Plant Cell, Tissue and Organ Culture (PCTOC) (2022) 149:281–293
https://doi.org/10.1007/s11240-022-02244-1

ORIGINAL ARTICLE

# Germplasm conservation of economically important medicinal plant *Nyctanthes arbor-tristis* L. through encapsulation technique and maintenance under slow growth condition

**Awadhesh Kumar Mishra[1] · Kavindra Nath Tiwari[1] · Pallavi Mishra[1,2] · Sunil Kumar Mishra[3] · Shailesh Kumar Tiwari[2]**

Received: 17 June 2021 / Accepted: 28 January 2022 / Published online: 5 February 2022


## Abstract

An efficient encapsulation and slow growth conservation protocol was developed for *Nyctanthes arbor-tristis* L. an antiviral medicinal plant of the family Oleaceae. A gel matrix with 3% sodium alginate and 100 mM calcium chloride ($CaCl_2 \cdot 2H_2O$) was found best for the encapsulation of nodal segments. The viability and shoot development potential of encapsulated nodal segments was optimized. Encapsulated nodal segments stored at 4 °C and 24 °C remained viable for up to 90 days and showed shoot development potential 42.89 ± 6.04% and 33.53 ± 7.15% respectively. Nodal segments maintained under slow growth conditions up to 180 days on one-eighth strength Murashige and Skoog (MS) medium supplemented with 0.5% sucrose was suitable for satisfactory viability (40.28 ± 2.04%), while further addition of 0.5 mg/l abscisic acid supported 40.36 ± 1.01% viability of the nodal segments. The best rooting response was achieved on half-strength MS medium supplemented with 4 mg/l indole-3-acetic acid. The field survival of rooted plants was 65%. The clonal fidelity of *in-vitro* derived plantlets was studied with start codon targeted primer profile, which showed the same banding mobility patterns as the source parent plant. The maximum banding profile was monomorphic and consistent, confirming the clonal stability of regenerated plants. The method developed will permit the *in-vitro* conservation of this species and facilitate an easy exchange of plant germplasm.

## Key message

3.0% sodium alginate and 100 mM $CaCl_2$ used for encapsulation of nodes of *N. arbortristis* L. For germplasm conservation nodal segments stored under slow growth conditions upto 180 days.

**Keywords** *Nyctanthes arbor-tristis* L. · Encapsulated propagules · Start codon targeted primer · Slow growth · Abscisic acid

## Abbreviations

| | |
|---|---|
| ABA | Abscisic acid |
| BAP | 6-Benzylaminopurine |
| IAA | Indole-3-acetic acid |
| MS | Murashige and Skoog |
| SA | Sodium alginate |
| SCoT | Start codon targeted |

Communicated by Ranjith Pathirana.

✉ Kavindra Nath Tiwari
kntiwari@bhu.ac.in; kntiwaribhu@gmail.com

Awadhesh Kumar Mishra
av84.mishra@gmail.com

Pallavi Mishra
pallavimishra23@gmail.com

Sunil Kumar Mishra
skmishra.phe@iitbhu.ac.in

Shailesh Kumar Tiwari
tiwarishailu@gmail.com

[1] Department of Botany, MMV, Banaras Hindu University, Varanasi, Uttar Pradesh 221005, India

[2] Crop Improvement Division, Indian Institute of Vegetable Research, Shahanshapur, Varanasi 221305, Uttar Pradesh, India

[3] Department of Pharmaceutical Engineering and Technology, Indian Institute of Technology, Banaras Hindu University, Varanasi, Uttar Pradesh 221005, India

## Introduction

Medicinal and aromatic plants are widely used in the traditional medicinal systems. The summation of over exploitation, long-term exposure to environmental stresses and current climate change affects the survival of valuable medicinal and aromatic plant species and place them at the risk of extinction (Joshi et al. 2019). Their conservation is hence urgently required and tissue culture techniques have focused on practical implementation and provide immediate strategies to conserve the medicinal plant species under controlled conditions (Singh et al. 2010).

Encapsulation technology offers an efficient means for conservation of desired plant species (Reddy et al. 2012). Encapsulated vegetative propagules can be used as an alternative to seeds for propagation (Chand and Singh 2004). Encapsulation also provides a viable tool for *in-vitro* germplasm conservation. During encapsulation embryonic or non-embryonic tissues are capable of conversion into plantlets either *in-vitro* or *ex-vitro* (Rihan et al. 2017). Earlier, the concept of encapsulated propagules was traditionally based on the encapsulation of somatic embryos which can be handled like a real seed for transport, storage and sowing (Rihan et al. 2017). However, in recent years the encapsulation of nodal explant was employed as a suitable alternative for germplasm maintenance (Prakash et al. 2018). This method is used for encapsulation of propagules in a wide range of species including ornamental (Hatzilazarou et al. 2020) and medicinal plants (Gupta et al. 2014). The recovery of plants through encapsulated propagules has been reported in other medicinally important plant species like *Sterculia urens* (Subhashini et al. 2014) and *Urginea altissima* (L.f.) Baker (Baskaran et al.2018).

Encapsulation technology is a convenient method for conservation of plant genetic resources without apparent risk of genetic instability using minimum space, less labour and maintenance costs and it is easy in transference (Rai et al. 2009). Short-term storage of germplasm can be achieved using encapsulated propagules at low temperature (Parveen and Shahzad 2014). An alternate method for storage of germplasm is to maintain the explants under slow growth conditions based on the manipulation of culture conditions and culture media which allow cultures to remain viable under slow growth rate (Zayova et al. 2017). Conservation under minimal growth conditions can be achieved by reducing the incubation temperature and modifying the culture medium composition (Tyagi et al. 2009). These storage techniques are applicable to a wide range of medicinal plant species and extend the ordinary subculture duration from a few weeks to several months.

*Nyctanthes arbor–tristis* is a valuable medicinal plant of the family Oleaceae. It is a woody, perennial deciduous small tree having tetragonal twigs and propagated through seeds. It is native to sub-tropical Himalayas of Nepal and southern parts of India along with South East Asian countries such as Thailand, Malaysia and Indonesia on rocky ground in dry hillsides and undergrowth in dry-deciduous forest (Agrawal and Pal 2013). The flower of *N. arbor-tristis* is axillary or terminal consisting of groups of two to seven flowers having two sub-sessile anthers inserted near the mouth of cylindrical orange red centered white corolla tube, with bifid cylindric style and has good fragrance (Mishra et al. 2016). Flowering generally occurs from late September to December, it blooms late in the evening and floral scents attract the insects for pollination (Raizada and Nangia 1989). The plant has a broad spectrum medicinal properties like ameliorative (Mishra et al. 2018) and antiviral (Gupta et al. 2005) activities. Due to over exploitation coupled with poor seed viability and germination, it is depleted in natural habitats (Sagar and Singh 2004). For germplasm conservation, Jahan and Anis (2015) reported storage of encapsulated nodal explants of *N. arbor-tristis* at low temperature (4 °C) for limited periods up to 6 weeks only. Against this background, it was required to develop a suitable protocol for the conservation of this species. To our knowledge, this is the first report on conservation of *N. arbor-tristis* by storage of encapsulated nodal segments and maintaining the nodal segments under slow growth conditions for a prolonged duration.

The objective of this study was to conserve the encapsulated nodal segments and to optimize the efficacy of their conversion into complete plantlets. Thus, we standardized short to medium term storage of encapsulated nodal segments and developed an approach to maintain the nodal segments under slow growth conditions. Recovery of the plants from stored nodal segments was optimized, and the clonal fidelity of in-vitro derived plants was established by start codon targeted (SCoT) molecular markers. SCoT markers are reproducible markers based on short conserved region in plant genes surrounding the ATG translation start or initiation codon (Collard and Mackill 2009).

