would produce true to type plants as the shoots are being proliferated from the preexisting meristem (Krishna et al. 2016). The development of synthetic seeds using unipolar propagules has been reported in various medicinal plant species, such as *Withania somnifera* (Singh et al. 2006), *Rauvolfia serpentina* (Ray and Bhattacharya 2008), *Bacopa monnieri* (Ramesh et al. 2009), *Cineraria maritima* (Srivastava et al. 2009), *Solanum nigrum* (Verma et al. 2010), *Vitex negundo* (Ahmad and Anis 2010), *Picrorhiza kurroa* (Mishra et al. 2011) etc. Synthetic seed formed by the encapsulation of in vitro derived unipolar axillary buds, can serve as an appropriate entity for in vitro conservation and propagation of *P. rosea*.

The plantlet conversion efficiency of the synthetic seeds determines its potential for in vitro conservation and subsequent propagation. The optimum concentration of the encapsulation matrix components viz., sodium alginate and calcium chloride determine the plantlet conversion efficiency of the synthetic seeds. Plant hormones, biofertilizers, pesticides, fungicides, nitrogen—fixing bacteria, antibiotics, or other essential additives are incorporated in the encapsulation matrix for promoting plant conversion and subsequent growth of the plants from synthetic seeds (Saiprasad 2001).

Hence, in the present study, it was attempted to standardize in vitro regeneration system via axillary shoot proliferation, which would serve as a source for axillary buds for synthetic seed production in *Plumbago rosea* and to evaluate the effect of varied concentrations of alginate

matrix components (sodium alginate and calcium chloride) on the morphogenetic response of encapsulated axillary buds of *P. rosea* and on its plantlet conversion efficiency.

Materials and methods

Explant collection, disinfection and culture establishment

Plumbago rosea var Agni, valued for high plumbagin content, was sourced from the Aromatic and Medicinal Plants Research Station (AMPRS), Odakkali, Ernakulam, Kerala, India and maintained for the collection of explants. Single node cuttings of 3–4 cm long were excised from vigorous growing shoots of 1-year old plants. The leaves were removed leaving a small portion of petiole on the cuttings. Nodal segments were given thorough 15 min wash under running tap water. The nodal segments were then immersed in detergent solution, Labolene for 30 min, followed by washing with distilled water. The nodal cuttings were then treated with a systemic fungicide solution, 0.1% carbendazim 50% wettable powder (W.P.), for 30 min followed by washing several times with distilled water. Further, the explants were aseptically sterilised with a dip for 2–3 s in absolute alcohol followed by sterilisation with disinfectant, 0.2% mercuric chloride for 5 min. These were then washed several times with sterile water to remove any trace of mercuric chloride. Single node cuttings of 2.5 cm were inoculated vertically on sterilized hormone free MS (Murashige and Skoog 1962) medium supplemented with 30 g/L sucrose and 5.7 g/L agar, for the establishment of culture. After 6 weeks of incubation, nodal cuttings (1.5 cm) with a single axillary bud were excised from in vitro established cultures for obtaining sterile explants for axillary shoot proliferation studies.

Axillary shoot proliferation

Nodal cuttings with a single axillary bud were cultured in basal MS media supplemented with different concentrations and combinations of auxins (NAA, IAA, IBA) and cytokinins (BA and Kn). The explants were inoculated on to thirty treatments involving different levels of BA (0.5–1.5 mg/L), Kn (0.5–1.0 mg/L), IAA (0.5–1.0 mg/L), IBA (0.5–1.0 mg/L) and NAA (0.5–1.0 mg/L) in basal MS medium supplemented with 30 g/L sucrose and 5.7 g/L agar. The media were dispensed to pre sterilised culture bottles, containing 30 mL of the media. The pH of the medium was adjusted to 5.7 using an electronic pH meter (Susima MP-1 PLUS). The media was sterilized by autoclaving at 121 °C and 1.06 kg/cm² pressure for 20 min using STERI horizontal cylindrical autoclave (Yoriko,

India). After 6 weeks of culture, the observations on number of days for bud initiation, shoots per explant, shoot length and nodes per shoot were recorded. The cultures were subcultured every 6 weeks and continued up to four subcultures.

