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RETRIEVAL OF PLANTLETS FROM CRYOPRESESRVED ALGINATE BUDS OF *NYCTANTHES ARBOR-TRISTIS* L.- AN EFFECTUAL APPROACH FOR GERMPLASM CONSERVATION

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ABSTRACT

The escalating exploitation of medicinal plants for the extraction of imperative pharmaceuticals amalgam dictated the need of using encapsulated vegetative propagules for germplasm conservation and propagation. The present report is the first attempt in the production of synseed and subsequently affirming retrieval of healthy plantlets of *Nyctanthes arbor-trisits* L. In the current exploration, best encapsulated nodal segments were developed in calcium alginate gel composed of 3% (w/v) sodium alginate and 100 mM $CaCl_2.2H_2O$. Maximum conversion response (76.66%) with maximum number of shoots (4.33 ± 0.57) was obtained on MS medium augmented with KN (2.5 µM) + NAA (0.5 µM). These encapsulated axillary buds ensured greatest retrieval (86.66%) of shoots after a period of 4 weeks spend in cold storage storage at 4 °C. Microshoots were then excised and rooted in half-strength MS medium (pretreated with 200 µM IBA). Well-developed plantlets were acclimatized successfully with 90% survival frequency.

INTRODUCTION

Nyctanthes arbor-tristis (Harsinghar) is one of the eminent medicinal plant belongs to the family-Oleaceae. It is a small tree native to southern Asia, widely distributed throughout India, grows upto 10 m in height with a flaky grey bark. It is also cultivated in gardens as an ornamental tree for its fragrant white flowers with bright orange corolla tube (Anonymous 2001). Iyer *et al*. (1999) investigated chemically the extract of plant which verified the presence of high amount of iridoid arbortristoside- A which is a major principle constituent of this plant, known to possess prominent anticancerous activities. The plant expresses varied pharmacological and biological behavior like antibacterial, antioxidant, anti-inflammatory, antipyretic, antimalarial and antidiabetic activities (Priya and Ganjewala 2007). The leaves and bark extracts can be engaged for the cure of a number of ailments like asthma, rheumatism, sciatica, piles, chronic fever, cough, inflammation, constipation, baldness premature graying of hairs, cancer and various nervous disorders.

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As a result of overexploitation, devastation of its habitation and uncertain troubles of seed viability and germination, the natural strands of this priority plant have been strikingly reduced (Rout *et al*., 2008). Hence, it is needed to extend measures for conservation of species from disappearance. Firming establishment of gene banks for ex situ conservation of plant germplasm in the form of field gene banks, seed gene banks, in vitro collections, and cryogenically preserved tissues is a recurrent procedure. The advances in competent means of biotechnology guarantee safe protection of elite plant species. The progress of biotechnology leads to the production of a new category of germplasm including clones obtained from elite genotypes, cell lines with special attributes and genetically transformed material. Synthetic seed technology has received significant awareness as a budding cost effective clonal propagation scheme. Fabrication of artificial seeds has unraveled new vista in plant biotechnology. The technology is designed to combine the advantages of clonal propagation with those of seed propagation and storage. Potential advantages of synthetic seeds include their designation as ‘‘genetically identical materials’’, ease of handling and transportation, along with increased efficiency of in vitro propagation in terms of space, time, labor and over-all cost (Nyende *et al*., 2003).

Earlier attempts of micropropagating *Nyctanthes* were based only on its rapid multiplication (Rout *et al*., 2008; Jahan *et al*., 2011 a and b) but neither of them were focused on germplasm conservation. The present study was designed with a particular outlook to develop an ex situ preservation strategy intended for *Nyctanthes arbor-tristis* plant for short term storage, stable supply and exchange of quality materials between laboratories. To the best of our knowledge this is the first report on synthetic seed production.

MATERIALS AND METHODS

Plant material and Explant Source

Nodal segments (approximately 1.0 cm long) excised from established regenerated shoots of *N. arbor-tristis* L. (Jahan *et al*., 2011a) were used as explants.

Encapsulation matrix

Sodium alginate (Qualigens, India) at different concentrations (w/v) (1, 2, 3, 4 and 5%) was added to liquid Murashige and Skoog's medium (MS 1962). For complexation, 25, 50, 75, 100 and 200 mM $CaCl_2.2H_2O$ solution (Qualigens, India) was prepared using liquid MS medium. The pH of the gel matrix and the complexing agent was adjusted to 5.8 prior to autoclaving at 121 °C for 20 min.

