

Encapsulation technology for short-term storage and germplasm exchange of *Vitex trifolia* L.

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Abstract The current study reports the encapsulation of nodal segment of *V. trifolia* L. excised from 2-month-old in vitro-raised cultures for short-term conservation and propagation. The encapsulation of nodal segments was significantly affected by the concentrations of sodium alginate (Na alginate) and calcium chloride ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$). The best gel complex using 3 % sodium alginate and 100 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ was found most suitable for the production of ideal Ca alginate beads. Maximum percent conversion response (84.9 %) was recorded on Murashige and Skoog (MS) basal medium supplemented with 5.0 μM 6-benzyladenine (BA), 0.5 μM α -naphthalene acetic acid (NAA) after 6 weeks of culture. The encapsulated nodal segments could be stored at 4 °C up to 8 weeks with 42.5 % regeneration efficiency. Plantlets obtained were rooted best on full-strength MS medium containing 0.5 μM α -naphthalene acetic acid (NAA) for the production of complete plantlets. The regenerated plantlets were successfully hardened and established in field where they grew well without any detectable variation with a survival rate of 92 %. The high frequency of plant re-growth from alginate-coated nodal segments coupled with high viability percentage after 4 weeks of storage is highly encouraging for the exchange of *V. trifolia* genetic resources.

Keywords Acclimatization · Encapsulation · Plant regeneration · Germplasm conservation

Abbreviations

$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	Calcium chloride
BA	6-Benzyladenine
IBA	Indole-3-butyric acid
NAA	α -Naphthalene acetic acid
MS	Murashige and Skoog (1962) medium
PGR	Plant growth regulator

1 Introduction

Preservation of the world's genetic resources is currently at the forefront of conservation activities and biotechnological techniques such as encapsulation technology is a highly promising tool for the management of transgenic, seedless plant and other plant species that are difficult to propagate through conventional propagation methods. Propagation using synthetic seeds has opened up new vistas in areas of plant germplasm characterization, acquisition, conservation, exchange and genetic resource management. This technology provides a viable approach for in vitro germplasm conservation as it combines the advantages of clonal multiplication with those of seed propagation and storage as “genetically identical materials” (Standardi and Piccioni 1998; Ara et al. 2000), ease of handling and exchange of axenic plant materials between laboratories (Rihan et al. 2011; Hung and Trueman 2012) along with increased efficiency of in vitro propagation in terms of space, time, labor and overall cost (Nyende et al. 2003). Synthetic seeds have been widely utilized for micropropagation and

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conservation of various medicinal plant species (Alatar and Faisal 2012; Parveen and Shahzad 2014; Fatima et al. 2013; Varshney and Anis 2014).

Vitex trifolia L. (Verbenaceae) is an aromatic shrubby tree, 1–6 m in height, sometimes prostrate or ascending in habit. The leaves are simple or 3-foliolate. It can be distinguished by its long-petioluled median leaflet and 3–5 leaflets. (Kulkari 2011). The flowers are numerous, and borne in terminal, oblong panicles 5–10 cm in length. The corolla is hairy, and lavender to blue. The fruit is rounded (Padmalatha et al. 2009). The genomic number of the species is $n = 32$ and no variation in chromosome number has been reported. The plant and its extracts are used to improve memory, relieve pain, cure fever, treating hair loss (Bhattacharjee and De 2005), and also regarded as anti-bacterial, anti-inflammatory, anti-diabetic, anti-cancerous and anti-HIV (Pullaiah and Naidu 2003; Woradulayapinij et al. 2005). Despite its well-known potential as a valuable medicinal plant, *Vitex trifolia* is not available in abundance in the wild and restricted distribution along beaches and sandy soils make this particular plant highly vulnerable due to human exploitation. Recently, the plant is Red listed by IUCN with low-risk status (Nagaveni and Rajanna 2013). Conventional propagation of *Vitex trifolia* is through vegetative cuttings and suckers which are slow growing, age and season dependent (Ahmed and Anis 2012). It can also be propagated through seeds, however, seed set and seed germination is poor (Hiregoudar et al. 2006). Thus, it is necessary to develop an alternative source of propagation and efficient method for easy distribution of in vitro-raised quality propagules in the form of small beads to meet the demand for this important medicinal plant. During last few years, several reports are available on its in vitro regeneration exploiting different strategies of micropropagation (Hiregoudar et al. 2006; Ahmed and Anis 2012, 2014a; Nagaveni and Rajanna 2013). However, till date no report is available on the synseed production and subsequent plant regeneration in *V. trifolia* using vegetative propagules. Therefore, the present study was undertaken to develop a method of short-term storage as well as germplasm exchange and distribution of *V. trifolia* using encapsulation technique.