Artificial seed synthesis

Nodal axillary buds from in vitro grown plantlets from the fourth subculture were used for artificial seed production. The encapsulation was attempted by suspending the axillary buds in modified MS medium (devoid of calcium chloride) supplemented with different concentrations (2.5, 3, 4, and 5% m/v) of sodium alginate (Sigma, St. Louis, USA) and 0.5 M sucrose. This mixture was dispensed with a 1 mL micropipette into different concentrations of calcium chloride solution (50, 75, 100 and 200 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 1.06 kg/cm² pressure for 20 min. The plant conversion potential of the beads after encapsulation were analysed by inoculating them on the medium which gave the maximum axillary shoot proliferation. After 6 weeks of culture, observations were recorded on number of days to bud initiation, shoots per explant, shoot length and nodes per shoot to determine the encapsulation matrix that exhibited maximum plant conversion potential. The cultures were subcultured every 6 weeks up to three subcultures.

In vitro rooting

The shoots of 3–4 cm length were separated individually from cultures and were transferred to MS medium with varying levels of IBA (0.1–1.5 mg/L), IAA (0.1–1.5 mg/L) and NAA (0.1–1.5 mg/L) to initiate the rooting process. After 6 weeks of culture, observations on percent cultures initiating roots, number of days for root initiation, number of roots and length of roots were recorded.

In all the above in vitro experiments, the cultures were incubated at a light intensity of 40 $\mu\text{mol}/\text{m}^2/\text{s}$ using white fluorescent tubes at 25 ± 2 °C temperature and 60% relative humidity. The photoperiod of 16 h of light and 8 h of darkness was maintained in the incubation room.

Planting out and acclimatization

Plantlets of 7–8 cm length each with 9–12 roots were removed from the bottles using forceps. The agar adhering to the roots was completely removed by washing under slow running tap water. The rooted plantlets were treated with 0.1% Carbendazim 50% W.P. for 1 h before planting out. The plants were then planted in trays filled with

potting media consisting of redsoil and coir pith (3:1) and redsoil and coirpith (2:1) supplemented with arbuscular mycorrhizal fungi (AMF) 1 g/plant. The plants were kept under 25% shade net house and irrigated twice a day maintaining field capacity. Observations were recorded on survival percentage after 6 weeks of planting out. Twenty plants each were planted in the two potting media and after 6 weeks of planting, observations on survival percentage was recorded.

