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Regeneration of *Viburnum dentatum* L. from Alginate-Encapsulated Shoot Explants after Short-Term Cold Storage and Assessment of Genetic Stability Using ISSR Analysis

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Abstract: The present study demonstrates an efficient protocol for alginate encapsulation, interim cold storing of artificial seeds and conversion to genetically stable plants of *Viburnum dentatum* L. "Lucidum Aiton". Explants of shoot tips and first-node segments, excised from in vitro-derived viburnum microshoots, were encapsulated in 2.5% sodium alginate mixed with liquid MS nutrient medium and hardened in 50 mM of calcium chloride producing solid, soft and uniform beads. These artificial seeds achieved 28.9% germination under light, forming 4.3 microshoots per bead. However, with 100 mM of calcium chloride for hardening, the beads were firm and of a uniform globular shape and suitable for handling and exhibited a germination response of 48.9%. Encapsulated shoot tip explants of viburnum, which were stored at 4 °C for 4, 8 or 12 weeks, showed a gradual decline in regeneration response (73.3, 62.2, 51.1%, respectively), while non-encapsulated explants, stored under same conditions, did not survive after the fourth week of cold storage. Microshoots from cold-stored encapsulated explants, which were rooted in solid MS nutrient medium with 0.5 μM of Indole-3-acetic acid (IAA) and transplanted to a substrate of peat-perlite (3:1, *v*/*v*), acclimatized successfully after application of 75 or 50% shading, which was gradually reduced, and were established with minimum losses in a greenhouse. For the genetic stability of the artificial seed-derived plantlets and compared with the mother plant, an assessment was conducted using Inter Simple Sequence Repeats (ISSRs) analysis. The ISSR profiles proved the genetic uniformity and clonal stability of the regenerated plantlets and their genetic resemblance to the mother plant. The present regeneration procedure could be used as an alternative method for the micropropagation of *V. dentatum*.

Keywords: arrowwood viburnum; artificial seeds; clonal fidelity; gDNA; germination; germplasm conservation; in vitro culture; micropropagation; synseeds; tissue culture

1. Introduction

V. dentatum, commonly called arrowwood viburnum, is a multi-stemmed deciduous shrub with glossy leaves bearing clusters of white flowers in spring which turn into bluish berries in autumn [1]. The plant, which is adapted to low and high temperatures, as well as drought and alkaline soil, is used in ornamental horticulture, particularly as a landscaping edging plant and also as a gardening container-grown plant [2]. Propagating viburnum by seed is a long process as it takes more than ten months to germinate, thus it is usually propagated by softwood shoot cuttings [3]. Yet, in vitro propagation protocols for *V. dentatum* have been developed, which could be used for large-scale production of abundant propagation materials for commercial use [4,5].

The encapsulation technique for producing artificial or synthetic seeds (synseeds) is an important application of micropropagation and can play a crucial role as an alternative to other conventional methods for large-scale propagation and long-term germplasm storage of plants [6,7]. Artificial seeds are defined as artificially encapsulated somatic embryos, shoot tips, shoot buds, nodal segments, cell aggregates or any tissue that can be used for sowing as a seed and that possess the ability to convert into a plant under in vitro or in vivo conditions [8–10]. Artificial seed technology mimics that of the conventional seed technology and combines the advantages of seed (sexual) propagation with those of vegetative (asexual) propagation [11–13]. By this method, the genetic uniformity among regenerated plantlets, in vitro mother culture and in the field-grown or in the greenhouse-grown mother plant is maintained in most cases, while elite genotypes can be stored for a short to long term and can be regenerated later upon demand for plant propagation material [14,15]. In addition, the method offers the easy handling of artificial seeds during storage, unhindered transportation among countries from customs barriers and/or quarantines and cost efficiency in comparison with the traditional method of in vitro culture, which requires frequent and costly subcultures [16–18]. Although many gel materials have been tried for artificial seed production, an alginate matrix was proved to be optimal for encapsulation towards artificial seed production, mainly due to the sensible thickness, weak spinability of solution, low expense and fast gelation [19,20]. Successful production of artificial seeds and retrieval of plants have been reported for a large range of plant species including ornamentals, fruit and forest trees, cereals, vegetables, and medicinal and aromatic plants [21–23], whereas there is no reference for *V. dentatum.*

Plantlets derived from in vitro cultures may exhibit somaclonal variation due to physical and chemical factors, type of explant source, longevity of the in vitro culture and genotype [24,25]. Thus, assessment for the genetic stability of the produced plantlets from artificial seeds is necessary using Random Amplification of Polymorphic DNA (RAPD) and/or Inter Simple Sequence Repeat (ISSR) analysis as it has been referred in a number of reports [14,25–27].

The aim of this work was to develop an effective procedure for encapsulating shoot explants in alginate in order to produce artificial seeds, alternatively to traditional micropropagation methods, for a fast, efficient and mass propagation of genetically uniform regenerants of *V. dentatum* for landscape use and also as a container-grown plant. Therefore, the selection of the proper shoot explants, the encapsulation procedure and the short-term storage at 4 °C of the artificial seeds as well as their subsequent germination followed by a stability assessment of the regenerants using ISSR analysis were investigated.

