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## Direct Somatic Embryogenesis and Synthetic Seed Production from *Tylophora indica* (Burm.f.) Merrill an Endangered, Medicinally Important Plant

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**Abstract:** An efficient reproducible system for direct somatic embryogenesis from *in vitro* propagated leaf explants of *Tylophora indica* have been developed using Murashige and Skoog (MS) medium supplemented with BA (6-benzylaminopurine), NAA ( $\alpha$ -naphthaleneacetic acid) and Kinetin (6-furfurylaminopurine) alone or in combination. The induced embryos were used for the synthetic seed production. The main objective of present investigation is to introduce the artificial seed technology for *Tylophora indica*. The highest induction frequencies of somatic embryos were obtained on Murashige and Skoog (MS) medium supplemented with NAA 1.0 mg L<sup>-1</sup> and kinetin 2 mg L<sup>-1</sup> and 3% sucrose. The induced somatic embryoids showed all the stages of embryogenesis i.e., globular, heart and torpedo shaped structures. Sub-culturing of mature embryos lead to their germination on MS medium supplemented with NAA 1.0 mg L<sup>-1</sup> and kinetin 2 mg L<sup>-1</sup> with a germination frequency of 77.2%. Somatic embryos obtained directly on leaf explants were used for encapsulation in MS and ½ MS medium containing different concentrations of sodium alginate. The encapsulated somatic embryos are subsequently germinated into plantlets. A 3% sodium alginate dissolved in ½ MS medium provided a uniform encapsulation of the embryos with survival and germination frequencies 22.4% and plant conversion 6.8%. A synthetic seed technology significantly offers an excellent scope for propagation of rare hybrids, elite genotypes and genetically engineered plants. These systems could be useful for the rapid clonal propagation and dissemination of synthetic seed material of *Tylophora indica*.

**Key words:** Direct somatic embryogenesis, synthetic seed production, *Tylophora indica*

### INTRODUCTION

*Tylophora indica* (Burm.f.) Merrill, commonly called Antamul or Indian ipecac, is an endangered medicinal plant belonging to the family Asclepiadaceae. It is a perennial, branched climbing shrub found on plains, hilly slopes and the outskirts of the forests of eastern and southern India. The roots and leaves of this plant possess stimulant, emetic, cathartic, expectorant, stomachic and diaphoretic properties and are used for the treatment of bronchial asthma, inflammation, bronchitis, allergies, dermatitis, rheumatic and gouty pains, hydrophobia and also an excellent medicinal remedy for psoriasis, seborrhea, anaphylaxis, leucopenia in addition to an inhibitory property to Schultz-Dale reaction (Bentley and Trimen, 1992; Jayanthi and Mandal, 2001; Faisal and Anis, 2003). The powdered leaves and roots of this plant contain the alkaloids tylophorine and tylophorinine (Gopalakrishnan *et al.*, 1980). The roots also contain a potential anti-tumor alkaloid, tylophorinidine (Mulchandani *et al.*, 1971). Several pharmaceutical

companies (Acron Chemicals, Mumbai, India; Sabinsa Corporation, Piscataway, NJ, USA) are marketing *T. indica* extracts as antiasthmatic herbal drugs (Faisal and Anis, 2007). Phenanthroindolizidine based *Tylophora* alkaloids have been reported to have potential anti-tumour, anti-immune and anti-inflammatory activities (Gao *et al.*, 2007).

Due to its huge demand by pharmaceutical industry, the plant has been over exploited, leading to a marked decline in the population of this species. Also, the conventional methods of propagation and preservation of this plant is not yet successful and wild population becoming extinct. Due to lack of proper cultivation practices, destruction of plant habitats and illegal and indiscriminate collection of plants from natural habitats, many medicinal plants are severely threatened. *Tylophora indica* is one that rapidly disappearing and is now listed as vulnerable to extinction plant species in India. Previous studies reported that this plant being conserved through axillary shoot bud sprouting (Faisal and Anis, 2007), callus mediated somatic embryogenesis from leaf

(Jayanthi and Mandal, 2001) and Stem (Rao, 1970; Rao and Narayanaswamy, 1972) of *T. indica* as explants, whereas root tissue has been proven to be a highly regenerative explants for *in vitro* propagation in a small number of species (Vinocur *et al.*, 2000). *Tylophora indica* is also conserved to some extent through callus mediated shoot organogenesis, shoot regeneration using petiole explants, somatic embryogenesis, secondary metabolite production and genetic transformation was also reported (Jayanthi and Mandal, 2001; Faisal *et al.*, 2005, 2006). Moreover propagation either by seed or by vegetative cuttings is rather difficult. Stem cuttings failed to produce proper root when treated with different growth regulators (Chandrasekhar *et al.*, 2006a,b).

