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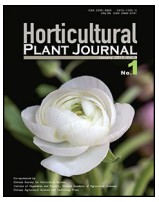

Artificial Seed Production of *Tylophora indica* for Interim Storing and Swapping of Germplasm

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A B S T R A C T

Our research work demonstrates the single bead alginate-encapsulation, interim storing and conversion of *Tylophora indica* (Burm. Fil.) Merrill. Most effective encapsulation of *in vitro* nodal segments [(4 ± 1) mm long], ensuing in sphere-shaped artificial seeds of similar morphology, was achieved through 75 mmol·L^{-1} calcium chloride ($CaCl_2 \cdot 2H_2O$) plus 3% (w/v) Na-alginate with 93.3% conversion frequency. The earliest conversion (within 7 days of incubation) of artificial seeds occurred in half-strength liquid Murashige and Skoog medium. Among the three different temperature regimes [(5 ± 1) °C, (15 ± 1) °C, and (25 ± 1) °C], storage of artificial seeds at (15 ± 1) °C executed the highest frequency of conversion (90%) after 15 days of storage. Lengthier storage significantly reduced the conversion frequency of artificial seeds irrespective of storage temperature. Nevertheless, the conversion frequency after 30 days of storage at (15 ± 1) °C was recorded at 70% without further decline even following 45 days of storage, which evidently suggests that lower temperature (15 ± 1) °C is apt for storage and subsequent conversion of *T. indica* artificial seeds. The present protocol could be expedient for short-term storing and swapping of *T. indica* germplasms between national and international laboratories.

Keywords: *Tylophora indica*; calcium chloride; conversion; encapsulation; germplasm swap; Na-alginate; nodal segment

1. Introduction

Tylophora indica (Burm. Fil.) Merrill is a threatened medicinal herb with several bioactive compounds such as alkaloids, saponins, tannins and flavonoids (Rao et al., 1971; Benjamin and Mulchandani, 1973). Usually, *Tylophora* is propagated through seeds; nonetheless, propagation by direct sowing of seeds in the field has been unsuccessful and germination frequency of sun-dried and stored seeds is poor (Thomas and Philip, 2005). Propagation by means of natural seeds might prove even unreliable, since variation in alkaloidal yield is under genetic control and might get reduced in successive progenies through adverse gene recombination.

Since recent past, the alginate-encapsulation-based artificial seed development became a competent option together with bulk-propagation plus interim storing of several medicinal plants with industrial importance (Gantait et al., 2015). This technique delivers a cost-effective and large-scale plant production system (Saiprasad and Polisetty, 2003). Hassle-free handling of plant germplasms during carrying or storing without losing the viability is the additional benefit of artificial seeds (Ghosh and Sen, 1994; Standardi and Micheli, 2012; Sharma et al., 2013). Alginate coating on the plant parts shields the tissues from mechanical damage and prevents unfavorable drying throughout storing (under optimized environment) and swapping of germplasms (Ara et al., 2000; Gantait et al., 2012; Reddy et al., 2012). There has been some

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discussion on alginate encapsulation in combination with its storage system but published work on *Tylophora* is limited to two only (Faisal and Anis, 2007; Devendra et al., 2011). None of these two works reported on encapsulation of *in vitro*-regenerated nodal segments (NS). Considering such perception, the involvement of a refined artificial seed production technique could be convenient, aiming at the distribution of this germplasm (which acts as the exclusive source of tylophorine) amid several research and extension centers along with pharmaceutical industries.

Considering this backdrop, the objectives of our present research were: to study the effect of Na-alginate (NA) and calcium chloride (CC) for development of artificial seeds through encapsulation of *in vitro*-derived NS explants; and to determine ambient temperature for storing of artificial seeds ensuring maximum post-storage conversion.

2. Materials and methods

2.1. Collection of plant material and initial establishment of in vitro culture

Ex vitro NS measuring 2–3 cm in length were isolated from 6-month-old *Tylophora* plants at Medicinal Plant Garden, Narendrapur Ramakrishna Mission Ashrama, Kolkata, India for *in vitro* multiple shoot cultures during November 2015. Collected explants were surface sterilized following the method reported by Gantait and Sinniah (2013) in the Plant Tissue Culture Laboratory of Ramakrishna Mission Vivekananda University, Kolkata, India. Surface sterilized and washed explants were inoculated in 500 mL culture vessels containing 30 mL of MS semi-solid medium (Murashige and Skoog, 1962) complemented by 3% (w/v) sucrose along with 2.5 $\mu mol \cdot L^{-1}$ N_6-benzyladenine (BA) + 0.5 $\mu mol \cdot L^{-1}$ α-napthalene acetic acid (NAA) + 100 $mg \cdot L^{-1}$ ascorbic acid (Faisal et al., 2007). The medium was attuned to pH 5.8 and was autoclaved for 15 min at 1.1 $kg \cdot m^{-2}$ (121 °C). The NS-derived multiple shoot cultures were maintained in the same medium at (25 ± 1) °C under 16 h photoperiod of 60 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetic photon flux density delivered by cool fluorescent tubes (Philips Lifemax, PHILLIPS, India). After 40 days of culture the multiple shoots were isolated from the clump and individual NS were excised and employed for encapsulation.