Encapsulation

Encapsulation was accomplished by mixing the nodal segments with sodium alginate solution using a pipette and by dropping them in $CaCl_2.2H_2O$ solution. The droplets containing the explants were held atleast for 20 min to achieve polymerization of the sodium alginate. The calcium alginate beads containing the nodal segments were retrieved from the solution with a tea stainer and rinsed twice with autoclaved distilled water to remove the traces of $CaCl_2.2H_2O$ and transferred to sterile filter paper in petridishes for 5 min under the laminar airflow cabinet to eliminate the excess of water and thereafter planted onto petridishes containing nutrient medium.

Planting media and culture conditions

The encapsulated nodal segments (calcium alginate beads) were transferred to wide mouth culture flask (Borosil, India) containing both Murashige and Skoog's medium (MS 1962) or MS medium supplemented with plant growth regulators viz., Benzyladenine, Kinetin or α-naphthalene acetic acid either singly (BA or KN) or in combination with auxin (NAA) as specified in results. The media were designated as A1 (MS + Kn (2.5 µM)), A2 (MS + BA (2.5 µM)), A3 (MS + Kn (2.5 µM) + NAA (0.5 µM)) and A4 (MS + BA (2.5 µM) + NAA (0.5 µM)). The culture medium was gelled with 0.8% (w/v) agar and pH was adjusted to 5.8 prior to autoclaving at 121 °C for 20 min. For root induction, individual microshoot isolated from regenerated encapsulated nodal segments was pulse treated for 24 h with 200 µM indole-3-butyric acid (IBA) followed by their transfer to ½ strength MS medium by the method adopted by Jahan *et al* (2011 b). Cultures were maintained at 24 ± 2 °C under 16/8 h light-dark conditions with a Photosynthetic Photon Flux Density (PPFD) of 50 $\mu molm^{-2}s^{-1}$ provided by cool white fluorescent tubes.

Low temperature storage

A set of 10 encapsulated nodal segments were transferred to MS basal medium (50 ml medium) and stored in refrigerator at 4 °C. Seven different low temperature exposure times (0, 1, 2, 3, 4, 5 and 6 weeks) were evaluated for regeneration. After each storage period, encapsulated nodal segments were cultured on MS medium supplemented with plant growth regulators (Kn (2.5 µM) + NAA (0.5 µM)) for their regeneration. Plantlets were formed within 4 weeks when microshoots were excised from above said culture and incubated in 200 µM indole-3-butyric acid (IBA) for 24 h followed by their shift to ½ strength MS medium. The percentage of encapsulated nodal segments forming shoots was recorded after 4 weeks of culture.

Acclimatization

Plantlets with well developed roots and shoots were removed from the culture medium and washed gently under running tap water to remove any adherent gel from the roots and transferred to thermocups containing sterile soilrite. These were kept under culture room conditions (16:8h photoperiod) covered with transparent polythene bags to ensure high humidity, irrigated after every three days with half strength MS salt solution (without vitamins and sucrose) for two weeks. Polythene membranes were removed after 2 weeks in order to acclimatize plantlets and after 4 weeks they were transferred to pots containing garden soil or a mixture of garden soil and vermicompost (1:1) and maintained in greenhouse under normal day length conditions (30 ± 2 °C; 60-65% RH).

Statistical analysis

All the experiments were conducted with a minimum of ten replicates per treatment and repeated three times. The data were analyzed statistically using SPSS Ver.10 (SPSS Inc, Chicago, USA). The significances of differences among means was carried out using Duncan's multiple range test at P = 0.05. The results are expressed as a means ± SE of three repeated experiments.

RESULTS AND DISCUSSION

Formation of ideal beads

The success of retrieving plants from encapsulated nodal beads depends upon its calcium-alginate mixture. The formation of firm and uniform beads encapsulating nodal segments was influenced by different concentrations and combinations of sodium alginate (1, 2, 3, 4 and 5%) and $CaCl_2.2H_2O$ (25, 50, 75, 100 and 200 mM). Gel encapsulation using sodium alginate and calcium salt was a useful technique as a method for encapsulation as they forms a good combination because the ions are non-damaging materials easy to use, have a low price and embryo to plant conversion occurs successfully (Redenbaugh *et al*., 1991). However, the formation of best firm, clear and uniform beads was achieved

