Micropropagation, encapsulation and growth of *Artemisia vulgaris* node explants for germplasm preservation

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**Abstract**

As an alternative to seed propagation, an efficient micropropagation system and subsequent rooting were developed for the medicinal plant *Artemisia vulgaris* (Asteraceae). A maximum of 32.8 shoots were produced from the nodal explants cultured on 4.9 μM 2iP. The effect of different types and concentrations of carbohydrates was tested for multiple shoot induction and we found that sucrose at 3% concentration resulted into a better response. Healthy plantlets were transferred to garden soil:farmyard soil:sand (2:1:1) mixture (growing medium) for acclimatization, which was successful and subsequent maturity was achieved under garden conditions. Nodal segments were excised from proliferating shoot cultures and encapsulated in high density sodium alginate hardened by 50 mM CaCl2. 2.0% sodium alginate was determined to produce the highest quality encapsulated nodal segments beads because of the viscosity produced by 2.0% sodium alginate solution. When encapsulated nodal segments were stored at 5 °C they did not grow in light or dark conditions. All encapsulated nodal segments survived 20 weeks of 5 °C storage. In fact, 85% of encapsulated mugwort nodal segments survived refrigerated storage for 11/4 years (60 weeks) and after 3 months on proliferation medium, the nodal segments produced a similar result as compared to encapsulated segments either not stored at 5 °C or stored for 20 weeks at 5 °C. Thus a simple micropropagation and a cost-effective cold storage protocol for alginate encapsulated vegetative (node) explants of mugwort was successfully developed for the germplasm preservation of this valuable medicinal plant.

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**Keywords:** *Artemisia vulgaris*; Germplasm preservation; Light; Mass propagation; Refrigeration; Sodium alginate; Sucrose

1. Introduction

*Artemisia vulgaris* L. (mugwort) belongs to the family Asteraceae and is a tall aromatic perennial herb, which grows in the hilly district of India in areas up to 2400 m elevation. The plant has been known not only as an edible plant (mostly as a spice) but also as a folk medicine resource. The investigations of mugwort extracts indicated a hepatoprotective activity and validated the traditional use of this plant for various liver disorders (Gilani et al., 2005). Mugwort is commonly used in traditional European medicine as a choleretic and for amenorrhoea and dysmenorrhoea (Teixiera Da Silva, 2004). In traditional medicine, this plant is being widely used for the treatment of diabetes and extracts of the whole plant is used for epilepsy and in combination for psychoneurosis, depression, irritability, insomnia and anxiety stress (Walter et al., 2003). The crude extract has been used as an antimalarial agent for thousands of years, and Sun et al. (1992) found that artemisinin extracted from *A. vulgaris* had antitumor activity. Mugwort essential oils are used for their insecticidal, antimicrobial and antiparasitical properties (Judzentiene and Buzelyte, 2006). The authors also reported that *A. vulgaris* essential oils have a significant fumigant and repellent effect on *Musca domestica*. Several medicinally active components of *A. vulgaris* have been identified including vulgarin, quercetin, coumarins, sesquiterpene lactones, volatile oils and insulin (USDA-ARS-NGRL, 2004).

Since the harvest of medicinal plants on a mass scale from their natural habitats is leading to a depletion of plant resources, the conservation of these valuable genotypes is imperative. A large-scale and unrestricted exploitation of these natural

**Abbreviations:** 2iP, 2-isopentenyl adenine; CaCl2, Calcium chloride; IAA, Indole 3-acetic acid; MS, Murashige and Skoog, 1962; PGR, Plant Growth Regulator.