Propagation through tissue culture offers a viable alternative for this species because it can also be used as a complimentary strategy for conservation and utilization of genetic resources. The present investigation reported here was carried out to establish the rapid and extensive propagation of *Tylophora indica* through direct somatic embryogenesis from leaf explants without involving a callus phase. We are also reporting a method for developing synthetic seeds that can convert to plants under *in vivo* conditions. In addition, the effect of storage on encapsulated embryos was investigated. The results of present investigation provide a practical means of mass clonal propagation for this vulnerable medicinal shrub. Hence, the produced synthetic seeds meet the requirement of pharmaceutical industries for the production of plant based medicines.

## MATERIALS AND METHODS

**Plant material:** Leaf segments (1.0 cm) of *Tylophora indica* served as explants. These were harvested from *in vitro* regenerated plantlets which had originated from leaf explants, cultured on MS medium.

**Medium composition and cultural conditions:** The basal medium consisted of MS mineral salts and vitamins supplemented with BA 1-2 mg L<sup>-1</sup> or kinetin 1-4 mg L<sup>-1</sup> alone or along with NAA 0.1-1 mg L<sup>-1</sup> and 3% sucrose (Table 1). The pH was adjusted to 5.6±0.2 before

sterilization by autoclaving at 121°C for 20 min. The growth regulators (BA, NAA and Kinetin) were filter-sterilized prior to being added to the autoclaved culture media. All cultures were maintained in a growth chamber set at 25°C, 70% relative humidity and a 16/8 h (light/dark) photoperiod with light supplied by cool-white fluorescent lamps at an intensity of 50-60 µmol m<sup>-2</sup>sec<sup>-1</sup>.

**Somatic embryo development:** Somatic embryos were induced within 4 weeks on MS media supplemented with NAA 1.0 mg L<sup>-1</sup> and kinetin 2 mg L<sup>-1</sup> (Table 1). The induced calli showed all stages of embryogenesis. *viz.*, globular, heart and torpedo shaped structures as well as small plantlets. Explants with globular embryos were subsequently transferred to MS media supplemented with various concentrations and combinations of BA, NAA and Kinetin for further growth and development of the somatic embryos. Maturation and germination of the somatic embryos were tested on the media with subculturing every 2 weeks. Six explants were cultured per petridish containing MS medium supplemented with various combinations and concentrations of BA, NAA and Kinetin. The number of globular, mature and germinated embryos formed directly on the surface of the explants were counted and recorded at the end of each subculture. Frequencies were the means of four experiments using 30 explants in each treatment.

**Encapsulation:** The somatic embryos (10 to 30 day old) induced from leaf explants were encapsulated in calcium alginate to get synthetic seeds. For encapsulation purposes, we tested 1.0, 2.0, 3.0 and 4.0% sodium alginate (w/v) in MS salt solution as solvent. Four weeks after, the beginning of a standard subculture on ½ MS medium, we isolated embryos (1.5-2.0 mm) by drawing them with suction into a 4-mm (internal diameter) pipette containing 0.15-0.20 mL of alginate mixture. Single-coated somatic embryos were then dropped into a solution of CaCl<sub>2</sub> (50, 60 and 80 mM) (w/v). Each drop containing a single embryo was maintained in CaCl<sub>2</sub> for 10, 30 and 60 min. Following the incubation periods, the beads were recovered by decanting the CaCl<sub>2</sub> and washing three times with MS basal medium supplemented with 3% sucrose.

Table 1: Effect of growth regulators on somatic embryogenesis from leaf explant on MS medium