## Materials and methods

### Preparation and source of explant

Immature fresh green seeds of *N. arbor-tristis* were sampled from ayurvedic garden of Institute of Medical Sciences (IMS), Banaras Hindu University, Varanasi, Uttar Pradesh, India (NL25°16′23″ and EL82°59′50″) in the month of January. A voucher specimen (DG/15/124) was preserved in herbarium library, Department of Dravyaguna, IMS, BHU for further reference. The seeds were sterilized by washing under running tap water and followed by agitating in 1% cetrimide solution for 5 min,

then they were treated with 0.1% (w/v) $HgCl_2$ (Hi-media, Mumbai, India) solution for 5 min and washed (six times) with sterile-distilled water. Murashige and Skoog (1962) nutrient medium(15 ml) supplemented with 3% sucrose (w/v) (Hi-media, Mumbai, India) and 0.8% (w/v) agar (Hi-media, Mumbai, India) was poured into 30 ml culture tubes and autoclaved (1.06 kg $cm^{-2}$) at 121 °C for 15 min. Immature embryos excised from sterilized seeds were aseptically inoculated on growth regulator free full-strength MS medium. The cultures were kept under a 16 h/8 h light/dark photoperiod at a light intensity of 50 µmol $m^{-2}$ $s^{-1}$ irradiance from cool-white fluorescent tubes (Philips, India) at 24 ± 2 °C. After 28 days of culture, the nodal segments (4–8 mm long) were excised from actively growing young seedlings and served as explant source for encapsulation and for slow growth conservation.

## Encapsulation of nodal segments

For encapsulation, solutions of different concentrations of sodium alginate (SA) (1.0, 2.0, 3.0, 4.0% w/v) (HiMedia-Mumbai, India) and calcium chloride ($CaCl_2 \cdot 2H_2O$) (HiMedia-Mumbai, India) (25, 50, 75 and 100 mM) were prepared using liquid MS medium. The pH of gel matrix (SA) and complexing agent (Calcium chloride) was adjusted at 5.8 and autoclaved at 121 °C at 1.06 kg $cm^{-2}$ for 15 min. For optimization of encapsulation protocol, (1) nodal segments (4–8 mm long) mixed in different concentrations (1, 2, 3 and 4%) of SA were poured gently with sterile pipette tips (7 mm) in the solution of 100 mM calcium chloride and (2) nodal segments mixed in 3% SA were poured gently with sterile pipette tips in different concentrations (25, 50, 75, 100 mM) of calcium chloride solution. Nodal segments containing droplets were left for 20 min with occasional shaking in the calcium chloride solution to achieve complete polymerization and proper cross-linking of SA. Thereafter, nodal segments encapsulated in isodiametric, easy-to-handle beads were selected, washed 3–4 times with autoclaved distilled water, and dried on sterile filter paper in Petri-dishes (Borosil, India) to remove the excess of water. These encapsulated nodal segments, considered as encapsulated seeds, were used for further study.

## Shoot development potential of encapsulated nodal segments on BAP supplemented medium

For optimization of efficient shoot development potential, encapsulated nodal segments were inoculated on MS medium supplemented with different concentrations (0.0–10.0 mg/l) of 6-Benzylaminopurine (BAP). The medium without BAP was used as control. Data of shoot development potential of the encapsulated nodal segments were recorded at the end of 4 weeks of culture in terms of percent shoot development potential, number and length (cm) of shoots.

## Short-term storage of encapsulated nodal segments and shoots development potential

Erlenmeyer flasks (Borosil, India) containing solid MS medium without sucrose were used for the storage of encapsulated nodal segments, at 4 °C and 24 °C for different periods (30, 60, 90 and 180 days) under dark conditions. At the end of storage duration, encapsulated nodal segments were transferred for shoot development to MS medium with 3% sucrose (w/v), 0.8% (w/v) agar and 5.0 mg/l BAP. The percent shoots development potential of encapsulated nodal segments and the number of shoots per encapsulated bead were recorded at the end of four weeks from culture initiation.

## Maintenance of nodal segments under slow growth conditions and recovery of shoots

Nodal segments were cultured on full-strength, half (1/2), quarter (1/4) and one-eighth (1/8th) strength MS medium supplemented with 0.5, 1.5 and 3.0% sucrose. Cultures were incubated for 30, 90 and 180 days at 24 ± 2 °C under a 16 h/8 h light/dark regime, under the illuminance of 50 µmol $m^{-2}$ $s^{-1}$ from white fluorescent tubes (Phillips, India). After 30, 90 and 180 days storage, the number of shoot /explant and length of shoots (cm) were recorded. To confirm the shoot recovery potential of nodal segments after 180 days storage, they were sub-cultured on shoot recovery medium (Full-strength MS medium + 3% sucrose + 5.0 mg/l BAP) for re-growth. Data on the number shoots/explant and length of shoots were recorded at the end of four weeks from culture initiation.

## Effect of abscisic acid (ABA) on slow growth storage of nodal segments and recovery of shoots

To study the effect of abscisic acid (ABA), nodal segments were cultured on 1/8th strength MS medium containing 0.5% sucrose and supplemented with four concentrations (0.0, 0.5, 1.0 and 2.0 mg/l) of ABA. ABA was filter sterilized through 0.45 µm Millipore filter before adding to the sterilized medium. Cultures were incubated for 30, 90 and 180 days at 24 ± 2 °C under a 16 h/8 h light/dark regime, with the illuminance of 50 µmol $m^{-2}$ $s^{-1}$ from white fluorescent tubes (Phillips, India). After storage, the number of shoots/

node and lengths of shoots were recorded. To confirm the shoot recovery potential of nodal segments after 180 days storage, nodal segments were transferred onto shoot recovery medium (full-strength MS medium supplemented with 3% sucrose and 5.0 mg/l BAP) for regrowth. Data based on regrowth, the responding frequency (%), mean number shoot/explant and mean length of shoots was recorded at the end of four weeks from culture initiation.

### In-vitro rooting, hardening and acclimatization of plantlets

For in-vitro rooting, green, actively growing healthy shoots (4-5 cm) developed from germinating encapsulated nodal segments or slow growth conserved nodal segments on shoot recovery medium were used. Individual shoots were cultured on half-strength MS medium supplemented with 1, 2, 4, 6 and 8 mg/l Indole-3-acetic acid (IAA), and medium without IAA was used as control. The root induction frequency (%), number of roots/shoot and roots length were noted after four weeks from culture initiation. The complete plantlets with 1 cm long roots were removed from rooting medium, washed gently with sterile distilled water to remove attached media on roots, and transferred to plastic cups containing a 1:1:1 mixture of sterilized commercial soil-rite (Kel Perlite, Vishwas Nagar, Karnataka, India), garden soil and sand. The cups were covered with clear polythene bags to maintain high humidity and irrigated daily with 1–2 ml of sterilized MS salt solution upto six days. The plants were maintained in a culture room at 24 ± 2 °C and 16-h/day illuminance of 20 µmol $m^{-2}$ $s^{-1}$ from cool-white fluorescent tubes. After two weeks bags were partially opened. The acclimatized juvenile plantlets were transferred to the potting mixture above and kept in a greenhouse with controlled temperature (27 °C) and natural sunlight conditions in to the open field.

### Assessment of clonal stability

For assessment of clonal stability, leaves were taken from acclimatized plants and frozen immediately in liquid nitrogen, were used for DNA extraction. A comparison between the start codon targeted (SCoT) profile of the source parent plant and five acclimated plants was performed. Doyle and Doyle (1990) method with modification was used for the isolation of DNA. Clonal fidelity was assessed by the PCR based 14 SCoT primers (SCoT 5, 6, 8, 9, 10, 11, 24, 25, 26, 27, 28, 29, 30, 31) analysis. The gel was prepared with 0.8% agarose and stained with ethidium bromide dye. The quality and quantity of isolated DNA were checked by Dyna Quant 200 Fluorimeter. The PCR reaction consists of 75 ng of template DNA, 0.5 µM primer, 2.5 mM $MgCl_2$, 0.2 mM dNTP (GeNeiTM, Bangalore, India) in 25 µl reaction volume, 2U Taq DNA polymerase (GeNeiTM, Bangalore, India) along with suitable buffer (100 mM Tris–Cl; pH 9.0, 50 mM KCl, 1 percent Triton X-100). A total of 35 cycles of denaturation at 94 °C for four minutes, annealing at72 °C for 1 min, and extension at 72 °C for 8 min were used for the PCR amplification. After agarose gel electrophoretic separation, amplicons were visualized by exposing the gel through ultra-violet rays under the Gel documentation system (Syngene Gel Doc, Syngene, Synoptics Ltd., UK) for scoring the bands. To assess the homogeneity of band profiles, PCR amplification was carried out in triplicate. Data analysis was carried out by scoring well-marked, high-intensity amplified bands. The size of the amplicons was determined by comparing them with a 1 Kb DNA ladder (GeNeiTM, Bangalore, India).