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resources to meet its ever-increasing demand by the pharmaceutical industry, coupled with limited cultivation and insufficient attempts for its replenishment, have culminated in the marked depletion of this medicinally important plant species (Pandey et al., 1993). The plant is at low risk of extinction as marked depletion of this medicinally important plant species provides the mechanical support needed to protect the tissue surrounding an explant slows the process of desiccation and growth must return to normal rates when placed back under storage to be successful, tissue dehydration must be prevented or proliferation cultures become infested with bacteria, fungi, or arthropods (West and Preece, 2006). Cold storage has the potential to reduce the need for transferring and subculturing, out of season (West et al., 2006). Encapsulation can also provide a means to reduce the need for transferring and subculturing, out of season (West and Preece, 2006). Cold storage has the potential to reduce the need for maintaining germplasm cultures because of the reduced need for manual labor due to less frequent subculturing. Cold storage also has lessened the possibility of genetic instability from frequent subculturing and adventitious regeneration (West and Preece, 2006). For cold storage to be successful, tissue dehydration must be prevented and growth must return to normal rates when placed back under standard incubation conditions. An alginate-embedded matrix surrounding an explant slows the process of desiccation and provides the mechanical support needed to protect the tissue within encapsulation medium during long-term storage. Embryogenic tissues of Santalum album cultured in vitro (Bapat and Rao, 1988) and adventitious buds of M. alba (Machii, 1992) have been encapsulated in alginate or agar beads and then stored for 34–80 days at 4 °C without loss of viability. Kinoshita and Saito (1992) reported that the encapsulated axillary buds of Betula platyphylla var. japonica could be stored at 4 °C for more than 80 days without loss of viability. Microcuttings of Eucalyptus grandis × E. urophylla, encapsulated in alginate beads and kept on a nutrient-free agar medium were stored for 10 months at 30/25 °C with a plant recovery rate of at least 52% after storage (Yuehua and Wenming, 1994).

The aim of the investigation reported here was to devise a micropropagation protocol that is easy to establish and maintain for the regeneration of in vitro shoots of A. vulgaris. Besides micropropagation, a cold storage protocol for alginate encapsulated vegetative explants of mugwort by evaluating alginate concentrations, presence of light, storage temperatures on nodal segments; growth and survival were evaluated thus enabling germplasm conservation of this valuable medicinal plant.

2. Materials and methods

2.1. Plant material and explant establishment

Young nodal explants (1–1.5 cm) were excised from stock plants (3 months old) of A. vulgaris cultivated in the Botanical Evaluation Garden, Department of Plant Science, Bharathidasan University, India. They were surface cleaned with 10% (v/v) dettol solution (Reckitt Benckiser, Kolkata, India) for 30 s, followed by rinsing three to five times in distilled water. Then they were surface sterilized with 0.1% (w/v) aqueous mercuric chloride (HgCl₂) for 1–6 min and finally rinsed with autoclaved distilled water (three to five changes) in a flow chamber. The surface sterilized explants were trimmed at the edges prior to inoculation.

2.2. Culture conditions

Surface sterilized nodal segments (1–1.2 cm) were cultured on MS (Murashige and Skoog, 1962) basal medium supplemented with 3% (w/v) sucrose (Himedia, Mumbai, India) for culture initiation and served as explant sources for subsequent experiments. The pH of the medium (supplemented with respective growth regulators) was adjusted to 5.7 with 1N NaOH or 1N HCl before gelling with 0.7% agar (Himedia, Mumbai, India). In all the experiments the chemicals used were of analytical grade (Himedia, Mumbai, India, Sigma-Aldrich, USA and E. Merck, Germany). The medium was dispensed into culture vessels (Borosil, India) and autoclaved at 105 K Pa and 121 °C for 15 min. The explants were implanted vertically on the culture medium (test tubes (150× 25 mm) containing 15 ml medium) and plugged tightly with non-absorbent cotton. All cultures were incubated at 25 ± 1 °C under a 16/8 (light/dark) photoperiod of 45–50 μmol m⁻²s⁻¹ irradiance provided by cool white fluorescent tubes (Philips, India) and with 55–60% relative humidity (RH). All the subsequent subcultures were carried out at 2 week intervals.

2.3. Multiple shoot induction and plantlet production

Nodal segments (1–1.2 cm) were inoculated vertically on MS medium containing 3% (w/v) sucrose, 0.7% (w/v) agar supplemented with different concentrations of 2iP (0.4−12.3 μM) for multiple shoot induction. For each treatment, a total of 10 replicates each with 2 explants were inoculated; therefore, 20 explants per treatment were tested. Multiple shoots initiated were subcultured at every 2 weeks for 2 months. A control group was maintained (basal medium without hormones) to record the frequency of response.

