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In vitro Plant Regeneration of an Endangered Sikkim Himalayan Rhododendron (*R. maddeni* Hook. f.) from Alginate-Encapsulated Shoot Tips

Kaushal Kumar Singh
G.B. Pant Institute of Himalayan, Sikkim Unit, Environment and Development,
Pangthang, P.O. Box 24, Gangtok, 737 101, East Sikkim, India

Abstract: Shoot tips obtained from *in vitro* cultures of *Rhododendron maddeni* Hook. f. were precultured on Anderson medium, then encapsulated in sodium alginate hydrogel containing Anderson Medium (AM). Conversion of encapsulated shoot tips into plantlets was significantly affected by the concentration of sodium alginate, duration of storage and planting media with different gelling agent. Amongst the two gelling agents used higher shoot proliferation as well as better growth were observed in cultures grown on phytagel in comparison to agar gelled medium. Encapsulated shoot tips exhibited the best shoot development on AM supplement with 7 mg L^{-1} 2iP, 100 mg L^{-1} PVP, 100 mg L^{-1} ascorbic acid, 10 mg L^{-1} citric acid, 3% sucrose and gelled with 0.3% phytagel. Size of the shoot tips was an important factor in producing multiple shoot. Encapsulated shoot tips demonstrated successful regeneration after different periods of cold storage at 5°C. Among the four different storage conditions (0, 5, 17 and 25°C), the beads stored for 30 days at 5°C showed maximum frequency (68%) of shoot proliferation when placed back to regeneration medium. Shoots regenerated from encapsulated shoot tips required 0.2 mg L^{-1} IBA for rooting. Plantlets developed from encapsulated shoot tips with well developed roots and shoots were transferred successfully to green house. *In vivo* transplants were observed growing best on peat moss; after one month these were transferred to polythene bags containing normal garden soil, 86% survival was recorded.

Key words: Calcium alginate, *in vitro*, encapsulation, germplasm storage, *Rhododendron maddeni*

INTRODUCTION

The genus *Rhododendron* is represented by 50 species in India and about 98% of the Indian species are found in the Himalayan region. Out of this 72% are found in Sikkim Himalayan region (Cowan and Cowan, 1929; Gamble, 1936; Pradhan and Lachungpa, 1990). It has been observed recently that the rhododendrons of the region are under pressure from various major quarters which are basically man-made and some of the species, for example, *R. micromeres*, *R. maddeni*, *R. niveum*, etc. are under serious threat of extinction (Krishna *et al*., 2002; Singh *et al*., 2003). *R. maddeni* is a beautiful and endangered rhododendron that has limited distribution. It is the only species found in the region which produces fragrant flowers. The regeneration status in the form of available seedlings/saplings is very poor due to the above situation for many of the rhododendrons. So far no attempt has been made to develop synthetic seeds and large scale production of rhododendron species which are under the threat of extinction.

During the last few years, considerable efforts have been made for *in vitro* regeneration of this endangered plant species from nodal segments and shoot tip culture (Mercure *et al*., 1998; Kumar *et al*., 2004). So far, there is no report on synthetic seed production and subsequent plant regeneration, using shoot tips in rhododendron species. The synthetic seed was first developed by Murashige (1978). The encapsulation technique for producing synthetic seeds or artificial seed has become an important asset in micropropagation. This technique is simple, does not need expensive cooling equipment and can be used to a wide range of plant materials (Nino and Sakai, 1992; Ganapathi *et al*., 2001; Faisal *et al*., 2006). Despite the above advantages, only a handful of reports on encapasulation of non-embryogenic micropropogules are presently available (Sharma *et al*., 1994; Hasan and Takagi, 1995; Ganapathi *et al*., 2001; Keng and Hoong, 2005).

In this study, we describe here for the first time the encapsulation of nodal cuttings of *R. maddeni* in calcium alginate beads and conversion of encapsulated shoot tips

Corresponding Author: K.K. Singh, G.B. Pant Institute of Himalayan Environment and Development, Sikkim Unit, Pangthang, P.O. Box 24, Gangtok, East Sikkim 737 101, India Tel: (91) 03592-237189 Fax: (91) 03592-237415

into plantlets. The effect of different storage duration on the morphogenic response of the encapsulated nodal segments has also been attempted.