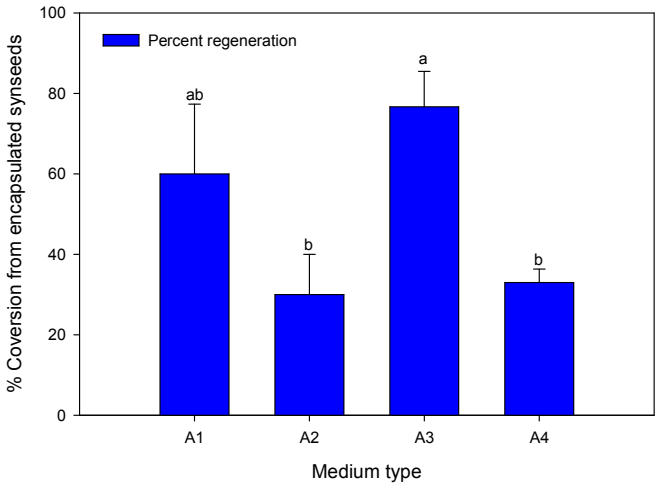
by using 3% sodium alginate solution complexed with 100 mM $CaCl_2.2H_2O$ for 30 min ion exchange duration (Fig 1 A). The present results corroborates with the findings of Mishra *et al*. 2011, Ahmad *et al*. 2012 and Varshney and Anis 2014 where 3% sodium alginate combined with 100mM $CaCl_2.2H_2O$ proved best for obtaining ideal beads. Higher concentrations of sodium alginate (4 and 5%) and $CaCl_2.2H_2O$ (200 mM) were not found suitable as the resulting beads were hard which results in considerable delay in germination while lower concentrations of both sodium alginate (1 and 2%) and $CaCl_2.2H_2O$ (25, 50 and 75 mM) results in fragile beads.

Plant regeneration from encapsulated nodal beads

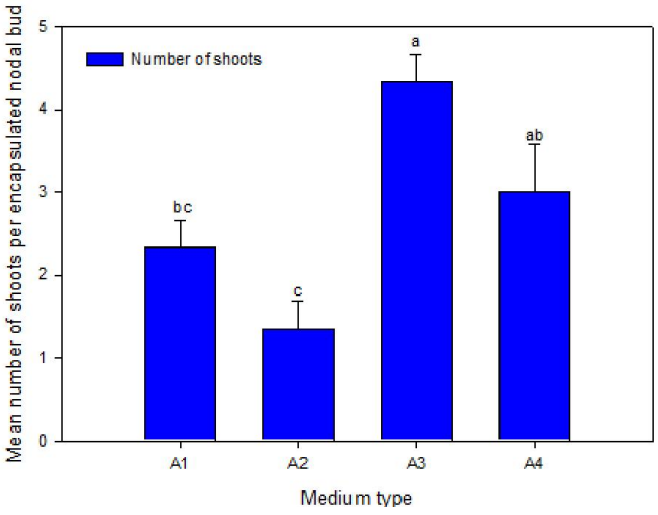
A new strategy of using unipolar plant propagules for the production of synthetic seed was added in this technology where somatic embryos are difficult to produce. In such cases, synthetic seed production from nodal segments can be used for cost-effective mass clonal propagation system, enduring germplasm storage practice, and for deliverance of tissue-cultured plants. Earlier, artificial seeds are most commonly described as encapsulated somatic embryos. But now, the concept of synthetic seeds has been set free from its bonds to somatic embryogenesis and links the term not only to its use (storage and sowing) and product formation (plantlet) but also to other techniques of micropropagation like organogenesis and enhanced axillary bud proliferation system. Apart from somatic embryos, other explants such as shoot tips, axillary buds have also been used in preparation of artificial seeds.

There are various advantages of artificial seeds such as better cloned plants could be propagated similar to seeds, preservation of rare plant species extending biodiversity could be realized, breeding of plants in which propagation through normal seeds is not possible, genetic uniformity, easy storage and transportation and more consistent and synchronized harvesting of important agricultural crops would become a reality, among many other possibilities. In addition, ease of handling, potential long-term storage and low cost of production and subsequent propagation are other benefits (Ravi and Anand, 2012). Axillary shoot buds were suitable for encapsulation studies as they were excellent plant material for the preparation of synthetic seeds, besides possessing great potential from the pre-existing meristematic tissues that ensures the genetic stability of regenerants.