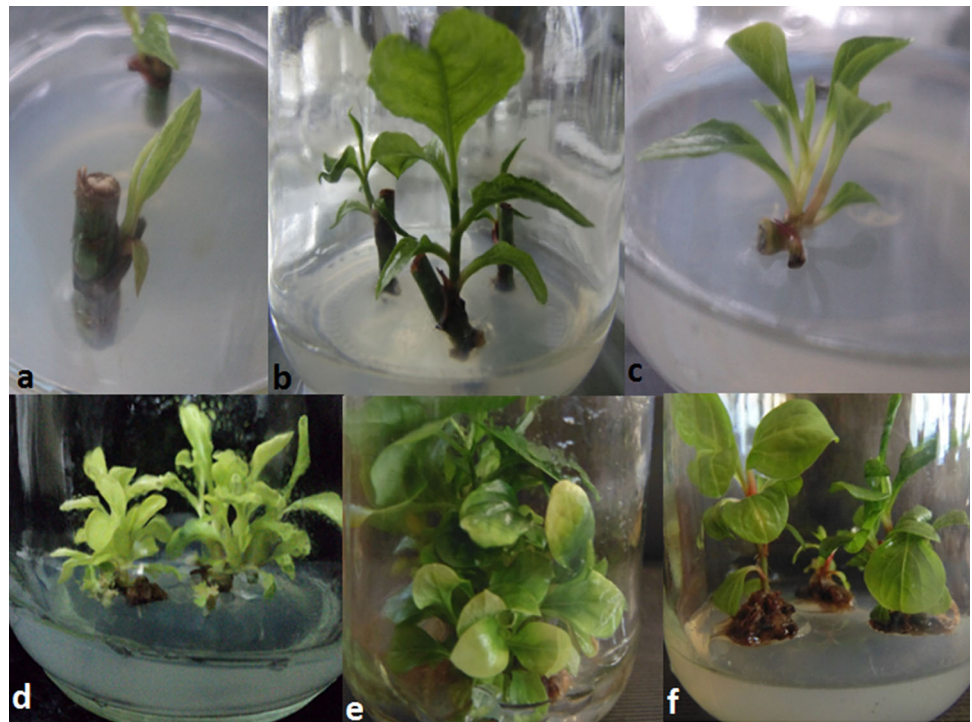
Statistical analysis

The experiment was conducted in a completely randomized design. The treatments were replicated six times with six explants/microshoots per replication. The data were represented as mean and standard deviation. Data were subjected to analysis of variance and significant difference between treatments were determined by Duncan Multiple Range Test.

Results and discussion

Synthetic seeds are encapsulated tissue culture derived micropropagules that possess plant conversion potential and is used for conservation, germplasm exchange and distribution. The use of axillary buds as micropropagule for synthetic seed production eliminates the chance of somaclonal variation. Synthetic seed production using axillary buds in *P. rosea*, a medicinal plant becoming extinct due to high commercial exploitation is not yet reported. In the study, nodal segments of 2.5 cm were used as explants for culture initiation. Nodal segments of 1.5 cm with a single axillary buds from in vitro cultures were inoculated on to MS medium supplemented with different combinations of plant growth regulators (BA, IAA, IBA, NAA and Kn) for axillary bud proliferation and further regeneration (Fig. 1). A cent per cent regeneration could be accomplished in all the different combinations. Results of axillary bud proliferation and regeneration are summarized in Table 1. The bud initiation was indicated by swelling of the preexisting buds and subsequent emergence of the shoot. The earliest bud initiation (7.94 ± 0.22 days) was obtained in MS medium supplemented with BA 1.5 mg/L and IAA 1.0 mg/L. Maximum shoot proliferation (5.28 ± 0.26 shoots), maximum shoot length (5.33 ± 0.19 cm) and maximum nodes per shoot (5.83 ± 0.14) were also obtained in the same medium. Satheeshkumar and Bhavanandan (1988) reported that calli induced from stem segment of *P. rosea*, gave shoot regeneration and multiplication on MS medium containing auxin and cytokinin; MS medium supplemented with 2, 4-D 2.5 mg/L and Kn 1.5 mg/L gave maximum callus induction while BAP 2 mg/L and NAA 1 mg/L induced shoot

Fig. 1 Culture establishment in MS medium **a** after 2 weeks in culture, **b** after 4 weeks in culture; Axillary shoot proliferation in MS + BA 1.5 mg/L + IAA 1.0 mg/L, **c** after 3 weeks in culture, **d** after 6 weeks in culture; **e** and **f** basal callusing in MS + BA 1.5 mg/L + NAA 1.0 mg/L



formation from the callus. In the present study, MS medium augmented with BAP 1.5 mg/L and NAA 1 mg/L gave basal callusing. Bud initiated very late (20.33 ± 0.58 days) in this medium. Higher ratio of BA (1.0–1.5 mg/L) to NAA (0.5–1.0 mg/L) exhibited excessive callusing at the base of the nodal explants. In contrast to this, Agastian et al. (2006) observed that when nodal explants were grown in MS medium with low concentrations of BAP 0.1 mg/L and higher concentration of NAA 1.0 mg/L, compact chlorophyllous calli with shoots in *Justicia gendarussa* were produced. BAP in combination with NAA was not effective in enhancing shoot proliferation but supported prolific callus growth at the basal end of the explants in *Sapindus trifoliatus* (Asthana et al. 2011). In our study, cytokinins solely supplemented in the MS medium (BA alone or BA and Kn in combination), did not give as much proliferation of shoots as with BA-IAA combination. This indicates the synergistic effect of BA and IAA on shoot proliferation as reported in *Bacopa monnieri* (Yelne et al. 1997), *Cardiospermum halicacabum* (Jayasheelan and Rao 1998), *Gardenia jasminoides* (Duhoky and Rasheed 2010) and *Lysimachia vulgaris* (Turker and Guner 2013). It was also observed in the study that multiple shoot induction was low in MS medium containing combinations of BA and NAA/Kn. When compared to BA-NAA/Kn combinations, MS medium supplemented with BA alone gave better shoot proliferation. The control treatment, MS medium devoid of growth regulators recorded least number of shoots (1.28 ± 0.16) as well as nodes (2.44 ± 0.16) per shoot.