2.4. Effect of carbohydrate source and concentration on shoot formation

To test their effect, explants were cultured on MS basal medium supplemented with 4.9 μM 2iP and different types of
carbon sources, including glucose, fructose maltose and sucrose at 1–5% (w/v) concentration.

2.5. Rooting

To induce rooting, individual shoots (6.8–10.8 cm long) were isolated and transferred to MS medium containing IAA (2.80–17.1 μM). One set of cultures was inoculated in basal MS medium without the addition of auxins and kept as control.

2.6. Acclimatization and field experiment

Plantlets with well-developed roots were removed from the culture medium and after washing the roots gently under running tap water to remove the adhering medium, plantlets were transferred to plastic cups (10 cm diameter) containing growing medium (autoclaved garden soil, farmyard soil and sand mixture (2:1:1)). Each was irrigated with distilled water every 2 d for 3 weeks followed by tap water for 2 weeks. The potted plantlets were initially maintained inside the culture room conditions (5 weeks) and later transferred to normal laboratory (33±1 °C) conditions (4 weeks). After 65 d, the plantlets were then transplanted to the field in the Botanical Evaluation Garden of Bharathidasan University, India and grown for 5 months. The morphological and growth characteristics were examined.
2.7. Alginate encapsulation and subsequent culture incubation

Nodal segments (4–6 mm long) were excised from proliferating microshoots and had their subtending leaves removed. The nodal segments were then coated with high viscosity sodium alginate (2%) (Sigma Chemical Co.) and placed into a sterile 50 mM CaCl₂ solution for 15–25 min. The encapsulated nodal segments were washed with deionized water 3 times and were placed individually in 25×150 mm borosilicate glass culture tubes containing proliferation (MS basal + 4.9 μM 2iP) medium. The nodal segments were then incubated for 3 weeks, unless otherwise noted, under cool white fluorescent lamps that provided a photon flux of approximately 45–50 μmol m⁻² s⁻¹ and a 16 h photoperiod at 25 °C.

2.8. Determination of suitable alginate concentration and light and temperature requirement

To determine the suitable alginate concentration on encapsulation the following different concentrations (1%, 1.5%, 2%, 2.5% and 3%) of alginate were tested. The encapsulated nodal segments were stored at 5±1 °C or 25±1 °C and placed in both light (45–50 μmol m⁻² s⁻¹ and a 16 h photoperiod) and dark conditions to evaluate the light and temperature requirements on growth of encapsulated nodal segments.

2.9. Long-term storage experiment

Encapsulated nodal segments were stored in the dark in a refrigerator at 5±1 °C for 0, 20, 40 and 60 weeks. Encapsulated nodal segments were produced at different times for storage. After 60 weeks from the date of the encapsulation, nodal segments (stored or not stored) were simultaneously placed individually into 25 ×150 mm borosilicate glass culture tubes containing proliferation medium for subsequent incubation. Nodal segments from all the three treatments were evaluated for the production of shoots and roots. Survival of nodal explants was evaluated by the nodal segment’s ability to produce shoots and roots and nodes not producing shoots and roots were considered to not have survived storage. Encapsulated nodal segments were subcultured at every 4 weeks for proliferation.

2.10. Data collection and statistical analysis

Experiments were setup in a randomized block design (RBD) and each experiment had 10 replications and was repeated 3–5 times. Observations were recorded on the frequency (number of

| Table 2 | Effect of IAA on root induction of microshoots of A. vulgaris |
|---------|---------------------------------------------------------------|
| IAA concentration (mM) | Frequency of rooting (%) | Number of shoots (Mean±SE) | Root length (cm) (Mean±SE) |
| Control | 0.0 | 0.0 | 0.0 |
| 2.8 | 69.4abc | 3.8±0.24abc | 3.0±0.28abc |
| 5.7 | 71.9abc | 5.6±0.32abc | 5.4±0.32abc |
| 8.5 | 82.6c | 9.2±0.42abc | 6.8±0.23abc |
| 11.4 | 100.0a | 10.0±0.44abc | 7.8±0.22abc |
| 14.2 | 98.9bc | 8.4±0.25abc | 7.0±0.44abc |
| 17.1 | 94.6b | 8.0±0.28bc | 6.2±0.20bc |

Data recorded at 15th day of culture.