Sl. No.	Growth regulators (mg L <sup>-1</sup> )			% of explants showing embryo genesis	Average number of somatic embryos/culture			Average number of embryoids converted into plantlets
	NAA	BA	Kn		Globular shaped embryo	Heart shaped embryo	Torpedo shaped embryo	
1	0	1	0	64.4±0.8 <sup>a</sup>	48.4±1.0 <sup>d</sup>	26.1±0.8 <sup>d</sup>	15.0±0.6 <sup>e</sup>	8.10±0.2 <sup>d</sup>
2	0	2	0	62.6±0.4 <sup>d</sup>	49.8±0.6 <sup>e</sup>	29.4±0.3 <sup>e</sup>	18.1±0.2 <sup>e</sup>	7.04±0.2 <sup>e</sup>
3	0	0	1	65.8±1.0 <sup>e</sup>	47.8±0.6 <sup>de</sup>	26.4±0.3 <sup>d</sup>	16.8±0.1 <sup>d</sup>	9.16±0.3 <sup>e</sup>
4	0.1	0	4	77.2±0.9 <sup>a</sup>	60.7±1.7 <sup>a</sup>	38.4±0.7 <sup>a</sup>	27.8±0.1 <sup>a</sup>	12.40±1.0 <sup>b</sup>
5	1.0	0	2	74.3±0.2 <sup>b</sup>	58.4±0.8 <sup>b</sup>	36.6±0.1 <sup>b</sup>	26.8±0.4 <sup>b</sup>	13.60±0.1 <sup>a</sup>

The data represent the means±standard error of 5 replicates was subjected to DMRT followed by ANOVA with significance of p<0.05

Non-encapsulated embryos were used as controls. Encapsulated and control embryos were transferred into vials containing peat and perlite (3:1) and placed under continuous light (cool-white fluorescent lamps; intensity:  $30 \mu\text{mol m}^{-2}\text{sec}^{-1}$ ) for germination evaluation. The survival and germination of both encapsulated and control embryos were recorded after 4 weeks on soil.

**Storage experiments of encapsulated somatic embryos:**

Two experiments were carried out to evaluate the effect of storage on the capacity of encapsulated embryos for germination. The encapsulated embryos were either directly transferred onto soil containing peat and perlite, or they were kept in petridishes sealed with parafilm and left for 30 days or 60 days in the dark at room temperature (20-22°C). The cold treatment (4°C) was applied to encapsulated and non-encapsulated embryos for 30 or 60 days. Following storage, the embryos were transplanted to vials containing peat and perlite (3:1) and incubated under the growth chamber conditions described earlier. Non-stored embryos (encapsulated or not) were placed directly into the vials. The cultures were evaluated after 4 weeks for germination.

**Statistical analysis:** For the direct somatic embryogenesis experiments, 30 explants were used and each experiment was repeated thrice. The data shown represent the mean±standard error of four independent experiments. ANOVA was performed on the results of each experiment and the data were analyzed using DMRT ( $p < 0.05$ ).

For the synthetic seed experiments, 30 seeds were used per treatment and each treatment was repeated thrice. Storage experiments were repeated thrice, each with 30 embryos per treatment. Data were analyzed by one-way ANOVA. The statistical significance of differences between means was estimated at the 5% level by the t-test.

## RESULTS

**Induction of somatic embryogenesis:** Leaf explants from 3 month old aseptically grown *T. indica* were initially cultured on MS medium supplemented with BA  $1-2 \text{ mg L}^{-1}$  or kinetin  $1-4 \text{ mg L}^{-1}$  alone or along with NAA  $0.1-1 \text{ mg L}^{-1}$ . An optimal response for direct somatic embryo induction from leaf explants were obtained after 7 days on MS medium supplemented with NAA  $0.1 \text{ mg L}^{-1}$  and kinetin  $4 \text{ mg L}^{-1}$  with 77.2% (Table 1). When smeared and viewed under microscope after 40 days, numerous small white, clumps of somatic embryos appeared on outer periphery of the friable calli

within 4 weeks. The induced calli showed all stages of embryogenesis. *viz.*, globular, heart and torpedo shaped structures as well as small plantlets (Fig. 1a-e). Globular somatic embryos developed directly from leaf explants were maintained on MS medium supplemented with kinetin  $4 \text{ mg L}^{-1}$  for regeneration (Fig. 1b).

**Maturation, germination of somatic embryos and plantlet regeneration:**

The induced globular, heart and torpedo shaped somatic embryos were matured on MS medium supplemented with NAA  $1.0 \text{ mg L}^{-1}$  and kinetin  $2.0 \text{ mg L}^{-1}$  at an average of 12.4 embryoids per explants after 4 weeks of inoculation (Fig. 3a, b). The induced single embryos germinated and regenerated into plantlets with both shoots and roots after 1 month of culture on MS medium supplemented with NAA  $0.1 \text{ mg L}^{-1}$  and Kinetin  $3 \text{ mg L}^{-1}$  (Fig. 1e).

