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In Vitro Plant Regeneration from Alginate-Encapsulated Microcuttings of *Rauvolfia tetraphylla* L.

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Abstract: Nodal segments obtained from *in vitro* cultures of *Rauvolfia tetraphylla* were encapsulated in calcium alginate hydrogel containing Murashige and Skoog (MS) medium. Morphogenic response of encapsulated buds to various planting media was evaluated. Encapsulated buds exhibited the best shoot development on MS medium supplemented with 10 μM BA, 0.5 μM NAA and 3% sucrose and gelled with 0.8% agar. Encapsulated nodal segments demonstrated successful regeneration after different periods (1-8 weeks) of cold storage at 4°C. Among the five different periods of cold storage, the beads stored for 4 weeks showed maximum frequency (86.7%) of shoot proliferation when placed back to regeneration medium. The regenerated shoots rooted on MS medium containing 0.5 μM IBA. Plantlets with developed shoot and roots were hardened off to survive *ex vitro* conditions and transferred to soil. The high frequency of plant regrowth from alginate-coated nodal segments coupled with high viability percentage after 4 weeks of storage is highly encouraging for the exchange of *R. tetraphylla* genetic resources.

Key words: Calcium alginate · encapsulation · germplasm storage · nodal segments · regeneration · synthetic seeds

INTRODUCTION

Rauvolfia tetraphylla L. (syn. *Rauvolfia canescens*) is an endangered, evergreen woody shrub belongs to family Apocynaceae. The plant is known in folk medicine as a treatment of snake poisoning and for external application in skin ailments. Several indole alkaloids have been reported from this plant, which grows worldwide [1-3]. Reserpine is a most potent alkaloid that depresses the central nervous system and produce sedation and lowers blood pressure [4]. The root of this plant is also used to stimulate uterine contraction and recommended for use in difficult child birth cases. The government of India has restricted the exports of its drugs to reduce its exploitation and thus conserve natural stands. This has resulted in a shortage of its alkaloids in the world market. During the last few years, considerable efforts have been made for *in vitro* regeneration of this endangered plant species from nodal segments [5-8] and shoot tip culture [9]. So far, there is no report on synthetic seed production and subsequent plant regeneration, using vegetative propagules in *R. tetraphylla*.

The encapsulation technique for producing synthetic seeds or artificial seed has become an important asset in micropropagation. Encapsulation of *in vitro* derived vegetative propagules to develop synthetic seeds has been employed in recent years as a suitable alternative to the use of somatic embryos [10-13]. The alginate coat protects micropropagules and thus has practical application for germplasm conservation of an elite plant species and exchange of axenic plant materials between laboratories [14, 15]. In addition, alginate-coated, non embryogenic micropropagules are relatively inexpensive to produce [16] and easy to handle, transport and plant. Furthermore, they can be used for cryopreservation via encapsulation dehydration and encapsulation vitrification [17, 18]. Despite these advantages, there are only few reports on encapsulation of non-embryogenic micropropagules [13,15,19-23].

We report here for the first time the encapsulation of nodal cuttings of *R. tetraphylla* in calcium alginate beads and conversion of encapsulated nodal segments into plantlets. The effect of different storage duration on the morphogenic response of the encapsulated nodal segments has also been attempted.

MATERIAL AND METHODS

Plant materials and explant source: Nodal segments from mature plants of *R. tetraphylla* were used to initiate *in vitro* cultures. The nodal segments were washed with running tap water for 1 h, immersed in aqueous solution

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of 5% (v/v) labolene for 5 min and rinsed with sterile distilled water. The explants were then surface-disinfected with an aqueous solution of 0.1% (w/v) $HgCl_2$ for 3 min followed by 5-6 rinses with sterile distilled water. The sterile explants (0.5-1 cm) were cultured *in vitro* according to Faisal and Anis [7] on Murashige and Skoog [24] medium containing 10 μM BA, 0.5 μM NAA, 3% (w/v) sucrose and solidified with 0.8% (w/v) agar. For all experiments, the pH of the media was set to 5.7 before autoclaving at 121°C for 20 min. Shoots that developed from nodal segments were transferred to fresh medium to establish proliferating shoot cultures. Cultures were maintained at 25±2°C under 16/8-h (light/dark) photoperiod with a light intensity of 50 μmol m^{-2} s^{-1} provided by cool white fluorescent lamps. Nodal segments approximately 3 mm long were dissected aseptically and used as explants for encapsulation.