MATERIALS AND METHODS

Plant materials and shoot tip culture: Mature seeds of *R. maddeni* were collected from Rate-chu, East Sikkim between, October-November (2005). Seeds were thoroughly washed with a detergent (Tween -80; 1.0%, v/v; 20 min) and surface disinfected with an aqueous solution of mercuric chloride (0.15%; w/v; 3 min) before use. Following washings (3 times) with autoclaved double distilled water under aseptic conditions, these were inoculated (10 seeds per culture test tube, 25 mm dia, 15 mL medium) onto Murashige and Skoog's (1962), basal medium containing 0.8% (w/v) agar. The *in vitro* germinated seeds were allowed to grow till the first leaf appeared and the seedlings had attained an average height of 2.5 cm (about 7 weeks after germination). Shoot tip portions (0.5 cm) were carefully excised from seedlings, under aseptic conditions and subcultured on AM containing isopentenyladenine (2iP) along with additives 100 mg L^{-1} polyvinyl pyrrolidone (PVP), 100 mg L^{-1} ascorbic acid and 10 mg L^{-1} citric acid. The pH of the media was adjusted at 5.6 prior to the addition of agar (0.8% w/v) and autoclaving at 121°C for 15 min at 1.05 kg cm^{-2} pressure. The cultures were incubated under white fluorescent light 60 μmol m^{-2} sec^{-1} photon flux, 16 h photoperiod at 17±1°C temperature and 60% relative humidity. The cultured were transferred to fresh medium after 4 week intervals.

Encapsulation: Encapsulation of cultured shoot tips was performed by preparing 3% (w/v) sodium alginate (Merck India Limited) solution in Anderson Medium (1975) containing 3% sucrose. For encapsulation, 60 mM Calcium chloride solution was prepared in distilled water. Both the sodium alginate and $CaCl_2.2H_2O$ solution were autoclaved at 121°C for 15 min after adjusting the pH to 5.6. Shoot tips collected from *in vitro* proliferated shoots were used for synthetic seed production. Encapsulation was accomplished by mixing the nodal segments into the alginate solution and dropping these into the $CaCl_2.2H_2O$ solution. Calcium alginate beads were incubated in the $CaCl_2$ solution for 30 min. Encapsulated shoot tips were taken out by decanting off the $CaCl_2$ solution followed by washing in sterilized double-distilled water. The beads were blot dried using sterile filter paper and thereafter cultured on different media.

Planting medium and culture conditions for plant regeneration: Freshly encapsulated shoot tips were directly planted onto conical flak (250 mL) containing six different culture media. The media was gelled with agar (0.8%) or with phytagel 0.3%) supplemented with and without 2iP along with additives (100 mg L^{-1} PVP, 100 mg L^{-1} ascorbic acid and 10 mg L^{-1} citric acid) used for multiplication of shoots and was adjusted to pH 5.6 before autoclave at 121°C for 15 min at 1.05 kg cm^{-2} pressure. The cultures were maintained at 17±1°C temperature and 60% relative humidity under 16/8 h (dark/light) photoperiod and a light intensity of 60 μmol m^{-2} sec^{-1} photon flux provided by cool-white fluorescent lamps.

Low temperature storage: Four different temperature and exposure times were evaluated for regeneration. The percentage of encapsulated shoot tips forming multiple shoots and the number of differentiated shoots per encapsulated bud was recorded after 15 days of culture to regeneration medium. In all experiments, each treatment consisted of 20 replicates and each experiment was repeated twice. Standard error of the mean was calculated. Least Significance Difference (LSD) at $p<0.05$ level was calculated following the method of Snedecor and Cochran (1967).

Rooting and acclimatization: The shoots of 2-4 cm length were isolated from the shoot clump and kept for rooting in AM. Anderson medium containing 0.20 mg L^{-1} IBA (optimum concentration) was used for filter paper bridge technique using liquid medium. After rooting, regenerated plantlets were washed carefully and planted in plastic pots (125 mL) containing autoclaved fresh peat moss and soil (1:3) enrich with Anderson nutrient salts. After 1 month, surviving plants were transferred to pots containing normal garden soil and maintained in greenhouse.