2.2. Alginate-encapsulation

In vitro NS [(4 ± 1) mm long)] were drenched for 10 min with autoclaved-sterilized 2%, 3%, 4% and 5% (*w/v*) NA gelling matrix dissolved in calcium-free 1/2 MS liquid medium. For development of artificial seeds, the aliquots (0.2 mL approx.) of NA solution, each containing single NS, were taken aseptically by a Pasteur pipette (5 mm-diameter) and softly dropped one-by-one in 100 mL of 75 or 100 $mmol \cdot L^{-1}$ autoclaved CC ($CaCl_2 \cdot 2H_2O$) liquid. For optimum polymerization the droplets were kept as such for 30 min in CC. The CC solution was then decanted and the artificial seeds were double-washed with sterile water. Immediately after, the artificial seeds were dried on filter paper and were incubated onto 15 mL 1/2 MS liquid medium (drenched in cotton) (Gantait et al., 2017) containing 3% sucrose (*w/v*), at the culture condition mentioned in section 2.1. The days required for the artificial seed conversion (extrusion of shoot and/or roots) and the conversion frequency (percentage) were recorded up to 4 weeks, based on daily observation.

2.3. Storage of artificial seeds

Artificial seeds were transferred into sterilized microcentrifuge tubes (Eppendorf, USA), each tube containing 5 seeds. Tubes were stored at temperature regimes of (5 ± 1) °C, (15 ± 1) °C, and (25 ± 1) °C for 15, 30, and 45 days without illumination. For storing at (5 ± 1) °C and (15 ± 1) °C, tubes were kept in apposite sections of refrigerator whereas for (25 ± 1) °C the tubes were maintained in culture room.

2.4. Assessment of post-storage conversion of artificial seeds

To assess the after-storage conversion efficiency following distinct storage periods, the artificial seeds were sowed in 1/2 MS liquid medium (drenched in cotton) having 3% sucrose (*w/v*) and maintained for 4 weeks at similar culture condition (section 2.1.). All the development and handling of artificial seeds along with their culture processes were accomplished in complete sterilized environments under laminar airflow chamber.

2.5. Statistical assessment

Experimentations were replicated thrice comprising 5 samples for each replication following completely randomized design. Data were statistically assessed through One-way analysis of variance (ANOVA). Percent data were transformed using *arc sine* preceding ANOVA then were reversed to original scale (Compton, 1994). Treatment data (Means ± *SE*) were evaluated based on Duncan's multiple range test (DMRT) (Duncan, 1955) ($P \leq 0.05$) through SPSS 19 software.

3. Results

3.1. Development of artificial seeds through alginate encapsulation

In our investigation (Fig. 1), concentrations of NA lower than 3% were not suitable as the beads were formed without a definite shape, too soft to handle and even lost the gelling ability (Fig. 2, A,E). For this reason, 2% NA (Fig. 2, A,E; Fig. 3, A,E) couldn't be considered owing to deformed bead in spite of showing the highest conversion frequency. Very firm, clear, isodiametric beads of viable, uniform size and shape, were obtained at and above the concentration of 3% NA upon complexation with both 75 $mmol \cdot L^{-1}$ and 100 $mmol \cdot L^{-1}$ CC (Fig. 2, B–D,F–H). The conversion frequency was higher in those artificial seeds, which were polymerized with 3% NA in 75 $mmol \cdot L^{-1}$ CC (*i.e.* 93.3%) in comparison to 100 $mmol \cdot L^{-1}$ CC (76.6%) (Fig. 1; Fig. 3, B,F). However, at higher (4%) NA concentrations, the conversion frequencies declined further to 46.6% and 13.3% at 75 $mmol \cdot L^{-1}$ and 100 $mmol \cdot L^{-1}$ CC, respectively (Fig. 3, C,G). At 5% NA, artificial seeds were most hard and formed tails (Fig. 2, D,H) and the conversion frequency was the lowest (23.3% at 75 $mmol \cdot L^{-1}$ CC and 3.3% at 100 $mmol \cdot L^{-1}$ CC) (Fig. 1; Fig. 3, D,H). Our investigation showed the polymerizing ability of NA varied markedly at different concentrations (2%–5%) when used to encapsulate the *in vitro*-derived NS.

3.2. Post-storage conversion of artificial seeds

With a preliminary assessment of post-storage conversion following 15 days of storage at (15 ± 1) °C we observed

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