Encapsulation: Different concentrations (1, 2, 3, 4 and 5%) of sodium alginate were prepared using MS liquid medium. For complexation 100 mM of $CaCl_2.2H_2O$ solution were prepared in liquid MS medium. Both the sodium alginate and $CaCl_2.2H_2O$ solution were autoclaved at 121°C for 20 min after adjusting the pH to 5.7. Encapsulation was accomplished by mixing the nodal segments into the alginate solution and dropping these into the $CaCl_2.2H_2O$ solution. The droplets containing the explants were held for at least 30 min to achieve polymerization of the sodium alginate. After 30 min, the alginate beads were collected and rinsed with sterile distilled water to wash away $CaCl_2.2H_2O$ residues and thereafter cultured on different medium.

Planting medium and culture conditions for plant regeneration: In order to induce sprouting *in vitro*, the encapsulated nodal segments were planted onto petri dishes containing the following culture media: (M1) Growth regulator free MS basal medium + 3% Sucrose; (M2) MS basal medium + 2.5 μM BA + 0.5 μM NAA + 3% sucrose; (M3) MS basal medium + 5 μM BA + 0.5 μM NAA + 3% sucrose; (M4) MS basal medium + 10 μM BA + 0.5 μM NAA + 3% Sucrose; (M5) MS basal medium + 20 μM BA + 0.5 μM NAA + 3% sucrose. After shoot development, the encapsulated buds were transferred to 250 mL culture flask containing the same medium (M1-M5) as that on which they developed shoots.

The medium was gelled with 0.8% (w/v) agar and the pH was adjusted to 5.7 before being autoclaved at 121°C for 20 min. Cultures were maintained at 25±2°C under a 16/8-h (dark/light) photoperiod and a light intensity of 50 μmol $m^{-2}s^{-1}$ provided by cool-white fluorescent lamps.

Low temperature storage: A set of encapsulated nodal segments were stored in a laboratory refrigerator at 4°C for various periods of time before being transferred for regeneration. Five different low-temperature exposure times (1, 2, 4, 6 and 8 weeks) were evaluated for regeneration. The percentage of encapsulated nodal segments forming shoots and the number of differentiated shoots per encapsulated bud was recorded after 6 weeks of culture to regeneration medium.

Rooting and acclimatization: For rooting, shoots about 4-5 cm long were excised and transferred to MS medium containing 0.5 μM. After rooting, regenerated plantlets were washed carefully to remove remnant agar and planted in plastic pots containing sterile soilrite. The potted plants were covered with transparent polyethylene bags to ensure high humidity. After 1 month, surviving plants were transferred to pots containing normal garden soil and maintained in greenhouse.

Statistical analysis: All the experiments were repeated thrice and twenty replicates were employed for each treatment. The data were analyzed using SPSS Version 10 (SPSS Inc., Chicago, USA). Analysis of variance (ANOVA) was used to test the statistical significance and the significance of differences among means was carried out using Tukey's test at 5% probability level.

RESULTS AND DISCUSSION

The encapsulated beads differed morphologically with respect to texture, shape and transparency, with different combinations of sodium alginate (1, 2, 3, 4 and 5%) and $CaCl_2.2H_2O$ (100 mM). The assessment of the effects of various concentrations of sodium alginate and calcium chloride was prerequisite in order to standardize the preparation of characteristics beads. An encapsulation matrix of 3% sodium alginate with 100 mM of $CaCl_2.2H_2O$ was found most suitable for the formation of ideal beads (Fig. 1A). Sodium alginate concentrations below 3% were not suitable for encapsulation because the resulting beads were without a defined shape and were too soft to handle, whilst at higher concentration the beads were isodiametric and hard to cause considerable delay in regeneration. Sodium alginate preparation at lower concentration became unsuitable for encapsulation, probably because of a reduction in its gelling after exposure to high temperatures during autoclaving [25, 26].

The beads encapsulated in 3% sodium alginate cultured on the five different media, described above,