RESULTS AND DISCUSSION

The seeds were used as explants for *in vitro* culture initiation and establishment. The sterilized seeds were germinated on hormone-free MS medium. Five weeks old seedling, cotyledonary nodal parts were used for shoots multiplication on Anderson acid and 10 mg L^{-1} citric acid. After 8 weeks, a well developed shoots were observed on this medium (Kumar *et al*., 2004).

The encapsulated beads differed morphologically with respect to texture, shape and transparency, with combinations of sodium alginate and $CaCl_2.2H_2O$. An encapsulation matrix of 3% sodium alginate with 60 mM of $CaCl_2.2H_2O$ was found most suitable for the formation of ideal beads (Fig. 1A). Freshly encapsulated shoot tips in 3% sodium alginate when directly inoculated on AM containing 7 mg L^{-1} 2iP and various gelling agents like agar (0.8%) and phytagel (0.3%) along with additives (100 mg L^{-1} PVP, 100 mg L^{-1} ascorbic acid and 10 mg L^{-1}

Fig. 1: Plant regeneration from encapsulated shoot tips of *Rhododendron maddeni*. (A) bead formed by the encapsulation of shoot tips using 3% Sodium alginate and 60 mM $CaCl_2$ H_2O; (B) Ruptured beads showing sprouting shoots on AM after two week of culture; (C, D) Well developed shoots derived from encapsulated shoot tips grown on AM supplemented with 0.3% phytagel after 8 weeks of culture; (E, F) Root induction from shoot tips on AM supplemented with 0.2 mg L^{-1} IBA and (G) Hardened plants of *R. maddeni* in green house

Table 1: Effects of different media/substrates on conversion of encapsulated shoot tips into palntlets in *R. maddenii*

Media/substrate	No. of shoots±SE	Shoots length (cm)±SE	Conversion of encapsulated shoot tips into plantlets (%)±SE
MS + 0.8% agar	2.00±0.217	0.70±0.061	24±2.74
MS +2iP + 0.8% agar	5.75±0.734	1.80±0.790	36±1.87
AM+ 0.8% agar	8.25±0.545	0.70±0.612	68±3.00
AM+2iP + 0.8% agar	11.75±0.645	2.80±0.612	84±1.41
AM + 0.3% phytagel	9.75±0.739	1.60±0.791	76±3.79
AM +2iP + 0.3% phytagel	15.50±0.250	4.00±0.079	96±0.71
LSD at the 5% level	5.90	0.80	16.7

SE: Standard error. Data were recorded 8 weeks after transfer to medium; each treatment consisted of 20 replicates and the experiment was repeated twice. Values are the mean±SE of two independent experiments

citric acid), showed growth initiation after second week. Subsequently, they emerged out by rupturing the alginate matrix and established contact with the media (Fig. 1B). Of the six different media tested, the frequency of shoot emergence from the encapsulated buds was highest (68-96%) on AM and the lowest (24-36%) on MS medium (Table 1). Well defined shoots obtained from encapsulated shoot tips grown on AM supplemented

Table 2: Effects of cold storage on *in vitro* regeneration from alginate-encapsulated shoot tips into plantlets in *R. maddeni*

Storage temperature (°C)	Storage duration (day)	No. of shoots	Shoots length (cm)±SE	Conversion of encapsulated shoot tips into plantlets (%) ± SE
0	15	2.00±0.35	0.70±0.13	24±1.58
	30	2.25±0.42	0.58±0.09	8±0.94
5	15	15.75±0.42	3.80±0.48	88±2.24
	30	10.25±0.74	2.00±0.16	68±1.84
17	15	8.00±0.79	1.60±0.15	40±2.74
	30	4.75±0.74	1.12±0.67	16±1.87
25	15	1.50±0.25	0.50±0.79	12±1.22
	30	-	-	-
LSD at the 5% level		4.90	0.70	12.9