### Statistical analysis

All the experiments were setup in a completely randomized block design. Each experiment was repeated thrice with twenty culture tubes in each. Analysis of variance (ANOVA) was used to examine the data and Tukey's t-test was used to determine the significance of differences between the treatment means at $P < 0.05$ level.

## Results

### Encapsulation of nodal segments, shoot development potential and short-term storage

Results revealed that lower concentrations of SA (1.0 and 2.0% w/v) were not suitable for the formation of calcium-alginate beads. The lower concentrations of SA resulted in beads of undefined shape and they were difficult to handle. The shoot emergence from these beads was not observed on MS basal medium (Table S1). The higher SA concentration (4.0%) produced firm and spherical beads that affect the shoot emergence from the encapsulated nodal segments. SA at 3.0% was found most ideal for the formation of desirable beads. These beads have a maximum shoot development potential (55.73 ± 1.58%) from encapsulated nodal segments. Similarly, a low concentration of calcium chloride (25 mM) did not support good beads preparation, and no shoot growth response was observed from these beads. Calcium-alginate beads prepared using 50 and 75 mM calcium chloride support the poor shoot emergence. Beads prepared using 100 mM calcium chloride was found most suitable for the high-frequency shoot development potential (55.73 ± 1.58%) from encapsulated nodal segments (Table S1). Thus, gelling matrix of 3.0% SA and 100 mM calcium chloride was found the ideal combination for the formation of calcium-alginate beads (Fig. 1A).

To evaluate the efficient shoot development response from encapsulated nodal segments, calcium-alginate beads were cultured on MS medium supplemented with various concentrations (0.0–10.0 mg/l) of BAP (Fig. 2). The results revealed that the response of the encapsulated nodal segments varied depend on the concentration of BAP. The maximum frequency (82.91 ± 7.70%) of shoot development (Fig. 2A), number (5.55 ± 0.26) of shoots emerged from each encapsulated node (Fig. 2B), and maximum (2.64 ± 0.21 cm) average shoots length (Fig. 2C) was recorded on MS medium supplemented with 5 mg/l BAP (Fig. 1B, C).

To assess the shoot emergence response from nodal segments encapsulated in calcium-alginate beads and stored at 4 °C and 24 °C upto 30, 60, 90 and 180 days, were subcultured on best shoot multiplication medium (MS + 5 mg/l BAP). The encapsulated nodal segments stored at 4 °C for 30 days showed the maximum frequency (80.24 ± 1.01%) of shoot development, and the number (6.07 ± 0.19) of shoots /encapsulated bead (Table 1; Fig. 1D). However, when storage duration was increased from 30 to 180 days, the viability and shoot development response of encapsulated nodal segments were gradually decreased (Table 1). Similarly,

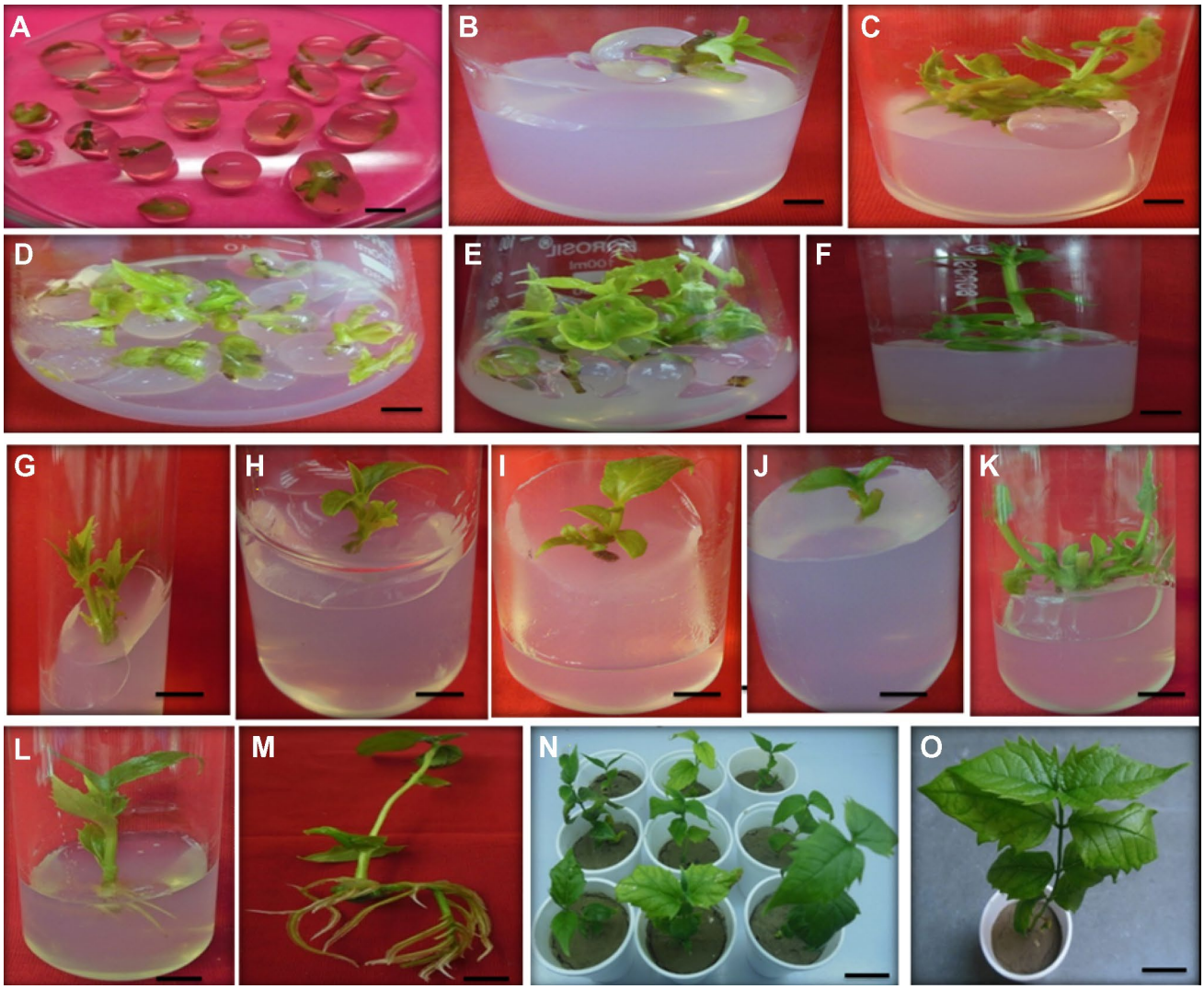


**Fig. 1** Encapsulated nodal segments of *N. arbor-tristis* L. and its shoot development potential; **A** Encapsulated nodal segments, **B**, **C** Shoot development response from encapsulated nodal segments on 5 mg/l BAP supplemented MS medium, **D**, **E** Shoot growth response in stored encapsulated nodal segments when cultured on 5 mg/l BAP supplemented MS medium, **F** Elongation of shoots, **G–I** Photographs showed the conservation of nodal segments under slow- growth condition on one-eighth strength MS medium + 0.5 percent sucrose up to 30 days **(G)**, 90 days **(H)**, 180 days **(I)**, **J** Nodal segments maintained up to 180 days on one-eighth strength MS medium containing 0.5 percent sucrose and 0.5 mg/l ABA, **K** Recovery of multiple shoots from nodal segments after 180 days storage on MS medium supplemented with 5 mg/l BAP, **L** In-vitro root induction in elongated shoot on half-strength MS medium supplemented with 4 mg/l IAA, **M** Four weeks old rooted plantlet, **N** Eight weeks old acclimatized plants, **O** Acclimatized healthy plant ready to transfer in to field.Scale 5.0 mm