2. Materials and Methods

2.1. Plant Material, Establishment of In Vitro Cultures

Terminal softwood shoots were cut from a viburnum (*V. dentatum* L. "Lucidum Aiton") plant, grown in the greenhouse, suitable for flowering container-grown plants, as well as for gardening plantation. Explants of shoot tips (1.0–1.5 cm in length) were excised from these terminal softwood shoots and dipped for 5 min in 0.1% (*w*/*v*) Mercuric chloride ($HgCl_2$) solution and then were surface-disinfested for 5 min in 2.0% (*v*/*v*) Sodium hypochlorite (NaOCl) solution under vigorous stirring plus 0.05% (*v*/*v*) Tween-20 (BDH Chemicals, Poole, England), followed by 3 rinses with sterilized distilled water under aseptic conditions. Afterwards, each explant was placed in a single glass culture tube (100 × 25 mm) containing 10 mL of Murashige and Skoog (MS) nutrient medium [28], with the addition of 5 μM of 6-Benzylaminopurine (BAP) (Sigma-Aldrich, St. Louis, MI, USA), 2% (*w*/*v*) sucrose and 0.65% (*w*/*v*) agar (Technobiochem, Athens, Greece). The pH of the medium was adjusted to 5.8 using 0.5N NaOH or 0.1N HCl prior to agar addition. The glass culture tubes were covered with aluminum foil and autoclaved at a temperature of 121 °C and under pressure of 122 kPa for 20 min. Two weeks later, shoot tip explants without any signs of contamination were subcultured in fresh solid MS nutrient medium,

of the same composition and sterilization, for multiple shoot formation. Microshoots developed in these subcultures, over a 4-week period, were the donors of various explants for encapsulation.

2.2. Source of Explants for Encapsulation

To determine the proper type of explants for encapsulation, based on the easiness of explant sprouting during germination, shoot tips (3–4 mm in length) or nodal segments of equal size from the 1st, 2nd 3rd and 4th node below the shoot tip, were aseptically cut from the microshoots of the above-described subcultures and transferred to solid MS nutrient medium containing 5 μM of BAP for shoot induction. At the end of the 4-week in vitro culture, the frequency of shoot formation (percentage of sprouted explants), and the number and length of produced shoots were recorded. Based on the response of the various explants in vitro, the shoot tips and 1st-node segments were selected, inequal numbers, for the encapsulation and production of artificial seeds, unless otherwise specified.

2.3. Encapsulation of Explants, Germination of Artificial Seeds

For determining the effect of the inclusion of nutrient medium and sucrose in the encapsulation matrix of viburnum explants, shoot tips and first-node segments were dipped into a liquid MS nutrient medium (lacking of Ca) supplemented with 2.5% sodium alginate, as a gelling agent, and with or without 1% sucrose. Apart from this, other explants were dipped into 2.5% sodium alginate solution deprived of liquid MS nutrient medium and sucrose. Then, explants from the three treatments were picked up by a forceps and placed separately into three different beakers containing 50 mM of calcium chloride ($CaCl_2.2H_2O$), as a complexing agent, with continuous stirring for 30 min in order to avoid bead sticking and complete the ion-exchange (Na^+/Ca^{2+}) reaction. The resultant alginate beads were collected and rinsed twice with sterilized distilled water to remove excess calcium chloride and then were placed in glass culture tubes (100 × 25 mm) containing solid MS nutrient medium supplemented with 5 μM of BAP. Thereafter, the bead cultures were kept in darkness or under light; to determine their germination success, after a 4-week period, the regeneration response (percentage of sprouted beads), and the number and length of produced shoots were recorded.

The optimal concentration of calcium chloride for bead hardening was identified, after encapsulation of shoot tips and first-node segments in 2.5% sodium alginate, by immersing the coated explants in solutions of calcium chloride at concentrations of 25, 50, 100 or 200 mM for 30 min. The resultant beads after rinsing with sterilized distilled water were placed in glass culture tubes with solid MS nutrient medium and maintained under light for germination, which was evaluated as previously described.

2.4. Germination after Storage of Artificial Seeds

Shoot tip explants encapsulated in 2.5% sodium alginate that was mixed with liquid MS nutrient medium plus 1% sucrose and hardened for 30 min in 100 mM of calcium chloride, as previously described, were maintained at 4 °C in darkness for 4, 8 or 12 weeks in order to evaluate the influence of cold storage duration on the conversion of artificial seeds into plantlets. The resultant beads were placed on moistened with sterilized distilled water filter paper in sealed Petri dishes covered by black polyethylene film. At the end of each cold storage period, the beads were transferred and cultured on fresh solid MS nutrient medium with the addition of 5 μM of BAP. After four weeks in vitro, the germination was evaluated and compared with non-encapsulated (naked) similar explants which were maintained under the light of the same cold conditions on hormone-free solid MS nutrient medium and after storage were subcultured on fresh MS nutrient medium supplemented with 5 μM of BAP.

2.5. Microshoot Rooting, Plantlet Acclimatization

V. dentatum microshoots, produced from the germinated beads after their storage at 4 °C for 12 weeks, were transferred aseptically for rooting to solid MS nutrient medium supplemented with

0.5 μM of Indole-3-acetic acid (IAA) (Sigma-Aldrich, St. Louis, MI, USA). After 4 weeks, rooted microshoots were taken out of the culture medium, washed thoroughly under running tap water to remove any agar medium remnants and transferred to the greenhouse where they were transplanted to a 1:1 (*v*/*v*) substrate of peat and perlite in pots. Afterwards, the young plantlets were placed for acclimatization under 75, 50 or 25% shading from a polyethylene net cover, equipped with a water-fogging system controlled electronically, for various periods of time. In the case of 75% shading, the plantlets remained for one week under these conditions and then were placed for one week under 50% shading followed for one more week under 25% shading and afterwards were moved to a greenhouse bench under full-light irradiance (approx. 320 $\mu mol.m^{-2}.s^{-1}$) for an additional week. In the treatment of 50% shading, the plantlets remained under these conditions for one week and then were placed under 25% shading for one more week and finally they were transferred to full-light irradiance. In the next two treatments, the plantlets were placed under 25% shading for one or two weeks and then were moved to full-light irradiance for three or two weeks, respectively. At the end of the 4-week acclimatization period the survival rate (percentage of healthy plantlets) of the viburnum plantlets was calculated. The ambient temperature during the acclimatization period was 24 ± 2 °C, while the natural photoperiod was 12–13 h. The relative humidity under the polyethylene net cover decreased gradually from 95% (under 75% shading) to 85% (under 50% shading) and finally to 75% (under 25% shading), while the light irradiance varied from 145 (under 75% shading) and 180 (under 50% shading) to 220 $\mu mol.m^{-2}.s^{-1}$ (under 25% shading). After the 4-week acclimatization period, the survived young viburnum plantlets were maintained for further growth in greenhouse conditions.