**Encapsulation and germination of encapsulated embryos:**

Somatic embryos (10 to 30 day old) induced directly from leaf explants were encapsulated in calcium alginate to get synthetic seeds. Somatic embryos encapsulated in 2% sodium alginate resulted in the formation of soft beads that were not suitable for handling. At a higher concentration of sodium alginate (4%), beads were harder but germination did not occur. At low concentrations of sodium alginate, uniform, sufficiently firm beads were not formed, resulting in a reduced frequency of germination. An optimal encapsulation, germination and subsequent plant conversion of somatic embryos occurred optimally with 3% sodium alginate prepared in MS salt solution and submerged for 30 min in 50 mM  $\text{CaCl}_2$  for hardening produced seeds with a coat firm enough for handling and also allowed the seeds to convert to plantlets (Fig. 2). 30 days old, somatic embryos responded efficiently for germination ( $20.2 \pm 4.8\%$ ) and subsequent plant conversion ( $5.2 \pm 1.3\%$ ) compared to other stages tested. Efficient plant conversion with  $22.4 \pm 2.8\%$  germination having ( $6.8 \pm 1.2\%$ ) conversion into plantlet from encapsulated somatic embryos was achieved on  $\frac{1}{2}$  strength MS medium compared to full-strength MS medium (Table 2). Non encapsulated somatic embryos (Control) cultured under identical condition exhibited  $80.6 \pm 1.2\%$  survival and germination rate than the encapsulated material.

The concentration of  $\text{CaCl}_2$ , also affected the germination frequency of encapsulated embryos. Although the complexing time was shorter (10 min) when 60 mM or 80 mM  $\text{CaCl}_2$  was used instead of 50 mM  $\text{CaCl}_2$  (30 min), the germination frequency of encapsulated embryos treated with the former was significantly reduced.

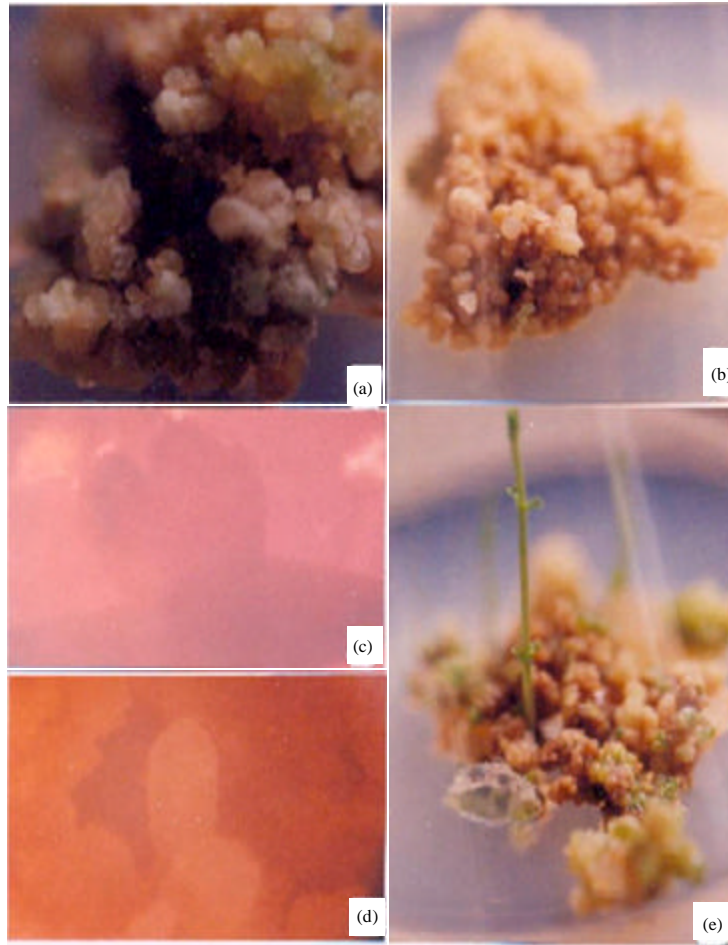


Fig. 1(a-e): Somatic embryogenesis from leaf explants of *Tylophora indica*, (a) Embryogenic calli, (b) Globular and heart shaped embryos, (c) L. S. of heart shaped embryo, (d) Torpedo staged embryo and (e) Shoot regeneration from somatic embryos