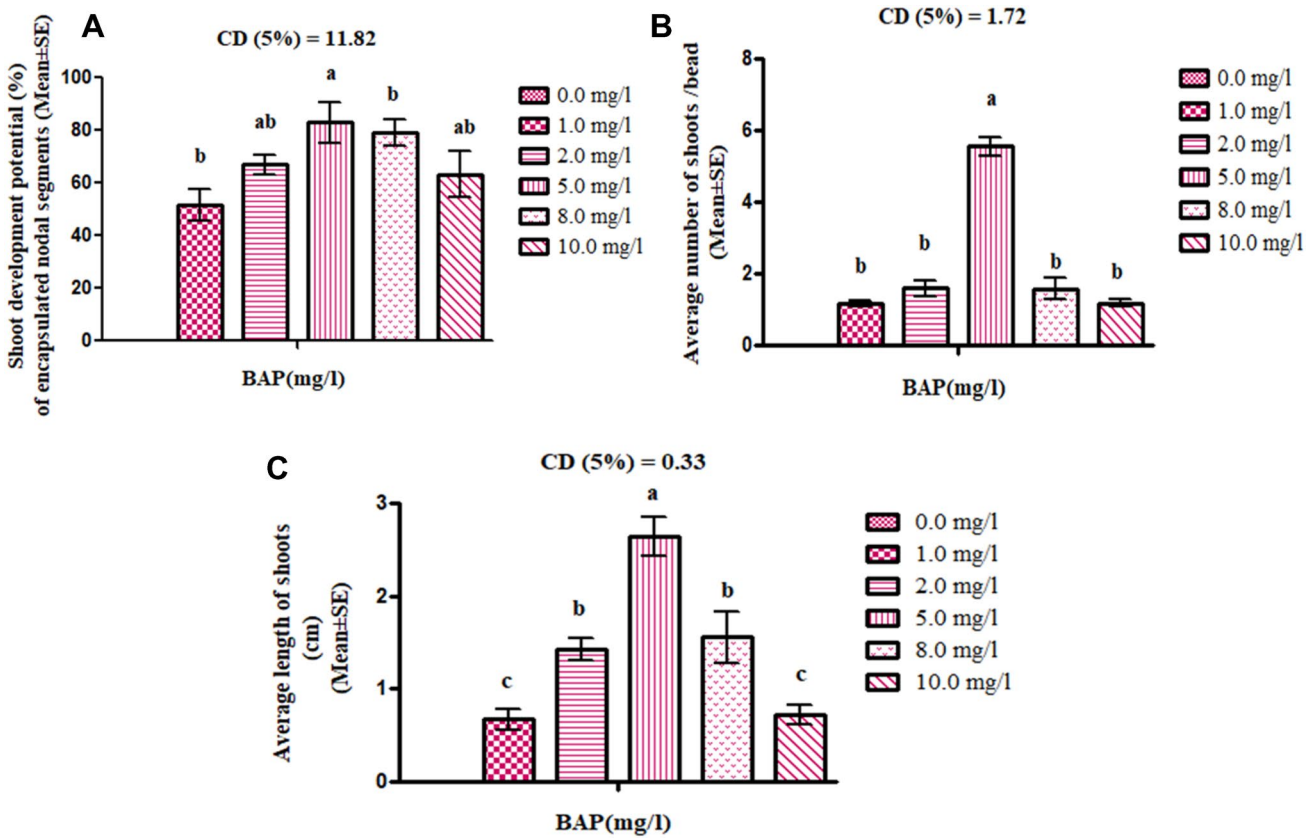


**Fig. 2** The percent shoot development potential of encapsulated nodal segments on MS medium supplemented with BAP **(A),** Average number of shoots / bead **(B)**, and average length (cm) of shoots **(C)**. Each mean is calculated from three replicates. Different letters above the bar indicate the significant differences from each other (Tukey's test, $P < 0.05$).Critical Difference (CD) at 5 percent level

encapsulated nodal segments stored at 24 °C upto 30 days showed maximum (68.81 ± 2.59%; Fig. 1E) shoot development potential and the number (5.47 ± 0.35; Fig. 1F) of shoots/bead (Table 1). The viability of encapsulated nodal segments and their shoot development response was drastically decreased with increasing the storage periods from 30 to 180 days (Table 1). In general, it was observed that encapsulated nodal segments stored at 4 °C were found more responsive for shoot development potential (Table 1). Encapsulated nodal segments stored up-to 180 days either at 4 °C or 24 °C were not responsive at all.

## Maintenance of nodal segments under slow growth conditions and recovery of shoots

The slow growth conservation of *N. arbor-tristis* was evaluated by maintaining the nodal segments on full, half (1/2), quarter (1/4) and one-eighth (1/8th) strength of MS medium supplemented with 3.0, 1.5 and 0.5% sucrose, and stored upto 30, 90 and 180 days at 24 ± 2 °C (Table 2). The percentage viability of explants and shoot regeneration response varied significantly. It was depend on

**Table 1** Effect of storage temperature and duration of encapsulated nodal segments and its shoot development potential after culture on MS medium supplemented with 5.0 mg/l BAP

| Storage temperature (°C) | Storage duration (Days) | Shoot development potential (%) | Number of shoots/bead |
|---|---|---|---|
| 4 | 30 | $80.24 \pm 1.01^{a}$ | $6.07 \pm 0.19^{a}$ |
| | 60 | $68.03 \pm 1.51^{b}$ | $4.43 \pm 0.40^{bc}$ |
| | 90 | $42.89 \pm 6.04^{cd}$ | $2.68 \pm 0.09^{d}$ |
| | 180 | 0.0 | 0.0 |
| 24 | 30 | $68.81 \pm 2.59^{b}$ | $5.47 \pm 0.35^{ab}$ |
| | 60 | $47.69 \pm 1.38^{c}$ | $3.13 \pm 0.38^{cd}$ |
| | 90 | $33.53 \pm 7.15^{d}$ | $1.80 \pm 0.25^{d}$ |
| | 180 | 0.0 | 0.0 |
| | CD | 6.10 | 0.86 |

Each mean is calculated from three replicates and represented as Mean ± SE. Different letters in the columns depict the significant differences from each other (Tukey's test, $P < 0.05$). Critical Difference (CD) at 5.0 percent level

the strength of the MS medium and sucrose concentration. The results showed that the percent viability of the explants gradually decreased with increasing the storage duration. The percent survival of nodal segments after 180 days of storage on different media, were as follows: Full-strength (30.33 ± 4.84% to 54.00 ± 4.00%) > half-strength (26.37 ± 3.48% to 46.49 ± 7.61%) > quarter-strength (16.81 ± 1.07% to 39.66 ± 1.54%) > one-eighth strength (18.28 ± 1.27% to 40.28 ± 2.04%) (Table 2). In general, the proliferations of axillary shoots from nodal segments were gradually decreased, when the concentration of sucrose was reduced from 3.0 to 0.5%. It also affects the length of the axillary shoots. The results revealed that when MS medium strength and sucrose concentration were reduced, the length of axillary shoots was gradually decreased. Nodal explants maintained upto 180 days on the one-eighth strength MS medium supplemented with 0.5% sucrose was found most suitable for reducing the number of shoot buds development (1.61 ± 0.28) and length (1.12 ± 0.32 cm) of axillary buds (Fig. 1G–I). As a result, one-eighth strength MS medium supplemented with 0.5% sucrose was found most effective for slow growth conservation of *N. arbor-tristis*, because it enhanced the dormancy of axillary shoot buds with the reduced number of shoot growth from nodal segments.

To promote the dormancy of axillary shoot buds, the one-eighth strength MS medium with a reduced concentration of sucrose (0.5%), was supplemented with 0–2 mg/l ABA (Table 3). After 180 days of storage, nodal explants maintained on the one-eighth strength MS media supplemented with 0.5 mg/l ABA exhibited good viability (40.36 ± 1.01%) with reduced number of shoots/ node (1.32 ± 0.11) and shoot length (0.50 ± 0.14 cm) (Fig. 1J).