2.6. Culture Conditions

All in vitro cultures of viburnum for microshoot formation and those of encapsulated explants designed for bead germination were kept in a growth chamber with a 16-h photoperiod from cool-white fluorescent lamps of 50 $\mu mol.m^{-2}.s^{-1}$ photon flux density at the culture level. The relative humidity in the growth chamber was 60–70%, while the temperature was set at 23 ± 0.1 °C.

2.7. Genomic DNA Extraction, Polymerase Chain Reaction (PCR) Amplification

Genomic DNA (gDNA) was extracted from the leaves of seven randomly selected artificial seed-derived plantlets of viburnum, four weeks after acclimatization, and the mother control plant using NucleoSpin Plant II (Macherey Nagel, Düren, Germany) following the instructions of the package. Purified gDNA was quantified and its quality was verified by nanodrop 2000 (Thermo Electron Corporation, Waltham, MA, USA), while each sample was diluted to 20 ng/μL in TE buffer (10mM Tris: 0.1mM EDTA; pH 8.0) and stored at −20 °C. Ten Inter Simple Sequence Repeat (ISSR) primers were used with numbers ISSR-UBC 808, 809, 810, 811, 812, 815, 816, 818, 821 and 834 (University of British Columbia, Vancouver, BC, Canada) as more appropriate, from a total number of 37 primers available in the laboratory. ISSR amplifications were performed in a volume of 20 μL containing 40 ng total DNA, Horse-Power™ Taq DNA Polymerase MasterMix (Canvax Biotech, Cordoba, Spain), which contained all PCR reaction components (dNTPs, PCR buffer, Mg^{2+} and Taq DNA Polymerase) and 1 μM from each primer.

PCR was performed at an initial denaturation of 94 °C for 4 min, followed by 35 cycles of 1-min denaturation at 94 °C, 1-min annealing at specific temperature for each primer and 2-min extension at 72 °C with a final extension of 72 °C for 10 min. The SimpliAmp™ Thermal Cycler (Life Technologies, Thermo Scientific, Waltham, MA, USA) was used for the PCR amplifications. DNA amplification fragments were separated in a 1.5% agarose gel with the application of electricity, using 1 × TAE buffer (Tris-acetate-EDTA), stained with ethidium bromide (0.001%) and analyzed using the gel documentation system Transilluminator UV light (Biostep, Burkhardtsdorf, Germany). The size of the amplification products was estimated by BrightMAX™ 1kb DNA Ladder (Canvax Biotech, Cordoba, Spain). Two independent PCR amplifications were performed for each sample.

For ISSR profiles, the clean-separated and consistently reproducible fragments ranging from 200 to 1400 bp were scored as present or absent. For detecting any genetic change, all the ISSR results were compared with each other for all the DNA samples. A similar procedure of genetic homogeneity confirmation among in vitro-derived plantlets has been published for other ornamental plant species such as gerbera [29], *Lilium longiflorum* [30], *Platanus acerifolia* [31] and *Phoenix dactylifera* [32].

2.8. Data and Statistical Analysis

The experiments were conducted twice in completely randomized designs. In the various experiments, 45 replicates per treatment were used with the exception of the plantlet acclimatization where 20 replicates were employed. The statistical analysis of the data was based on analysis of variance (ANOVA). Data in percentages were subjected to an arcsin transformation prior to statistical analysis and were transformed back to percentages for presentation in tables. The separation among means was carried out with Duncan's multiple range test and significance was determined at $p \leq 0.05$. The statistical analysis was conducted using the SPSS 22 software (SPSS Inc. Statistical Package for the Social Sciences, Chicago, IL, USA).

3. Results and Discussion

3.1. Explant Source

Shoot tip and first-node explants, excised from in vitro-derived microshoots that were produced in viburnum subcultures, formed new microshoots at frequencies of 100% (Table 1). Significantly lower microshoot formation frequencies were observed on the explants of the second, third and fourth node (66.7, 55.7 and 40.0%, respectively). Moreover, the shoot tip as well as the first and second-node explants produced more (2.7 and 2.4 microshoots, respectively) and longer microshoots than the rest of the nodal segment explants (Table 1).

Table 1. Influence of type of explant on shoot formation frequency (%), number and length of shoots of *V. dentatum* cultured on solid MS nutrient medium with the addition of 5 μM of 6-Benzylaminopurine (BAP).

Explant Type	Shoot Formation (%)	Number of Shoots	Length of Shoots (cm)
Shoot tip	100a [1,2]	2.7 ± 0.6a [1,2]	0.4 ± 0.1a [1,2]
1st node	100a	2.4 ± 0.6a	0.4 ± 0.1a
2nd node	66.7 ± 17.6b	2.4 ± 0.6a	0.3 ± 0.1ab
3rd node	55.7 ± 10.2bc	1.7 ± 0.6ab	0.2 ± 0.1b
4th node	40.0 ± 6.7c	1.0 ± 0.0b	0.2 ± 0.1b
ANOVA (F)	***	**	**

[1] Mean (±SD) of 45 explants per treatment, after 4 weeks of culture. [2] Different letters within columns indicate statistically significant differences according to Duncan's multiple range test at $p \leq 0.05$. **, *** Significant at the 0.01 and 0.001 level, respectively.