* Treatment means followed by different letters in their superscript are significantly different from each other (p<0.05) according to Duncan’s multiple range test.

* Values shown were proportion of three replicates of 25 microplants

Fig. 3. The frequency of ex vitro survival and growth of acclimatized microplants of A. vulgaris.

Fig. 4. Effect of alginate concentration on root growth from encapsulated A. vulgaris nodal segments that were first stored in darkness at 5±1 °C for 4 weeks then cultured in vitro on proliferation medium under cool white fluorescent lamps and 25 °C for an additional 4 weeks.

| Table 3 Effect of light, storage temperature on shoot and root growth from encapsulated A. vulgaris nodal segments after 4 weeks |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Storage temperature | Light condition | Shoot number (Mean±SE) | Shoot length (cm) (Mean±SE) | Root number (Mean±SE) | Root length (cm) (Mean±SE) |
| (°C) | | (Mean±SE) | (cm) | (Mean±SE) | (cm) |
| 5 | Dark | 1.2±0.12bc | 4.0±0.45ab | 1.0±0.00b | 1.8±0.24ab |
| | Light | 1.8±0.24ab | 5.5±0.32a | 4.0±0.29a | 5.8±0.24a |

Treatment means followed by different letters in their superscript are significantly different from each other (p<0.05) according to Duncan’s multiple range test.
cultures responding for multiple shoot proliferation and root development) and the number of shoots per explant, shoot length, number of roots per shoot, root length, survival % and increase in plant height after successive hardening, respectively. The analysis of variance (ANOVA) appropriate for the design was carried out using 2iP as the hormone source. Nodes (1–1.2 cm) of *A. vulgaris* were cultured on PGR free media and media containing various concentrations of 2iP for shoot regeneration. PGR free media did not respond well for shoot flushing. On MS medium supplemented with 4.9 µM 2iP, 98.9% of shooting response was observed after 40 d with an average of 32.8 shoots per nodal explant (Table 1). This was significantly higher than that of our previous report (Sujatha and Ranjitha Kumari, 2007). A similar phenomenon was observed by Burza and Malepszy (1995). 2iP was reported to induce maximum number of shoots in *Digitalis purpurea* and *Asparagus officinalis* (Cellarova and Hochvar, 2004; Pontaroli and Camadro, 2005). In the present study, higher concentrations of cytokinins reduced the number of shoots. Hu and Wang (1983) reported that higher concentrations of cytokinins reduced the number of micropropagated shoots. A similar phenomenon was also observed in our previous studies (Sujatha and Ranjitha Kumari, 2007). MS medium fortified with 7.3 µM 2iP attained the longest shoot length of 10.8 cm after 40 d of culture in the subsequent subcultures. The medium containing concentrations of 2iP higher than 4.9 µM decreased shooting frequency (Table 1). A similar phenomenon was observed by Baskaran and Jayabalain (2005).

### 3.3. Carbon source and shoot proliferation

Several research reports have demonstrated that the carbon source influences *in vitro* morphogenesis of different plant species (Fuentes et al., 2000). It is also known that carbon source in the culture medium is an essential component of the medium as a source of energy and for maintaining the osmoticum (Cuenca and Vieitez, 2000). In this study, organogenesis of the explant was affected by the carbon source (Fig. 2) Among the four different carbon sources tested, sucrose at 3% concentration proved to be the best for shoot proliferation than fructose, glucose and maltose (Fig. 2). Similar results were also observed in direct organogenesis of Anattto (De Paiva Neto et al., 2003) and Socotran fig (Krostrup et al., 2005). Sucrose has been commonly used as a carbon source in tissue culture media. This is due to its efficient uptake across the plasma membrane (Borkowska and Szezebra, 1991). Plant development, physiology and metabolism are regulated by inputs from a number of signaling/response pathways. However, there are a few reports where other carbohydrates are superior to sucrose in stimulating morphogenesis. In explants of *Prunus avium*, the use of maltose stimulated the formation and development of somatic embryos (Reidiboym-Talleux et al., 1999). In shoot explants of *Anacardium occidentale*, replacing sucrose (2%) with maltose or glucose improved shoot formation (Boggetti et al., 1999).