To evaluate the shoot re-growth response from 180 days stored nodal segments maintained either on one-eighth strength MS medium containing 0.5% sucrose or one-eighth strength MS medium supplemented with 0.5% sucrose and 0.5 mg/l ABA, further sub-cultured on optimum shoot induction medium (MS + 5 mg/l BAP). The nodal segments maintained on one-eighth strength MS medium supplemented with 0.5% sucrose showed the maximum shoot re-growth frequency of 80.26 ± 4.26%, the maximum number of shoots/node 4.67 ± 1.10, an optimum length of shoots 2.25 ± 0.45 cm on the recovery medium after four weeks from culture initiation (Fig. 1K). Similarly, the nodal segments stored on one-eighth strength MS medium supplemented with 0.5% sucrose and 0.5 mg/l ABA, when sub-culture on re-growth medium, showed a maximum responding frequency 74.54 ± 1.85%; 3.07 ± 0.38 number of shoots/

**Table 2** Percent survival of nodal segments, that were maintained on full, half, quarter and one-eighth strength MS medium supplemented with various sucrose concentrations for slow growth conservation

| MS medium strength | Sucrose concentration (%) | Percent survival (Days) | | | Average number of shoots/explant | Average length of shoots (cm) |
|---|---|---|---|---|---|---|
| | | 30 | 90 | 180 | | |
| Full | 3.0 | 94.13 ± 1.87$^{a}$ | 80.56 ± 0.46$^{a}$ | 30.33 ± 4.84$^{de}$ | 2.49 ± 0.53$^{a}$ | 1.81 ± 0.49$^{a}$ |
| | 1.5 | 95.07 ± 2.82$^{a}$ | 74.88 ± 1.25$^{a}$ | 44.00 ± 3.45$^{abc}$ | 1.95 ± 0.26$^{a}$ | 1.70 ± 0.47$^{a}$ |
| | 0.5 | 89.71 ± 2.31$^{abc}$ | 76.62 ± 0.85$^{a}$ | 54.00 ± 4.00$^{a}$ | 1.94 ± 0.37$^{a}$ | 1.42 ± 0.30$^{a}$ |
| Half (1/2) | 3.0 | 94.09 ± 1.69$^{a}$ | 76.25 ± 0.80$^{a}$ | 26.37 ± 3.48$^{ef}$ | 2.17 ± 0.30$^{a}$ | 1.75 ± 0.68$^{a}$ |
| | 1.5 | 84.68 ± 2.29$^{bc}$ | 81.66 ± 9.27$^{a}$ | 36.33 ± 2.96$^{bcde}$ | 1.93 ± 0.37$^{a}$ | 1.31 ± 0.39$^{a}$ |
| | 0.5 | 90.84 ± 2.54$^{ab}$ | 77.22 ± 4.93$^{a}$ | 46.49 ± 7.61$^{ab}$ | 1.90 ± 0.38$^{a}$ | 1.12 ± 0.34$^{a}$ |
| Quarter (1/4) | 3.0 | 95.14 ± 2.76$^{a}$ | 75.03 ± 1.48$^{a}$ | 16.81 ± 1.07$^{fg}$ | 1.95 ± 0.26$^{a}$ | 1.83 ± 0.43$^{a}$ |
| | 1.5 | 76.59 ± 1.31$^{d}$ | 46.35 ± 3.47$^{b}$ | 33.38 ± 3.71$^{cde}$ | 1.76 ± 0.25$^{a}$ | 1.68 ± 0.37$^{a}$ |
| | 0.5 | 94.39 ± 2.88$^{a}$ | 79.58 ± 2.44$^{a}$ | 39.66 ± 1.54$^{bcd}$ | 1.40 ± 0.20$^{a}$ | 1.56 ± 0.49$^{a}$ |
| One-eighth (1/8th) | 3.0 | 88.88 ± 0.69$^{abc}$ | 53.76 ± 1.88$^{b}$ | 18.28 ± 1.27$^{fg}$ | 1.89 ± 0.44$^{a}$ | 1.71 ± 0.35$^{a}$ |
| | 1.5 | 87.30 ± 0.51$^{bc}$ | 79.38 ± 2.06$^{a}$ | 15.00 ± 0.57$^{h}$ | 1.61 ± 0.28$^{a}$ | 1.38 ± 0.26$^{a}$ |
| | 0.5 | 83.92 ± 0.90$^{c}$ | 70.58 ± 1.54$^{a}$ | 40.28 ± 2.04$^{bcd}$ | 1.61 ± 0.28$^{a}$ | 1.12 ± 0.32$^{a}$ |
| CD | – | 3.50 | 5.84 | 6.01 | 0.60 | 0.71 |
| Analysis of interaction performed by three-way ANOVA | MS medium strength × Sucrose concentration | Sum of squares = 599.955* | Sum of squares = 2859.406* | Sum of squares = 447.674 | Sum of squares = 0.259 | Sum of squares = 0.174 |
| | | Df = 6 | Df = 6 | Df = 6 | Df = 6 | Df = 6 |
| | | Mean square = 99.993 | Mean square = 476.56 | Mean square = 74.612 | Mean square = 0.043 | Mean square = 0.029 |
| | | F = 7.715 | F = 13.227 | F = 1.952 | F = 0.102 | F = 0.053 |

Mean value is calculated from three replicates and represented as Mean ± SE. Different letters in the columns depict the significant differences from each other (Tukey's test, $P < 0.05$). Critical Difference (CD) at 5.0 percent level. *Df* degrees of freedom, *Significant at $P < 0.05$

**Table 3** Response of nodal segments stored for different time on one-eighth strength MS media containing 0.5% sucrose and different concentrations of ABA

| Concentration of ABA (mg/l) | Percent survival (Days) | | | Average number of shoots | Average length of shoots (cm) |
|---|---|---|---|---|---|
| | 30 | 90 | 180 | | |
| 0.0 | $76.00 \pm 2.84^{b}$ | $50.22 \pm 1.76^{a}$ | 0.0 | $2.30 \pm 0.46^{a}$ | $1.30 \pm 0.08^{a}$ |
| 0.5 | $84.66 \pm 2.35^{a}$ | $55.00 \pm 2.88^{a}$ | $40.36 \pm 1.01^{a}$ | $1.32 \pm 0.11^{b}$ | $0.50 \pm 0.14^{b}$ |
| 1.0 | $74.33 \pm 2.35^{b}$ | $35.00 \pm 1.60^{b}$ | $28.70 \pm 1.75^{b}$ | $1.46 \pm 0.08^{b}$ | $0.50 \pm 0.08^{b}$ |
| 2.0 | $53.96 \pm 1.88^{c}$ | $34.16 \pm 2.20^{b}$ | $26.88 \pm 1.68^{b}$ | $1.91 \pm 0.16^{a}$ | $0.57 \pm 0.12^{b}$ |
| CD | 4.39 | 4.58 | 2.15 | 0.25 | 0.23 |

Mean value is calculated from three replicates. Different letters in the columns depict the significant differences from each other (Tukey's test, $P < 0.05$). Critical Difference (CD) at 5.0 percent level

node, and $1.50 \pm 0.17$ cm length of shoots after four weeks from culture initiation.

## In-vitro rooting, hardening and acclimatization of plantlets

Elongated shoots (4–5 cm) were sub-cultured on a half-strength MS medium supplemented with various concentrations (1, 2, 4, 6 and 8 mg/l) of IAA for root induction (Table 4). The shoots cultured on a half-strength MS medium without IAA were unable to induce the root formation. Results revealed that the percentage frequency (%) of root induction, number of roots/shoot and root length was depend on the concentration of IAA. The best rooting response with maximum rooting frequency, efficient number of roots/shoot, and proper root length were achieved on a half-strength MS medium supplemented with 4 mg/l IAA (Fig. 1L, M; Table 4). The higher concentration of IAA induced green compact callus development at the base of the shoots without root formation. The well-rooted plants were taken out from the culture vials, washed gently with sterile distilled water and transferred into plastic cups filled with a mixture of garden soil, soil-rite, and sand for hardening. These cups were kept for acclimatization of plants under green-house conditions (Fig. 1N, O) and further transferred into the field. These plants in the field had a survival rate 65%.

## Assessment of clonal stability

The SCoT markers were used to determine the clonal stability of the plants transferred in to field. To validate the clonal stability, five acclimatized plants were randomly selected from the field, along with the source parent plant (P) and subjected to the genetic stability analysis. The finding suggests that, the banding profiles of recovered plants were similar to the parent plant and no genetic variation was observed (Fig. 3). A total of 78 distinct bands were scored from 14 SCoT primers. The number of the scorable bands varied between 2 to 8 per SCoT primers. A total of 444 bands were amplified using SCoT primers (Table 5). In comparison with the source parent plant, the maximum bands were consistent; uniform with no distinct differences was observed. The identical banding pattern revealed the genetic homogeneity among the recovered plants with source parent plant (P). Based on binary matrices data of the SCoT profile of presence (1) or absence (0) of bands, a genetic similarity index was analyzed and subjected to cluster analysis using the unweighted pair group and arithmetic averages (UPGMA) clustering methods.