Encapsulated axillary buds of *Nyctanthes arbor-tristis* show signs of shoot regrowth after 2 weeks of culture on MS medium supplemented with different cytokinins–auxin regimes either with BA (2.5 µM) or Kn (2.5 µM) alone or in combination with NAA (0.5 µM) as depicted in garph 1 and 2. The best conversion response (76.66%) with maximum number of shoots (4.33 ± 0.33) was obtained on MS medium augmented with A3-Kn (2.5 µM) + NAA (0.5 µM) after 4 weeks as evident from graph 1 and 2 (Fig. 1B). After 8 weeks, well developed shoots were observed on this medium which was phenotypically normal with distinct nodes and internodes. The encapsulated beads cultured on other medium showed the emergence of weak and stunted shoots. Micropropagation through encapsulation of vegetative propagules has also been reported in a few medicinally important plant species like *Spilanthus acmella* (Singh *et al*., 2009), *Eclipta alba* (Singh *et al*., 2010), *Ruta graveolens* (Ahmad *et al*., 2012) and *Balanites aegyptiaca* (Varshney and Anis, 2014).



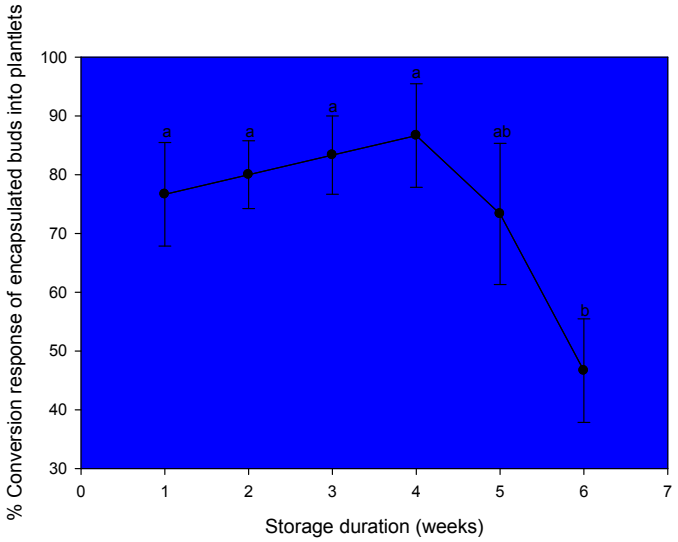
Graph 1. Effect of different media on conversion of encapsulated nodal segments of *Nyctanthes arbor-tristis* after 4 weeks of culture on MS medium. The bars represents data of percent regeneration from encapsulated synseeds which are not significantly different (P = 0.05) using DMRT



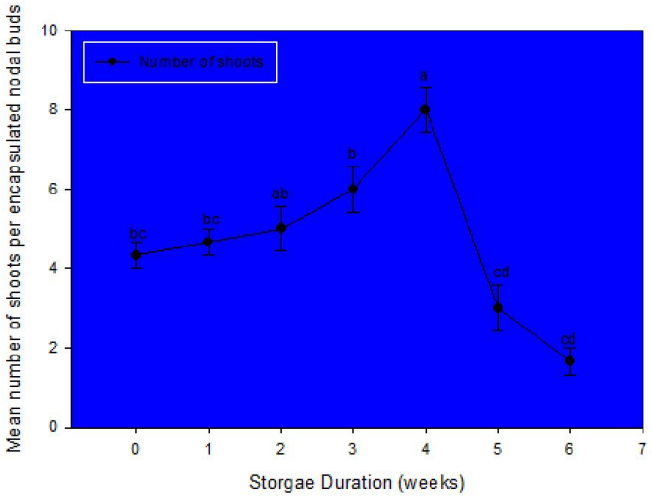
Graph 2. Effect of different medium on in vitro shoot formation from sodium-alginate encapsulated nodal segments of *N. arbor-tristis* after 4 weeks of culture. The bars represent mean ± SE. Bars denoted by the same letter within response variables are not significantly different (P = 0.05) using DMRT

Low temperature storage

One of the most remarkable features of encapsulated synseeds is their aptitude to preserve viability in terms of sprouting and conversion potential even after a considerable period of storage, which is essential for their use in germplasm exchange or field application. The alginate covering provides necessary ingredients which served as an artificial endosperm, thereby providing nutrients to the encapsulated explants for regrowth. The conversion response of encapsulated nodal segments of *Nyctanthes* after 1, 2, 3, 4, 5 and 6 weeks of storage duration at 4 °C are accessible in graph 3 and 4.