In the present study, 3% of sucrose, glucose, maltose and 2% of fructose were found to be optimal in inducing maximum number of shoots than compared to other treatments tested. The

| Weeks in 5 °C | Weeks in proliferation culture | Survival (%)** | Nodal segment number** | Shoot number | Shoot length (cm) | Root number | Root length (cm) |
|--------------|-------------------------------|---------------|------------------------|-------------|------------------|-------------|-----------------|
| 0            | 4                             | 100           | 8.4bc                 | 2.0μ         | 10.8ab           | 4.9ab       | 6.7ab           |
| 8            | 100                           | 8.8ab         | 2.8ab                 | 11.0a        | 4.7b             | 6.8bc       | 5.7bc           |
| 12           | 100                           | 10.2a         | 3.4a                  | 11.1a        | 5.0a             | 7.2a        |                 |
| 20           | 4                             | 100           | 6.8μ                  | 2.0μ         | 7.4e             | 3.4e        | 5.4μ            |
| 8            | 100                           | 7.2d          | 2.4c                  | 7.8bc        | 3.5e             | 5.7ce       |                 |
| 12           | 100                           | 7.5c          | 2.6bc                 | 7.6bc        | 3.5e             | 5.9f        |                 |
| 40           | 4                             | 95            | 4.3f                  | 1.0f         | 5.6c             | 2.7f        | 4.7f            |
| 8            | 95                            | 4.5f          | 1.4f                  | 5.3f         | 2.8f             | 4.8f        |                 |
| 12           | 95                            | 4.6e          | 1.6e                  | 5.8f         | 2.9f             | 4.8e        |                 |
| 60           | 4                             | 85            | 2.1f                  | 0.8f         | 3.4f             | 1.1f        | 2.4f            |
| 8            | 85                            | 2.7f          | 1.0f                  | 3.5f         | 1.5f             | 2.5f        |                 |
| 12           | 85                            | 2.8f          | 1.0f                  | 3.7f         | 1.9f             | 2.8e        |                 |

**Treatment means followed by different letters in their superscript are significantly different from each other (p<0.05) according to Duncan’s multiple range test.**

** Mean number of nodal segments that could be excised from shoots for subculturing and using for encapsulation or micropropagation.
The maximum number of shoots obtained was 32.8, 29.7, 23.9 and 30.8 respectively (Fig. 2). The difference between the ability of various concentrations and types of carbohydrates to affect a variety of morphogenetic pathways has been ascribed to various mechanisms. One explanation for the frequently observed morphogenetic effect is the osmotic stress that has been shown to affect cell division and cell wall extension (Seijo et al., 1997). In this study, the decrease in the number of shoots following the increasing concentration of carbohydrates may well be explained by a corresponding increase in media osmolality (Krogstrup et al., 2005). This stresses the importance of using molar concentration for carbohydrate concentrations as stated by Neto and Otoni (2003), as it isolates the osmotic effect from the nutritional effect.

Finally, the various effects of carbohydrates could be attributed to a basic regulatory role in gene expression in a manner similar to classical plant growth regulators (Blane et al., 2002).