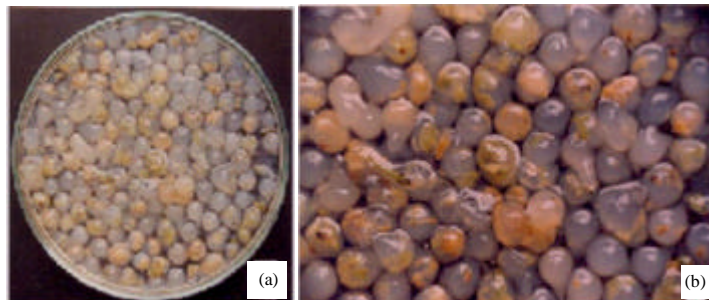


Fig. 2(a-b): Synthetic seeds of *Tylophora indica*. (a) Synthetic seeds and (b) Magnified view of synthetic seeds

The duration of exposure to  $\text{CaCl}_2$  during the hardening process significantly affected the frequency of germination from encapsulated embryos. After 10 min, although beads did form, they were not firm as strong

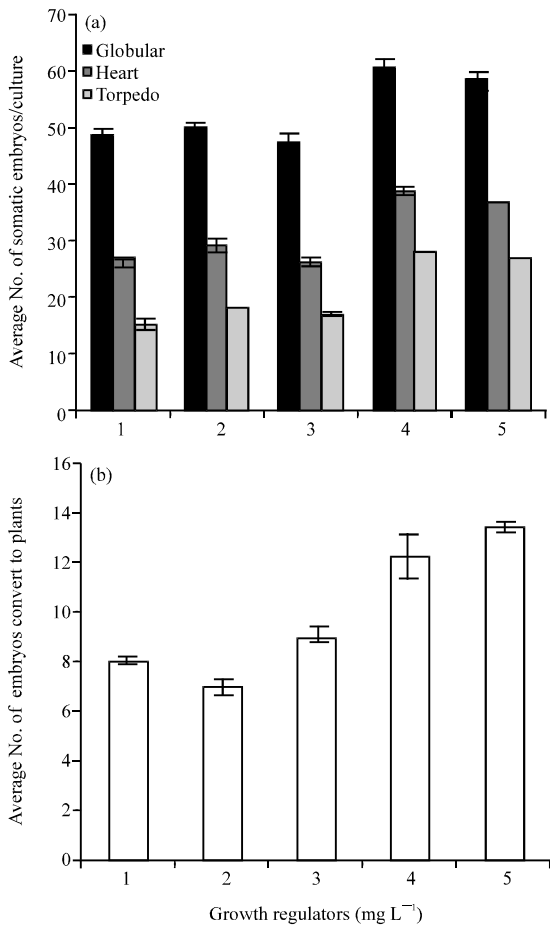


Fig. 3(a-b): Effect of growth regulators on % of somatic embryogenesis from leaf explants of *Tylophora indica* on MS medium. (b) Effect of growth regulators on Somatic embryogenesis from leaf explants on MS medium

Table 2: Germination and conversion of different stages of somatic embryos of *Tylophora indica* encapsulated in calcium alginate

Age (days)	MS medium		½ MS medium	
	Germination (%)	Conversion (%)	Germination (%)	Conversion (%)
10	Nil	Nil	Nil	Nil
15	Nil	Nil	Nil	Nil
20	09.2±2.5	Nil	10.1±2.8	Nil
30	20.2±4.8	5.2±1.3	22.4±2.8	6.8±2.4

Data were analyzed by T-test followed by one-way ANOVA. The significance of differences between means was estimated at the 5% level

enough to facilitate transfer with forceps, while after a 1 h exposure, the beads tended to be very hard. Ultimately leading to a lower percentage of regeneration into plantlets.

**Storage experiments:** After 30 or 60 days of storage at room temperature (20-22°C), the survival and germination frequencies of encapsulated embryos had fallen from 80% to 15% when compared with encapsulated embryos directly transferred on soil containing peat and perlite. The present investigation revealed that low temperature (4°C) storage gave promising results for survival and germination frequencies for both naked and encapsulated embryos relative to those of non-stored control embryos but the reduction was much greater for naked embryos. In contrast, the germination frequency of encapsulated embryos after a 60 days storage was lower than that after a 30 days storage at 4°C. The plantlets germinated were morphologically identical to the donor material and developed normally under greenhouse conditions.

### DISCUSSION

A new procedure for synthetic seed technology was reported through the direct somatic embryogenesis from an *in vitro* propagated leaf. All the stages of the somatic embryos from globular shaped through cotyledonary embryos developed on the surface of the leaf when *in vitro* regenerated leaf inoculated on to MS medium supplemented with NAA 1.0 mg L<sup>-1</sup> and kinetin 2 mg L<sup>-1</sup>. The leaf explants were more efficient in somatic embryogenesis than any other explants (Ipekci and Gozukimizi, 2003). The somatic embryos induced from leaf explants of *Tylophora indica* were successfully regenerated but in some plants somatic embryos do not germinate into complete plantlets (Mohanraj *et al.*, 2009). In such cases, synthetic seeds can be produced from leaf explants for cost-effective mass clonal propagation, potential long-term germplasm storage and delivery of tissue-cultured plants (Andlib *et al.*, 2011).