2 Materials and methods

2.1 Plant material

Nodal segments approximately (0.8–1.0 cm) long dissected aseptically from in vitro-grown 8-week-old proliferating shoot (developed by Ahmed and Anis 2012 of *Vitex trifolia* L.) were used as explants for encapsulation.

2.2 Encapsulation matrix and complexing agent

Different concentrations of sodium alginate (Central Drug House, India) viz. 1, 2, 3, 4 and 5 % (w/v) were prepared using either liquid MS medium (Murashige and Skoog 1962) or DDW, both supplemented with 3 % sucrose. For complexation, 25, 50, 75, 100 and 200 mM CaCl_2 solution was prepared. Both the gel matrix and complexing agent were sterilized by autoclaving at 1.06 kg cm^{-2} ($121 \text{ }^\circ\text{C}$) for 15 min after adjusting the pH (MS medium) to 5.8.

2.3 Planting media and culture conditions

Encapsulation was accomplished by mixing the nodal segments from in vitro-regenerated shoots of *V. trifolia* into the sodium alginate mixture and dropping them into the calcium chloride solution. The droplets containing the explants were held for at least 30 min to achieve polymerization of the sodium alginate. The alginate beads were then collected, rinsed with sterile liquid MS medium and transferred to sterile filter paper in Petri dishes for 5 min under the laminar air flow hood to eliminate the excess of water and thereafter planted into wide mouth flask (Borosil) containing sowing medium composed of MS nutrient medium with the different concentrations of BA (0.0, 1.0, 2.5, 5.0, 7.5 μM) either singly or in combination with NAA (0.5 μM). The medium was gelled with 0.8 % (w/v) bacteriological grade agar (Qualigens Fine Chemicals, Mumbai, India) and sterilized by autoclaving as described above. All the cultures were incubated for 6 weeks in growth room at $25 \pm 2 \text{ }^\circ\text{C}$ at 16-h photoperiod (irradiance of $50 \mu\text{mol m}^{-2} \text{ s}^{-1}$) and air humidity of 55–65 % for germination.

2.4 Low-temperature storage

Encapsulated nodal segments were transferred in Petri dishes containing agar medium and stored in a laboratory refrigerator at $4 \text{ }^\circ\text{C}$. Five different low-temperature exposure times (0, 1, 2, 4, 6 and 8 weeks) were evaluated for re-growth. After each storage period, encapsulated nodal segments were placed on MS medium with or without growth regulators for conversion into plantlets. The percentage of encapsulated nodal segments forming shoot were recorded after 0, 1, 2, 4, 6, 8 weeks of culture on re-growth media.

2.5 In vitro rooting and acclimatization

Individual synseed-raised microshoots (4–5 cm) were excised from culture and transferred to MS medium containing different concentrations of auxins, NAA or IBA (0.1, 0.5, 1.0 and 2.0 μM). Rooted plantlets were removed

from the culture medium, washed gently with tap water and transferred to thermocol cups (Shalimar Thermocol Products, Mumbai, India) containing sterile Soilrite™ (Keltech Energies Ltd., Bangalore, India), moistened with half-MS medium lacking organic supplements and placed under diffused light (16:8 h photoperiod) conditions. Potted plantlets were covered with a transparent polythene membrane to maintain high humidity and watered every 3 days for 2 weeks. The polythene membranes were removed after 2 weeks to acclimatize plants to field conditions. After 4 weeks, properly hardened plants were transferred to pots containing normal garden soil, maintained in a greenhouse conditions.

2.6 Photosynthetic pigment estimation

The chlorophyll (*a* and *b*) and carotenoid contents were determined after extraction of the pigment with 80 % acetone (Arnon 1949). Fresh mass of leaves (200 mg) was ground in small volumes of acetone solutions and filter with Whatman No. 1 filter paper. The extract obtained was diluted to a final volume of 10 ml. Absorbance of chlorophyll content at 663, 645 nm and carotenoid content at 480 and 510 nm was estimated on UV–VIS Spectrophotometer (UV-1700 Pharma Spec, Shimadzu, Kyoto, Japan).