The type of explant plays a crucial role not only during encapsulation but also during artificial seed maintenance and development and most significantly during germination and shoot formation responses. Thus, various explants, such as somatic embryos, shoot tips, nodal segments, and axillary buds have been used for artificial seed production with different degree of success [11,21,22]. In this work, the shoot tip and the first-node explants exhibited high microshoot formation rates and yielded, in general, more and longer microshoots than the other explants of nodal segments. This better performance could be attributed to the higher juvenility of their tissues. Hence, they were chosen as the potent explant source for producing artificial seeds in *V. dentatum*. Shoot tips and nodal segments, as the most efficient material for explant encapsulation, have been reported for "Kober 5BB"grapevine rootstock [12], while nodal segments have been used in *Viburnum odoratissimum* [23], *Punica granatum* [33] and *Cassia angustifolia* [18]. On the other hand, in the majority of conifers, the somatic embryos seemed

to be the best explant source for artificial seed production [34], even though the produced artificial seeds had variable and in general low conversion rates compared to viable plants [35].

3.2. Encapsulation of Explantsand Germination of Artificial Seeds

The shoot tip and nodal segment explants of *V. dentatum* were successfully encapsulated in a solution of 2.5% sodium alginate with or without the addition of liquid MS nutrient medium and/or sucrose (1%) and further hardening for 30 min in 50 mM of calcium chloride. The produced beads were solid and soft with a defined shape that was easy to handle (Figure 1A). The addition of liquid MS nutrient medium, alone or with 1% sucrose, in the encapsulation matrix with 2.5% sodium alginate did not affect the regeneration response (%) of artificial seeds kept under the light but in the darkness it doubled the regeneration response (Table 2). The number of shoots produced per bead, after artificial seed germination, was higher with the addition of liquid MS nutrient medium and sucrose (1%) in the encapsulation matrix of the germinated artificial seeds both under light and darkness maintenance (Table 2). The length of the shoots was restricted in darkness in all treatments tested (Table 2). The addition of nutrients to the capsule is an essential factor for a successful artificial seed production technique, increasing in many cases the competence of germination and viability of these seeds [20]. These elements are considered as artificial endosperm and they also play an important role in the artificial seed storage capacity [36]. In other words, the capsule may serve as a pool of nutrients which probably improves explant survival and hastens its growth [37,38]. However, in this research, inclusion of MS nutrient medium in the alginate cover affected the germination response of the artificial seeds of viburnum only when they were kept in the darkness and enhanced the shoot formation on the germinated artificial seeds (Table 2).

Table 2. Influence of inclusion of MS nutrient medium and sucrose (1%) in the encapsulation matrix (with 2.5% sodium alginate and 50-mM calcium chloride) on artificial seed germination of *V. dentatum*, in darkness or under light.

Treatments	Regeneration Response (%)	Number of Shoots/Bead	Length of Shoots (cm)
	Light		
Alginate	26.7 ± 6.7a [1,2]	2.7 ± 0.6b [1,2]	0.3 ± 0.1a [1,2]
Alginate + MS	17.8 ± 3.9ab	2.3 ± 0.6b	0.2 ± 0.1ab
Alginate + MS + Sucrose	28.9 ± 3.8a	4.3 ± 0.6a	0.2 ± 0.1ab
	Darkness		
Alginate	6.7 ± 3.9d	0.7 ± 0.6c	0.1 ± 0.0b
Alginate + MS	15.5 ± 3.9bc	2.0 ± 1.0b	0.1 ± 0.0b
Alginate + MS + Sucrose	11.1 ± 3.9cd	4.0 ± 1.0a	0.1 ± 0.0b
ANOVA (F)	***	***	**

[1] Mean (±SD) of 45 artificial seeds per treatment, after 4 weeks of culture. [2] Different letters within columns indicate statistically significant differences according to Duncan's multiple range test at $p \leq 0.05$. **, *** Significant at the 0.01 and 0.001 level, respectively.