3.4. Effect of auxins on rooting of shoots

From our previous study, we concluded that IAA is more suitable for rooting in A. vulgaris (Sujatha and Ranjitha Kumari, 2007) even though IBA was stated to be more optimal for root induction in A. judaica (Liu et al., 2003). Excised shoots were rooted on MS medium with different concentrations of IAA (2.8–17.1 μM). MS medium supplemented with different concentrations of IAA induced roots within 2 weeks of culture. Among the various concentrations of IAA tested, the number of roots and root lengths varied (Table 2). Plantlets significantly developed lengthy roots and root induction was strengthened within 2 weeks of culture. MS medium supplemented with IAA (11.4 μM) was found to be more effective for root induction (Table 2). Roots were visible within 5–10 d following transfer of elongated shoots to the rooting medium. After 2 weeks, plantlets developed primary and secondary root system. Frequency of rhizogenesis was 100%. In agreement with our findings, IAA was reported as potential auxins for rooting in Arachis stenosperma, A. villosa, Sesbania drummondii and Salvia numerosa (Vijayalakshmi and Giri, 2003; Cheepala et al., 2004; Ewa and Halina, 2004). Growth on medium containing IAA at concentration greater than 11.4 μM resulted in callusing at the base of shoots. The average number of roots per shoot ranged from 3.8–10 and the maximum root length observed was 7.8 cm (Table 2). Shoots did not produce roots when transferred to basal medium containing no growth regulators.

3.5. Acclimatization of rooted plantlets and examination of morphological characteristics

A crucial aspect of in vitro propagation is to acquire regenerated plants that are capable of surviving outside the sterile and protected in vitro environment. A substantial number of micropropagated plants do not survive transfer from in vitro conditions to greenhouse or field environment. The greenhouse and field have substantially lower relative humidity, higher light level and septic environment that are stressful to micropropagated plants compared to in vitro conditions. The benefit of any micropropagation system can, however, only be fully realized by the successful transfer of plantlets from tissue-culture vessels to the ambient conditions found ex vitro (Hazarika, 2003).

In the present study, the successfully rooted plantlets were transferred to plastic cups containing growing medium for hardening. Plantlets were acclimatized without growth chamber facilities. Plantlets were maintained in the culture room (25±1 °C) conditions initially for 5 weeks and after transferred to normal laboratory conditions and maintained for about 4 weeks. Finally the plantlets were transferred to Botanical Evaluation Garden and maintained. 100% of plantlet survival was seen after hardening on growing medium for 6 weeks. However, the survival ability decreased to 98% and 78% after 10 and 20 weeks of acclimatization, respectively (Fig. 3). The initial growth rates of plant height were 18.3±0.30 cm during first 2 weeks of acclimatization. However, in the following 3–20 weeks, substantial increase in plant height was observed (Fig. 3). There was no branching of shoots observed. The stem was slender and growth of minute hairs on the stem and on the lower surface of the leaves was observed. There was no detectable variation among the acclimatized plants with respect to morphological, growth characteristics. All the micropropagated plants were free from external defects.

3.6. Effect of alginate concentration on encapsulation

In the control treatment (0% alginate), the nodal segments had been placed in petri dishes without being encapsulated. After 4 weeks of dark storage at 5 °C, these control nodal segments were dead and had completely dried up. A similar result was noticed by West et al. (2006). Because of this, the control nodal segments were omitted from the data analysis.

Alginate concentration affected complete encapsulation and the growth of root (Fig. 4). When alginate concentration of 3.0% was used, root length was less than with the other alginate concentrations. There are two factors that may have caused this effect: the actual physical barrier of the denser matrix may have delayed the emergence of the root or the higher concentrations of sodium from the sodium alginate caused a change in water potential, resulting in less water for root growth. 3% alginate was reported to be more suitable for encapsulation in Centaurium rigualii (Benito et al., 1997). Timbert et al. (1995) reported that 2.5–3.0% alginate increased bead density and had a negative effect on germination of carrot somatic embryos because they were unable to rupture and emerge from the encapsulated bead as compared to 1.5–2.0% alginate having higher germination frequency. In agreement, we found that 2.0% sodium alginate was more effective in inducing complete encapsulation and subsequent growth (Fig. 4). Whereas, higher concentrations (2.5 and 3.0%) were extremely viscous and were difficult to decant and use for encapsulation. However, low concentrations (1.0 and 1.5%) of sodium alginate had lower viscosity and coated the nodal segments poorly. An optimum of 2.0% sodium alginate concentration was determined to be the best because it coated the nodal segments very effectively and
was the easiest to drop into the calcium chloride solution. The resulting encapsulation beads held the nodal segments in place and still provided enough resistance to external mechanical pressure for ease of handling. Therefore, 2.0% sodium alginate was used in all subsequent experiments. Alginate is one of the most common polymers used for immobilization of plant cells and production of manufactured seeds because it is available in large quantities, is inert, non-toxic, cheap, and easily manipulable (Endress, 1994).