2.7 Statistical analysis

All the experiments were conducted in a completely randomized block design (CRD) with ten explants per treatment and each treatment was repeated three times. The results are expressed as a mean \pm SE of three independent experiments. The cultures were observed periodically and morphological changes were recorded at regular intervals. Data on percentage of conversion of encapsulated nodal segments were recorded after 8 weeks of culture. Hardening of plantlets was evaluated after 4 weeks of transfer to pots and percentage of survival of plantlets was recorded for different potting mixtures. All the data were subjected to analysis of variance (ANOVA) followed by Duncan's multiple range test at $P = 0.05$ using SPSS software version 16 (SPSS Inc., Chicago, IL, USA).

3 Results and discussion

3.1 Synseed production and recovery of complete plantlet

Hydrogel encapsulation is the most successful and widely adopted system to produce synseeds; in the present study in vitro-raised nodal segments of *V. trifolia* were encapsulated in sodium alginate and calcium chloride. However,

the evaluation of the effects of various concentrations of sodium alginate and calcium chloride was prerequisite to standardize the preparation of characteristic beads. The encapsulated beads differed morphologically with respect to texture, shape and transparency with different combination and concentrations of sodium alginate (2–5 %) and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (25–200 mM). A 3 % Na alginate with 100 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ was found to be the best combination for hydrogel complexation which produced firm, clear and isodiametric beads or capsules in liquid MS exhibiting 84.9 % regeneration response on MS basal medium containing BA (5.0 μM) + NAA (0.5 μM) (Table 1; Fig. 1b). The lower concentration of sodium alginate (1 or 2 %) and CaCl_2 (25 or 50 mM) not only prolonged the ion exchange (polymerization) duration but also resulted in the formation of fragile beads that were difficult to handle. Sodium alginate preparations at lower concentrations (1, 2 %) were not suitable because beads were fragile and difficult to handle during transfer whereas, concentrations above 3 %, produced isodiametric beads which were hard enough to cause considerable delay in germination. This is in consonance with the findings of other plant species such as *Tylophora indica* (Faisal and Anis 2007); *Withania somnifera* (Fatima et al. 2013) and *Balanites aegyptiaca* (Varshney and Anis 2014). Nevertheless, an encapsulation matrix of 5 % sodium alginate with 50 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ was found most suitable for the formation of ideal beads in *Cannabis sativa* (Lata et al. 2009) which is contrary to our results. The synseeds prepared by dissolving sodium alginate in DDW failed to regenerate on all the treatments applied. The most desirable property of the encapsulated

Table 1 Effect of different plant growth regulators on regeneration of encapsulated nodal segments of *V. trifolia* after 6 weeks of culture on MS medium

PGRs (μM)	Regeneration response (%)	No. of shoots/bead	Shoot length (cm)
MS + BA (1.0)	67.5 + 0.2e	1.3 + 0.2e	1.3 + 0.1f
MS + BA (2.5)	69.5 + 0.2d	1.6 + 0.1de	2.1 + 0.1de
MS + BA (5.0)	72.4 + 0.7c	2.5 + 0.0c	2.8 + 0.1bc
MS + BA (7.5)	65.8 + 0.4f	2.0 + 0.1c	1.7 + 0.1e
MS + BA (1.0) + NAA (0.5)	70.4 + 0.2d	1.9 + 0.2d	2.4 + 0.1cd
MS + BA (2.5) + NAA (0.5)	77.9 + 0.5b	3.5 + 0.2b	3.1 + 0.0b
MS + BA (5.0) + NAA (0.5)	84.9 + 0.4a	5.9 + 0.1a	3.6 + 0.1a
MS + BA (7.5) + NAA (0.5)	78.1 + 1.0b	3.9 + 0.2b	1.1 + 0.0f

Data recorded after 6 weeks

Values represent mean \pm SE of three repeated experiments with ten replicates each. Means followed by the same letter within columns are not significantly different ($P = 0.05$)