The synthetic seeds are surrounded by a protective coating, which provides necessary protection during storage, handling and transportation. The coating could incorporate plant nutrients or plant growth regulators that aid its conversion into plantlet. In the study, axillary buds from the in vitro cultures were encapsulated using sodium alginate (2.5, 3, 4 and 5% m/v) in modified MS medium and calcium chloride (50, 75, 100 and 200 mM). The beads were formed by exchange of ions, Na^+ ions of sodium alginate being exchanged with Ca^{++} ions forming calcium alginate. Encapsulation hardness is determined by the optimal ion exchange of Na^+ and Ca^{++} ions, which may vary with propagules as well as with plant species (Rai et al. 2009; Benelli 2016). After giving 20 min of complexing time, the beads (artificial seeds) formed were cultured on the MS medium with BA 1.5 mg/L and IAA 1.0 mg/L, that gave the best axillary shoot proliferation. All the sixteen encapsulation treatments showed 100% survival and regeneration. The results are presented in Table 2. The days to bud initiation was the earliest (9.19 ± 0.39 days) in the beads formed using the encapsulation matrix, E3 (SA 2.5% in modified MS + CaCl_2 100 mM) which was significantly different from all the other treatments. The beads from the same treatment gave the maximum number of shoots (2.31 ± 0.16) per explant, shoot length (3.35 ± 0.21 cm) and nodes (3.75 ± 0.08) per shoot. The regeneration of the plants from encapsulated beads are illustrated in Fig. 2. Similar concentrations of sodium alginate and calcium chloride were used for

Table 1 Effect of cytokinins and auxins on enhanced release of axillary buds from nodal explants