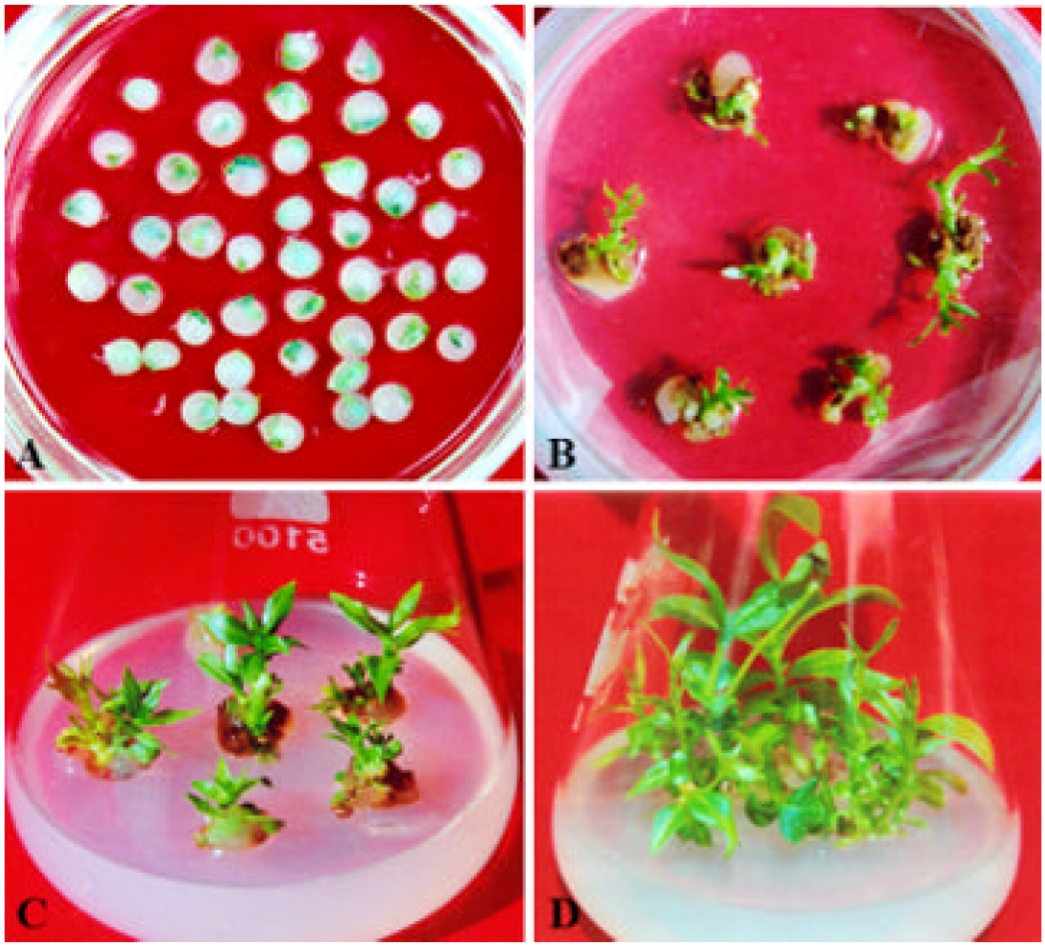

Fig. 1: A-D: Plant regeneration from encapsulated nodal segments of *R. tetraphylla* L. (A) Beads formed by the encapsulation of nodal segments using 3% sodium alginate and 100 mM $CaCl_2.2H_2O$; (B, C) Ruptured beads showing sprouting of shoots on M4 medium after 2 weeks of culture; (D) Well defined shoots derived from encapsulated nodal segments grown on M4 medium after 6 weeks of culture

showed the emergence of shoots 2-3 weeks after incubation (Fig. 1B and C). The frequency of shoots development on these culture media varied according to medium composition (Fig. 2). MS medium supplemented with 10 μM BA with 0.5 μM NAA (M4) gave the maximum frequency (75.3%) of conversion of encapsulated buds into multiple shoots. After 6 weeks, a well developed shoots were observed on this medium (Fig. 1D). Shoots were phenotypically normal with distinct nodes and internodes. There was no regeneration occurred on hormone free medium (M1). The encapsulated beads cultured on M5 medium showed the emergence of weak shoots with stunted growth.

Figure 3 shows the conversion of encapsulated nodal segments, after 1, 2, 4, 6 and 8 weeks of storage duration at 4°C. Storage at 4°C resulted in high rate of shoot proliferation (86.7%) if the beads containing microshoots were transferred back to the medium (M4) within 4 weeks. Longer storage, for 6 and 8 weeks significantly decreases the number of explants able to grow and multiply. In the present study, the conversion of encapsulated nodal segments into plantlets after considerable period of storage could be attributed to the inclusion of MS salts in encapsulation matrix which serves as an artificial nutrient to the encapsulated explants for regeneration [27-29]. The data from our experiment with cold-stored encapsulates nodal segments were in accordance with the study of Tsvetkov and Hausman [23] concerning the rates of encapsulated segments with axillary buds in *Quercus* spp. stored at 4°C. Similarly, M-26 apple root-stock encapsulated microcuttings cold stored for 60 days were found to display better performance and regeneration