Graph 3. Effect of low temperature storage (4 °C) on % conversion of encapsulated nodal segments into plantlets of *N. arbor-tristis* on A_3 medium. The line represent mean conversion response of buds and these variables are not significantly different (P = 0.05) using DMRT



Graph 4. Effect of storage duration on conversion of encapsulated nodal buds into shoots of *Nyctanthes arbor-tristis* after 4 weeks of culture. Lines represent means ± SE. Means followed by the same letter within columns are not significantly different (*P=0.05*) using Duncan's multiple range test

As observed from data, synseeeds stored at 4 °C resulted in high rate of shoot proliferation (86.66%) producing maximum number of shoots (8.00 ± 0.57) if the beads containing nodal segments were transferred back to regenerative media containing MS medium supplemented Kn (2.5 µM) + NAA (0.5 µM) within 4 weeks (Fig. 1C). During cold storage, encapsulated nodal segments requires no transfer to fresh medium, thus reduces the cost of maintaining germplasm cultures (West *et al*., 2006). Our results are in accordance to the reports of Faisal *et al*. (2006) on *Rauvolfia tetraphylla*, Singh *et al*. (2010) on *Eclipta alba*, Ahmad *et al*. (2012) on *Ruta graveolens* and Varshney and Anis (2014) on *Balanites aegyptiaca* where encapsulated nodal buds of these plants successfully regenerated after different periods of storage at 4 °C.

However, longer storage duration for more than 4 weeks significantly decreased the growth and multiplication of explants. Conversion response of encapsulated axillary buds decreased gradually after storage at 4 °C when the storage duration was increased beyond 4 weeks. The decline in the conversion of encapsulated propagules stored at low temperature may be due to inhibited respiration of plant tissues because of alginate cover as described by Redenbaugh *et al*. (1991).

Fig. 1. A. Encapsulated nodal buds of *N. arbor-tristis* in calcium-alginate mixture; B. Emerging shoot buds from encapsulated beads on MS + Kn (2.5 µM) + NAA (0.5 µM) after 4 weeks; C. Multiplication of shoots obtained from stored encapsulated nodal buds at 4 °C for 4 weeks after being transferred back to regenerative media i.e., MS + Kn (2.5 µM) + NAA (0.5 µM); D. An *in vitro* rooted plantlet of *Nyctanthes arbor-tristis* on ½ strength hormone free MS media after being treated with 200 µM IBA for 24 hrs; E. Acclimatized plantlet obtained from synthetic seeds of *Nyctanthes arbor-tristis*

Rooting and Acclimatization

The success of a micropropagation protocol depends strongly on the rooting efficiency of regenerated shoots and their subsequent acclimatization to the field condition. *De novo* formation of root meristems involves complex changes in the metabolism and it is evident that endogenous factors interact in developmental shift leading to adventitious root formation both at biochemical and at molecular levels (Caboni *et al*., 1997). Auxins played a major role in rooting process and their efficiency depends on several factors such as the affinity for auxin receptor protein involved in rooting, the concentration of free auxin that reaches target competent cells, the amount of endogenous auxin and the metabolic stability (De Klerk *et al*. 1999). A two step rooting procedure is involved here by involving a short term exposure of microshoots to high concentration of IBA (200 µM) for 24 hrs followed by their

transfer to hormone free ½ strength MS medium stimulates root differentiation within 4 weeks (Fig. 1D). The distinctiveness of IBA which proves its efficiency is its slow movement and slow degradation that facilitates its better localization near the site of application and thus proves its better function in inducing roots (Nickell and Kirk-Othmer, 1982). The auxin IBA is being widely used for such procedures as an effective plant growth substance as employed by Feyissa *et al*. (2005) in *Hagenia abysscinica*, Siddique and Anis (2007) in *Cassia angustifolia*, Singh *et al*. (2008) in *Simmondsia chinensis* and Jahan *et al*. (2011 b) in *Nyctanthes arbor-tristis*. Plantlets with well developed fully expanded leaves were successfully hardened off inside growth room in a sterile soilrite with 90% survival rate (Fig 1 E). The micropropagated plantlets survived did not show any morphological detectable differences in their characteristics.

Conclusions

The currently designed encapsulation procedure was the first concrete attempt of synthetic seed production in *Nyctanthes arbor-tristis* using axillary buds as vegetative propagules for encapsulation studies. The approach described here makes available an alternative system for the conservation, rapid multiplication and reintroduction of *N. arbor-tristis* plants to natural state. Artificial seeds offer great opportunity to store plants with single outstanding combination of genes that could not be maintained by conventional approaches due to genetic recombination exists in every generation for seed multiplication. The successful retrieval of plantlets from cold stock seeds indicates that this method could be used for preservation for germplasm for future use and to exchange between laboratories. The protocol described here offers great potential not only for rapid multiplication of existing stock of plant species but also for the conservation of important elite and endangered germplasm.

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