Treatment no.	Plant growth regulators (mg/L)					Survival (%)	Days for bud initiation	Shoots per culture	Shoot length (cm)	Nodes per shoot
	BA	IAA	IBA	NAA	Kn					
T1	0.5	0.5	–	–	–	100	9.39 ± 0.54 ^a	1.89 ± 0.19	4.64 ± 0.22	5.22 ± 0.37
T2	0.5	1	–	–	–	100	10.00 ± 0.77	1.56 ± 0.11 ^b	4.31 ± 0.13	4.50 ± 0.22
T3	1	0.5	–	–	–	100	10.16 ± 0.73	2.11 ± 0.29	3.92 ± 0.30	5.11 ± 0.20
T4	1	1	–	–	–	100	9.78 ± 0.54 ^a	3.00 ± 0.17	3.89 ± 0.47	4.72 ± 0.26
T5	1.5	0.5	–	–	–	100	11.05 ± 0.92	2.22 ± 0.11	3.28 ± 0.32	4.22 ± 0.29
T6	1.5	1	–	–	–	100	7.94 ± 0.22 ^a	5.28 ± 0.26 ^a	5.33 ± 0.19 ^a	5.83 ± 0.14 ^a
T7	0.5	–	0.5	–	–	100	11.28 ± 0.78	1.39 ± 0.10 ^b	2.81 ± 0.17	3.06 ± 0.10
T8	0.5	–	1	–	–	100	11.72 ± 0.42	1.33 ± 0.09 ^b	2.92 ± 0.18	2.94 ± 0.28 ^b
T9	1	–	0.5	–	–	100	12.83 ± 1.04	1.39 ± 0.16 ^b	3.11 ± 0.29	3.39 ± 0.16
T10	1	–	1	–	–	100	11.50 ± 1.28	1.44 ± 0.19 ^b	3.08 ± 0.31	3.22 ± 0.24
T11	1.5	–	0.5	–	–	100	10.83 ± 0.83	1.56 ± 0.17 ^b	3.22 ± 0.28	2.72 ± 0.20 ^b
T12	1.5	–	1	–	–	100	10.83 ± 0.59	1.67 ± 0.12 ^b	2.69 ± 0.12 ^b	2.83 ± 0.14 ^b
T13	0.5	–	–	0.5	–	100	14.66 ± 1.21	1.45 ± 0.11 ^b	3.19 ± 0.12	3.78 ± 0.19
T14	0.5	–	–	1	–	100	16.55 ± 1.23	1.50 ± 0.14 ^b	2.31 ± 0.21 ^b	3.11 ± 0.19
T15	1	–	–	0.5	–	100	16.16 ± 0.67	1.33 ± 0.15 ^b	2.22 ± 0.18 ^b	2.94 ± 0.13 ^b
T16	1	–	–	1	–	100	17.28 ± 0.62	1.83 ± 0.14 ^b	3.14 ± 0.16	3.83 ± 0.19
T17	1.5	–	–	0.5	–	100	20.05 ± 0.64 ^b	1.56 ± 0.17 ^b	2.86 ± 0.10	3.67 ± 0.23
T18	1.5	–	–	1	–	100	20.33 ± 0.58 ^b	1.72 ± 0.20 ^b	2.83 ± 0.10	3.44 ± 0.20
T19	0.5	–	–	–	0.5	100	14.16 ± 0.59	1.33 ± 0.15 ^b	2.92 ± 0.20	3.61 ± 0.13
T20	0.5	–	–	–	1	100	15.33 ± 0.53	1.39 ± 0.13 ^b	2.36 ± 0.11 ^b	3.39 ± 0.22
T21	1	–	–	–	0.5	100	15.66 ± 0.40	1.78 ± 0.22 ^b	2.64 ± 0.15 ^b	3.17 ± 0.25
T22	1	–	–	–	1	100	16.50 ± 0.85	1.83 ± 0.19 ^b	2.39 ± 0.24 ^b	3.28 ± 0.18
T23	1.5	–	–	–	0.5	100	14.72 ± 0.49	2.00 ± 0.12 ^b	2.36 ± 0.07 ^b	3.33 ± 0.12
T24	1.5	–	–	–	1	100	15.50 ± 0.31	2.11 ± 0.25	2.14 ± 0.13 ^b	3.00 ± 0.21
T25	0.5	–	–	–	–	100	9.94 ± 0.22 ^a	1.86 ± 0.32 ^b	2.17 ± 0.23 ^b	2.83 ± 0.21 ^b
T26	1	–	–	–	–	100	11.17 ± 0.67	2.75 ± 0.17	2.92 ± 0.20	3.20 ± 0.18
T27	1.5	–	–	–	–	100	10.72 ± 0.57	2.72 ± 0.57	2.64 ± 0.15 ^b	3.16 ± 0.11
T28	2	–	–	–	–	100	10.56 ± 0.56	3.47 ± 0.37	2.81 ± 0.17	2.95 ± 0.10 ^b
T29*	1	1	–	–	–	100	9.50 ± 0.24 ^a	4.50 ± 0.27	4.81 ± 0.12 ^a	5.08 ± 0.12
Control	–	–	–	–	–	100	19.61 ± 0.90 ^b	1.28 ± 0.16 ^b	2.17 ± 0.22 ^b	2.44 ± 0.16 ^b
CD (5%)	–	–	–	–	–	–	2.02	0.60	0.59	0.56
FV (5%)	–	–	–	–	–	–	23.06	19.31	15.33	17.96

Basal culture medium: MS, *1/2 MS; Data represented as mean ± standard deviation; ‘a’ and ‘b’ denotes the highest and the lowest on par values

synthetic seed formation in *Clitoria ternatea* and *Indigofera tinctoria* as reported by Nair and Reghunath (2007, 2009). The days to bud initiation though varied significantly and was late (11.67 ± 0.59 days) in E6 (SA 3% in modified MS + CaCl₂ 75 mM), it gave on par results with shoots per culture. Padro et al. (2012) and Ai et al. (2012) reported effective bead formation using sodium alginate 3% solution and CaCl₂ 100 mM, in *Morus alba* and *Rabdosia rubescens*, respectively. In the study, it is observed that encapsulated explant took more number of days to initiate bud compared to non-encapsulated explant.