**Table 4** Rooting of elongated shoots cultured on half-strength MS medium supplemented with different concentrations of IAA

| Concentration of IAA (mg/l) | Response | | |
|---|---|---|---|
| | Rooting frequency (%) | Average number of roots/shoot | Average length of roots (cm) |
| 0.0 | 0.0 | 0.0 | 0.0 |
| 1.0 | $31.66 \pm 4.40^{b}$ | $1.74 \pm 0.29^{c}$ | $2.13 \pm 0.07^{a}$ |
| 2.0 | $43.22 \pm 2.43^{a}$ | $1.82 \pm 0.09^{c}$ | $1.87 \pm 0.19^{ab}$ |
| 4.0 | $54.00 \pm 3.05^{a}$ | $3.25 \pm 0.14^{b}$ | $1.11 \pm 0.05^{c}$ |
| 6.0 | $45.00 \pm 2.88^{a}$ | $4.66 \pm 0.56^{a}$ | $1.29 \pm 0.27^{bc}$ |
| 8.0 | Callus | Callus | Callus |
| CD | 6.17 | 0.62 | 0.33 |

Mean value is calculated from three replicates and represented as Mean ± SE. Different letters in the columns depict the significant differences from each other (Tukey's test, $P < 0.05$).Critical Difference (CD) at 5.0 percent level

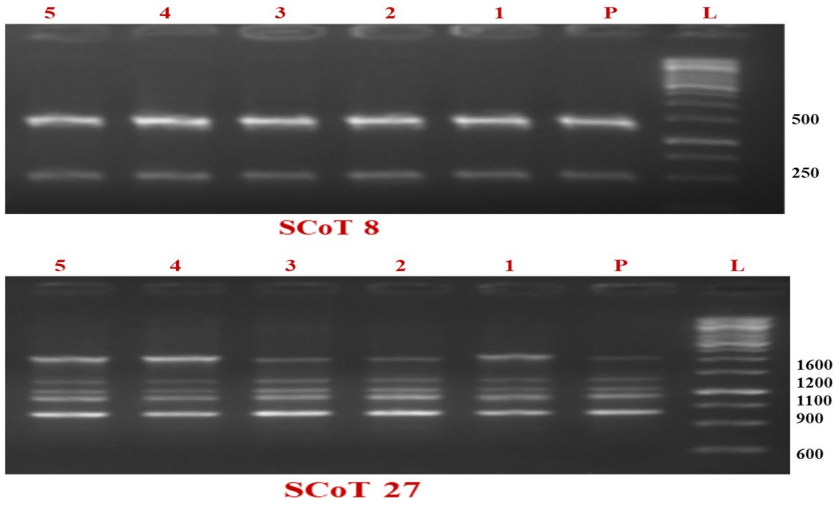


**Fig. 3** Gel picture depict SCoT amplification banding pattern. In photographs, an alphabetical letter (L) represents 1 Kb ladder, letter (P) denotes the source parent mother plant of *N. arbor-tristis* L., and numeric number 1 to 5 denotes bands of tissue culture recovered acclimatized hardened plants

The average Jaccard's similarity coefficient was 0.92–0.99 with the mean value of 0.96 in two clustered groups were shown through UPGMA matrix-based phenogram (Fig. 4). The different numbers in dendrogram hierarchical clustering showed that 4 and 6 have most genetic closeness. The numbers 4, 6 and 5 are almost similar to each-other as well as 1, 3 and number 2 are similar to all.

## Discussion

Germplasm conservation of elite plant species was achieved through storage of encapsulated propagules (Shilpha et al. 2021) and maintenance of explants on suitable media under slow growth conditions (Nasiruddin and Rafiul Islam 2018). The encapsulation protocol was influenced by the effective concentration of SA and calcium chloride used during the preparation of calcium-alginate beads (Saeed et al. 2018). Calcium-alginate beads varied morphologically in texture, shape and transparency. It depends on the concentration of SA and calcium chloride used during the preparation of calcium-alginate beads. The lower concentrations of SA (1–2%) and calcium chloride (25–75 mM) was not found suitable for calcium-alginate bead preparation. It may be due to a reduction in the gelling capacity. A higher concentration of SA (4%) produced firm and isodiametric beads that delayed the shoot emergence from encapsulated nodal segments. This delay in the emergence of shoots may be due to the hardness of the beads. In the case of *N. arbor-tristis* 3% SA and 100 mM calcium chloride were used in the preparation of calcium-alginate beads. It was highly effective for the emergence of shoots from encapsulated nodal segments.

**Table 5** Details of SCoT primer, sequence of primer, annealing temperature (°C), number of scorable and total amplified bands for clonal fidelity analysis

| S No | SCoT Primer | Sequence (5′–3′) | Tm (°C) | Amplified number of scorable bands/primer | Total bands |
|---|---|---|---|---|---|
| 1 | SCoT 5 | CAACAATGGCTACCACGA | 54 | 6 | 36 |
| 2 | SCoT 6 | CAACAATGGCTACCACGC | 56 | 8 | 46 |
| 3 | SCoT 8 | CAACAATGGCTACCACGT | 54 | 2 | 12 |
| 4 | SCoT 9 | CAACAATGGCTACCAGCA | 54 | 6 | 36 |
| 5 | SCoT 10 | CAACAATGGCTACCAGCC | 56 | 6 | 34 |
| 6 | SCoT 11 | AAGCAATGGCTACCACCA | 50 | 4 | 20 |
| 7 | SCoT 24 | CACCATGGCTACCACCAT | 56 | 5 | 28 |
| 8 | SCoT 25 | ACCATGGCTACCACCGGG | 61 | 6 | 34 |
| 9 | SCoT 26 | ACCATGGCTACCACCGTC | 58 | 8 | 44 |
| 10 | SCoT 27 | ACCATGGCTACCACCGTG | 58 | 5 | 30 |
| 11 | SCoT 28 | CCATGGCTACCACCGCCA | 61 | 8 | 46 |
| 12 | SCoT 29 | CCATGGCTACCACCGGCC | 63 | 5 | 30 |
| 13 | SCoT 30 | CCATGGCTACCACCGGCG | 63 | 4 | 22 |
| 14 | SCoT 31 | CCATGGCTACCACCGCCT | 61 | 5 | 26 |
| Total | | | | 78 | 444 |

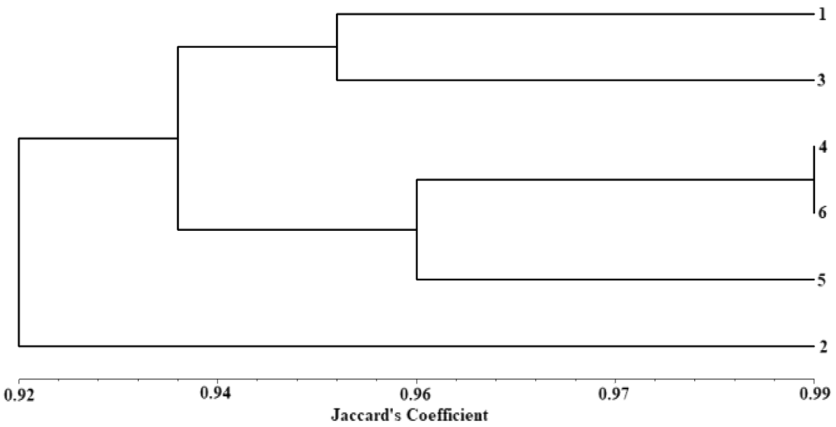


**Fig. 4** Picture of maximum likelihood tree based on Jaccard's average similarity co-efficient originated through UPGMA matrix of tissue culture recovered acclimatized plants of *N. arbor-tristis* L.

Similar concentrations were used previously by Ahmad et al. (2012) in *Ruta graveolens* and by Varshney and Anis (2014) in *Balanites aegyptiaca*. The developments of multiple shoots from encapsulated explants depend on the species and media used during the germination of calcium-alginate beads. Singh et al. (2006) reported multiple shoots development from encapsulated shoot tips of *Withania somnifera* on MS medium without cytokinin, while in *N. arbor-tristis* MS medium supplemented with BAP supported multiple shoot development from encapsulated nodal segments. Hatzilazarou et al. (2020) found that BAP supplemented media was highly effective for the emergence of several shoots from encapsulated explants in *Viburnum dentatum* L.