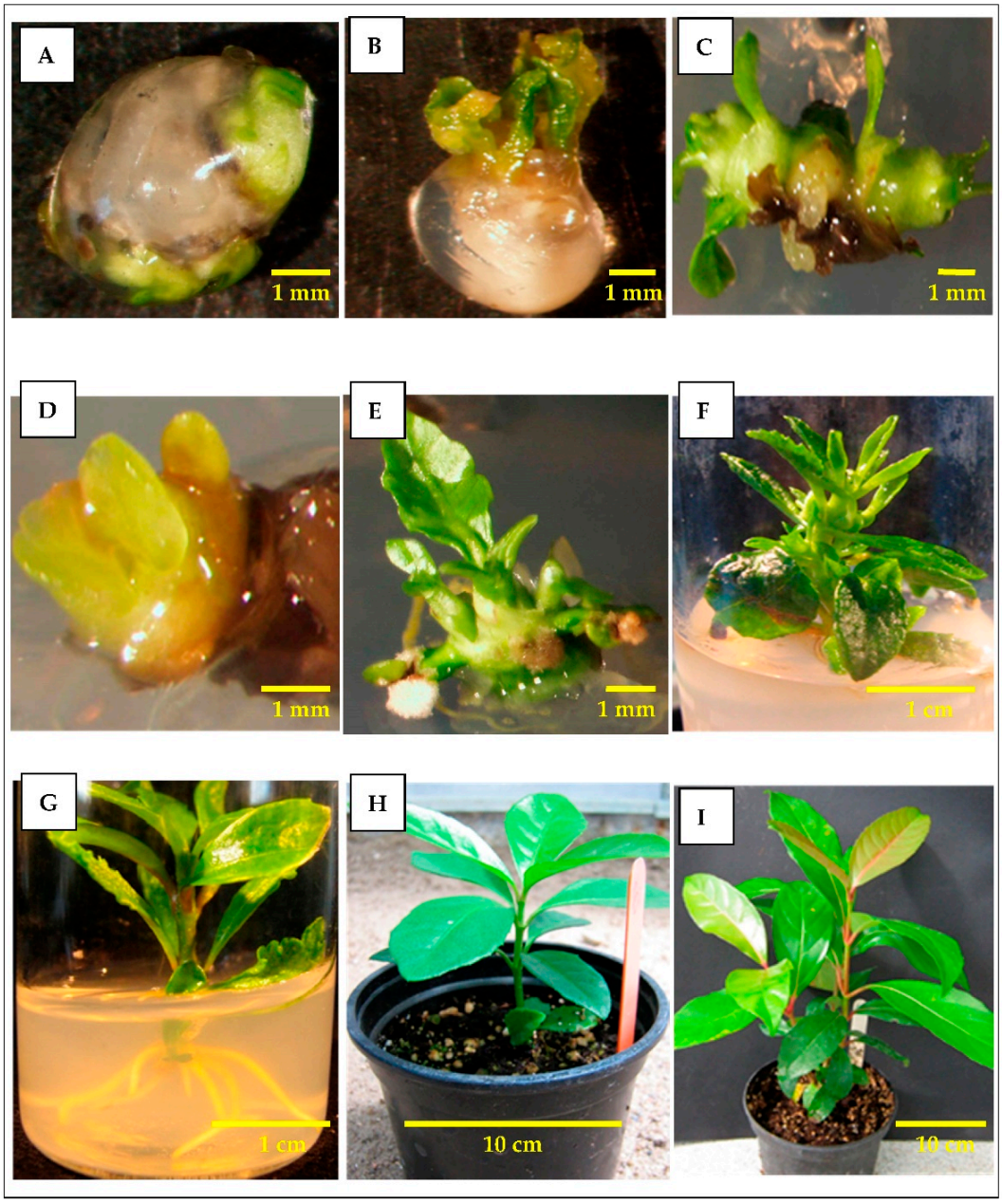

Figure 1. Encapsulated explants of *V. dentatum* and their conversion into plantlets: (**A**) Encapsulated first-node explant in 2.5% sodium alginate followed by exposure for 30 min to 50-mM calcium chloride. (**B**) Germination commencement of an alginate bead (of shoot tip explant) formed in 2.5% sodium alginate and 100-mM calcium chloride, after 1 week of culture. (**C**) Germination of an alginate bead produced in 2.5% sodium alginate and 50-mM calcium chloride, after 4 weeks of culture. (**D**,**E**) Germination of artificial seeds stored at 4 °C for 12 weeks after 1 (**D**) and 3 (**E**) weeks of culture. (**F**) Formation of multiple shoots (in 4 weeks) on germinated artificial seed after storage at 4 °C for 12 weeks. (**G**) Rooted microshoot (derived from culture of case **F**) in solid MS nutrient medium with 0.5 μM of Indole-3-acetic acid (IAA). (**H**,**I**) Microplants established in the greenhouse, after being acclimatized under 75%shading of weekly gradual reduction, 4 (**H**) and 8 (**I**) weeks from the end of the acclimatization period.

The addition of a carbon source (i.e., sucrose) in the gelling matrix was mainly used in somatic embryo explants, such as in conifers [34], whereas in other species (i.e., *Solanum melongena*) the type of carbon source was related to the explant type [39]. Thus, when somatic embryos were used as explants,

sucrose plus sorbitol (1:1) promoted the germination and regeneration response, while in nodal segment explants sucrose alone exhibited a better performance. The inclusion of 1% sucrose in the gelling matrix of viburnum explants, in combination with MS nutrient medium, enhanced the formation of shoots after artificial seed germination, probably through its nutritive capacity. Light could be a stress factor for artificial seeds maintained for a long time, as it was found in *Taraxacum pieninicum* [25]. In our experiments, the 4-week light period was not stressful for the artificial seeds of viburnum; on the contrary, it promoted the regeneration response and increased the number of shoots formed in comparison with the maintenance in the darkness (Table 2).

The concentration of the calcium chloride of the complexing agent influenced the texture of beads and their subsequent germination (Tables 3 and 4). At low concentrations of calcium chloride (25 mM), soft and very fragile beads with no defined shape were formed, whereas in high concentration (200 mM), the beads were isodiametric in shape but too hard. Intermediate concentrations of calcium chloride and especially that of 100 mM, in combination with 2.5% sodium alginate, resulted in firm, clear and uniform beads of globular shapes, suitable for handling (Table 3). The regeneration response in subsequent germination varied among the different concentrations of calcium chloride used. Thus, the lowest regeneration response was achieved with 25 mM of calcium chloride (15.5%), while the application of higher concentrations (50, 100 and 200 mM) favored the regeneration response of the explants (42.2, 48.9, 42.2%, respectively) (Table 4, Figure 1B). The greatest number of shoots per explant and the longest shoots occurred at the concentration of 100 mM of calcium chloride (2.7 shoots of 1.9 cm in length, Figure 1C). In the artificial seed production of *V. odoratissimum*, the best results were reported with the mixture of 4% sodium alginate and 2% calcium chloride for 30 min [23].

Table 3. Influence of different concentrations of calcium chloride ($CaCl_2.2H_2O$), mixed with 2.5% sodium alginate, on the type of bead formation of *V. dentatum*.