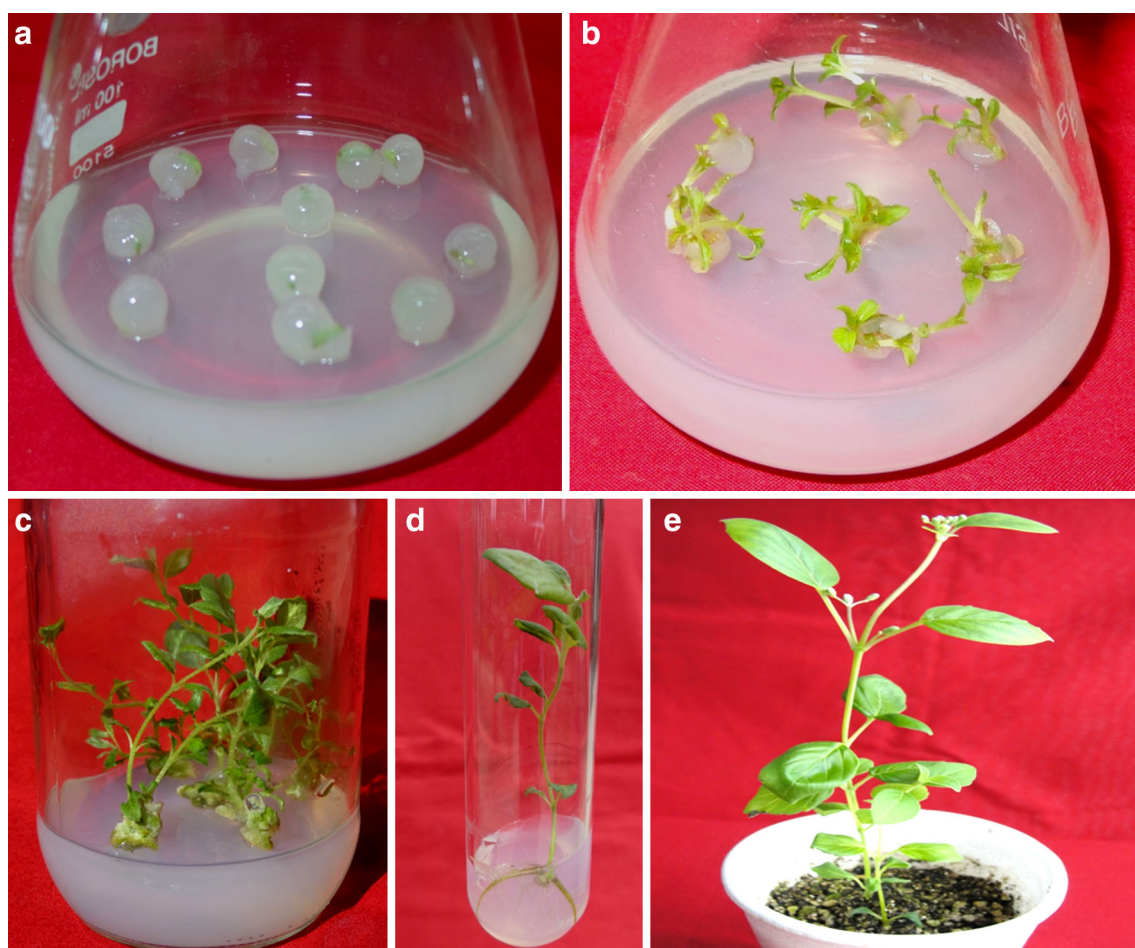


Fig. 1 Plant regeneration from encapsulated nodal segments of *V. trifolia*. **a** Artificial seeds of *V. trifolia* obtained by the encapsulation of nodal segments in 3 % sodium alginate and 100 mM calcium chloride. **b** Culture showing shoot emergence from synthetic

seeds after 2 weeks of culture. **c** Germinated synthetic seed with shoot on MS + BA (5.0 μ M) + NAA (0.5 μ M) after 6 weeks of culture. **d** In vitro rooting on MS + 0.5 μ M NAA. **e** Acclimatized plantlets derived from encapsulated nodal segments