The globular structures induced from leaf was passed through heart, torpedo and cotyledonary developmental stages with an efficacy of 25% globular structures converted into cotyledonary embryos, similar results were reported for *Pennisetum americanum* with 1 mg L<sup>-1</sup> 2, 4-D at early stages of embryo development (Chandrasekhar *et al.*, 2006a) but there is no response obtained with 2, 4-D in combination with TDZ for somatic embryogenesis of *Paspalum scrobiculatum* (Vikranth and Rashid, 2002) (Fig. 1).

The successfully induced somatic embryos, separated from inoculated explant during late maturation stage were encapsulated as artificial seeds. Encapsulation of somatic embryos was influenced by the concentration of sodium alginate and calcium chloride. The highest rate of synthetic seed production and plantlet regeneration was achieved with 3.0% sodium alginate and 2.5%

CaCl<sub>2</sub>·2H<sub>2</sub>O. The embryos encapsulated in sodium alginate prepared in MS salts were germinated on ½ MS medium, whereas MS medium supplemented with BAP and IBA was also suited for germination was also reported (Andlib *et al.*, 2011). There are some reports that the addition of ½ MS nutrients in the gelling matrix enhanced the germination and conversion frequency in *Carica papaya* (Castillo *et al.*, 1998). Sodium alginate preparation at lower concentration (1-2%) becomes unsuitable for encapsulation, probably because of reduction in its gelling capacity. At higher concentrations of sodium alginate (4-5%), beads were hard which may have suppressed the emergence of shoots and roots. The differential responses may be due to a synergistic effect of alginate and calcium chloride concentrations, respectively. The present investigation results for artificial seed production with sodium alginate and calcium chloride have also been supported by previous reports on different plant species such as on Orchid (Saiprasad and Polisetty, 2003), *Hibiscus moscheutos* (West *et al.*, 2006), *Rauvolfia tetraphylla* (Faisal *et al.*, 2006), *Coelogyne breviscapa* (Mohanraj *et al.*, 2009) etc.

The development of artificial seed production technology is an efficient and alternate method for the propagation of commercially important plant species (Saiprasad and Polisetty, 2003). Non-encapsulated somatic embryos of *Tylophora indica* responded by plantlet regeneration under *in vitro* conditions on MS medium supplemented with NAA (1 mg L<sup>-1</sup>) and Kinetin (2 mg L<sup>-1</sup>), however their encapsulation helps for storage, transport and exchange of germplasm between laboratories and pharmaceutical industries with at wide distances.

During present investigation, encapsulated somatic embryos showed higher resistance to storage at 4°C than non-encapsulated embryos but the complete germination rate of the former was reduced to half after 2 months storage when compared to non stored encapsulated embryos. Exceptionally the storage life of synthetic *Alfalfa* seeds is quite short even at 4°C (Ipekci and Gozukimizi, 2003). Generally the stored encapsulated embryos resulted in the reduction of their germination rates but encapsulated and non-encapsulated somatic embryos of interior and black sprouts survived 1 month of storage at 4°C with no loss in germination capacity (Lulsdorf *et al.*, 1993). The decline in plant recovery from stored encapsulated somatic embryos may be due to both oxygen deficiency in the calcium alginate beads and its rapid drying (Redenbaugh *et al.*, 1987; Bazinet *et al.*, 1992).

## CONCLUSION

The present investigation was concentrated for the standardization of protocols established for an efficient direct somatic embryogenesis from *in vitro* regenerated leaf explants of *Tylophora indica* and production of its synthetic seeds. The effect of plantlets regenerated from encapsulated somatic embryos on MS medium incorporated with plant growth regulators was observed. Preserving the viability of encapsulated embryos even after 40-60 days of storage at low temperature offers the possibility of using synthetic seed technology for short-term germplasm conservation of this medicinally important plant species. Such work could be useful for germplasm preservation, micropropagation of diseases free plant species and also for production of their synthetic seeds. The artificial seed technology is an ultimate source for the conservation of an important medicinal plant species in order to prevent the somaclonal variations which are common for general micropropagation protocols.

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