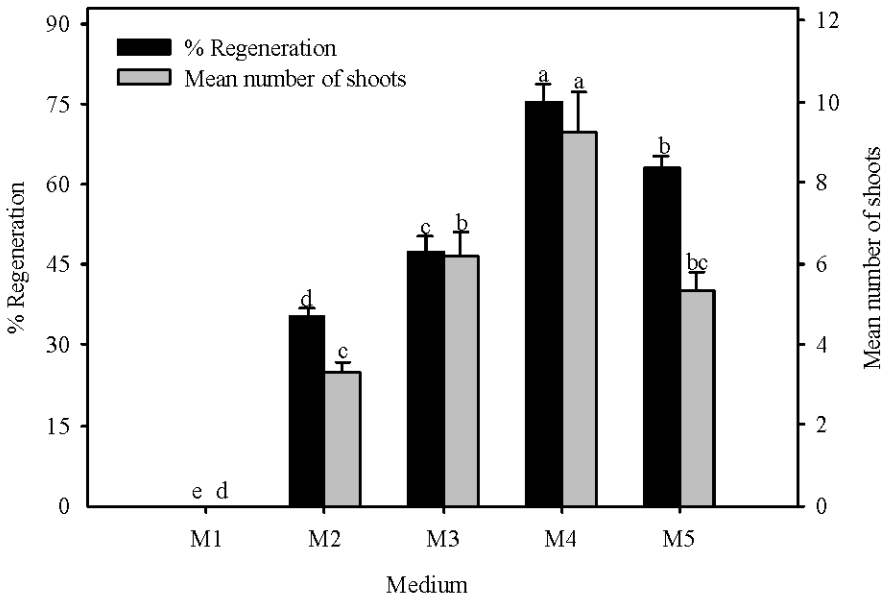

Fig. 2: Effect of different medium on *in vitro* regeneration from alginate-encapsulated nodal cuttings of *R. tetraphylla* after 6 weeks of culture. Bars represents the mean±SE. Bars denoted by the same letter are not significantly different by the Tukey's test at 5% probability level

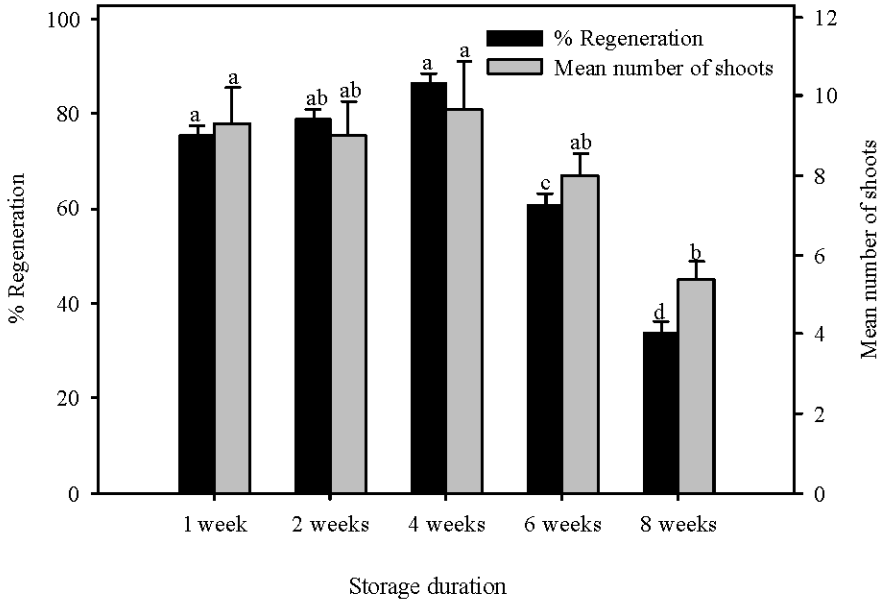

Fig. 3: Effect of cold storage (4°C) on *in vitro* regeneration from alginate-encapsulated nodal cuttings of *R. tetraphylla* after 6 weeks of culture to M4 medium. Bars represents the mean±SE. Bars denoted by the same letter are not significantly different by the Tukey's test at 5 % probability level

indices than non-stored one [30]. A possible explanation is that cold treatment of capsules help in faster and/ or more complete elimination of bud dormancy.

The regenerated shoots rooted when excised and subjected to MS medium containing 0.5 μM IBA. The plantlets with developed shoot and roots were transferred to plastic pots containing sterile soilrite and covered with transparent polyethylene bags. After 1 month these were planted in earthen pots containing normal garden soil and maintained in greenhouse.

To the best of our knowledge, this is the first report for encapsulation of *R. tetraphylla* nodal segments into a alginate matrix followed by successful *in vitro* regeneration. The high frequency of shoot recovery from encapsulated nodal segments after 4 weeks of storage could be used as nodal delivery system for germplasm exchange and storage. The result described could be considered as a perquisite for exploring the encapsulation technique for development of a procedure for production of synthetic seed from non-embryogenic explants.

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