explants is their capacity to retain viability in terms of regrowth and conversion abilities after encapsulation (Adriani et al. 2000; Micheli et al. 2007). In the present study, the ideal beads produced by encapsulating NS in 3 % sodium alginate and 100 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ were cultured on MS basal medium with various concentrations of BA either singly or in combination with NAA. Synseeds cultured on MS basal medium supplemented with BA (5.0 μ M) produced an average of 2.5 ± 0.0 shoots/bead was produced with 72.4 % regeneration response after 6 weeks of culture (Table 1; Fig. 1b). The regenerated microshoots failed to develop into complete plantlets on the same medium. Addition of NAA (0.5 μ M) with optimal concentration of 5.0 μ M BA also did not help in the induction of roots from the microshoots, conversely, further improved the regeneration response (84.9 %) (Table 1, Fig. 1c). Although capsula failed to induce rooting on regrowth media, therefore, an additional experiment was required to induce rooting in microshoots. The best rooting

was achieved on full-strength MS medium comprising 0.5 μ M NAA (Fig. 1d; Table 2). Similarly, shoots that developed from encapsulated buds of *Morus australis*, *Morus cathyana* and *Morus nigra* failed to root on any planting media tested. Rooting was induced from the regenerated shoots of *M. australis* and *M. cathyana* on half-strength MS medium containing 5.7 μ M IAA, 4.9 μ M indole-3-butyric acid (IBA) and 5.3 μ M IPA (indole-3-propionic acid), while that of *M. nigra* required only 4.9 μ M IBA (Pattnaik and Chand 2000). Gangopadhyay et al. (2005) devised a two-step method to achieve maximum recovery of complete plantlets from Ca alginate beads in *Ananus comosus*; first, shoots were retrieved from capsules and in the second step, microshoots were rooted in liquid medium (supplemented with IBA and Kn) supported with Luffasponge. Bekheet (2006) and Lata et al. (2009) achieved rooting in *Allium sativum* and *Cannabis sativa* on MS medium containing IAA and IBA, respectively. In contrast, Swamy et al. (2009) reported rooting on PGR-free

half-strength MS basal medium in microshoots retrieved from encapsulated nodal segments of *P. cablin*.

3.2 Low-temperature storage

Storage duration (1, 2, 4, 6 and 8 weeks) was also found to influence the regeneration frequency of encapsulated axillary buds at 4 °C. A highly desirable feature of encapsulated nodal segment is their ability to retain viability in terms of re-growth potential even after a considerable period of storage required for germplasm exchange. The effect of different storage duration on encapsulated nodal segment at 4 °C is summarized in Table 3. Short-term storage of germplasm of *Withania somnifera* at 4 °C has also been reported by Fatima et al. (2013). However, the temperature requirement for optimum viability varies from plant to plant. Generally, 4 °C temperature is found to be most suitable for alginate bead storage (Ahmad et al. 2012; Sharma et al. 2013; Parveen and Shahzad 2014). An alginate matrix also served as an artificial endosperm, thereby providing nutrients to the encapsulated explants for re-growth. Antonietta et al. (1999) reported that the synthetic endosperm should contain nutrients and a carbon source for germination and conversion.

The regeneration potential of the encapsulated explants reduced gradually and after 4 weeks of cold storage dropped to 74.5 %, after 6 weeks of culture. Beyond 4 weeks of cold storage, a sudden decline in regeneration potential was observed as after 8 weeks of storage only 42.5 % beads could show regeneration. This decline in the conversion response could be attributed to the inhibition of tissue respiration by the alginate matrix (Redenbaugh et al. 1984) or a loss of moisture due to the partial desiccation during storage (Danso and Ford-Lloyd 2003). Encapsulated

Table 2 Effect of NAA and IBA on rooting in synseed-raised microshoots of *V. trifolia* cultured on full-strength MS media after 4 weeks of culture

PGRs (μM)		% Response	Mean no. of roots per shoot	Mean root length (cm)
NAA	IBA			
0.0	0.0	0	0.0 ± 0.0 ^f	0.0 ± 0.0 ^d
0.1		80	3.4 ± 0.2 ^{de}	1.5 ± 0.1 ^{bc}
0.5		90	5.7 ± 0.4 ^a	2.1 ± 0.2 ^a
1.0		85	4.8 ± 0.4 ^b	1.7 ± 0.1 ^{ab}
2.0		65	3.9 ± 0.1 ^{cde}	1.5 ± 0.2 ^{bc}
	0.1	65	3.1 ± 0.3 ^e	1.0 ± 0.1 ^c
	0.5	80	4.6 ± 0.1 ^{bc}	1.9 ± 0.1 ^{ab}
	1.0	75	4.1 ± 0.4 ^{bcd}	1.6 ± 0.1 ^b
	2.0	60	3.2 ± 0.0 ^e	1.4 ± 0.1 ^{bc}