Encapsulation technology for short-term storage of encapsulated explants for germplasm conservation was achieved by the scientists (Micheli et al. 2007). Using this technology, germplasm can be stored for a few months (Bhattacharyya et al. 2018) to a year (Kaminska et al. 2018). Storage of encapsulated explants is a crucial factor that regulates the recovery of plantlets after transfer to regrowth media. In present study, the recovery of shoots from encapsulated nodal segments stored at 4 °C was found better than those stored at 24 °C. The retention of viability of encapsulated explants stored at low temperature may be due to the availability of nutrients in the gel matrix for longer period. At this temperature, the encapsulated explant slows down its metabolic activities. Hence, they remained in a quiescent state for a longer period. Thus, it will be helpful in conserving the encapsulated explants during cold storage. The encapsulated explants of *Pseudostellaria heterophylla* were stored at 4 °C was most favourable for the recovery of plantlets (Ma et al. 2011). Encapsulated explant stored at a higher temperature showed a significant decrease in the rate of shoot development on recovery media. It might be due to reduced respiration of plant material due to oxygen deficiencies in SA beads or the beads desiccating under low-humidity conditions (Ahmad and Anis 2010).

Slow growth is an important biotechnological tool for medium-term conservation of germplasm under *in-vitro* conditions. This procedure was successfully employed for the conservation of *Cynara cardunculus* var *scolymus* L. (Tavazza et al. 2015). Slow growth is usually achieved by modifying the salt concentrations of culture media. Media supplemented with osmotic agents and growth inhibitors enhanced the slow growth periods. The modification of medium strength was found to be effective for maintaining the explant as such for longer period in pear (Ahmed and Anjum 2010) and *Elettaria cardamomum* (Tyagi et al. 2009). Sucrose is a major component of plant tissue culture media. It acts as a carbon energy source and osmotic agent (Yaseen et al. 2013). The variation in the sucrose concentration in the media, which acts as an osmoticum, was effective in extending the storage life of *in-vitro* grown tissues (Jo et al. 2009; El-Bahr 2016). Reducing the sucrose concentration in MS medium was helpful in promoting the axillary buds dormancy and maintaining the nodal segments as such up to 180 days in *N. arbor-tristis*.

Kaminska et al. (2016) found that media supplemented with ABA was beneficial in promoting the shoot bud dormancy, which helped in maintaining the explant as such for a longer duration under *in-vitro* condition. ABA is an important growth inhibitor and is commonly used in in-vitro studies (Cid et al. 2008). It regulates several physiological and developmental processes in the plants (Vishwakarma et al. 2017) and its inhibitory effects could be attributed to interactions with auxin and cytokinin level in the host (Sah et al. 2016). Media supplemented with ABA was highly effective for slow growth storage of viable explant of *Tetrastigma hemsleyanum* (Peng et al. 2015). ABA was also found effective for the storage of nodal segments under slow growth condition in *N. arbor-tristis*.

The process of hardening of rooted plantlets and their transfer into the field is very crucial for their growth and survival. It is the slow adaptation of plants to their natural environment. Hardened plants, when transferred into the

field, compete with abiotic and biotic stress. The ultimate survival of hardened plants in the field depends on their ability to overcome the stress induced on them during the field establishment process (Teixeira da Silva et al. 2017). Plantlet of *N. arbor-tristis* had a survival rate of 65% when transferred to the field. All the plants that survived in the field had no phenotypic variations.

Clonal fidelity assessment of plants recovered from stored explants is highly important. Several DNA based molecular markers were recommended to study the genetic stability of recovered plants. These molecular markers have been used to detect somaclonal variations induced in regenerates (Kalia et al. 2011). Among these markers, the SCoT marker is frequently used to detect genetic variation among recovered plants (Seth et al. 2017). The recovered plants of *N. arbor-tristis* showed clonal fidelity to the source parent plant, as evidenced by the SCoT marker, which revealed no polymorphism in any of the bands. The genetic relatedness was calculated and determined using the Jaccard Similarity co-efficient**.** The similarity coefficient revealed the *in-vitro* recovered plants were true-to-type and they were genetically related to the source parent plant. Start codon targeted (SCoT) DNA polymorphism is a type of DNA marker based on the conserved region flanking translation start codon 'ATG' (Collard and Mackill 2009). The assessment of clonal stability by SCoT markers has been studied in several medicinal plants like *Cleome gynandra* (Rathore et al. 2014) and *Citrullus lanatus* (Vasudevan et al. 2017) by other workers.

## Conclusion

In conclusion, we have optimized the germplasm conservation protocol for *N. arbor-tristis,* which is an economically important antiviral medicinal plant. The germplasm in the form of encapsulated nodal segments is maintained at low temperature for 90 days. The nodal segments maintained under slow growth conditions up to six months showed good viability of explants and recovery of plantlets. ABA enhanced the dormancy of axillary buds and helped in maintaining the explant as such under slow growth conditions. The rooting of recovered shoots (4–5 cm) was achieved on half-strength MS medium supplemented with 4.0 mg/l IAA. The rooted plants were acclimatized successfully and showed 65% survival when transferred into the field. The hardened plants did not show any morphological variations from the parent plant. SCoT primers were used to assess clonal fidelity of recovered plants, which confirmed the genetic stability of these plants, and no variation was observed with respect to the parent plant. In summary, the outcome of optimized protocol provides improved scope for short-term conservation of germplasm of this economical antiviral plant. The methods open up avenues to protect this species from habitat loss.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s11240-022-02244-1.

**Acknowledgements** The author Awadhesh Kumar Mishra (AKM) is thankful to University Grants Commission (UGC), New Delhi, India for fellowship support throughout the study of this work. The author Kavindra Nath Tiwari acknowledges Institute of Eminence (IoE), Banaras Hindu University, Varanasi, India (Scheme 6031) for supporting the research work.

**Author contributions** Author AKM, design, writing the manuscript and completed all experimental work. PM and SKT technically help in the analysis of data. KNT and SKM, is involve in proofreading, intellectual input and finally revise and agreed to the publication of manuscript.

**Funding** The authors have not disclosed any funding.

## Declarations

**Conflict of interest** There is no conflict of interest in contributing authors for publication.

## References

Agrawal J, Pal A (2013) *Nyctanthes arbor-tristis* Linn: a critical ethnopharmacological review. J Ethnopharmacol 146:645–658

Ahmad N, Anis M (2010) Direct plant regeneration from encapsulated nodal segments of *Vitex negundo*. Biol Plant 54:748–752

Ahmad M, Anjum MA (2010) *In-vitro* storage of some pear genotype with the minimal growth technique. Turk J Agric for 34:25–32

Ahmad N, Faisal M, Fatima N, Anis M (2012) Encapsulation of microcuttings for propagation and short-term preservation in *Ruta graveolens*: a plant with high medicinal value. Acta Physiol Plant 34:2303–2310

Baskaran P, Kumari A, Staden JV (2018) In-vitro propagation via organogenesis and synthetic seeds of *Urginea altissima* (L.f.) Baker: a threatened medicinal plant. 3 Biotech 8:18. https://doi.org/10.1007/s13205-017-1028-7

Bhattacharyya P, Kumar V, Staden JV (2018) *In-vitro* encapsulation based short term storage and assessment of genetic homogeneity in regenerated *Ansellia africana* (Leopard orchid) using gene targeted molecular markers. Plant Cell Tissue Organ Cult 133:299–310

Chand S, Singh AK (2004) Plant regeneration from encapsulated nodal segments of *Dalbergia sissoo* Roxb.: a timber-yielding leguminous tree. J Plant Physiol 161:237–243

Cid B, Carvalho LPY, Luiz LCB (2008) Importance of abscisic acid (ABA) in the *in-vitro* conservation of cassava (*Manihot esculenta* Crantz). Chil J Agri Res 68:304–308

Collard BCY, Mackill DJ (2009) Start codon targeted (SCoT) polymorphism: a simple noval DNA marker technique for generating gene –targeted marker in the plants. Plant Mol Biol Rep 27:86–93