3.7. Light and temperature requirements

There was a significant interaction between light and storage temperature on shoot and root growth of stored encapsulated nodes (Table 3). Encapsulated nodal segments that were stored cold (5 °C) did not develop any shoots or roots during storage. Whereas at 25 °C shoots grew longer in both darkness and under fluorescent lamps. Growth from encapsulated nodal segments stored at 25 °C was dependent on light. When exposed to light, the nodal segments developed shoots and roots, similar to their in vitro response on proliferation medium. When placed in darkness, the encapsulated nodal segments developed longer, etiolated shoots with no roots. As growth during storage is not desirable and because darkness is easy to obtain during refrigeration, all subsequent storage treatments were in darkness for 5 ± 1 °C. The influence of light intensity during storage has been studied for different species. Best results have been observed when storage was performed in darkness for Populus (Hausman et al., 1994), or in low levels of illumination for Quercus petrea and Q. robur (Janeiro et al., 1995).

3.8. Long-term storage experiment

Nodal segments responded differently during each month of culture on proliferation medium depending on how long they were stored previously under dark refrigerated conditions (Table 4). Nodal segments that had not been refrigerated produced shoots that were significantly longer and had more nodal segments during the first month on proliferation medium as compared to nodal segments refrigerated for 20 or 60 weeks. However, both refrigerated and non-refrigerated nodal segments stored for 20 weeks produced shoots sufficiently long after 4 weeks under proliferation conditions. The most apical 10 mm long nodal segments from these treatments were transferred to fresh proliferation medium for further growth and analysis. After the second month in vitro (8 weeks) culture, new growth from recultured nodes from non-refrigerated and nodal segments stored for 20 weeks was not significantly different, indicating full recovery of the growth from nodal segments that had been cold stored for 20 weeks.

Nodal segments that were stored for 40 and 60 weeks were slower to recover than those not refrigerated or stored for 20 weeks. The growth was retarded and the new shoots were not sufficiently long for subdivision after 4 weeks under proliferation conditions. However, after 8–10 weeks under proliferation conditions, there was sufficient new shoot growth from these encapsulated nodal segments for subdivision. During the last 4 weeks under proliferation conditions (12 weeks), mean shoot length, mean root number and length produced by nodal segments from encapsulated nodal segments stored for 60 weeks was nearly the same as new growth from nodal segments that were not refrigerated or stored for 20 weeks, indicating recovery.

All nodal segments that were neither refrigerated nor stored for 20 weeks survived to the end of the experiment. A survival rate of 85% is remarkable for nodal segments that were stored in dark refrigerator for 11/4 years (60 weeks) with no addition of water or fresh medium. Those that did not survive for 11/4 years grew more slowly during the first 3 months under proliferation conditions than those stored for 20 weeks or not refrigerated. These long-term stored nodal segments showed steady linear growth over 3 months under proliferation conditions (Table 4). This indicates that these nodal segments slowly recovered from any damage that occurred during long-term storage. Others have also reported that it takes time for explants to recover from cold storage stresses after return to proliferation culture conditions (Ballester et al., 1997; West et al., 2006).

4. Conclusion

A. vulgaris is well adapted to alginate encapsulation utilizing 2.0% high viscosity sodium alginate. These can be stored at 5 °C for up to 11/4 years with no maintenance with reduced shoot and root vigor. Recovery from the stress of storage may take 1–3 months under proliferation conditions, depending on the length of the storage time. Long-term storage under commonly available refrigerated conditions (5 °C) is easier and less costly than cryopreservation that requires deep freezing or vitrification. Common refrigeration at 5 °C for storage of small, light weight propagules may be useful for micropropagation laboratories for maintaining cultures. This study has thus demonstrated that micropropagation can be relied upon for producing large quantities of plantlets of A. vulgaris for use in conservation work or for its commercial exploitation, thereby reducing the collection pressure on the remaining wild populations. This is the first report stating micropropagation and germlasm preservation of this valuable medicinal plant.

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