Values represent mean ± SE. Means followed by the same letter in the same column are not significantly different by the Duncan's multiple range test at 5 % probability level

Table 3 Effect of storage at 4 °C for different time periods on conversion of encapsulated and non-encapsulated nodal segments of *V. trifolia* on MS medium containing BA (5.0 μM) + NAA (0.5 μM)

Storage period (weeks)	Regeneration response (%)	No of shoots/bead	Shoot length (cm)
0	92.3 ± 0.1a	5.9 ± 0.1a	3.5 ± 0.2a
1	88.2 ± 0.1b	3.9 ± 0.0b	2.7 ± 0.1b
2	81.2 ± 0.1c	3.2 ± 0.1c	2.2 ± 0.0bc
4	74.5 ± 0.3d	2.0 ± 0.0d	1.8 ± 0.1 cd
6	65.1 ± 0.1e	1.5 ± 0.0e	1.4 ± 0.0de
8	42.5 ± 0.2f	1.0 ± 0.2f	1.1 ± 0.1e

Data recorded after 6 weeks

Values represent mean ± SE of three repeated experiments with ten replicates each. Means followed by the same letter within columns are not

Significantly different ($P = 0.05$)

nodal segments were viable up to (42.5 %), even after 8 weeks of cold-dark storage (Table 3). Our results are in corroboration with the earlier findings of Parveen and Shahzad (2014) who also reported the efficient conversion of encapsulated NS of *Cassia angustifolia* up to 4 weeks of cold storage at 4 °C.

3.3 Acclimatization

Fully developed *V. trifolia* plantlets with 4–5 fully expanded leaves and well-developed roots were successfully hardened off inside the growth room in a selected planting substrate for 4 weeks (Fig. 1e) and were eventually established in natural soil. Of the four different types of planting substrates examined, percent survival of the plantlets was highest (92 %) in soil rite (Table 4). About 92 % of the micro-propagated plants survived following transfer from soil rite to natural soil and did not show any detectable variation in respect to morphology or growth characteristics. This observation is in agreement with several earlier findings (Fatima et al. 2013; Parveen and Shahzad 2014).

3.4 Physiological changes during acclimatization

Unfortunately, ex vitro transfer in plant tissue culture-mediated micropropagation restricted by the high percentage of plants lost or damaged during the acclimatization to greenhouse or open field often limit the commercial application (Pospisilova et al. 1999). The plantlets are susceptible to various stresses. A switch to autotrophy and changes in stomata functioning and cuticle compositions has been reported during acclimatization (Huylenbroeck et al. 1998). Considering the importance of this step of acclimatization, various physiological parameters, such as chlorophyll and carotenoids contents were also studied.

Table 4 Evaluation of different planting substrates for hardening off synseed-raised plantlets of *Vitex trifolia* for their acclimatization and establishment in natural conditions

Planting substrates	No. of plants	No. of acclimatized plantlet transferred plants survival	(%)
Soil rite	25	23.0	92
Vermiculite	25	19.0	76
Vermiculite–garden soil mixture (1:1)	25	20.0	80
Garden soil	25	16.2	65