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12:13–15

El-Bahr MK, Abd El-Hamid A, Matter MA, Shaltout A, Bekheet SA, El-Ashry AA (2016) *In- vitro* conservation of embryogenic

cultures of date palm using osmotic mediated growth agents. J Gen Eng Biotechnol 14:363–370
Gupta P, Bajpai SK, Chandra K, Singh KL, Tandon JS (2005) Antiviral profile of *Nyctanthes arbor-tristis* L. against encephalitis causing viruses. Indian J Exp Biol 43:1156–1160
Gupta AK, Harish RMK, Phulwaria M, Agarwal T, Shekhawat NS (2014) *In-vitro* propagation, encapsulation, and genetic fidelity analysis of *Terminalia arjuna*: a cardio-protective medicinal tree. Appl Biochem Biotechnol 173:1481–1494
Hatzilazarou S, Kostas S, Joachim M, Economou A (2020) Regeneration of *Viburnum dentatum* L from alginate-encapsulated shoot explants after short-term cold storage and assessment of genetic stability using ISSR analysis. Agronomy 10: 1660; doi:https://doi.org/10.3390/agronomy10111660.
Jahan AA, Anis M (2015) Retrieval of plantlets from cryopresesrved alginate buds of *Nyctanthes arbor-tristis* L.: an effectual approach for germplasm conservation. Int J Dev Res 5:4397–4402
Jo EA, Tewari RK, Hahn EJ, Paek KY (2009) *In-vitro* sucrose concentration affects growth and acclimatization of *Alocasia amazonica* plantlets. Plant Cell Tissue Organ Cult 96:307–315
Joshi K, Bahukhandi A, Parihar N, Rawat S, Bhatt ID (2019) Promoting conservation of threatened plant species in West Himalayan region through cultivation- a participatory approach. ENVIS Bull Himal Ecol 27:45–48
Kalia RK, Rai MK, Kalia S, Singh R, Dhawan AK (2011) Microsatellite markers: an overview of the recent progress in plants. Euphytica 177:309–334
Kaminska M, Skrzypek E, Wilmowicz E, Tretyn A, Trejgell A (2016) Effect of light conditions and ABA on cold storage and post-storage propagation of *Taraxacum pieninicum*. Plant Cell Tissue Organ Cult 127:25–34
Kaminska M, Gołębiewski M, Tretyn A, Trejgell A (2018) Efficient long-term conservation of *Taraxacum pieninicum* synthetic seeds in slow growth conditions. Plant Cell Tissue Organ Cult 132:469–478
Ma XM, Wu CF, Wang GR (2011) Application of artificial seeds in rapid multiplication of *Pseudostellaria heterophylla*. Afr J Biotechnol 10:15744–15748
Micheli M, Hafiz IA, Standardi A (2007) Encapsulation of *In-vitro* derived explants of Olive (*Olea europaea* L.cv.Moraiolo) II. Effect of storage on capsule and derived shoot performance. Sci Horticult 113:286–292
Mishra AK, Upadhyay R, Chaurasia JK, Tiwari KN (2016) Comparative antioxidant study in different flower extracts of *Nyctanthes arbor-tristis* L. (Oleaceae): an important medicinal plant. Braz J Bot 39:813–820
Mishra AK, Upadhyay P, Dixit J, Kumar P, Tiwari KN, Mishra SK, More R, Singh J (2018) Ameliorative activity of ethanolic flower extract of *Nyctanthes arbor-tristis* L. against scopolamine-induced amnestic effect and profiling of active compounds using gas chromatography–Mass spectrometry and ultra-performance liquid chromatography-quadrupole-time-of-flight mass spectrometry. Pharmacog Mag 14:596–604
Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497
Nasiruddin M, Rafiul Islam AKM (2018) In-vitro slow-growth conservation for two genotypes of Solanum tuberosum l. Bangladesh J Bot 47:369–380
Parveen S, Shahzad A (2014) Encapsulation of nodal segments of *Cassia angustifolia* Vahl. for short-term storage and germplasm exchange. Acta Physiol Plant 36:635–640
Peng X, Ji Q, Wu H, Li Y (2015) Slow-growth conservation and clonal fidelity of *Tetrastigma hemsleyanum* microplants. In Vitro Cell Dev Biol Plant 51:463–470
Prakash AV, Nair DS, Alex S, Soni KB, Viji MM, Regunath BR (2018) Calcium alginate encapsulated synthetic seed production in *Plumbago rosea* L. for germplasm exchange and distribution. Physiol Mol Biol Plants 24:963–971
Rai MK, Asthana P, Singh SK, Jaiswal VS, Jaiswal U (2009) The encapsulation technology in fruit plants: a review. Biotechnol Adv 27:671–679
Raizada U, Nangia A (1989) Cyclic nocturnal opening and abcission of *Nyctanthes* flowers with a note on visiting thrips species. Curr Sci 58:93–96
Rathore NS, Rai MK, Phulwaria M, Rathore N, Shekhawat NS (2014) Genetic stability in micropropagated *Cleome gynandra* revealed by SCoT analysis. Acta Physiol Plant 36:555–559
Reddy MC, Murthy KSR, Pullaiah T (2012) Synthetic seeds: a review in agriculture and forestry. Afr J Biotechnol 11:14254–14275
Rihan HZ, Kareem F, El-Mahrouk ME, Fuller MP (2017) Artificial seeds (principle, aspects and applications). Agronomy 7: 71; doi: https://doi.org/10.3390/agronomy7040071.
Saeed T, Sahzad A, Ahmad N, Praveen S (2018) High frequency conversion of non-embryogenic synseeds and assessment of genetic stability through ISSR markers in *Gymnema sylvestre*. Plant Cell Tissue Organ Cult 134:163–168
Sagar R, Singh JS (2004) Local plant species depletion in a tropical dry deciduous forest of northern India. Environ Conserv 31:55–62
Sah SK, Reddy KR, Li J (2016) Abscisic acid and abiotic stress tolerance in crop plants. Front Plant Sci 7:571. https://doi.org/10.3389/fpls.2016.00571
Seth S, Rath SC, Rout GR, Panigrahi J (2017) Somatic embryogenesis in *Abutilon indicum* (L.) Sweet and assessment of genetic homogeneity using SCoT markers. Plant Biosyst 151:704–714
Shilpha J, Pandian S, Largia MJV, Sohn SI, Ramesh M (2021) Short-term storage of *Solanum trilobatum* L. synthetic seeds and evaluation of genetic homogeneity using SCoT markers. Plant Biotechnol Rep 15:651–661
Singh AK, Varshney R, Sharma M, Agarwal SS, Bansal KC (2006) Regeneration of plants from alginate-encapsulated shoot tips of *Withania somnifera* (L.) Dunal, a medicinally important plant species. J Plant Physiol 163:220–223
Singh SK, Rai MK, Asthana P, Sahoo L (2010) Alginate-encapsulation of nodal segments for propagation, short-term conservation and germplasm exchange and distribution of *Eclipta alba* (L.). Acta Physiol Plant 32:607–610
Subhashini DP, Satyanarayana B, Arundhati A, Raghava Rao T (2014) Plant regeneration from encapsulated nodal segments of *Sterculia urens* Roxb. an endangered gum-yielding tree. J Crop Sci Biotechnol 17:21–25
Tavazza R, Rey NA, Papacchioli V, Pagnotta MA (2015) A validated slow-growth *in-vitro* conservation protocol for globe artichoke germplasm: a cost-effective tool to preserve from wild to elite genotypes. Sci Horticult 197:135–143
Teixeira da Silva JA, Hossain MM, Sharma M, Dobranszki J, Cardoso JC, Songjun Z (2017) Acclimatization of *in-vitro*-derived *Dendrobium*. Hort Plant J 3:110–124
Tyagi RK, Goswami R, Sanayaima R, Singh R, Tandon R, Agrawal A (2009) Micropropagation and slow growth conservation of cardamom (*Elettaria cardamomum*, Maton). In Vitro Cell Dev Biol-Plant 45:721–729
Varshney A, Anis M (2014) Synseed conception for short-term storage, germplasm exchange and potentialities of regeneration genetically stable plantlets of desert date tree (*Balanites aegyptiaca* Del.). Agrofor Syst 88:321–329
Vasudevan V, Subramanyam K, Elayaraja D, Karthik S, Vasudevan A, Manickavasagam M (2017) Assessment of the efficacy of amino acids and polyamines on regeneration of watermelon (*Citrullus lanatus* Thunb.) and analysis of genetic fidelity of regenerated plants by SCoT and RAPD markers. Plant Cell Tissue Organ Cult 130:681–687

Vishwakarma K, Upadhyay N, Kumar N, Yadav G, Singh J, Mishra RK, Kumar V, Verma R, Upadhyay RG, Pandey M, Sharma S (2017) Abscisic acid signaling and abiotic stress tolerance in plants: a review on current knowledge and future prospects. Front Plant Sci 8:161. https://doi.org/10.3389/fpls.2017.00161

Yaseen M, Ahmad T, Sablok G, Standardi A, Hafiz IA (2013) Review: role of carbon sources for in vitro plant growth and development. Mol Biol Rep 40:2837–2849

Zayova E, Nedev T, Dimitrova L (2017) *In vitro* storage of *Stevia rebaudiana* Bertoni under slow growth conditions and mass multiplication after storage. Bio Bullet 3:30–38