Concentration of $CaCl_2.2H_2O$ (mM)	Grading of Bead Formation	Texture/Shape of Beads
25	+[1]	Soft and very fragile, no defined shape
50	++	Soft, solid, uniform shape
100	+++	Firm, clear, uniform of globular shape
200	+++	Very hard, isodiametric, globular shape

+[1] Poor quality, ++ Good quality, +++ Optimal quality.

Table 4. Influence of different concentrations of calcium chloride ($CaCl_2.2H_2O$), in combination with 2.5% sodium alginate, on subsequent artificial seed germination of *V. dentatum*.

Concentration of $CaCl_2.2H_2O$ (mM)	Regeneration Response (%)	Number of Shoots/Bead	Length of Shoots(cm)
25	15.5 ± 3.9b [1,2]	1.3 ± 0.6b [1,2]	0.3 ± 0.1d [1,2]
50	42.2 ± 3.9a	1.7 ± 0.6ab	1.6 ± 0.1b
100	48.9 ± 3.8a	2.7 ± 0.8a	1.9 ± 0.1a
200	42.2 ± 3.9a	1.9 ± 0.6ab	1.3 ± 0.1c
ANOVA (F)	***	*	***

[1] Mean (±SD) of 45 artificial seeds per treatment, after four weeks of culture. [2] Different letters within columns indicate statistically significant differences according to Duncan's multiple range test at $p \leq 0.05$. *, *** Significant at the 0.05 and 0.001 level, respectively.

Many studies report that the concentration ofcalcium chloride and the time duration of its application, for the ion-exchange reaction to occur, result in the formation of artificial seeds of different textures, shapes and transparencies [25,38]. In *Rauvolfia tetraphylla*, 100 mM of calcium chloride produced firm, clear and uniform beads within an ion exchange duration of 30 min, whereas a higher concentration (200 mM) resulted in hard-texture beads and lower concentrations (25 or 50 mM) in fragile ones [14]. In our investigation, the use of 100 mM of calcium chloride for 30 min led to the production of suitable beads and a good regeneration response. Similar results were obtained for a number of plant species such as *Saintpaulia ionatha* [37], *C. angustifolia* [18], *Plumbago rosea* [40],

T. pieninicum [25] and *Nerium oleander* [38]. Additionally, Ara et al. [41] mentions that generally 2% sodium alginate gel upon complexation with 100-mM calcium chloride is the best combination for artificial seed technology.

3.3. Germination after Storage of Artificial Seeds

The non-encapsulated (naked) and encapsulated viburnum shoot tip explants without cold storage scored the highest regeneration response (97.8 and 75.5%, respectively), while encapsulated explants stored at 4 °C for 4, 8 or 12 weeks exhibited a gradual decline over storage time (Table 5, Figure 1D,E). On the other hand, the non-encapsulated explants showed a sharp decline in the regeneration response after a 4-week period of cold storage (42.2%), whereas no regeneration response was recorded in the explants of 8- and 12-week cold storage (Table 5). Moreover, the encapsulated explants were significantly more prolific for both the control and the 4-week cold storage (5.3 and 5.0 shoots/bead, respectively) than non-encapsulated (4.0 and 2.3 shoots/bead, respectively) (Table 5, Figure 1F). In the case of 8 and 12 weeks of cold storage, the encapsulated explants produced 4.0 and 3.0 shoots/beads, respectively (Table 5). Storage at 4 °C for 4 weeks drastically diminished the shoot length of non-encapsulated explants to half of the control (0.8 and 1.7 cm, respectively), while a slight decline in the shoot length was recorded for the explants kept at 4 °C from 4 to 12 weeks (from 1.5 to 1.3 cm) (Table 5).

Table 5. Influence of cold storage (4 °C) duration on subsequent regeneration of encapsulated and non-encapsulated shoot tip explants of *Viburnum dentatum* on solid MS nutrient medium with 5 μM of BAP.

	Storage Duration (Weeks)	Regeneration Response (%)	Number of Shoots	Length of Shoots (cm)
Non-encapsulated explants (naked)	0 (control)	97.8 ± 3.9a [1,2]	4.0 ± 1.0ab [1,2]	1.7 ± 0.1a [1,2]
	4	42.2 ± 3.9e	2.3 ± 0.6c	0.8 ± 0.1c
	8	0f	0d	0d
	12	0f	0d	0d
Encapsulated explants	0 (control)	75.5 ± 3.9b	5.3 ± 0.6a	1.7 ± 0.1a
	4	73.3 ± 6.7b	5.0 ± 1.0ab	1.5 ± 0.1ab
	8	62.2 ± 3.9c	4.0 ± 1.0ab	1.3 ± 0.1b
	12	51.1 ± 3.8d	3.0 ± 0.0b	1.3 ± 0.1b
ANOVA (F)		***	***	***

[1] Mean (±SD) of 45 replicates (explants) per treatment, after 4 weeks of culture. [2] Different letters within columns indicate statistically significant differences according to Duncan's multiple range test at $p \leq 0.05$. *** Significant at the 0.001 level.

Encapsulated viburnum explants showed higher resistance to storage at 4 °C than non-encapsulated explants. After storage for 8 or 12 weeks, the regrowth response of the encapsulated explants was 62.2 and 51.1%, respectively, whereasthe non-encapsulated explants did not respond at all. This better regrowth of encapsulated explants compared to non-encapsulated ones could be attributed to the protective coating of the gel matrix which, furthermore, acts as a pool of nutrients for the explants, confirming the effectiveness of the gel matrix [12,18,42]. The beneficial role of the protective coating of the alginate matrix and the nutrients it provides to the explants, facilitating survival and growth, have also been reported for many plant species [12,18,23,42,43].