Data were recorded after 4 weeks of transfer to planting substrates

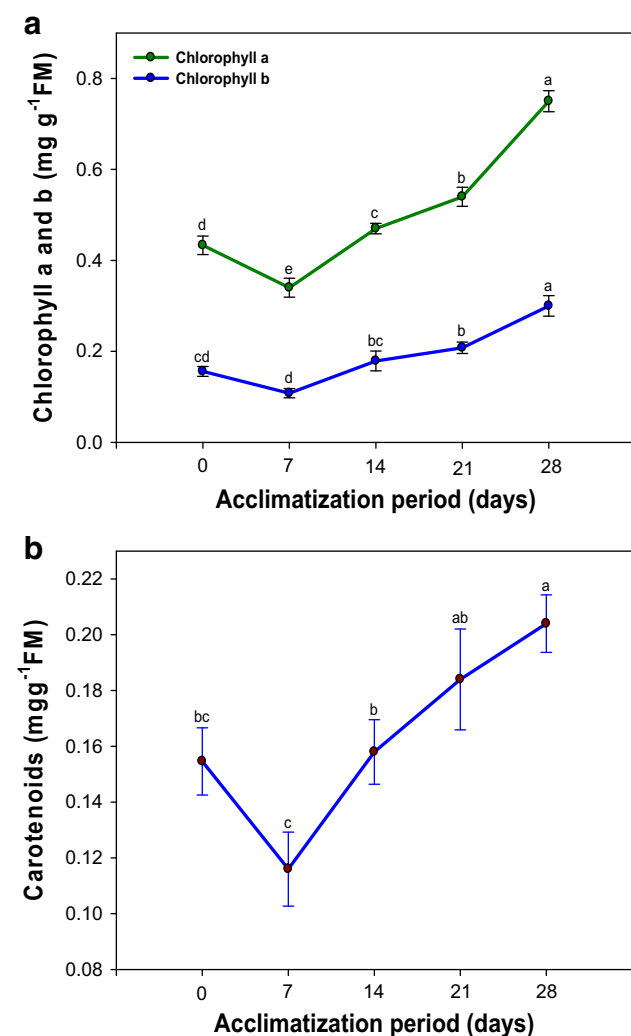


Fig. 2 Changes in levels of photosynthetic pigments (chl *a* and *b*) and carotenoid content and **a** Chlorophyll *a* and *b*, **b** Carotenoids in micropropagated plantlets of *V. trifolia*. Lines denoted by the same letter within response variables are not significantly different ($P = 0.05$) using Duncan's multiple range test

During transfer of tissue culture-raised plantlets from in vitro to ex vitro condition, the change in pigment concentration (chlorophyll and carotenoid contents) was estimated

and it was observed that with an increase in number of days of acclimatization, the pigment contents increased significantly. The Chl *a* content was low (0.43 ± 0.02) mg g^{-1} on 0 days of acclimatization whereas, it was increased up to (0.54 ± 0.02) mg g^{-1} after 21 days and was maximum (0.75 ± 0.02) mg g^{-1} at 28 days of transfer (Fig. 2a). Decrease in chlorophyll level during the first week of transplantation might be attributed to the poorly developed chloroplast and disorganized grana. Increment in pigment contents may be attributed to the induction of chlorophyll synthesis enzyme required for chlorophyll biosynthesis. Similar reports are available where an initial abrupt decrease in chlorophyll contents during the starting days followed by a continuous and subsequent increase was noticed as in *Ocimum basilicum* (Siddique and Anis 2008); *Coleus forskohlii* (Sahai and Shahzad 2013) and *Cassia alata* (Ahmed and Anis 2014a), *Vitex trifolia* (Ahmed and Anis 2014b) towards the final days of acclimatization.

Carotenoid plays an important role in protection of chlorophyll pigments under stress conditions (Kenneth et al. 2000) which might be generated during acclimatization. Carotenoid contents increased gradually during the period of transplantation. The maximum carotenoid level (0.20 ± 0.01) mg g^{-1} was observed after 28 days of acclimatization (Fig. 2b). Such an increase in carotenoids further reflects the functional response of photosynthetic apparatus to the different light environment, since the carotenoids play protective role in plants against photo-oxidative damage (Van Huylbroek et al. 2000).

4 Conclusion

In conclusion, the present encapsulation approach provides an alternative method for the short-term storage of encapsulated nodal segments, conservation, rapid multiplication and reintroduction of *V. trifolia* plants to natural condition. The protocol not only provides economy of time and space but also gives greater output and allows further augmentation of elite disease-free propagules. Encapsulated nodal segments of *V. trifolia* resumed growth immediately upon transfer to culture medium. Preservation of encapsulated explants is also simpler than cryopreservation and less labor intensive than conventional storage of non-encapsulated propagules under minimal growth conditions. The high frequency of plantlet retrieval from encapsulated nodal segments of *V. trifolia* after 4 weeks of storage at low temperature could be used as a delivery system for germplasm exchange and offers the possibility of using this method for ex situ conservation of this medicinal plant.

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