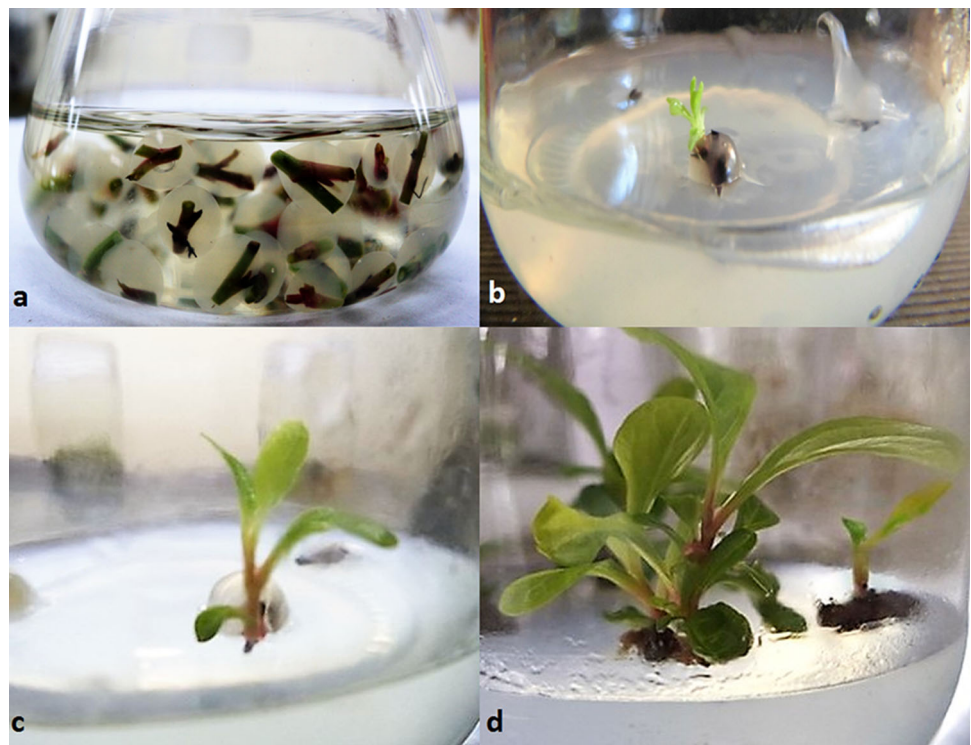
Shoots per culture, shoot length and nodes per shoot were higher in non-encapsulated axillary buds after 6 weeks of observation. The encapsulation of axillary buds resulted in late bud initiation and subsequent shooting. Higher the sodium alginate concentrations (4, 5%) and calcium chloride concentration (100 mM), firmer were the beads, taking longer period for bud initiation and subsequent shoot emergence. Bud initiated late (17.53 ± 1.28 days) in the beads formed from the matrix E12 (SA 4.0% in modified MS medium + CaCl₂ 200 mM). The low shoot proliferation and elongation in encapsulation treatments indicated

Table 2 Effect of encapsulation using different concentrations of sodium alginate and calcium chloride on plant conversion of synseeds