The gradual decline (from 73.3 to 51.1%) observed in the regeneration response and the shoot-forming capacity of the encapsulated viburnum explants after being stored from 4 to 12 weeks at 4 °C could be ascribed to the inhibited respiration of plant tissue imposed by the alginate matrix or to a loss of moisture due to partial desiccation during storage [14,44–46]. Additionally, the regeneration decline of artificial seeds due to prolonged cold storage has been reported for *N. oleander* [38], *Celastrus paniculatus* [47], *Sphagneticola calendulacea* [15], grapevine rootstock "Kober 5BB" [12], *C. angustifolia* [18], and other species.

3.4. Microshoot Rooting, Microplant Acclimatization

The in vitro rooting of viburnum microshoots, generated from germinated encapsulated explants after 12 weeks of cold storage, took place overapproximately 4 weeks (Figure 1G). The addition of 0.5 μM of IAA to the agar-solidified MS nutrient medium resulted in a high rooting rate (95%, data not shown) and the formation of an adequate root system (Figure 1G). This is in agreement with a previous work conducted by Hatzilazarou et al. [38] on *V. dentatum* microshoot rooting in vitro, achieving 100% rooting with the inclusion of 0.5 μM of IAA in the agar-solidified MS nutrient medium. It was evident from the present study that viburnum microshoots derived from encapsulated explants, which were germinated after cold storage for 12 weeks, sustained their rooting capacity at a very high level. Similar results on microshoot rooting, after cold storage of encapsulated artificial seeds, were also reported for *N. oleander* [38], *P. rosea* [40] and *Tylophora indica* [46].

After transplantation to soilless substrate, the plantlets were placed under different shading conditions for acclimatization. The percentage of shading in combination with the duration of its application drastically affected the survival rate of the acclimatized young viburnum plantlets in greenhouse conditions (Table 6). Plantlets kept for one week under 75% shading and thereafter moved every week gradually to 50 and 25% shading and finally to natural light irradiance for one more week achieved the highest survival rate of 95% (Table 6). A lower survival rate (85%) was noticed in the plantlets placed consecutively under 50 and 25% shading for one week each and then transferred to natural light irradiance for another two weeks. When plantlets were kept under 25% shading, for two weeks or one week, and then moved to natural light irradiance for the rest of the two or three weeks, their survival rate dropped to 30 and 5%, respectively (Table 6).

Table 6. Influence of shading treatments during acclimatization of in vitro-produced plantlets of *Viburnum dentatum*, derived from regenerant microshoots of encapsulated shoot tip explants which had stored at 4 °C, on their survival rate (%).

Shading (in Weeks)				Survival (%)
75%	50%	25%	0%	
1 wk	1 wk	1 wk	1 wk	95 ± 13.3a [1,2]
-	1 wk	1 wk	2 wks	85 ± 13.3a
-	-	2 wks	2 wks	30 ± 7.3b
-	-	1 wk	3 wks	5 ± 13.3c
ANOVA (F)				***

[1] Mean (±SD) of 20 plantlets per treatment. [2] Different letters within column indicate statistically significant differences according to Duncan's multiple range test at $p \leq 0.05$. *** Significant at the 0.001 level

The passage from the in vitro to ex vitro conditions is a stressful process for the microplants, which try hard to survive and therefore must be hardened enough prior to facing the natural environment [48]. Acclimatization of micropropagated plantlets to a greenhouse or field environment is essential to take place in order to avoid desiccations leading to low survival rates [49,50]. The use of a heavy shading net over the plantlets from the start, which gradually decreases, results in a gradual increase in the light irradiance and at the same time the relative humidity around the plantlets gradually decreases from a high to a low level; these are the usual environmental requirements for a successful acclimatization [48]. In this study, following these environmental requirements and especially by gradually reducing the shading over the viburnum plantlets, from 75 to 25%, during a 3-week period and controlling the relative humidity resulted in a 95% survival rate. Such acclimatized plantlets were established well in the standard greenhouse conditions with normal growth and morphology and grew further successfully (Figure 1H,I). High survival rates after an efficient implementation of acclimatization to microplants, which derived from germinated artificial seeds, have been reported for several plant species, among them *C. angustifolia* [18], *R. tetraphylla* [14], *Stevia rebaudiana* [9], *Momordica dioica* [51] and *T. pieninicum* [25].

3.5. ISSR Analysis, Genetic Stability

In order to confirm the genetic uniformity, a comparison of ISSR patterns for seven plantlets and their mother plant of viburnum was carried out. The screening with 10 ISSR primers (Table 7) generated 68 scorable band classes, ranging in size from 200 to 1400 bp. The number of bands for each primer varied from 5 (UBC 812) to 9 (UBC 834) with an average of 6.8 bands per ISSR primer. A total of 544 bands (numbers of plantlets analyzed x number of band classes with all the ISSR primers) were generated, giving rise to monomorphic patterns across all the plantlets analyzed. No polymorphic bands were observed, indicating that there is genetic uniformity among the seven randomly selected viburnum plantlets (G1–G7) and the mother plant (MP) (Figure 2A,B). The results indicated that viburnum plantlets derived from encapsulated shoot tips, after 12 weeks of cold storage at 4 °C, exhibited genetic stability after 4 weeks of acclimatization and resembled the mother plant based on ISSR profiles. These findings are in accordance with earlier reports of the genetic fidelity of plantlets generated from artificial seeds of *Cineraria maritima* [26], *Picrorhiza kurrooa* [27] and *R. tetraphylla* [14].