SE: Standard error, Data were recorded 8 weeks after transfer to Anderson medium; each treatment consisted of 20 replicates and the experiment was repeated twice. Values are the mean±SE of two independent experiments

with 0.3% phytagel after 6 weeks of culture (Fig. 1C, D). Highest yield of shoots was obtained with 7.0 mg L^{-1} 2iP on AM with phytagel in comparison to cultures grown on agar gelled media. The occurrence of some inhibitory compounds in agar has been reported by Nairn *et al.* (1995) and thus the relatively reduced growth of cultures on agar gelled media was observed. The number of subcultures also exhibited higher proliferation between 8-10 weeks and the effect in proliferation rate of shoots was remarkable. Shoots were phenotypically normal with distinct nodes and internodes. Anderson medium supplemented 7.0 mg L^{-1} 2iP with 0.3% phytagel gave the maximum frequency (96%) of conversion of encapsulated shoot tips into planlets (Table 1).

Encapsulated shoot tips stored at 0°C showed regeneration of 24 and 8% following 15 and 30 days of storage, respectively (Table 2). This could due to injury to the encapsulated shoot tips at low temperature storage. Storage at 5°C resulted in high rate of shoots proliferation if the beads containing microshoots were transferred back to the medium (AM) within 30 days. When storage duration exceeded 30 days, encapsulated shoot tips significantly decreases the number of plantlets. After 30 days in storage no regeneration occurred at 25°C, while 68% regeneration still regarded at 5°C. Visual observations showed that shoot tips progressively turned brown during storage (faster in 17 and 25°C than at 5°C). To obtain maximum shoots proliferation, the ranking among storage temperature, temperature was 5 >17>25 >0°C for *R. maddeni.* In the present study, the conversion of encapsulated nodal segments into plantlets after considerable period of storage could be attributed to the inclusion of AM in encapsulated matrix which serves as an artificial nutrient to the encapsulated explants for regeneration.

Rooting was successfully induced in the regenerated shoot when transferred to Anderson liquid medium (Anderson, 1978) containing activated charcoal (1.0%, w/v) and 0.2 mg L^{-1} indole-3-butyric acid (IBA). IBA was the only hormone effective in induction of roots in regenerated shoots. Lower concentration of IBA

Table 3: Effect of different auxins incorporated in Anderson medium on rooting regenerated shoots of *R. maddeni*

Auxins (mg L^{-1})	No. of roots±SE	Rooting (%)	Callusing
Control	1.25±0.22	12	C++
IBA			
0.05	3.25±0.55	28	C++
0.10	3.75±0.41	40	C++
0.20	7.00±0.61	52	C+
0.30	4.75±0.54	36	C++
0.50	2.25±0.41	28	C+++
IAA			
0.05	-	-	C++
0.10	4.00±0.50	14	C+
0.20	7.00±0.35	36	C++
0.30	3.75±0.42	28	C+++
0.50	-	-	C+++
LSD at the 5% level		1.30	

SE: Standard error, Data were recorded 5 weeks after transfer to Anderson medium; each treatment consisted of 20 replicates and the experiment was repeated twice. Values are the mean±SE of two independent experiments. C+: indicates less callusing, C++: indicates more callusing, C+++: indicates profuse callusing

inefficient for rooting and higher concentration showed inhibition of roots and initiation of compact non-embryogenic callus from shoot. Root initiation took more than 5 weeks on Anderson medium without agar (Fig. 1E, F). The roots were thick and healthy and new shoots continued to regenerate from the rooted basal portion of the plant on prolonged cultured. α- naphthaleneacetic acid (NAA) and indole-3-acetic acid (IAA) were ineffective at all concentrations employed in inducing rooting, whereas IBA could induce rooting response in 52% shoots (Table 3).

Well-developed plantlets were transferred in thermocole cups containing a mixture of autoclaved fresh peat moss and soil (1:3) and placed for hardening under high relative humidity (80%) in the mist chamber of a greenhouse (25°C). After one month these were planted in polythene bag containing normal garden soil, 86% survived under green house conditions (Fig. 1G).

In the present study we report a suitable protocol for obtaining plantlets from encapsulated shoot tips of *R. maddeni*. Application of this protocol would be useful in promoting conservation of rhododendron spp. in natural populations. This is the first report for encapsulation of *R. maddeni* from apical shoots into an

alginate matrix followed by successful *in vitro* regeneration where large number of plant have been successfully produced and transferred to the field.

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