Treatment no.	SA (%)	CaCl ₂ (mM)	Survival (%)	Days for bud initiation	Shoots/culture	Shoot length (cm)	Nodes/shoot
E1	2.5	50	100	12.08 ± 0.38	1.83 ± 0.19 ^{ab}	2.51 ± 0.20 ^b	2.94 ± 0.26 ^b
E2	2.5	75	100	11.44 ± 0.38	1.78 ± 0.10 ^{ab}	2.40 ± 0.26 ^b	3.00 ± 0.22 ^b
E3	2.5	100	100	9.19 ± 0.39 ^a	2.31 ± 0.16 ^a	3.35 ± 0.21 ^a	3.75 ± 0.08 ^a
E4	2.5	200	100	11.83 ± 0.50	2.08 ± 0.25 ^a	1.89 ± 0.20 ^b	2.53 ± 0.14 ^b
E5	3.0	50	100	12.75 ± 0.60	1.56 ± 0.19 ^b	1.93 ± 0.30 ^b	2.47 ± 0.25 ^b
E6	3.0	75	100	11.67 ± 0.59	2.06 ± 0.27 ^a	2.56 ± 0.11	3.00 ± 0.17 ^b
E7	3.0	100	100	11.72 ± 0.39	1.89 ± 0.31 ^{ab}	2.83 ± 0.43 ^a	3.36 ± 0.29 ^a
E8	3.0	200	100	13.47 ± 0.42	1.61 ± 0.16 ^b	2.04 ± 0.23 ^b	2.78 ± 0.28 ^b
E9	4.0	50	100	16.36 ± 0.70 ^b	1.44 ± 0.13 ^b	2.36 ± 0.15 ^b	2.83 ± 0.17 ^b
E10	4.0	75	100	15.83 ± 0.85 ^b	1.47 ± 0.18 ^b	1.90 ± 0.15 ^b	2.47 ± 0.10 ^b
E11	4.0	100	100	16.42 ± 0.72 ^b	1.56 ± 0.19 ^b	2.67 ± 0.08 ^a	3.08 ± 0.08 ^b
E12	4.0	200	100	17.53 ± 1.28 ^b	1.39 ± 0.18 ^b	1.81 ± 0.23 ^b	2.53 ± 0.18 ^b
E13	5.0	50	100	12.28 ± 0.69	1.86 ± 0.21 ^{ab}	2.79 ± 0.41 ^a	3.31 ± 0.33 ^a
E14	5.0	75	100	11.25 ± 0.51	1.44 ± 0.11 ^b	3.29 ± 0.33 ^a	3.72 ± 0.28 ^a
E15	5.0	100	100	13.61 ± 0.74	2.06 ± 0.13 ^a	3.10 ± 0.34 ^a	3.72 ± 0.39 ^a
E16	5.0	200	100	16.08 ± 1.16 ^b	1.72 ± 0.13 ^b	3.01 ± 0.22 ^a	3.44 ± 0.16 ^a
CD (5%)	–	–	–	1.96	0.53	0.73	0.65
FV (5%)	–	–	–	11.79	2.11	3.90	3.86

Culture medium: MS supplemented with BA 1.5 mg/L and IAA 1.0 mg/L; Data represented as mean ± standard deviation; 'a' and 'b' denotes the highest and the lowest on par values

Fig. 2 **a** Synthetic seeds made from nodal axillary buds formed by complexing with SA 2.5% + CaCl₂ 100 mM; **b–d** regeneration from synthetic seeds after 2, 4 and 6 weeks of culture



the resistance offered by the firmness of the coating of the axillary buds, on bud initiation and shoot emergence. Encapsulated beads formed by sodium alginate 2.5% and

calcium chloride 100 mM, gave maximum shoots per explant and shoot length among different encapsulation

matrices tried but were less compared to non-encapsulated buds.

After 8 weeks of culture in the rooting medium, 5–6 cm long shoots were excised and inoculated on to rooting medium. Twelve levels of different auxins, IBA (0.1–1.5 mg/L), IAA (0.1–1.5 mg/L) and NAA (0.1–1.5 mg/L) were tried for in vitro root formation. Result of the study are presented in Table 3. All the different levels of auxins in MS medium gave 100% root initiation except for the control treatment devoid of growth regulators. No root initiation was observed in MS medium devoid of growth regulators. Earliest (7.28 ± 0.27 days) root initiation, maximum number of roots (10.67 ± 0.94 roots per plant) and maximum root length (5.42 ± 0.29 cm) were recorded in MS medium augmented with NAA 1.0 mg/L (Fig. 3). Agastian et al. (2006) reported the formation of long chlorophyllous roots in medium supplemented with NAA (0.2–2.0 mg/L) in *Justicia gendarussa*. Gopalakrishnan et al. (2009) reported the root initiation in IBA supplemented MS medium in leaf derived plantlets of *P. rosea*, while root did not initiate in NAA supplemented MS media. This is contrast to our finding, wherein root initiated in NAA supplemented medium but the plantlets were derived from the axillary buds in the nodal segment.