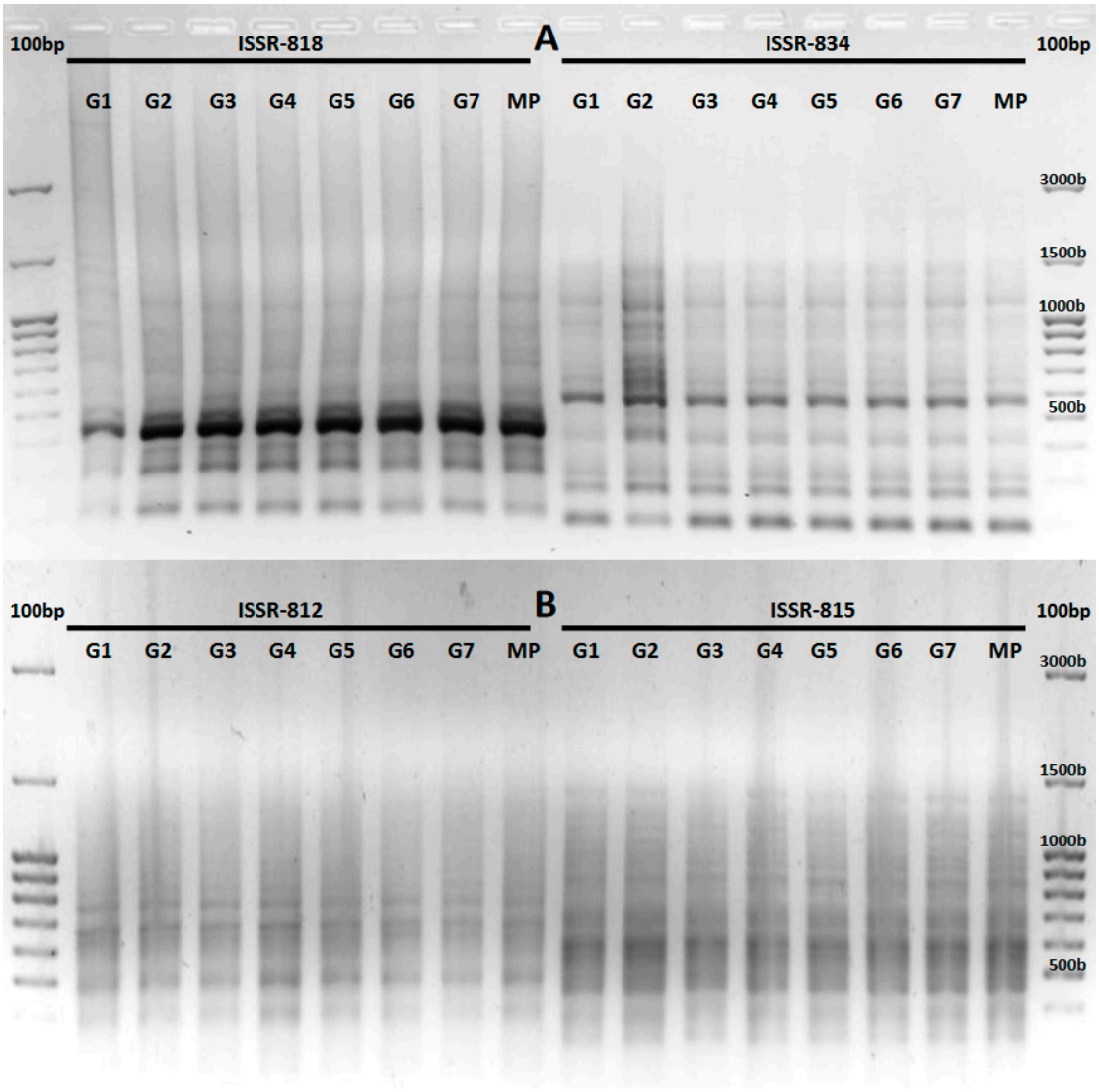

Figure 2. Inter Simple Sequence Repeats (ISSR) amplification pattern, obtained for gDNA of seven micropropagated *V. dentatum* plantlets (G1–G7) and one *V. dentatum* mother plant (MP), generated by the primers ISSR-UBC 818, 834 (**A**) and 812, 815 (**B**). The size of the amplified zones was calculated using 100-bp DNA ladder (100–3000 bp) and the image has been converted to black and white and negative.

Table 7. Sequence of 10 Inter Simple Sequence Repeat (ISSR) primers used to determine the genetic stability of *V. dentatum* plantlets produced from encapsulated shoot tip explants.

Primer Code	Primer Sequence (5′–3′)	Annealing Temperature
UBC 808	AGA GAG AGA GAG AGA GC	58 °C
UBC 809	AGA GAG AGA GAG AGA GG	58 °C
UBC 810	GAG AGA GAG AGA GAG AT	52 °C
UBC 811	GAG AGA GAG AGA GAG AC	54 °C
UBC 812	GAG AGA GAG AGA GAG AA	50 °C
UBC 815	CTC TCT CTC TCT CTC TG	50 °C
UBC 816	CAC ACA CAC ACA CAC AT	54 °C
UBC 818	CAC ACA CAC ACA CAC AG	56 °C
UBC 821	GTG TGT GTG TGT GTG TT	56 °C
UBC 834	AGA GAG AGA GAG AGA GYT	56 °C

4. Conclusions

The results of this study demonstrated the possibilities of encapsulating viburnum apical shoots and first-node segments in alginate beads, short-term storage at a low temperature and subsequent regrowth at high frequencies producing genetically stable plants. The artificial seed production protocol presented in this paper, with some further improvements, could offer potential for preserving germplasm for a short/medium period of time avoiding costly subcultures, for distributing desirable and uniform clones at a large-scale and for using as an efficient alternative method for *V. dentatum* micropropagation.

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