An effective micropropagation system should combine a successful planting out protocol. In such a protocol, the survival percentage of ex vitro established plants is crucial. In the study, the in vitro rooted plants were cautiously

removed from the culture vessels and planted out in two different potting media, red soil and coir pith (3:1) and red soil and coir pith (2:1) supplemented with AMF. In vitro plantlets when transferred to ex vitro conditions were highly susceptible to dehydration and wilting. To overcome these problems and to improve the survival per cent, AMF was included in the potting media. Krishna et al. (2006) reported that inoculation with mycorrhizal fungi at the time of planting out, enabled successful hardening and ex vitro establishment of plantlets, as it would alleviate transplanting shock in micropropagated plantlets and minimize the mortality rate. The survival rate of plants was estimated after 6 weeks of culture. Fig 3d and e illustrates the ex vitro establishment of the plants. There was no significant difference between the effects shown by two potting media. The plants showed a survival rate of 72 and 76% in the treatments PM1 [red soil:coir pith (3:1)] and PM2 [red soil:coir pith (2:1) supplemented with AMF], respectively.

The synthetic seeds developed using axillary buds of *P. rosea* gave cent per cent plantlet conversion under in vitro condition. The regenerated plantlets from synthetic seeds, when transferred to ex vitro conditions, recorded a survival rate of 76%. Hence, this technology can be considered as a promising strategy for the exchange and distribution of *P. rosea* between laboratories. This technology also can be effectively utilized for the in vitro conservation of this plant species.

Table 3 Effect of auxins on in vitro rooting

Treatment no. (cm)	Auxins (mg/L)			Rooting (%)	Days for root initiation	Roots/culture	Root length
	NAA	IBA	IAA				
R1	0.1	–	–	100	9.22 ± 0.34	7.17 ± 0.45^b	3.69 ± 0.16
R2	0.5	–	–	100	7.78 ± 0.27^a	6.56 ± 0.79^b	3.36 ± 0.21^b
R3	1.0	–	–	100	7.28 ± 0.26^a	10.67 ± 0.94^a	5.42 ± 0.29^a
R4	1.5	–	–	100	8.83 ± 0.32	9.33 ± 0.33^a	3.58 ± 0.22
R5	–	0.1	–	100	11.39 ± 0.25^b	10.39 ± 0.61^a	3.53 ± 0.19^b
R6	–	0.5	–	100	9.22 ± 0.39	9.06 ± 0.44^a	3.53 ± 0.17^b
R7	–	1.0	–	100	10.94 ± 0.65^b	9.06 ± 0.56^a	3.72 ± 0.35
R8	–	1.5	–	100	10.83 ± 0.49^b	9.78 ± 0.70^a	2.94 ± 0.14^b
R9	–	–	0.1	100	10.22 ± 0.62^b	8.06 ± 0.54^b	3.17 ± 0.12^b
R10	–	–	0.5	100	10.00 ± 0.38	8.33 ± 0.46	3.92 ± 0.10
R11	–	–	1.0	100	10.42 ± 0.35^b	8.11 ± 0.48^b	4.08 ± 0.33
R12	–	–	1.5	100	10.36 ± 0.54^b	8.17 ± 0.41^b	3.50 ± 0.11^b
Control	–	–	–	0.00	0.00	0.00	0.00
CD (5%)	–	–	–	–	1.21	1.75	0.61
FV (5%)	–	–	–	–	8.86	4.46	8.21

Basal culture medium: MS; Data represented as mean \pm standard deviation; 'a' and 'b' denotes the highest and the lowest on par values



Fig. 3 In vitro rooting in MS + NAA 1.0 mg/L, **a** after 2 weeks in culture, **b** and **c** after 6 weeks in culture, **d** and **e** ex vitro establishment in red soil and coir pith (2:1) supplemented with AMF 1 g per plant

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